Amyloidosis: Diagnostic investigations, clinical categories, prognosis and management

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Declaration

I, Ayesha Shameem Mahmood, confirm that the work presented in this thesis is my own work. I have acknowledged or declared work if derived from other sources.

Abstract

Background

Amyloidosis is a very rare disorder of protein misfolding characterised by the deposition of certain proteins in an abnormal fibrillary form within the extracellular space, which disrupts the normal structure and function of organs throughout the body. Amyloid deposition may be systemic or localised, though there have been few systematic clinical studies of the latter. Treatment depends on the respective amyloid fibril type, and comprises chemotherapy regimens derived from myeloma for the most prevalent systemic monoclonal immunoglobulin light chain (AL) type. The clinical features of systemic AL amyloidosis are protean, commonly including a variety of poorly understood coagulation abnormalities and fatigue symptoms of uncertain cause. Measurement of serum free light chains (FLC) has been a very important advance in guiding treatment of systemic AL amyloidosis. Novel treatment approaches include the serum amyloid P component (SAP) depleting drug ((R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl] pyrrolidine-2 carboxylic acid which has shown promise in a pilot study in patients with hereditary fibrinogen amyloidosis.

Aims

The hypothesis of this thesis was to explore the diagnostic investigations, categories including localised amyloidosis and prognosis and management of certain types of amyloidosis. To compare the performance of two commercially available serum free light chain assays and study the prognostic utility of each in systemic AL amyloidosis. To investigate the underlying bleeding and coagulation abnormalities, associated prognostic implications, endothelial dysfunction and implications for the possibility of light chain toxicity. To explore the sleep disordered breathing morbidity in amyloidosis. To investigate the incidence, patient characteristics and survival outcomes in patients with localised AL

amyloidosis. To explore a subgroup of localised amyloidosis: tracheobronchial and laryngeal amyloidosis from a clinical and proteomic perspective. To examine two types of treatment in systemic amyloidosis: the use of lenalidomide based chemotherapy with prior use of Thalidomide/Bortezomib treatment in systemic AL amyloidosis and CPHPC treatment.

Results and conclusions

Both Freelite™ and N Latex assays have high sensitivity for detecting abnormal FLC in patients with systemic light chain amyloidosis, showing an excellent correlation between the assays for identifying the abnormal light chain subtype but with discordance in the absolute values.

Coagulation abnormalities in systemic AL amyloidosis were frequent and included the following abnormalities: elevated concentration of fibrinogen in 42 (56.8%), elevated FVIII 67 (90.5%) and vWF Ag 67 (90.5%). Kaplan Meier estimates showed that vWF Ag (p=0.039) and FVIII (p=0.01) thresholds greater than 280IU associated with a significant survival disadvantage. A fall in the vWF Ag levels following chemotherapy in those achieving a clonal response suggests potential light chain toxicity implications. Albumin concentration lower than 25g/L correlated with coagulation factors which are prothrombotic, implying that anticoagulation may be an important consideration in newly diagnosed systemic AL. Thus these findings suggest the potential prognostic utility of vWF Ag levels and thrombotic risks associated with newly diagnosed systemic AL patients.

Recurrent overnight oxygen desaturations proved to be frequent in patients with cardiac and/or soft tissue amyloidosis, although the occurrence of sleep disordered breathing (SDB) needs confirmation with formal polysomnography. Patients with poor right heart ventricular systolic function score high with SDB questionnaires, which was associated with adverse outcome in newly diagnosed cardiac AL amyloidosis.

Localised AL amyloidosis is a very different disease from systemic AL amyloidosis, with a far superior prognosis. Local surgical resection is adequate in most patients with localised amyloidosis in whom treatment is needed, and radiotherapy can have a useful role in some patients whose disease cannot be controlled by local measures. Progression to systemic AL amyloidosis is extremely rare except among patients with lymph node involvement. Patients with lymph node involvement and those with isotypic specific circulating free light chains warrant closer follow up for development of systemic amyloidosis. Most patients with localised AL have excellent long term outcomes.

Laryngeal and tracheobronchial amyloidosis is a subtype of localised amyloidosis, in which hoarseness and dyspnoea are the predominant symptoms, the 2 year OS 93% and 90% respectively. Proteomic analysis of amyloid dissected from biopsies showed the presence of the amyloid signature proteins, apolipoprotein A1 (in greater amounts protein) and insulin-like growth factor binding protein complex in all samples compared with patients with systemic AL or transthyretin amyloidosis. Of interest, apolipoprotein A1 has been described within the respiratory tract and insulin growth factor has been postulated to play a role in inflammation, which may be relevant with respect to the pathogenesis and effects of airways amyloidosis.

Lenalidomide and dexamethasone combination treatment following prior proteasome inhibitor based therapy produced an overall haematologic response rate of 61%, including 20% complete responses. Renal responses among patients who received prolonged treatment were surprisingly frequent; twenty one out of 38 (55%) evaluable patients achieved a renal response (40% on an ITT basis) – 7 (18%) at 6 months, 7 (18%) at 12 months and an additional 7 (18%) patients at 18 months by long term follow up. This raises the possibility that immunomodulatory effects of lenalidomide therapy might enhance the otherwise slow natural regression of amyloid deposits.

CPHPC depletes circulating Serum amyloid P (SAP) component as a treatment for systemic amyloidosis.[1] Our study of 10 patients suggested a significant reduction in the natural progression of renal decline and renal survival along with an excellent safety profile; this was supported by our QoL assessments using SFv36 questionnaires.

The work in this thesis has thus contributed to improved characterisation and clinical management of various types of amyloidosis, and has identified several avenues of therapy that merit further investigation in larger populations and randomised clinical trials.

Ethical Approval

All patients with data used in these clinical research studies described in this thesis gave explicit informed consent, by signing a consent form whilst visiting the National Amyloidosis Centre. The consent form was approved by the Royal Free Hospital Ethics Committee (REC Ref 06/Q0501/42). The dosage and administration of radioactive isotopes were approved by the Administration of Radioactive Substances Advisory Committee of the Department of Health.

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Publications – from this thesis

Update on treatment of light chain amyloidosis. Shameem Mahmood, Giovanni Palladini, Vaishali Sanchorawala, Ashutosh Wechalekar. Haematologica. 2014, 99: 209-221 *(Original article)*

Comparison of free light chain assays: Freelite™ and N Latex in diagnosis, monitoring and predicting survival in light chain amyloidosis. Shameem Mahmood, Nancy L Wassef, Simon J Salter, Sajitha Sachchithanantham, T Lane, D Foard, Carol J Whelan, Helen J Lachmann, Julian D Gilmore, Philip N Hawkins, Ashutosh D Wechalekar, American Journal of Clinical Pathology, 2016;146(1):78-85. *(Original article).*

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Natural history and outcomes in localised immunoglobulin light-chain amyloidosis: a long-term observational study. Shameem Mahmood, Frank Bridoux, Christopher P Venner, Sajitha Sachchithanantham, Janet A Gilbertson, Dorota Rowczenio, Thomas Wagner, Rabya Sayed, Ketna Patel, Marianna Fontana, Carol J Whelan, Helen J Lachmann, Philip N Hawkins, Julian D Gillmore, Ashutosh D Wechalekar. Lancet Haematology. 2015; 2(6):e241-50. *(Original article)*

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Publications in process

High von Willebrand factor and factor VIII levels as a novel marker of prognosis and light chain induced endothelial dysfunction in systemic AL amyloidosis. Shameem Mahmood, Anne Riddell, Sajitha Sachchithanantham, Carol J Whelan, Helen J Lachmann, Julian D Gillmore, Philip N Hawkins, Pratima Chowdary, Keith Gomez, Ashutosh D Wechalekar. Haematologica, submitted August 2016 *(Original article)*

Oral presentations

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Localised amyloidosis. 6th UK Amyloidosis Network Workshop. London. 2014.

Sleep Apnoea – a newly identified problem in AL. 7th UK Amyloidosis Network Workshop. London. March 2015.

Bleeding diathesis and prothrombotic tendencies in newly diagnosed systemic light chain amyloidosis: important clinical implications in management. Bursary winner for 15th IMW 2015. Presentation at UK Myeloma Autumn Day Nov 2015.

Contents

Abbreviations

Systemic amyloid A amyloidosis	AA
Hereditary apolipoprotein AI amyloidosis	AApoAI
Hereditary apolipoprotein AII amyloidosis	AApoAII
Angiotensin converting enzyme	ACE
A disintegrin-like and metalloprotease with Thrombospondin type 1 repeats	ADAMTS13
Atrial fibrillation	AF
Hereditary fibrinogen A α-chain amyloidosis	AFib
Antigen	Ag
Gelsolin amyloidosis	AGel
Light chain amyloidosis	AL
Hereditary lysozyme amyloidosis	ALys
Alkaline phosphatase	ALP
Activated prothrombin complex concentrates	aPCC
Activated partial thromboplastin time	APTT
Assisted servo ventilation	ASV
Autologous stem cell transplantation	ASCT
Hereditary systemic transthyretin amyloidosis	ATTRm
Senile systemic amyloidosis	ATTRwt
Bence Jones proteins	BJP
Body mass index	BMI
Blood pressure	BP
Bodily pain	BP*

β-2 microglobulin	β2M
Collagen binding	CB
Confidence interval	CI
Chronic kidney disease	CKD
Cardiac magnetic resonance imaging	CMR
Chronic obstructive pulmonary disease	COPD
R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl] pyrrolidine-2-carboxylic acid	CPHPC
Cerebrovascular accident	CVA
Complete clonal response	CR
C-reactive protein	CRP
Central sleep apnoea	CSA
Difference between involved and uninvolved free light chains	dFLC
Deoxyribonucleic acid	DNA
Dialysis related amyloidosis	DRA
Electrocardiogram	ECG
Eastern Co-operative Group	ECOG
Ethylenediaminetetraacetic acid	EDTA
Event free survival	EFS
Estimated glomerular filtration rate	eGFR
End stage renal failure	ESRF
Epworth Sleepiness Score	ESS
Familial amyloid polyneuropathy	FAP
F18-flurodeoxyglucose positron emission tomography	FDG-PET/CT

Fresh frozen plasma	FFP
Free light chain	FLC
Familial Mediterranean fever	FMF
Factor VIII	FVIII
Factor X	FX
Food and Drug Administration	FDA
Glycosaminoglycans	GAGs
Gamma-glutamyl transpeptidase	GGT
Gastro-intestinal	GI
General health	GH
Haemoglobin	Hb
High density lipoprotein	HDL
Heart rate	HR
Intercellular adhesion molecule	I-CAM
Implantable cardioverter-defibrillator	ICD
Interleukin-1	IL-1
Interleukin-6	IL-6
Inter-quartile range	IQR
Interventricular septal thickness in diastole	IVSd
Laser capture micro dissection and mass spectrometry	LDMS
Late gadolinium enhancement	LGE
Monoclonal gammopathy of undetermined significance	MGUS
Mental health	MH
Major histocompatibility complex	MHC
Medicines and Healthcare Products Regulatory Agency	MHRA
Myocardial infarction	MI

^{123}I-MIBG – Metaiodobenzylguanidine	MIBG
Number	n
UK National Amyloidosis Centre	NAC
National Health Service	NHS
Nitric oxide	NO
No response	NR
N terminal pro brain natriuretic peptide	NT-proBNP
New York Heart Association Classification	NYHA
Oxygen desaturation index	ODI
Office of National Statistics	ONS
Orthotopic liver transplantation	OLT
Overall survival	OS
Obstructive sleep apnoea	OSA
Polymerase chain reaction	PCR
Positron emission tomography	PET
Physical functioning	PF
Progression free survival	PFS
Partial response	PR
Patient-reported outcomes	PRO
Prothrombin time	PT
Quality of life	QoL
Rheumatoid arthritis	RA
Role emotional	RE
Role physical	RP

Serum amyloid A protein	SAA
Serum amyloid P component	SAP
Sleep disordered breathing	SDB
Social functioning	SF
Strategic Health Authority	SHA
Small interfering RNAs	siRNAs
Tricuspid annular pulmonary systolic excursion	TAPSE
99mTc-3, 3-diphosphono-1, 2-propanodicarboxylic acid	Tc-DPD
Transient overexpression of transcription factor EB	TFEB
Treatment related mortality	TRM
Thyroid stimulating hormone	TSH
Thrombin time	TT
Transthyretin	TTR
University College London	UCL
United Network for Organ Sharing	UNOS
Visual analogue scale	VAS
Very good partial response	vGPR
Vitality	VT
Vascular cell adhesion molecule	V-CAM
Ventricular tachycardia	VT
Von Willebrand factor	vWF

Figures

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Tables

Chapter One: Introduction

This chapter is written in the context of my publication: Update on treatment of light chain amyloidosis. Shameem Mahmood, Giovanni Palladini, Vaishali Sanchorawala, Ashutosh Wechalekar. Haematologica. 2014, 99: 209-221, copyright permission obtained from Haematologica office for use in my thesis.

Amyloidosis is a rare systemic disorder characterised by misfolding of aberrant precursor proteins causing formation of unstable auto aggregates leading to amyloid fibril formation in a predominant β-pleated sheet structure.[5] These fibrils are deposited in different organs, progressively affecting the organ's architecture and function.[6] The unstable protein may be hereditary or acquired, with 25 different proteins to form amyloid fibrils,[7] The most common organs involved include the heart, kidneys, liver, gastrointestinal tract, autonomic and peripheral nervous system. Amyloidosis varies in its disease phenotype with a clear differentiation between a localised deposit of amyloid such as in localised amyloidosis and systemic amyloidosis, the latter depending on the underlying fibril type, organ involved and extent of amyloid deposition.

Amyloid proteins and fibrillogenesis

Early studies show that a vital part of the process of protein fibrillogenesis is partial unfolding of the protein.[8] Accelerated fibrillation in protein deposition diseases are associated with certain mutations, and allow destabilisation of the native structure, resulting in an increased quantity of steady-state concentration of partially folded conformers. Transthyretin in solution has been shown to undergo dissociation to a monomer influenced by the temperature, pH, ionic strength and protein concentration. Amyloid fibril formation related to some TTR variants may be triggered by tetramer dissociation to a compact non-native monomer with low conformational stability.[9] The structure of the monomer may form a partially unfolded monomeric structure and soluble aggregates. Variable domains have been implicated in systemic AL amyloidosis, with initial hypothesis suggesting that proteolysis occurred. Light chains are susceptible to aggregation due to a number of factors: (1) certain somatic mutations are thought to de-stabilise the protein and hence favour the formation of amyloid fibrils, (2) reduced thermodynamic stability and (3) the cellular environment. Structural experiments have shown that most variable domains from AL amyloidosis patients form crystals as monomers or dimers with the beta-pleated sheet. Oxidative stress is also important, having been associated with amyloid fibril deposits and cell death.[10] Internalisation studies have shown that immunoglobulin light chains initially internalise into the cardiomyocytes by migrating into the lysosomal compartments. Little is known as to the underlying reason why certain organs are affected with these dynamic amyloid deposits resulting in impairment of the affected organ,[11] even with slight variations within family members affected by the same hereditary variant protein. Previous experiments have shown that development of systemic AA amyloidosis occurs when a mouse is injected with protein from an AA amyloidosis mouse with an inflammatory precipitant.[12] This raises the hypothesis which proposes that once amyloid fibrils deposit in

a tissue, a particular template or backbone is formed. The ongoing deposition of further precursor proteins allowing further amyloid deposits to be laid down within this template.

Regression of amyloid deposits is less well understood, with earlier studies showing that this process is partly macrophage driven. Macrophage experiments in murine models have shown the complete degradation of Aβ amyloid fibrils in vitro.[13, 14] It has been postulated that these macrophages infiltrating the amyloid deposits consequently result in the formation of multinucleated giant cells, encircling the amyloid prior to engulfing the amyloid deposit. Initial studies have explored this concept proposing that the clearance of amyloid may be antibody mediated,[15] with antibody-amyloid specific administration successful in reducing the amyloid load in those diagnosed with Alzheimer's disease[16] or systemic AL.[17] One method of translating this concept visually is by use of I^{123}SAP scintigraphy, whereby the amyloid load may be assessed. It is variable as to which patients achieve amyloid regression by this technique independent of the clonal or inflammatory suppression attained – with clearly many factors which contribute to this phenomenon.

Epidemiology

Amyloidosis is a very rare condition, with the initial incidence based on deaths/post-mortem information, initially quoted as 4.5/1000000[18] and an estimated 500 new cases seen at the National Amyloidosis Centre each year. The subtle symptoms often masked as other medical conditions are likely to underestimate this condition. Multiple myeloma patients may have incidental amyloid deposits with no organ dysfunction in 38% of cases,[19] with organ dysfunction present in 3-7% of patients.[20] The incidence of systemic AA amyloidosis typically depends on the underlying inflammatory condition, with the prevalence increasing from 18% to 30% at post-mortem.[21] The incidence of hereditary amyloidosis varies greatly between countries and remains little studied. Familial Amyloid Polyneuropathy varies between countries with different mutation variants prevalent in different locations, with the

most common FAP variant in the UK and Ireland the T60A variant.[22] Earlier studies have estimated the V122I variant present in 3-4% of the Afro-Caribbean population, clinically similar to the wild type variant ATTRwt.[23, 24]

Types of amyloidosis

Localised amyloidosis

Localised AL amyloidosis is characterised by amyloid deposits at a single site (commonly: bladder, skin, larynx, lung) due to local production of light chains and no evidence of systemic involvement. It has excellent prognosis with generally no need for systemic therapy.[25] We will elaborate more on this type of amyloidosis in chapter 7.

Systemic amyloidosis

This group comprises of systemic light chain (AL) amyloidosis, systemic amyloid A (AA) amyloidosis, senile systemic amyloidosis (ATTRwt) and dialysis related amyloidosis (DRA). Systemic light chain (AL) amyloidosis is the most common type; in which the amyloidogenic protein is a monoclonal light chain secreted by a underlying clonal plasma cell (or rarely B lymphoid) dyscrasia.[6] Other hereditary amyloidoses are due to amyloidogenic mutations in fibrinogen, Apolipoprotein A1 and A2, lysozyme and Gelsolin genes. AA amyloidosis occurs due to deposition of serum amyloid A protein (an acute phase protein) in a spectrum of disorders causing prolonged inflammation and treatment focuses upon reducing that inflammatory drive. Table 1.1 illustrates the common types of systemic amyloidosis.[26]

Table 1.1: Common types of systemic amyloidosis[27]

Type	*Abbreviation*	*Precursor Protein*	*Site of Synthesis*	*Clinical Symptoms (in order of frequency of organ involvement)*	*Specific Treatment*
Immunoglobulin Light Chain Amyloidosis	AL	Monoclonal Light chain	Bone marrow plasma cells or B cell clone	Renal, cardiac, PNS/ANS, GI, soft tissue	Chemotherapy, ASCT, organ transplant
Senile Systemic Amyloidosis	SSA (ATTR – wild Type)	Wild type transthyretin	Liver	Cardiac, carpal tunnel syndrome	Supportive (optimal CHF control), Doxycycline*, Diflunisal*
Hereditary transthyretin Amyloidosis	ATTR - mutation	Greater than 100 variant mutations	Liver	PNS/ANS, cardiac, vitreous Involvement, leptomeninges	Liver transplantation (V30M mutation), supportive (cardiac and symptomatic PNS/ANS) Diflunisal*, Tafamidis*
Systemic AA	SAA	Serum amyloid A	Liver	Renal, GI, liver	Suppression of Inflammatory disorder Eprodisate*
Fibrinogen Amyloidosis	AFib	Fibrinogen α chain	Liver	Renal, liver	Renal replacement Therapy, renal (&/or liver) transplant
Apolipoprotein A1	AApoA1	Apolipoprotein A1	Liver, intestine	Renal, liver, cardiac, larynx	organ transplantation Supportive.

AL, light chain amyloidosis; SSA, senile systemic amyloidosis, ATTR, amyloidogenic transthyretin mutations; SAA, systemic amyloidosis A, AFib, fibrinogen amyloidosis; AApoA1, Apolipoprotein A1, PNS, peripheral nervous system; ANS, autonomic nervous system; GI, gastro-intestinal; ASCT, autologous stem cell transplant; CHF, congestive heart failure * denotes treatments currently in clinical trials

Systemic AA amyloidosis

The amyloid fibrils in systemic AA amyloidosis arise from serum amyloid A protein in the context of an underlying inflammatory condition. The most common inflammatory arthropathies account for 50% in the Western world, whereas in the developing world the majority of cases are secondary to infection. Hereditary periodic fever syndromes are associated with increased inflammatory stimulus and hence the risk of systemic AA amyloidosis. Table 1.2 describes the majority of inflammatory conditions.[28] There are many contributing factors which remain unexplained as to why AA amyloidosis and the production of variable levels of SAA occur in this disease, with genetic polymorphisms possibly contributing to this phenomenon.[29]

We recognise that systemic AA amyloidosis typically affects the kidneys in over 95% of patients, clinically presenting with proteinuria in approximately 10% of patients in end stage renal failure (ESRF) at diagnosis. Other organs involved can include the spleen (often seen by ^{123}I SAP scintigraphy, adrenal glands, liver and gastrointestinal involvement, with less dysfunction of these organs.[28] Approximately one third of patients will progress to ESRF with the risk of renal decline very much dependent on the underlying inflammatory disorder and treatment options, in recent years including newer biological agents. Renal transplantation has been performed in selected patients with excellent outcomes.[30]

Table 1.2: Conditions associated with underlying systemic AA amyloidosis[28]

Inflammatory Arthritis Adult Still's Disease Ankylosing Spondylitis Juvenile Idiopathic Arthritis Psoriatic Arthropathies Reiter's Syndrome Rheumatoid Arthritis Gout Chronic Infections Bronchiectasis Chronic Cutaneous Ulcers Chronic Pyelonephritis Leprosy Osteomyelitis Q Fever Sub-acute Bacterial Endocarditis Tuberculosis Whipples Disease Immunodeficiency States Common Variable Immunodeficiency Cyclic Neutropenia Hyperimmunoglobulin M Syndrome Hypogammaglobulinaemia HIV/Aids Other Conditions Predisposing To Chronic Infections Cystic Fibrosis Epidermolysis Bullosa Injected Drug Abuse Jejuno-Ileal Bypass Kartagener's Syndrome Paraplegia Sickle Cell Anaemia	Hereditary Periodic Fevers Cryopyrin associated periodic fever syndrome (CAPS) Familial Mediterranean fever (FMF) Mevalonate Kinase Deficiency (MKD or HIDS) TNF receptor associated periodic syndrome (TRAPS) Inflammatory Bowel Disease Crohn's disease Ulcerative colitis Neoplasia Adenocarcinoma of the lung, gut, urogenital tract Basal cell carcinoma Carcinoid tumour Castleman's disease Gastrointestinal stromal tumour Hairy cell leukaemia Hepatic adenoma Hodgkin's disease Mesothelioma Renal cell carcinoma Sarcoma Systemic Vasculitis Behcet's disease Giant cell arteritis Polyarteritis nodosa Polymyalgia rheumatic Systemic lupus erythematosis Takayasu's arteritis Additional Atrial myxoma Inflammatory abdominal aortic aneurysm Retroperitoneal fibrosis Sarcoidosis Sinus histiocytosis with massive lymphadenopathy

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Dialysis related amyloidosis

This condition may arise secondary to long term dialysis with the underlying fibril β-microglobulin (β_2M). This molecule is typically filtered via the kidneys, specifically the glomerulus and resorbed by the proximal tubular cells.[31, 32] The majority of patients present clinically following a period of 10 years on dialysis with symptoms of carpal tunnel syndrome, spondyloarthropathies, arthralgia, subchondral bone cysts and fractures. The incidence of DRA is lower with higher flux dialysis membranes with renal transplantation the only means of reducing the former clinical symptoms.[33]

Wild type transthyretin amyloidosis, senile systemic amyloidosis

Amyloidosis caused by deposition of misfolded transthyretin (ATTR) is the next most common, either hereditary (due to amyloidogenic ATTR mutations) or a disease of aging; due to wild type ATTR deposition (senile systemic amyloidosis), with the fibril wild type transthyretin.[34] The latter is typically seen in older men, with the majority describing previous Carpal Tunnel symptoms. Amyloid deposits with this fibril type can occur in the heart, and found in 25% of autopsy findings in those older than 80 years of age,[35] with deposits also found in other tissues including the bladder, gastro-intestinal tract and soft tissue.[36] Cardiac manifestations of this disease are typically demonstrated by echocardiography and cardiac MRI and tissue proof the ultimate test with wild type transthyretin gene sequencing. There is limited data as to the natural progression of this disease, typically slower with the mainstay of treatment centred on optimisation of heart failure treatment.

Hereditary Transthyretin amyloidosis

Hereditary Transthyretin amyloidosis accounts for the majority of hereditary amyloidosis with 100 mutations; with clinical presentation with progressive autonomic or peripheral neuropathy. Cardiac involvement is present in the majority of patients, with other tissue involvement including vitreous, gastrointestinal and less frequently the central nervous system usually in the third decade; with variability due to the mutation involved.[37-39]

Transthyretin is produced in the liver in 95% of patients, with the rest produced in the choroid plexus and retina.[40] The most prevalent transthyretin mutation involves the substitution of methionine for valine at position 30 (ATTRV30M). Clinical presentation is usually with an ascending sensorimotor peripheral neuropathy, with cardiac involvement rare. The age of onset occurs by the age of 30-40 years in the Portuguese, and presents approximately 20 years later in the Swedish one. Whilst the most common variant in the UK and Ireland is T60A; clinical presentation including autonomic neuropathy and cardiac involvement by the age of 50 years.[22, 41] Another transthyretin variant present in the Afro-Caribbean population (3-4%) is V122I variant with clinical cardiac disease after the age of 60 years.[24] Evaluation of family members at risk is also an issue, with genetic counselling important.

Orthotopic liver transplantation (OLT) was used initially in the 1990s with the premise that the mutant TTR is produced in the liver.[41, 42] Symptoms of peripheral neuropathy improved when chosen earlier in the time course of the disease, with criteria such as age less than 60 years, limited polyneuropathy or autonomic neuropathy, no significant renal or cardiac dysfunction important.[43] However the mortality for this procedure is high.[41, 44] There are newer anti-amyloid therapies, targeting stabilisation of the soluble TTR in the blood and inhibiting its production through silencing RNA and anti-sense oligonucleotide approaches.

Hereditary Aα-chain fibrinogen amyloidosis (AFib)

Hereditary fibrinogen amyloidosis accounts for the most common cause of renal amyloidosis first described in 1993, with a variable penetrance and no family history described.[45] There are 9 reported variants with E526V the most common.[46] Clinical presentation is with proteinuria, hypertension and renal impairment at the age of 60 years and ESRF usually within 5 years from diagnosis. The renal biopsy shows a characteristic abundant glomerular amyloid infiltration with almost complete obliteration of the normal architecture but little or no vascular or interstitial deposits.[47]

Combined renal and liver transplantation has been a treatment option for a selected cohort of patients, given the amyloidogenic protein is produced exclusively by the liver. The long term outcomes have been excellent but this procedure carries a high mortality risk.[48] Renal transplantation is another option with the median graft survival 7 years with recurrent amyloid deposits typically the cause for further renal decline.[47]

Hereditary apolipoprotein A1 amyloidosis (AApoA1)

Apolipoprotein A1 is an HDL important in the role of cholesterol transport which is produced in the liver (50%)[49, 50] and intestines, with the liver and kidneys being major sites for Apo A1 catabolism. Thirteen variants have been reported, with the underlying pathogenesis involving proteolytic cleavage at amino terminal 83-93 residues incorporated into the amyloid fibrils.[51] Each variant is associated with varying phenotypes and again differences which occur within the family. The main organ involved is the kidney, and consequently chronic renal failure, with neurological, cardiac, and hepatic dysfunction also reported. The phenotype of the following six variants: Gly26Arg, Trp50Arg, Leu60Arg, Del70-72, Leu75Pro and Leu64Pro involve renal involvement with hypertension and proteinuria with the clinical presentation of hepatosplenomegaly.[52] Other AApoAI variants (Leu90Pro, Arg173Pro, Leu174Ser and Leu178His) have been reported with skin and cardiac amyloid deposits with

death usually occurring due to progressive cardiomyopathy within 10 years of diagnosis.[53, 54] The exact underlying aetiology of this disease remains uncertain, with the hypothesis of mutations destabilising the native structure and facilitating fibrillogenesis and consequent proteolysis.[55, 56]Renal transplantation has offered one therapeutic option with renal graft survival exceeding 10-15 years irrespective of recurrent amyloid in the transplanted organ.[57] Reduction in the variant ApoA1 by 50%, can be sufficient to facilitate extra-hepatic amyloid regression in a certain cohort of patients, such as in liver transplantation.[58]

Apolipoprotein AII amyloidosis (AApoAII)

Apolipoprotein AII is an HDL apolipoprotein with Apoliprotein AII amyloidosis described initially in 1973 by Weiss and Page,[59] with 4 amyloidogenic variants reported to date, but less well elucidated.[60] Patients typically present with proteinuria and progressive renal decline.

Hereditary Gelsolin amyloidosis (AGel)

The amyloid fibrils occur due to cleavage fragments of the variant Gelsolin initially described by the Finnish ophthalmologist Jouko Meretoja in 1969.[61] There are 2 main variants: G654A described in Portugal, Japan and Iran, with G654T reported in countries including France, Czech Republic and Denmark.[62] Gelsolin is an actin-modulating protein facilitating the migration into other cells, with the mutated form unable to bind to calcium and hence susceptible to proteolysis and fibril formation.[63-65] Patients typically present with corneal lattice dystrophy during middle age with slowly progressive cranial neuropathies. The clinical phenotype can vary greatly from a slight sensory neuropathy to severe ataxia, from mild visual impairment to total blindness.[66] Interestingly renal amyloid deposits are seen by ^{123}I SAP scintigraphy with no corresponding renal functional decline.

Lysozyme amyloidosis (ALys)

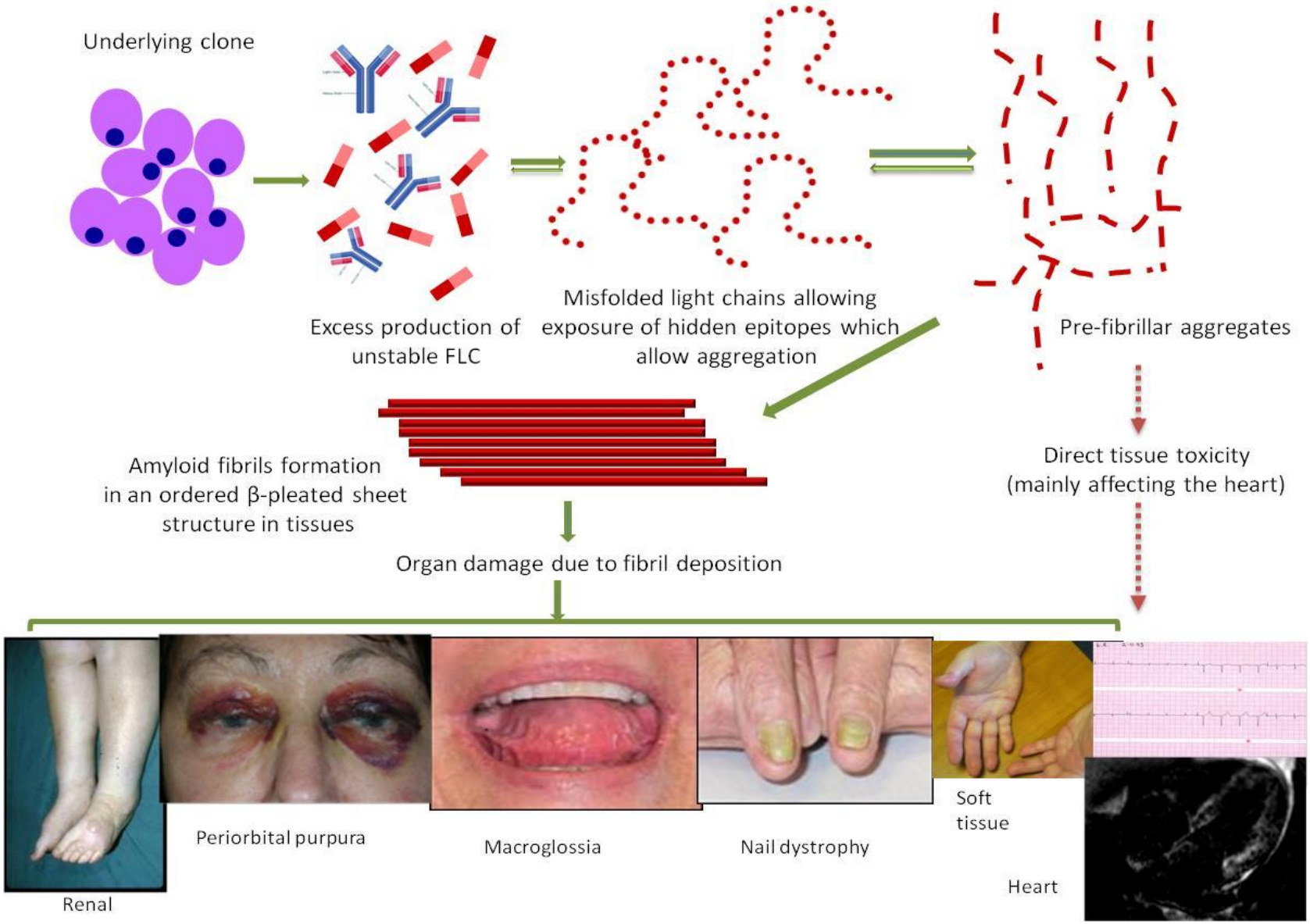
Lysozyme is a ubiquitous bacteriolytic enzyme typically found in high quantities in the liver, articular surfaces, saliva and tears and expressed in granulocytes, monocytes and bone marrow precursor cells. It is typically inherited in an autosomal dominant fashion. Pepys et al first described Lysozyme amyloidosis,[67] with seven amyloidogenic mutations described including: Ile56Thr, Phe57Ile, Trp64Arg, Asp67His, Trp112Arg, Tyr54Asn and D67G with patients presenting in their 3rd and 4th decade. Previous studies show that fibril formation by human wild type lysozyme was accelerated by fibrils of the variant proteins, with wild type lysozyme deposits significantly much lower in concentration compared to amyloidogenic variants.[67, 68] Patients clinically present with a slowly progressive decline in renal function with organ involvement also involving liver, spleen, gastrointestinal tract and lymph node involvement. Interestingly certain variants including Try64Arg and Asp67His and lung and thyroid tissue involvement with the Ile56Thr variant describe Sicca syndrome due to salivary gland amyloid deposits.[69]

Systemic light chain (AL) amyloidosis

The presenting symptoms of AL amyloidosis have a wide spectrum: dyspnoea, lethargy, weight loss, bleeding tendency, swelling of lower limbs, frothy urine, orthostatic hypotension or peripheral neuropathy. Macroglossia and peri-orbital bruising are almost pathognomonic, occurring only in a third of all cases (Figure 1.1). The diagnosis of AL amyloidosis is often delayed as presenting features are subtle or mimic other more common conditions. Table 1.1 shows the most common other types of systemic amyloidosis.

Figure 1.1: Pathogenesis and presentation of AL amyloidosis: Direct deposition of amyloid fibrils lead to the typical clinical features depicted: peri-orbital bruising; macroglossia with indentation of teeth marks of the tongue; nail dystrophy; lower limb oedema with nephrotic syndrome; soft tissue infiltration of hands bilaterally; ECG showing small QRS complexes and late gadolinium enhancement of cardiac MRI. The pre-fibrillar light chain aggregates (and possibly the misfolded light chains) can have direct tissue toxicity. Cardiac toxicity of light chains appears to be a significant contributor to myocardial dysfunction seen in AL amyloidosis. This may also be the reason for rapid improvement in NT-proBNP which parallels a haematological response to therapy often without any evidence of structural cardiac improvement but correlating with clinical improvement in the patients' cardiac symptoms.[27]

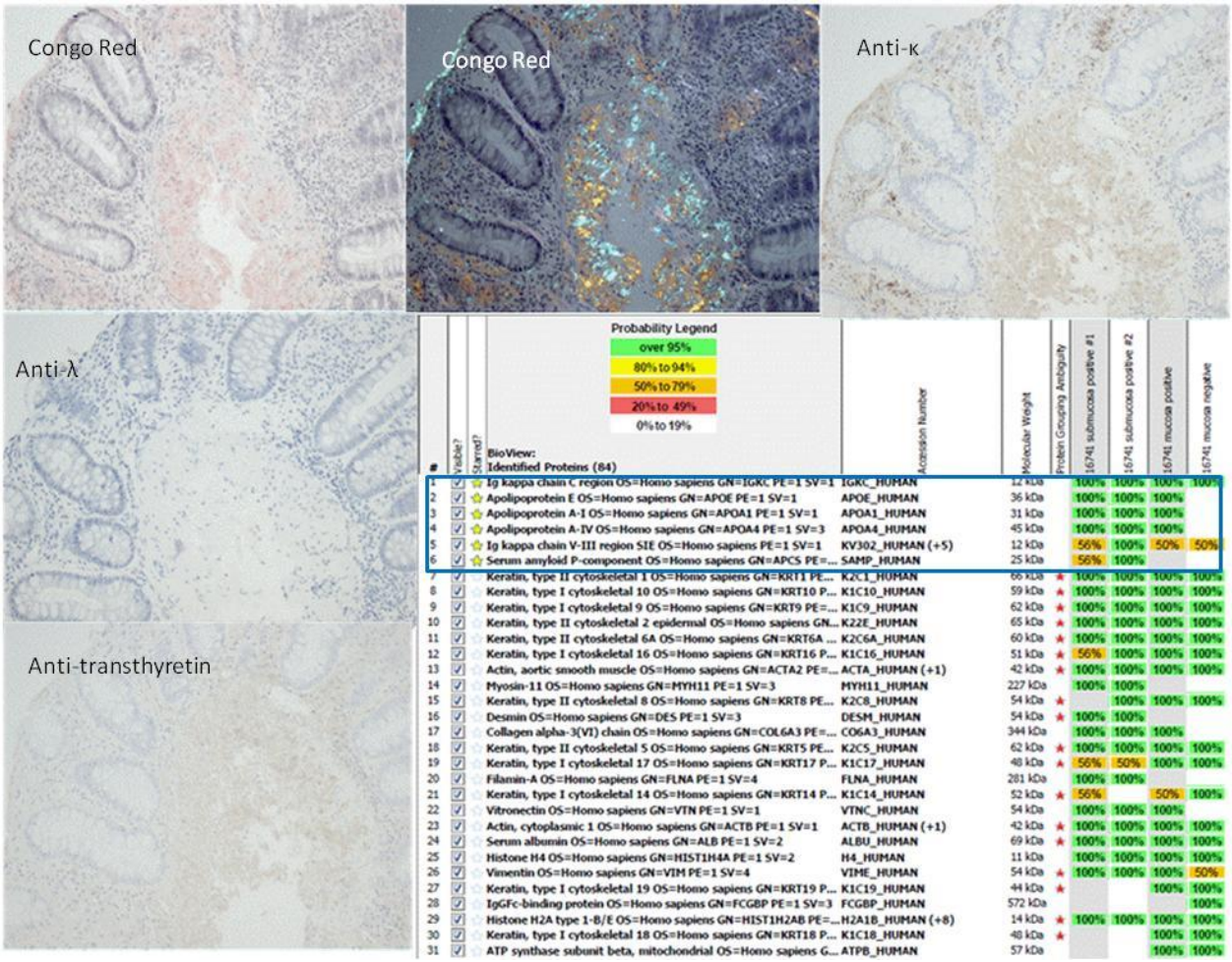
Figure 1.1


Underlying clone
Excess production of unstable FLC
Misfolded light chains allowing exposure of hidden epitopes which allow aggregation
Pre-fibrillar aggregates
Amyloid fibrils formation in an ordered β-pleated sheet structure in tissues
Direct tissue toxicity (mainly affecting the heart)
Organ damage due to fibril deposition
Renal
Periorbital purpura
Macroglossia
Nail dystrophy
Soft tissue
Heart

Advanced organ dysfunction has often ensued prior to a clinical diagnosis of amyloidosis although monoclonal gammopathy (MGUS)[70] or myeloma usually predates a diagnosis of amyloidosis. Fifteen percent of patients with myeloma have symptomatic AL amyloidosis and up to 30% may have "incidental" deposits, which may become clinically significant with improving long term outcomes in myeloma.[71] Patients with MGUS and an abnormally elevated free light chain (FLC) should be additionally monitored at each visit by measurement of serum brain natriuretic peptide (BNP or its N-terminal fragment, NT-proBNP) and urine for albuminuria – abnormal presence of either may herald development of amyloidosis[5] before advanced, symptomatic organ damage thus significantly reducing the early deaths which are still observed.

Confirmation of diagnosis needs demonstration of amyloid deposition; pathognomonic apple green birefringence by Congo red staining using crossed polarised light on histological tissue sections of either the affected organ, bone marrow, rectum or abdominal fat aspirate (latter being an easy bedside procedure available for all patients including those with haemostatic impairment).[72] Fibril typing is critical in deciding appropriate therapy and performed by immunohistochemistry (widely available but specific only in 75-80% of cases of AL),[73] immuno-electron microscopy (highly specific but limited availability)[74] or lately, mass spectrometry of amyloid deposits obtained by laser capture (rapidly becoming an invaluable adjunct)[75] (Figure 1.2). Detecting the underlying clone requires serum and urine electrophoresis and immunofixation, serum free light chain analysis, bone marrow examination and imaging for presence of myeloma related bone disease.

Figure 1.2: Confirming the diagnosis and fibril typing in a patient with AL amyloidosis due to underlying kappa light chain secreting plasma cell dyscrasia. Congo red staining demonstrates characteristic staining and apple green birefringence under cross polarised light. Immunostaining with antibodies to kappa light chains is positive and there is no staining with antibodies to lambda or transthyretin (or SAA (not shown). Proteomic analysis of the amyloidotic tissue shows presence of kappa light chains in addition to other proteins known to be present in amyloid fibrils (blue box). Also note the presence of keratin which a common contaminant from the operators skin showing the need for meticulous specimen preparation to avoid false positive results.[27]



Baseline assessment of organ function (Table 1.3) is important for prognosis and selection of therapy. Formal testing for autonomic and peripheral neuropathy may be needed in selected cases.

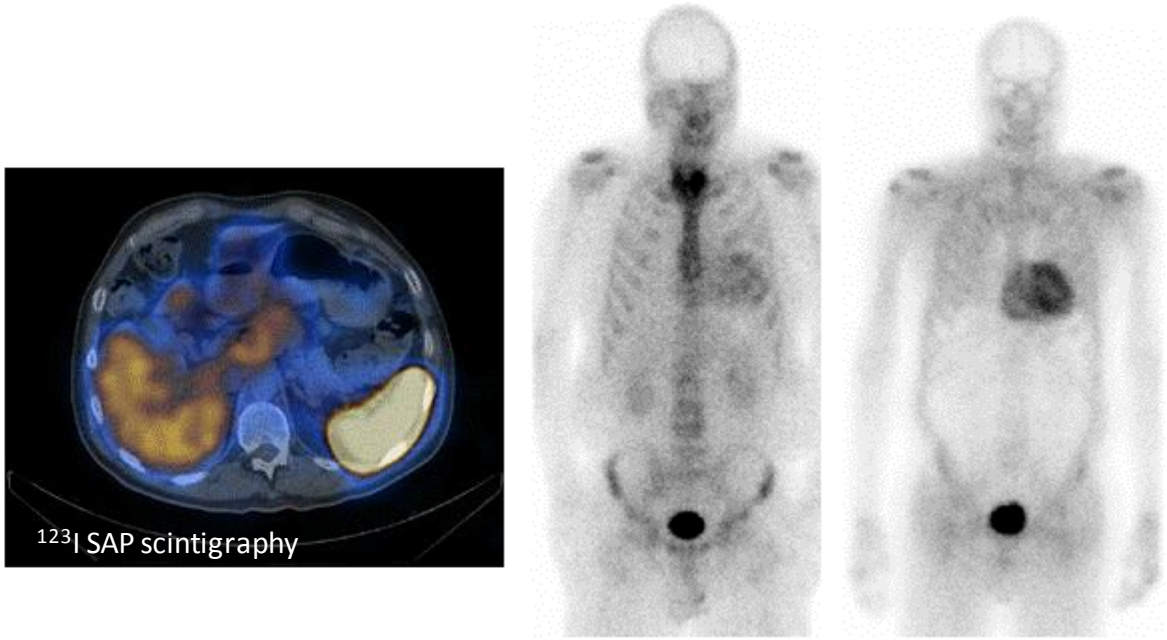
Table 1.3: Diagnostic and Staging Investigations for systemic AL amyloidosis[27]

Tissue diagnosis	Abdominal fat aspirate Salivary gland or rectal biopsy Biopsy of involved organ
Amyloid typing	Immunohistochemistry (immuno-electron microscopy if available) Mass spectrometry DNA analysis (if indicated)
Studies to detect an underlying plasma/B cell Clone	Serum and urine electrophoresis and immunofixation Serum Free light chain measurement Bone marrow aspirate / biopsy (plus FISH) Imaging studies for bone disease
Assessing of organ involvement and staging	Cardiac NT-proBNP (or BNP), cTnT (or hs-cTnT, or cTnI) Echocardiography (plus strain imaging) ECG (plus Holter ECG) Cardiac MRI (if indicated) ^{99m}Tc-DPD scan (if indicated) Renal 24 h urinary protein Serum creatinine (and eGFR) Liver Liver function tests Liver US / CT scan Nerves Nerve conduction studies (if indicated) Autonomic testing Whole body amyloid load ^{123}I labelled SAP scintigraphy (if available)

FISH - fluorescent in situ hybridisation; NT-proBNP - N-terminal prohormone of brain natriuretic peptide; BNP – brain natriuretic peptide; cTnT or cTnI – troponin T or I; hs-cTnt – high sensitivity troponin T; ECG – electrocardiograph; ^{99}mTc-DPD scan - ^{99m}Tc-dicarboxypropane diphosphonate scan; eGFR – estimated glomerular filtration rate; US – ultrasound; CT – computed tomography; MRI – magnetic resonance imaging; SAP – serum amyloid P.

^{123}I labelled serum amyloid P component (SAP) scintigraphy (if available), is useful for diagnosis, quantification and especially valuable in serial monitoring of amyloid deposits (Figure 1.3).[76] Cardiac magnetic resonance imaging (CMR) is more sensitive and specific than echocardiography for diagnosis of cardiac amyloidosis, showing a characteristic subendocardial late gadolinium enhancement. A cardiac biopsy maybe needed in a select cohort of patients with isolated cardiac amyloidosis and MGUS to differentiate AL amyloidosis from senile systemic amyloidosis/ATTR amyloidosis due to V122I mutation. Bone scintigraphy tracers, ^{99m}Tc-dicarboxypropane diphosphonate (^{99m}Tc-DPD) and ^{99m}Tc-pyrophosphate (^{99m}Tc-PYP), are avidly taken up in cardiac ATTR amyloid deposits but not in AL[77,78]– an emerging non-invasive means of differentiating the two conditions (Figure 1.3).

Figure 1.3: Radionuclide imaging in amyloidosis. ^{123}I labelled serum amyloid P component scintigraphy showing uptake in the spleen and liver in a patient with AL amyloidosis (left). The middle panel shows low grade cardiac uptake of ^{99m}Tc-DPD in a patient with AL amyloidosis compared with marked cardiac uptake of ^{99m}Tc-DPD in a patient with wild type transthyretin (senile cardiac) amyloidosis (right panel).[27]



Risk stratification is an essential part of the diagnostic workup – cardiac involvement determines the risk. The Mayo clinic group, using NT-proBNP and troponin T or I (or more recently using high sensitivity troponin[79]) and more recently serum free light chains,[80] defined stages in AL amyloidosis depending on none, one or both being greater than the threshold levels (median survival of 26.4, 10.5 and 3.5 months respectively)[81] – even with newer therapies Mayo stage III disease still has a poor median survival of 7 months.[81] NT-proBNP >8500 ng/L and systolic blood pressure <100mmHg identify a subgroup of stage III patients with a very high risk of early death.[81] Confounding factors, like renal failure, impact

the concentration of NT-proBNP, and measurement of BNP may be preferred in this setting.[82]

Treatment of AL amyloidosis

Goals of therapy

The aim of treatment in AL amyloidosis is eradicating the fibril precursor protein by suppressing production of free light chains as rapidly as possible by targeting the underlying clonal plasma/B cell dyscrasia, whilst minimising treatment related mortality (TRM) and morbidity[5] with supportive measures to preserve organ function. Since patients with AL amyloidosis have a small clonal burden[83] and lack the high risk cytogenetic features seen in myeloma such as t(4:14) or del17p,[84] shorter courses of dose adapted chemotherapy may be adequate to achieve good haematologic responses.[85]

Response assessment has two components: haematologic and organ response – the latter follows the former.[86, 87] dFLC measurement is a key marker to assess clonal disease response.[87] Consensus criteria for haematologic response assessment have recently been published (Table 1.4).[88] A very good partial response (VGPR) (defined as dFLC less than 40mg/L) or better is associated with an OS of 80-90% at 3 years,[88] and is currently considered the minimum goal of therapy.

In addition to standard measures of organ function, cardiac biomarkers such as a reduction in NT-proBNP of 30% and 300 ng/L from baseline following completion of therapy usefully defines a cardiac organ response.[87] Lack of such a decrease in NT-proBNP (in patients with heart involvement) identifies a subgroup of patients achieving less than CR who need a more profound haematologic response. Factors such as worsening renal failure or treatment with immunomodulatory drugs may lead to elevated cardiac biomarkers, confounding response assessment.[89]

Table 1.4: Consensus Haematologic Response in systemic light chain (AL) amyloidosis[87]

Haematologic Response	***Criteria***
Complete Response (CR)	Normal serum free light chain ratio with negative serum and urinary immunofixation
Very good partial response (VGPR)	The difference in the free light chains (dFLC) less than 40mg/L
Partial Response (PR)	A reduction in the dFLC greater than 50%
No response	A less than 50% response in dFLC

CR - Complete response; VGPR – Very good partial response; PR – partial response; dFLC – difference between involved and uninvolved free light chains.

Supportive care

Supportive treatment, aimed at improving or palliating organ function, maintaining quality of life, and prolonging survival whilst anti-plasma cell therapy has time to take effect, has an important impact upon survival and is a fundamental part of an integrated treatment strategy. It requires the coordinated expertise of several specialists familiar with the disease. Patient education with daily weights, judicious diuretic use, low salt diet, salt-poor albumin, cautious angiotensin converting enzyme-inhibitors, use of thigh-high stockings, midodrine for postural hypotension and close multidisciplinary monitoring make lifesaving differences. Diarrhoea, malabsorption and malnutrition may be ameliorated by antimicrobial therapy for bacterial overgrowth, reduced gut motility with opioids (codeine phosphate and loperamide) with addition of Octreotide in non-responsive cases; prokinetic agents for gastro paresis, PEG feeding in those with marked macroglossia impairing swallowing or parental feeding in malabsorption. Amiodarone may have a role in cardiac arrhythmias, a common cause of death in AL. Implantable cardioverter defibrillators (ICD) are increasing considered in patients with life threatening ventricular arrhythmias but there is no definite evidence of survival advantage at present.[78]

Autologous stem cell transplantation

High dose melphalan and autologous peripheral blood stem cell transplantation (ASCT) has been routinely used as treatment for AL amyloidosis dating back to first reports in the mid 1990's at Boston University[90, 91] (Table 1.5). Contrary to the common experience with ASCT in multiple myeloma, the toxicity of the procedure can be higher in AL e.g. a 15% incidence of major complications during stem cell mobilisation and collection, and mortality 2-10%[92] in patients with cardiac or multi-organ involvement. Over the last decade, complications of ASCT in AL patients have been well appreciated and addressed - appropriate patient selection is the key to reduction in morbidity and mortality. A small French randomised controlled trial failed to show a difference in survival with ASCT over conventional chemotherapy with oral melphalan and dexamethasone, with clonal haematologic response rates similar; 67% versus 68% respectively[93] but major limitations included high TRM of 24% in the ASCT arm and small sample size.[94] Dose adapted melphalan strategy, with dose reduction to 100, 140 and 200mg/m^2 depending on renal, cardiac parameters and age, increases a potentially suitable patient population, with lower doses potentially offering a reduced toxicity, but also reduced haematologic responses.[95]

The largest transplant experience in AL amyloidosis comes from the Mayo clinic and Boston University. At Boston University, assessment by a multi-disciplinary team and use of risk stratification is employed to select patients for ASCT. Eligibility criteria include histological proof of amyloidosis, clonal plasma cell dyscrasia, age greater than 18 years, performance status (PS) 0-2 (Southwest Oncology Group or Zubrod), left ventricular ejection fraction >40%, oxygen saturations >95% on air and supine BP > 90mmHg.[5] Of 421 consecutive patients treated with ASCT in this centre from July 1994 to December 2008, 55% received 200mg/m^2 of high-dose melphalan and 45% received modified dose melphalan (100-140mg/m^2). On an intention-to-treat basis, 34% achieved a haematologic CR with an OS of

6.3 years. Of 340 evaluable patients, 43% achieved a CR and 78% achieved organ responses. Comparison of those achieving a CR versus less than CR, the median event free survivals (EFS) and OS were 8.3 and 13.2 years in the former and 2 and 5.9 years in the latter group respectively. The overall TRM was 11.4%, with 5.6% in the latter 5 years, suggesting further refinement to patient selection with added investigations and staging criteria lowered the TRM.[5]

Table 1.5: Chemotherapy regimens and ASCT studies in AL amyloidosis[27]

Chemotherapy / Reference	Number of patients	Haematologic Response % (CR %)	Overall survival (months) or 1-3 year OS (%)
Conventional chemotherapy			
Dex *(Dhodapkar et al [96])*	93	53 (24)	31
Cyclo/Thal/Dex (*Wechalekar et al [85])*	75	74 (21)	41
MDex *(Palladini et al [97, 98])*	46	67 (33)	61
MDex *(Jaccard et al [93])*	50	68 (47)	56.9
Bortezomib containing regimens			
Bortezomib *(Reece et al [99])*	70	OW: 68.8 (37.5) TW: 66.7 (24.2)	OW: 94% (1 yr OS) TW: 84% (1 yr OS)
Bor/Dex*(Kastritis et al [100])*	94	71 (25)	76% (1 yr OS)
Cyclo/Bor/Dex *(Venner et al [101])*	43	81.4 (41.9)	97.7% (2 yr OS)
Cyclo/Bor/Dex *(Mikhael et al [102])*	17	94 (71%)	Not specified
Bor/Mel/Dex – 33 Cyclo/Bor/Dex -17 *(Palladini et al [103])*	50	67 (27) stage I & II 40 (5) stage III	Not reached 58% (1 yr OS projected)
Lenalidomide containing regimens			
Len/Dex *(Sanchorawala et al [104])*	34	67 (29)	Not specified
Len/Dex *(Dispenzieri et al [105])*	23	41	Not specified
Cyclo/Len/Dex *(Palladini et al [106])*	21	62 (5)	36
Cyclo/Len/Dex *(Kastritis et al[89])*	37	55 (8)	41% (2 yr OS)
Cyclo/Len/Dex *(Kumar et al [107])*	35	60 (11)	37.8
Mel/Len/Dex *(Moreau et al [108])*	26	58	80.8% (2 yr OS)
Mel/Len/Dex *(Dinner et al[109])*	25	58 (8)	58% (1yr OS)
Mel/Len/Dex *(Sanchorawala et al [110]*	16	50 (7)	Not reached
Other Regimens			
Bendamustine/Pred *(Palladini et al [111])*	36	47 (3)	65% (3 yr OS)
Pomalidomide/Dex *(Dispenzieri et al [112])*	33	48 (3)	76% (1 yr OS)
ASCT			
ASCT *(Jaccard et al [93])*	50	67 (61)	22.2
ASCT *(Vesole et al [113])*	107	16 (1 yr)	56% (3 yr OS) 30 day TRM 18%
ASCT *(Cibeira et al [5])*	421	34% CR	75.6 100 day TRM 11.4%
ASCT *(Goodman et al [114])*	92	58% CR	63.6 100 day TRM 23%
ASCT *(Venner et al [115])*	88	28% CR	Not reached 100 day TRM 6.8%
ASCT & Thal/Dex consolidation (*Cohen et al [116]*)	45 total 31 TD	21% CR 39% CR (1 yr)	84% (2 yr OS) TRM 4.4%
ASCT & Vel/Dex consolidation (*Landau et al [117]*)	40 total 23 VD	27% CR 58% CR (1 yr)	82% (2 yr OS) 100 day TRM 10%

Mel - melphalan; Pred, prednisolone; Dex - dexamethasone; Cyclo - cyclophosphamide; Bor - Bortezomib; Thal - thalidomide; Len - lenalidomide;; ASCT - autologous stem cell transplantation; TD - thalidomide and dexamethasone consolidation; VD - velcade and dexamethasone consolidation; OS - overall survival; yr, year; CR - complete remission; PR - partial remission; OW – once weekly; TW – twice weekly; TRM – transplant related mortality

A landmark analysis at 1 year in 140 patients with evaluable FLC results, showed a superior OS in those achieving a CR and vGPR versus those achieving a PR and NR (Figure 1.4A).[2] The Mayo Clinic reported a series of 422 patients receiving an ASCT from March 1996 to December 2009. Transplant eligibility included age < 70 years, PS 0-2, troponin T <0.06ng/mL, creatinine clearance >30mls/min (unless on dialysis), New York Heart Association class I-II and less than 2 organ involvement. The focus of this analysis was to examine the TRM before and after January 2006, with TRM pre 2006 as 12% and 7% post-2006.[118] Troponin T >0.06ng/L and NT pro-BNP>5000pg/ml were associated with a high TRM, whilst patients with both markers below the thresholds had a TRM of 1%.[119] Refining patient selection has allowed ASCT to be done safely, but eligibility of patients for ASCT has reduced outside of centres with extensive transplant experience.

First major organ responses in AL were initially reported after ASCT in 2001 – 36% of patients achieved a renal response at 12 months defined by the amyloidosis consensus criteria[5, 92, 95, 118] proving that clonal responses in AL translate into organ responses. The depth of haematologic response strikingly correlated with renal responses. 71% of patients in CR had renal responses compared to 11% with persistence of the plasma cell dyscrasia. Over the last decade, improvements in quality of life, hepatic and cardiac responses have been published. Similar to renal responses, clinical responses in other organ systems are more evident with deeper haematologic responses. Organ responses can take up to 6-12 months or longer to occur. Haematologic CR occurs in just under half of all patients undergoing ASCT; strategies to improve haematologic CR rates following ASCT are an important focus. These include induction therapy prior to ASCT, novel conditioning and consolidation therapy. A current phase II trial to evaluate the role of induction treatment prior to ASCT using Bortezomib/Dex is ongoing at Boston. Among the first 22 patients treated, by intention-to-treat analysis, haematologic responses occurred in 79% (53% CR and 26%

VGPR) of patients (for evaluable patients 67% CR).[120] Tandem cycles of HDM have been shown to improve the proportion of patients who ultimately achieve a haematologic CR, leading to overall CR rate of 67%.[121] A pilot study incorporating Bortezomib with HDM has shown promising results with high haematologic response rates.[120] Consolidation with thalidomide and dexamethasone is too toxic for routine use[116] but Bortezomib and dexamethasone as consolidation give high responses and durable response rates including CR in 58% at 12 months,[117] with larger studies needed.

Combination chemotherapy

Alkylators and steroid based regimens

Alkylating agents have formed the backbone of treatment of AL for over 40 years with melphalan and cyclophosphamide used in many current therapies. The first randomised controlled trials proved the efficacy of melphalan and prednisone[122] in this disease, but novel treatment options make this regimen less attractive given few and slow haematologic responses, poor survival of 18 months and rarer organ responses.[122] Similarly, high dose single agent dexamethasone[96] and vincristine, Adriamycin and dexamethasone (VAD),[123] although effective, have been superseded due to toxicity and ease of administration, respectively, with recent regimens.

Melphalan-Dexamethasone (MDex) regimen, pioneered by the Italian amyloidosis group, is well tolerated and associated with good haematologic and organ response rates of 67% and 33% respectively, a third of patients achieving a complete clonal response. The median PFS and OS are 3.8 and 5.1 years respectively;[98] results confirmed prospectively in subsequent studies (Figure 1.4B).[3, 93] This regimen is usually well tolerated, with 10-15% experiencing severe adverse events, mainly fluid retention and cytopenias. Oral MDex is generally considered as the standard of care for patients outside of clinical trials in a number of countries. However, the role of MDex in patients with advanced cardiac disease is uncertain

where the median survival is 10.5 months[124] and response rates (11% CR and 33% PR in 61 patients with cardiac AL) - another study reporting a median survival of 17 months and 28% mortality in the first three months in patients treated with this same regime.[125] But high early mortality makes this group difficult to treat regardless of the regimen. Intravenous intermediate dose melphalan (25mg/m^2) had a 12% TRM in a UK study with prolonged remission in the responders[126] but a recent prospective trial found this too toxic for routine use.[127]

Reduced toxicity of low dose dexamethasone containing regimens in myeloma,[128] make these appealing in AL amyloidosis. Melphalan and once weekly dexamethasone was associated with lower responses[129] than standard MDex[98] - raising doubts as to this approach in AL where rapid and deep responses are desirable.

Bendamustine, an alkylator with a novel mechanism of action, with prednisone is useful in relapsed/refractory disease and achieved haematologic responses in 47% (n=17) with a survival advantage in responders in a 3 months landmark analysis (p=0.036). Three patients achieved a VGPR or better in a heavily pre-treated patient group with a 3-year OS of 65%.[111] Grade 3 or greater adverse events (seen in 33%), predominantly cytopenias, were manageable. Bendamustine should be explored earlier in the disease course.

Immunomodulatory agents

The efficacy of immunomodulatory (IMiD's) agents in plasma cell dyscrasias opened up another treatment avenue for AL amyloidosis. Thalidomide, the first of these agents, has a limited role in AL amyloidosis as monotherapy due to unacceptable toxicity at higher doses and limited efficacy.[130] The combination of thalidomide and dexamethasone (median thalidomide dose 300mg) was effective and with clonal response rates of 48% (n=31), CR

in 19%, and organ response in 26%. The median time to response was 3.6 months[131] but 60% experienced grade ≥3 toxicity.

In the UK, a risk adapted strategy based on the patient's clinical status allowed use of dose adapted CTD (cyclophosphamide / thalidomide / dexamethasone) with good and rapid haematologic responses[85] seen in 74%, with CR and PR in 21% and 53% respectively; and organ responses in 33%. Recent analysis of a larger cohort of 202 patients confirmed these findings[4] (Figure 1.4C). Prospective analysis suggests that toxicity of CTDa still remains high with 60% experiencing grade 3 or greater toxicity – mainly fluid overload.[132] This regime was the standards of care in the UK but Bortezomib based regimes are increasingly being used.

Figure 1.4: Overall survival in AL amyloidosis stratified by haematological response in patients treated with high dose melphalan and autologous stem cell transplantation in a landmark analysis of 140 patients showing superior OS in those achieving a CR and vGPR (median OS not reached; no significant difference between the groups p=0.13) versus those achieving a PR and NR (median OS 77 and 50 months respectively; with no significant difference; p=0.39).[2] (A); oral melphalan dexamethasone[3] (B); dose adapted cyclophosphamide-thalidomide-dexamethasone in 202 patients with the median OS 42 months; not reached at 60 months in patients achieving a CR; 50 months and 33 months for those achieving a PR and non-responders respectively[4] (C). The survival is best in patients who achieve complete or very good partial response with either treatment modality. Note: These survival curves describe different cohorts of patients with varying selection criteria and are not directly comparable to each other. NR, no response; PR, partial response; VGPR, very good partial response; CR, complete response, dFLC; difference in involved and uninvolved free light chains.[27]

Figure 1.4A

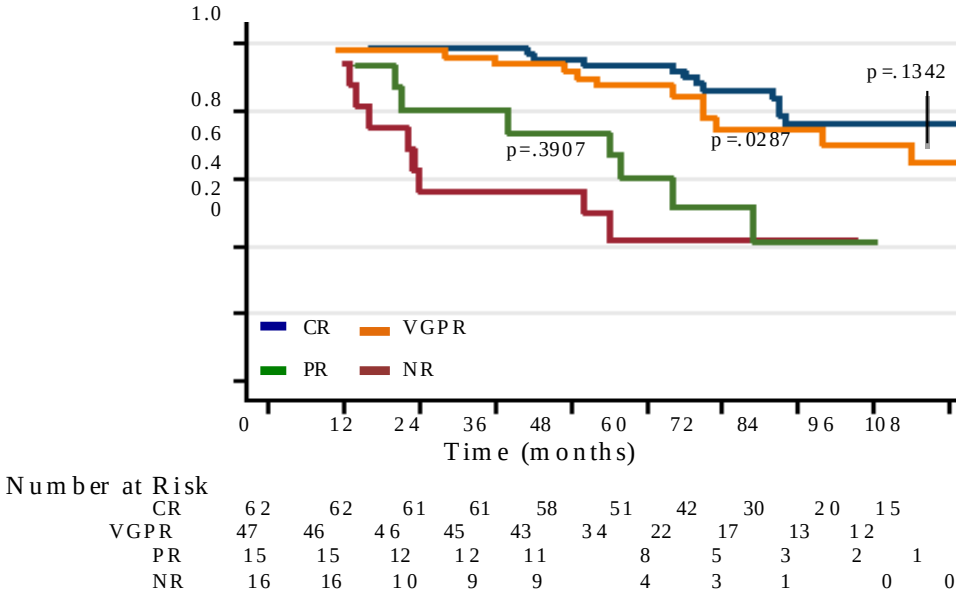


Figure 1.4B

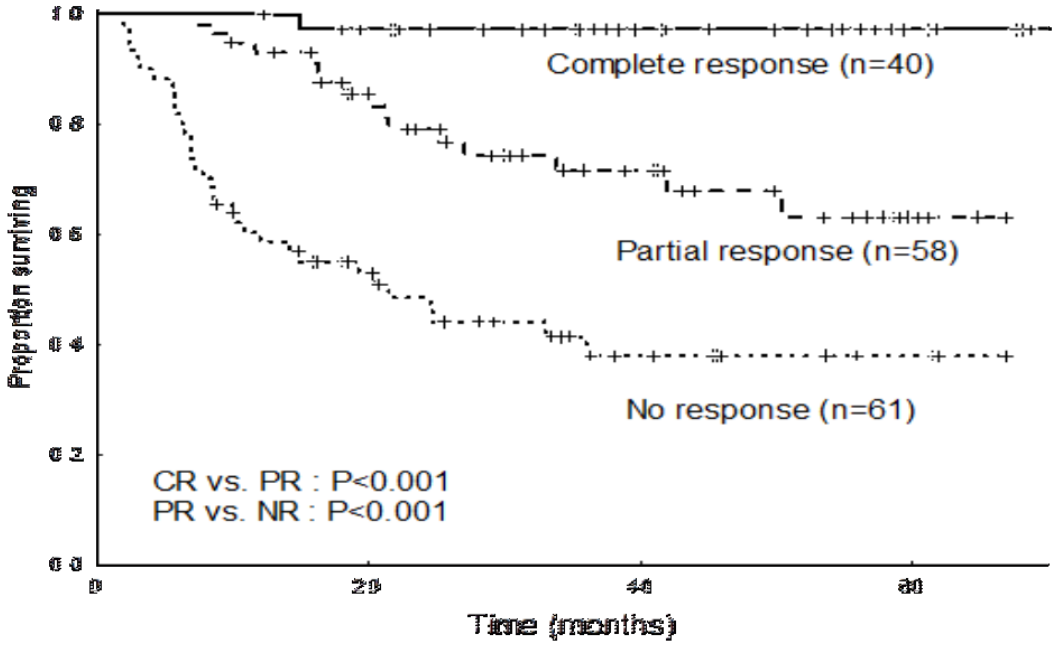
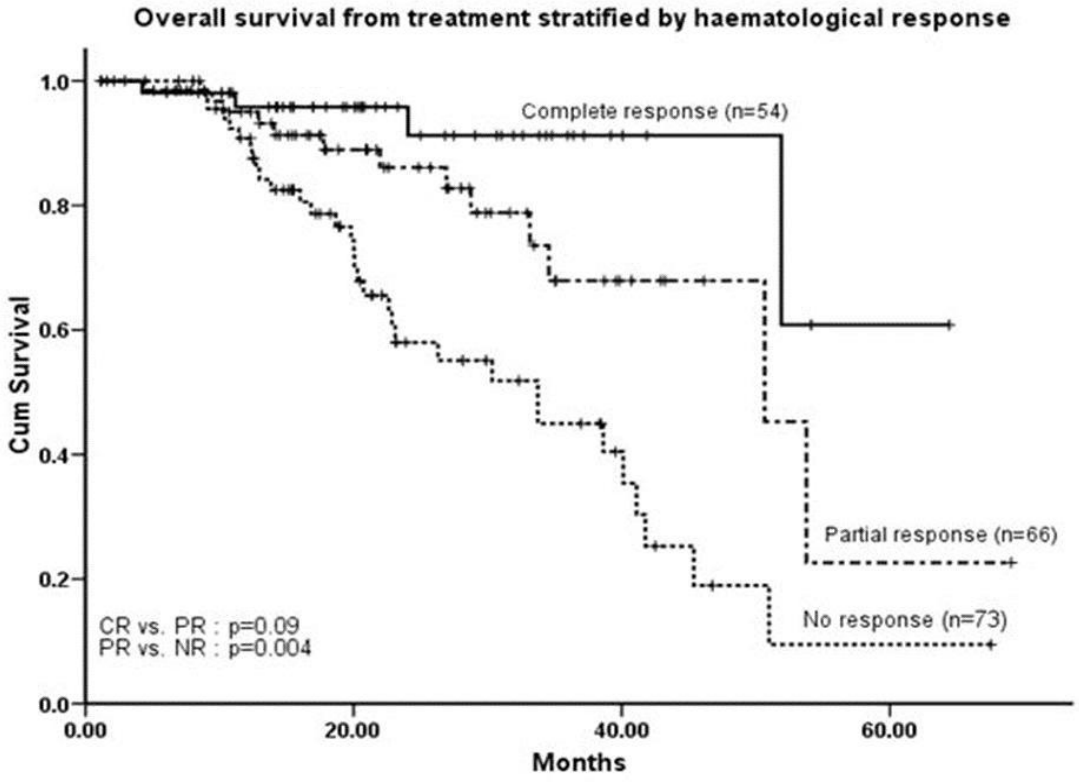


Figure 1.4C



Lenalidomide is a second generation IMiD with greater anti-myeloma efficacy and favourable toxicity profile. Two phase II studies reported that lenalidomide-dexamethasone (Len/Dex) treatment showed good haematological responses in 67% (n=24), with CR and PR in 29% and 38% respectively.[104, 105] Standard lenalidomide doses of 25 mg were poorly tolerated with much better tolerance with 15 mg daily. The most common side effects were fatigue and myelosuppression accounting for 35%, and thromboembolism in 9% (n=3). Skin rashes were experienced in 43%.[133] Lenalidomide combined with dexamethasone has been used in a small cohort of patients refractory to alkylators, Bortezomib and thalidomide, with a 41% response rate and survival advantage in responders, indicating that IMiD's can have a role in salvage treatment,[134] with no increased incidence of secondary malignancies.[135]

Complete response rates remain low with lenalidomide based regimes and a number of phase II studies have focused on addition of an alkylator to improve the response rates.[89, 106-109] Studies with additional cyclophosphamide or melphalan report overall haematological response rates of 55-60% and CRs of approximately 20%. The toxicity of the combination is high with nearly two thirds of patients reporting grade 3 or greater toxicity including haematologic in 46%; other side effects including fatigue, oedema and gastro-intestinal problems. The response rates in advanced stage patients are poor.[89, 109] The exact place of a lenalidomide-alkylator combination and advantages offered over Len/Dex remains to be clearly defined.

Pomalidomide (a third generation IMiD effective in myeloma refractory to lenalidomide) with dexamethasone achieved haematologic responses in 48% of patients with refractory AL[112] with organ responses in 5 patients. The median OS and PFS were 28 months and 14 months respectively. A third of all patients withdrew from the study due to adverse events. Pomalidomide shows promise as salvage therapy in relapsed refractory patients and its combination with proteasome inhibitors needs to be explored further.

IMiD's, although effective and an important part of the treatment in AL amyloidosis, for unknown reasons, are poorly tolerated in AL compared to multiple myeloma with fluid retention, fatigue and a recently identified phenomenon of paradoxical increase in cardiac biomarkers during therapy[136] – all needing further study. The latter is of particular significance given its role in assessing cardiac responses in AL amyloidosis although a rise in the NT pro-BNP following the first cycle of lenalidomide was not associated with poor survival or renal deterioration.[89]

Proteasome inhibitor based regimes

Bortezomib-based regimens have changed the treatment paradigm in AL amyloidosis over the last few years. Plasma cells in AL amyloidosis appear to be especially sensitive to proteasome inhibitors in a preclinical model[137] – the accumulation of pre-fibrillar light chains after proteasome inhibition adding to the cellular toxicity. The high response rates and good tolerance have led to Bortezomib combinations being adopted as front line therapy in AL amyloidosis. Prospective evidence of superiority or better tolerability over current standard regimens, particularly in elderly subjects with advanced cardiac disease, is still lacking.

A multicentre retrospective analysis of 94 patients showed high haematologic response rates (71%) achieved rapidly (a median response time of 52 days) with associated organ responses in 30%. The one year survival rate was 76%. One third experienced grade 3 toxicity and the most common non-haematological side effects included peripheral sensory neuropathy, orthostatic hypotension, gastro-intestinal disturbance or peripheral oedema.[100] A prospective phase I/II trial confirmed the high response rate and reported similar efficacy in patients with relapsed refractory AL amyloidosis with both once (OW) or twice (TW) weekly schedules in 70 patients.[99] Haematologic response, median time to best response, one year PFS and ≥ grade 3 toxicities were 68.8%/66.7%, 3.2/1.2 months, 72.2/74.6% and 50%/79% respectively in the OW and TW groups.[99]

A number of groups, including our own, have now studied Bortezomib with additional cyclophosphamide (CyBorD) or melphalan (BMDex) in AL amyloidosis.[102, 138] Of 17 patients receiving CyBorD at the Mayo clinic (58% with symptomatic cardiac involvement), a clonal response was achieved in a median of 2 months in 94%, with 71% and 24% achieving a CR and PR respectively.[102] In the UK, 43 patients received CyBorD (bi-weekly Bortezomib) (74%;cardiac involvement and 46% stage III by the Mayo cardiac staging), with overall haematologic response rates of 81% (CR - 42%).[101] The estimated 2 year PFS was 66% and 41% for front-line treatment and relapsed setting respectively. The estimated 2 year OS was 98%.[101]

BMDex has been studied in a prospective trial in the US[139] and in a retrospective cohort in Italy.[92] An early report from the prospective study has shown a 94% haematologic response rate with 38% CRs. However, in the retrospective study haematologic response rates were 48% (CR 18%, VGPR 21%) with BMDex and the absolute dFLC decrease in responders 95%; significantly greater than those treated with MDex (median 83%, p=0.018). The lower overall response rate in this study was due to early deaths in cardiac patients and similar results now reported with larger CyBorD treated cohorts[140] – highlighting a possible concern using Bortezomib in advanced cardiac disease. A randomised prospective trial is ongoing in Europe and Australia comparing MDex with BMDex. Ixazomib and carfilzomib are novel proteasome inhibitors appearing to have greater efficacy of proteasome inhibition compared with Bortezomib with a more favourable toxicity profile. A phase I study of Ixazomib has been completed with good tolerance and responses seen in multiply refractory patients.[141] A phase III trial is ongoing.

Allogeneic stem cell transplantation

Allogeneic stem cell transplantation is not widely used in AL amyloidosis. Following the first successful case was reported in 1998,[142] a European Group for Blood and Marrow

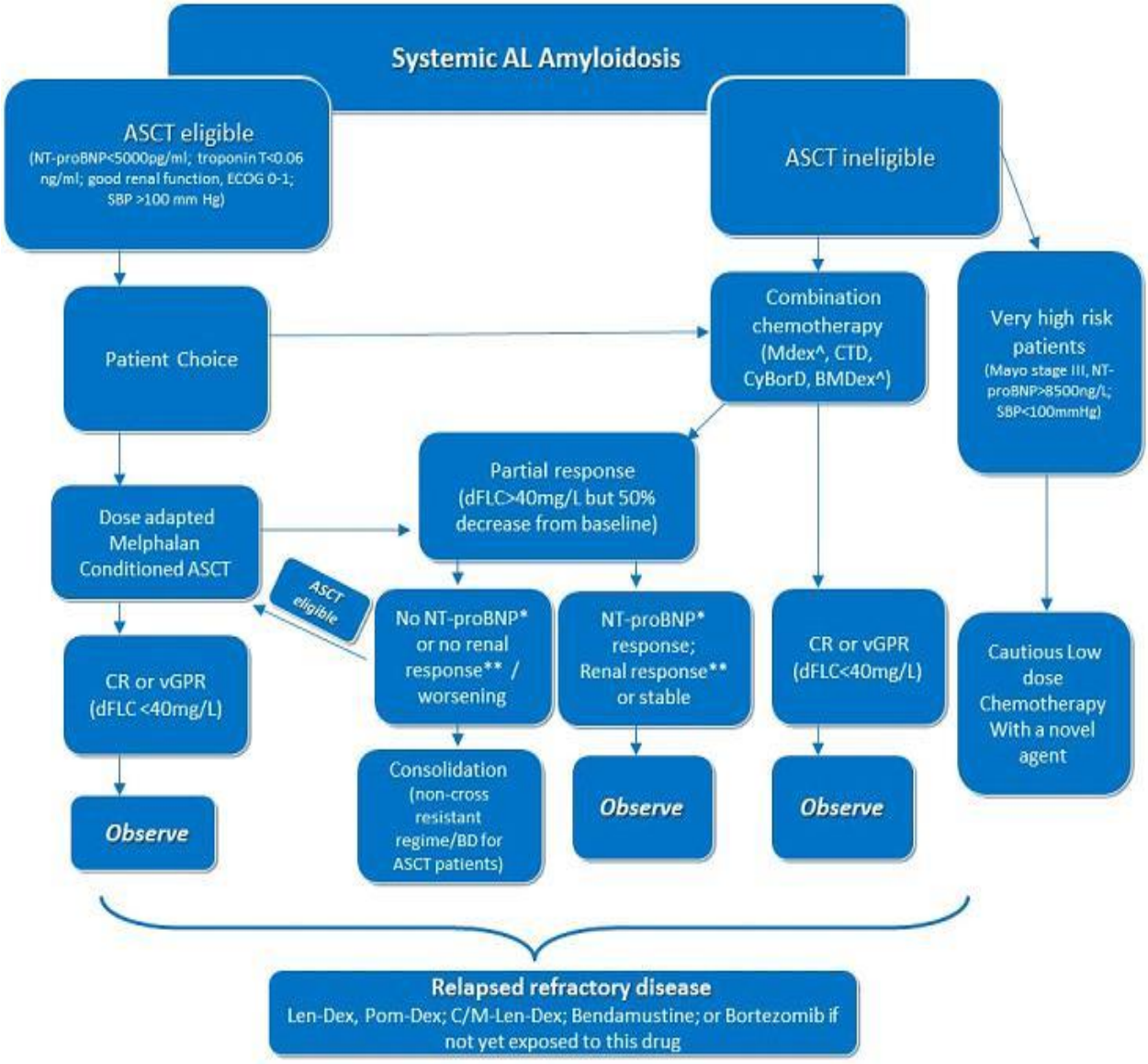
Transplantation (EBMT) registry study in 2005, reported 19 patients with AL amyloidosis (7 patients full intensity conditioning; 8 reduced intensity conditioning (RIC)). The overall and progression free survival was 60% and 53% respectively at one year, with TRM of 40% (TRM 50% in patients receiving total body irradiation). Ten patients achieved haematologic responses and 8 attaining organ responses.[143] Anecdotal reports suggest RIC-SCT is possible in AL.[144] Currently, allogeneic stem cell transplantation may have a role in highly selected young fit patients with relapsed disease but, ideally, should only be considered in the context of a clinical trial.

An approach to treatment

The treatment strategy in a patient with AL amyloidosis is shaped by poignant background factors including age, co-morbidities, performance status, contraindications to drugs and patient wishes deciding the ultimate choice of the regimen, especially in the lack of clear randomised evidence. Figure 1.5 provides a suggested treatment algorithm for patients treated outside of clinical trials. The choice of upfront treatment is between autologous stem cell transplantation (ASCT) and combination chemotherapy – one not necessarily precluding the other. Patients with good performance status, limited organ involvement, good renal function, cardiac ejection fraction >50%, CO diffusion capacity > 50%, cardiac troponin T <0.06 ng/L and NT-proBNP <5000 ng/L appear to have a <5% TRM during ASCT[119, 145, 146] and should be offered ASCT as a treatment choice. Borderline patients treated with a stem cell sparing regime as improved organ function may allow ASCT at a later date. Bortezomib has emerged as a key backbone in induction regimens – CyBorD or BMDex are becoming the regimens of choice in most non-neuropathic patients. Cautious low dose regimens are needed in advanced cardiac involvement (especially with NT-proBNP >8500ng/L). Patients with neuropathy pose a dilemma, with melphalan/dexamethasone or lenalidomide/dexamethasone as suitable upfront treatments. Response reassessment at

the 3 month point interval is key – patients with a poor response considered for either dose increments or an additional drug to improve the response.

Figure 1.5: Therapy algorithm for treatment for immunoglobulin light chain (AL) amyloidosis.[27] Reassessment post 3 cycles of chemotherapy should be undertaken to optimise haematologic response according to consensus criteria.[87]



*NT-proBNP response - >30% and >300ng/l decrease in patients with baseline NT-proBNP ≥650ng/l
** Renal response maybe delayed with no immediate correlation with haematologic response.
^ Avoid melphalan containing regimens if eligible for ASCT

ASCT - Autologous stem cell transplantation; CTD - cyclophosphamide, thalidomide, dexamethasone; CyBorD - cyclophosphamide, Bortezomib, dexamethasone; MDex - melphalan and dexamethasone; BMDex - Bortezomib, Melphalan, Dexamethasone; C/M – Len-Dex – Cyclophosphamide or melphalan with lenalidomide and dexamethasone; VGPR - very good partial response; CR – complete response; dFLC - difference in involved and uninvolved free light chains; NT-proBNP - N-terminal prohormone of brain natriuretic peptide; SBP – systolic blood pressure

Best organ responses occur in those achieving a haematologic CR/VGPR, but partial responders may also achieve an organ response. Lenalidomide and Pomalidomide based regimens or Bendamustine are useful in the relapsed/refractory setting. The value of alkylator based regimes in patients relapsing after a novel agent based regime is uncertain.

IgM associated AL amyloidosis

4-7% of all patients with amyloidosis have an IgM secreting (mainly) lymphoplasmacytic lymphoma (LPL) as the underlying cause of AL.[147, 148] Treatment should be directed toward the LPL clone. Single agent alkylators have limited efficacy. Regimes such as melphalan/dexamethasone (n=14), purine analogues (n=17) confer good haematologic responses of 64% and 73% respectively.[147, 148] Recently, Rituximab/Bortezomib/Dexamethasone shows promise, with overall haematologic responses of 78% (n=7).[149] Larger clinical trials are needed to evaluate this further.

Organ transplantation

Organ transplantation can be considered in patients attaining a VGPR or better with irreversible end-stage organ function, or in a situation to facilitate aggressive chemotherapy; not otherwise feasible due to organ dysfunction. Long term control of the underlying clonal disorder is needed to prevent recurrence after organ transplantation or progression in other organs.

Cardiac transplantation may be the only option to improve survival in younger patients with advanced isolated heart involvement, accounting for 0.14% of heart transplants nationwide according to the United Network for Organ Sharing (UNOS).[150] A UK study reported 24 patients from 1984 to 2002 undergoing heart transplantation in 6 UK cardiac transplant

centres, 17 diagnosed with AL amyloidosis. The 1 and 5 year OS was 50% and 20% respectively not receiving chemotherapy versus 71% and 36% who did.[151] Another multicentre study included 69 patients receiving heart transplants for amyloidosis (all types), reported 1 and 5 year survival as 74.6% and 54% respectively.[150] Control of the underlying clone is important, and ASCT is generally considered most appropriate treatment. One US series between 1994 and 2005 included 11 patients undergoing the two procedures serially; 9 patients surviving both with 3 eventually dying due to progressive amyloidosis; the earliest 55 months post ASCT. The 1 and 5 year survival following heart transplantation in this cohort was 82% and 65% respectively;[139,152] a marked improvement over the otherwise median survival of advanced cardiac AL of 3-8 months. Scarcity of organs, risk of amyloid recurrence and higher mortality of amyloid patients undergoing heart transplants still makes cardiac transplantation a contentious issue.[150]

Renal transplantation in AL amyloidosis is considered to improve long term survival and quality of life. The largest UK series reports 22 patients undergoing renal transplantation; (19 cadaveric donors and 3 live donors), and 3 with extra-renal organ dysfunction. Nineteen patients received chemotherapy or ASCT; 74% achieving a haematologic response (11 PR and 3 CR) with no graft failures secondary to amyloid recurrence and 1 and 5 year OS 95% and 67% respectively.[153] The Mayo group reported 19 patients receiving renal transplantation (1 cadaveric donor and 18 live donors), 12 having extra-renal involvement from 1999 to 2008. All attained a haematologic response (18 CR and 1 PR); 8 receiving renal transplantation followed by ASCT, 6 receiving ASCT followed by renal transplantation and 5 receiving non-myeloablative chemotherapy followed by renal transplantation, with no significant difference between these groups. The 1 and 5 year OS was 84% and 76% respectively.[153] Good long-term outcomes appear to be associated with renal transplantation in AL in highly selected cases.

Outcomes with orthotopic liver transplantation (OLT) for advanced liver AL remain poor. A UK study from 1984 to 2009 included 9 patients undergoing an OLT with the 1 and 5 year survival from transplantation 33% and 22% respectively.[153]

In summary, AL Amyloidosis is rare. It is frequently diagnosed late due to the subtle signs and symptoms of the disease. Increasing awareness of this disease poses major challenges. Improved diagnostic techniques have enabled better detection of this disease. Risk stratification using cardiac biomarkers has refined treatment selection and reduced morbidity and TRM. Diagnosis and treatment planning in AL is complex and best considered in specialist centres availing expertise of a multi-disciplinary team. Novel agent based chemotherapy especially with Bortezomib has improved chemotherapy responses and is being explored pre and post ASCT. Bendamustine, lenalidomide and Pomalidomide are valuable therapies for relapsed refractory patients. Good haematologic responses (attaining at least a VGPR being the goal of therapy[87]) translates into organ responses and improved survival. While cardiac and haematologic responses can be simultaneous, responses of other organs may evolve over months or years. Early mortality of patients with advanced disease persists. Future directions involving therapies targeting the amyloid deposits - anti-fibril antibodies are in early phase trials. Exploring the role of minimal residual disease, the importance of eradicating this, gaining insight of the microenvironment is needed. Ultimately, the most important factor is increasing the awareness to ensure early diagnosis to allow therapy before extensive organ involvement.

Novel therapies for amyloidosis

A number of therapies are in development targeting various components of the pathophysiology of amyloid fibrillogenesis. Glycosaminoglycans are a universal constituent of all amyloid deposits and inhibition of the interaction between GAGs and amyloid fibrils which seems a promising therapeutic approach AA type – which may also have merit in AL. Eprodisate (Kiacta®, Bellus Health, Canada) is a negatively charged, sulfonated molecule of low molecular weight is undergoing phase III trials.[154] There is growing interest in developing therapeutic antibodies to directly target amyloid deposits. Mu 11-1F4 is a chimeric antibody reactive with many AL fibrils,[155] localisation of which has been studied in patients with PET imaging.[156] Its administration as a therapeutic is planned. mAb2A4 is another monoclonal antibody, binding AL/AA fibrils and human AL amyloid extracts,[157] reported to cause amyloid regression in mouse models of AA and AL amyloidosis. This antibody has been further developed as NEOD001 (Onclave Therapeutics Limited, California), recently commencing phase I trials in systemic amyloidosis in the United States. Pre-clinical development of small interfering RNAs (siRNAs) have also been explored as a treatment in reducing the expression of the amyloid precursor protein, with *in vitro* studies showing inhibition of synthesis of light chains in transfected cells, and *in vivo* reducing the production and circulating free light chains.[158]

R-1-[6-[R-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid (CPHPC) is a drug which cross-links pairs of SAP molecules in vivo[159] resulting in rapid clearance of SAP from the liver and almost complete depletion of plasma SAP.[160] Some SAP persists on the amyloid deposits and lack of plasma SAP allows administration of an anti-SAP antibody which can now target the amyloid deposits. Following promising results in transgenic mouse model of AA amyloidosis, using anti-SAP immunoglobulin-G (IgG) antibodies and CPHPC,[15]

early phase clinical trials are underway to explore this further with initial patient recruitment commencing this year.

Aims and scopes of this thesis

The introduction of this thesis provides a general overview of the different types of amyloidosis with a comprehensive overview of systemic light chain amyloidosis and treatment options available. This rare disease is often a delayed diagnosis, with the clinical presentation masked by many other medical conditions. More recently, advances have been paved with use of targeted treatment, monoclonal antibodies or accessibility of newer chemotherapeutic agents. Prognostic stratification has emerged as an important tool in treating amyloidosis patients.

The premise of this thesis focuses upon areas of interest. Firstly section one concentrates on the investigations important in diagnosing, monitoring, and assessing the prognostic utility and potential complications of patients diagnosed with systemic amyloidosis. Chapter 3 concentrates on the comparison of the utility of free light chain assays currently important in diagnosing and monitoring these patients. The current serum Freelite™ assay measures immunoglobulin free light chains using polyclonal sheep antibodies directed against hidden epitopes on the light chain molecule giving an accurate measurement of serum free kappa and lambda and forms a standard part of the baseline and serial follow up assessments in systemic AL amyloidosis[161, 162], and is part of the revised Mayo staging system and standard international consensus criteria for disease response assessment.[80, 86, 87] A group in the Netherlands working with Siemens, Germany, has developed this technology using a mixture to two kappa and two lambda monoclonal antibodies to the hidden epitopes of the constant region of the immunoglobulin light chain molecules.[163] With increasing adoption by laboratories of the novel assay, it is important to assess the utility of the new assay compared to the current reference Freelite™ assay. We report a comparison of serum free light chains analysing the serum samples by both immunoassays at diagnosis and three

further time points in during the initial chemotherapy for patients with systemic AL amyloidosis.

Chapter 4 outlines the coagulation abnormalities seen in systemic AL. Systemic light chain (AL) amyloidosis is also known to be associated with a bleeding diathesis[164], which may range from small cutaneous bruising, pathgnomic “racoon eyes” or life threatening bleeds. The crucial role of endothelial dysfunction in systemic AL may assist in understanding the basis of the light chain toxicity in this disease. We designed a prospective study to investigate the endothelial dysfunction in newly diagnosed systemic AL patients, using Von Willebrand factor (vWF) antigen (Ag) and factor VIII to serve as a surrogate marker and examine its relationship with light chain toxicity. Other coagulation factors were also examined. This was performed examining the clinical bleeding manifestations using the Royal Free Hospital v4 adapted bleeding questionnaire in conjunction with extensive laboratory investigations such as factor assays, protein S, protein C, Anti-thrombin III, vWF:Ag and VWF:Ricof and ADAMTS13 assays. Endothelial function was explored using vWF antigen (Ag) and factor VIII to serve as a surrogate marker and examine its relationship with light chain toxicity. The secondary aim was to assess the prognostic utility of these investigations.

Chapter 5 focuses on a new area of interest which may pose a potential issue in patients with systemic amyloidosis – sleep disordered breathing. Cardiac involvement and/or macroglossia with soft tissue deposits, risk factors for central and obstructive sleep apnoea, are common features of systemic AL amyloidosis. Little data exists on the occurrence of sleep disordered breathing (SDB) or recurrent nocturnal hypoxia in amyloidosis. All were screened for SDB using Epworth Sleepiness Score (ESS) and STOP BANG questionnaires. We report here the results of a pilot study of overnight continuous pulse oximetry in patients with systemic light chain (AL) amyloidosis, based on the hypothesis that recurrent nocturnal

hypoxaemia (likely due to sleep disordered breathing OSA and/or CSA) could occur in patients with amyloidosis reporting a high incidence of recurrent nocturnal oxygen desaturations and raise a question whether these desaturations may be the trigger for sudden cardiac mortality.

The second section focuses on a rare type of amyloidosis - localised amyloidosis and a subgroup – tracheobronchial amyloidosis. Chapter 6 elaborates on localised amyloidosis. Localised deposits of amyloid can occur in various tissues in the body and are usually presumed to be of AL type, with the consequent presence of a focal monoclonal B cell dyscrasia within the affected tissue. As such, the clinical effects of the localised amyloid deposits depend on their precise anatomical location, and can result in substantial morbidity. Localised amyloidosis is much rarer than systemic types, and consequently remains very poorly studied; with most knowledge arising from individual case reports or small series of less than 20 patients. There are commonly reported sites include the urinary tract, respiratory tract, larynx, skin and eyelids.[165] Currently, data on long term outcomes and progression to systemic disease is lacking with the need for further exploration in this field. We have examined the clinical features and outcomes of a large series of 606 patients with localised AL amyloidosis highlighting the striking differences from systemic AL amyloidosis with respect to the lack of progression, benefit from debulking procedures, limited need for cytotoxic chemotherapy therapy and excellent overall long term outcomes. Chapter 7 examines a subgroup of localised amyloidosis – laryngeal and tracheo-bronchial amyloidosis. This group of patients have a more refractory control of their disease. We examined the clinical features, treatment options and also sought to explore the laboratory aspect of proteomic analysis in these biopsies to attempt to gain insight into any underlying instigating factors. Although amyloid fibril formation and aggregation occurs when high concentrations of the key partially folded intermediate is present, other factors including the

pH, temperature, amino acid sequence and concentration of the intermediate play a role. The presence of ApoA1 and insulin-like growth factor binding protein complex in the LTBA proteomic analysis suggests the potential involvement in the development of localised amyloid in the laryngeal and tracheobronchial cases. Further studies are needed to elucidate the function of these factors before a significant pathogenesis is attributed to the latter.

The third section focuses on 2 types of treatments in systemic amyloidosis – a chemotherapeutic option (lenalidomide/dexamethasone post novel agents) and ((R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl] pyrrolidine-2 carboxylic acid (CPHPC)) which has emerged as a potential therapeutic target to deplete SAP.

Chapter 8 relays our experience with a chemotherapeutic drug option – lenalidomide/dexamethasone. We report a substantial cohort of patients treated with lenalidomide in the relapsed/refractory setting, following the use of Bortezomib and/or thalidomide with long term follow up assessing the impact of previous therapies on haematologic responses.

Chapter 9 outlines a small cohort of patients treated with compassionate use of CPHPC. Fibrinogen related amyloidosis has predominant renal involvement with end stage renal failure within 5 years of presentation with proteinuria.[47] Currently there are no definitive therapeutic cures for this condition and we used CPHPC on a compassionate basis to try and improve the renal survival duration in these patients, with the use of QoL questionnaires to gain a better insight into the patients' perspective. We also assessed its use in patients with dialysis related amyloidosis.

Chapter Two: Materials and methods

Declaration

I have designed the studies, collected the data and performed analysis of the data using 2 software programs – SPSS v20 (IBM SPSS) and Graph pad prism (version 5). This thesis includes 3 prospective studies: the coagulation study, sleep disordered breathing study and the CPHPC study. I recruited patients for the Coagulation study, collected the data and performed the statistical analysis as a clinical research fellow based at the National Amyloidosis Centre, University College Medical School (Royal Free Campus). I also recruited patients for the sleep disordered breathing study, again collected the data and performed the statistical analysis and sent the overnight pulse oximetry downloads to Dr Milind Sovani (Respiratory physician) to analyse. I recruited patients for the CPHPC study following discussion at our multidisciplinary meeting for suitability for compassionate use of CPHPC in these patients. Ms Thirusha Lane, a clinical lead nurse at the National Amyloidosis Centre used the QoL software (SF36) in giving the output for the quality of life data. Various diagnostic methods were undertaken by other individuals in the department and external sources as follows:

Measurement for biochemical serum and urinary investigations were performed by the Royal Free Hospital laboratory services.

Frozen serum blood samples for the serum free light chain assay comparison were collected by Wendy Taylor and Lois Cook, based at the National Amyloidosis Centre.

Coagulation assays were performed by the Haemophilia department at the Royal Free Hospital and these investigations overseen by Ms Anne Riddle, Dr Keith Gomez and Dr Pratima Chowdary.

Histological and immunohistochemical studies were performed by Janet Gilbertson and Karen Boniface, both based at the National Amyloidosis Centre. Laser capture techniques were undertaken by Janet Gilbertson and Nicola Botcher, both based at the National Amyloidosis Centre. Proteomic analysis was undertaken using the Scaffold program by Graham Taylor and Nigel Rendell, both based at the Wolfson Centre Research Institute, National Amyloidosis Centre. Analysis of proteomic data was performed by Dr Julian Gillmore and Janet Gilbertson.

The spinning and storing or serum samples for patients on compassionate use of CPHPC were performed by the clinical nurses Thirusha Lane, Charlene Kearney and Randolph Gaudia.

Genetic sequencing was performed by Dorota Rowczenio and Hadija Trojer, based at the National Amyloidosis Centre.

Echocardiography was performed by Babaita Pawarova, Oliver Manalo, and Sevda Ozer, based at the National Amyloidosis Centre.

^{123}I-SAP scintigraphy was performed by David Hutt, Dorothea Gopaul and Stephanie McKnight, based at the National Amyloidosis Centre.

Pulse oximetry output using the Minolta 300I pulse oximeter was read by Dr Milind Sovani, a respiratory physician at Nottingham University hospital.

Patient selection

All of the patients recruited and studied in this thesis have been seen at the National Amyloidosis Centre, London. Medical records were retrieved and an electronic database has recorded details of all patients attending the centre, which I used. All patients included in this thesis provided explicit informed consent. The database

recorded dates of deaths according to the Office of National Statistics, updates from family members and details of the causes of deaths were specifically sought for by contacting local general practioners or hospital consultants caring for the patients. Only anonymised data was used.

Immunoassays

Serum free light chain assays

Kappa and lambda serum free immunoglobulin (FLC) concentrations were measured using a latex-enhanced immunoassay (Binding Site, Birmingham, UK) on an Ehring BNII auto analyser (Dade Behring Marburg, Germany). Antibodies were directed against the FLC epitopes within the whole immunoglobulin molecules, with the sensitivity quoted as<5mg/L for this assay. The reference range as provided by the manufacturers for Freelite^{TM}: kappa 3.3-19.4mg/L, lambda 5.7-26.3mg/L and kappa/lambda ratio 0.26-1.65. The N Latex (Siemens, Germany) immunoassay is a turbid metric assay based on monoclonal antibodies. The reference range as provided by the manufacturers for N Latex: kappa 6.7-22.4mg/L, lambda 8.3-27mg/L and kappa/lambda ratio 0.31-1.56mg/L. In chapter 3, in total, 240 serum blood samples were measured in duplicate using the Freelite^{TM} (Human kappa and lambda free kits, Binding site Ltd, Birmingham, UK) and N Latex (Siemens Healthcare Diagnostics, Germany) from stored frozen samples.

Histology

Congo red staining

Biopsies were logged into our database once received and the formalin fixed de-paraffinised tissue sections 6-8µg thick were rehydrated and stained with

haematoxylin under running tap water. The Congo red method as described in Puchtler el al[166] was adopted with sections using this method. Increasing ethanol concentrations to xylene were used to dehydrate the sections and eventually mounted with DPX mounting medium. The stained slides once dry were viewed in bright field under cross polarised light by microscopy.[167] A known Congo red positive block validated by laser micro dissection and proteomic analysis was used as a positive control.

Immunohistochemistry

Immunohistochemical staining techniques were used to type the amyloid. Formalin fixed de-paraffinised 2µm sections of amyloidotic tissue was used. These sections were cleansed; washed with water and endogenous peroxidise activity was quenched by incubation in aqueous (0.3%) hydrogen peroxide (H_2O_2) for approximately 30 minutes. Rinsing was performed using a phosphate-buffered saline (PBS) containing 0.05% Tween (Calbiochem) solution. 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). These sections were incubated overnight with primary antisera at 4^{o}C, and then rinsed with PBS containing 0.05% Tween (Calbiochem) and labelled with secondary antibodies. Each section was washed in PBS and bound enzyme-antibody bound complexes were consequently visualised using a metal-enhanced DAB (Fisher Scientific solution).

Immunohistochemistry was possible with 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. Immunohistochemical stained sections were counterstained in haematoxylin, 'blued' under running tap water and stained with Congo-red.[168]

When performing TTR staining, pre-treatment was performed for enhanced antigen retrieval using a 10 minute incubation with 1% sodium periodate. The slides were then washed and incubated for a further 10 minutes with 0.1% sodium metabisulphate, and washed again and incubated for 5 hours at room temperature with 6M Guanadine in 0.9% sodium chloride.

Laser capture micro dissection and mass spectrometry

(Also described in chapter 7)

Laser capture micro dissection and mass spectrometry (LDMS) techniques were also used to identify proteins from formalin-fixed, paraffin embedded tissues and described in detail for chapter 7.[169] 10µm sections were cut from formalin fixed tissue on the Director *Expression Pathology 50001-024.* Stained slides with Congo red (*Putchler et al)* were left to dry after clearing with xylene.

Visualisation and locating the amyloid was done using bright field and Rhodamine filter sets (TRITC) by the Zeis Palm Micro beam Laser capture microscope, with amyloid typically yellow on a "red" background. Delineation of the areas of interest were drawn around , appearing yellow under a Tritc florescent filter or under bright field and "cut" out using laser capturing in to 0.5ml micro-centrifuge adhesive cap tubes (Zeiss) whilst checking the cap to ensure that some tissue has been captured. A 10x concentration stock solution of the following reagents were constituted: (1) **TRIS**: 0.1 M; use 1.2114g in 100ml, (0.6057g in 50 ml), (2) **EDTA**: 10 mM; use 0.37224g in 100ml, (0.18612g in 50ml) and (3) **Zwittergent** (Z3/16): 0.02% w/v; use 0.02g in 100ml,

(0.01g in 50ml). A 1ml aliquot of each buffer is placed into 1.5 ml microfuge tubes and stored in a separate snap seal bag, at -20°C. **TEZ Buffer (Tris, EDTA, and Zwittergent)** and constituted by diluting each buffer in 1:10 in MQ. A glass vial of 100µg trypsin gold was reconstituted with 50mM Acetic acid and 5 µl aliquots were stored in microfuge tubes at -80°C (>for 1 month 1mg/ml). To constitute working strength trypsin, 5µl of stored frozen trypsin solution was added to 245µl of TEZ buffer (1x)(4 fold concentration of trypsin) and made just prior to use. To constitute the Dithiothreitol, 10 mg ml^{-1} solution in MilliQ water aliquot in 100ul volumes was used and stored in a snap seal bag freezer.

35µl of TEZ buffer was added into each sample and then closed tightly, and the sample vortexed. The sample was inverted and the left on the bench for 20 minutes. The capped tubes were inverted, flicked to transfer the liquid to the cap and left to stand for approximately 20 minutes at room temperature. The samples were centrifuged at 5000 rpm x 5 min (Eppendorf bench, 24 fixed positions) to recover the liquid into the bottom of the vial.

The cap lock was fitted to the tubes, with care taken not to disturb the liquid at the bottom of the vial and heated at 99°C x 90 minutes, mixing every 20-25 minutes. Cooling next and centrifuged at 5000 rpm x 5 min to recover as much condensate as possible from the tube lids. The Sonicater was cleaned with 70% alcohol (fume hood) and refilled with fresh MQ water. The cap lock was fitted to tubes and sonicated for 60 minutes at room temperature. Samples were centrifuged at 5000 rpm x 5 min. Working Trypsin Solution was made by taking 5µl of stored frozen trypsin solution and add to 245µl of TEZ buffer (1x) (4 fold concentration of trypsin). 1.5µl of working trypsin was added to each sample, (total volume 36.5µl), and each sample vortexed. Again, centrifuging at 5000 rpm x 5 min, with the cap locks fitted to the tubes and allowed to

digest overnight at 37ºC. Samples were centrifuge at 5000 rpm x 5 min. Addition of 5µl DTT to the Trypsin digested samples (reduced with Dithiothreitol (DTT) - (0.008M). Centrifuged at 5000 rpm x 5 min. Cap locks were fitted and sample heated for 5 minutes at 98ºC (total volume 41.5 µl). The samples are cooled, then centrifuged at 5000 rpm x 5 min. The samples are taken to the equipment room to unfasten the caps and place in freezer at -80.

On the freeze drier turn the drain valve and the air admittance valve clockwise. Ensure the mode selector on the pump is at zero (0). Switch pump on at side and leave for 20 minutes. Dials should be set at 3mb and -40 temp. Select a small dissector and outlet valve is turned fully anticlockwise and attach rubber pipe from manifold to outlet valve. On manifold turn tail switch to on position (down) and check the dials move to the right before falling again. This is removing the air from the rubber tube. Remove samples from -80 and place in dissector ensuring lid is in place correctly. Quickly turn outlet valve clockwise until the screw thread is just visible. Dials should be fully to the right. Check that a vacuum has been created by gently lifting the dissector with the lid – the bottom should be raised off the bench if not correct. Check that the dials are falling. After 60 minutes check that the dials are at 3mb and -40 temp. Leave to freeze dry. The person loading the MS will remove the samples however if this has not happened within 24 hours. Remove the samples by reversing the steps and place in -80 with the lids tightly shut.

The residues were reconstituted with 1% MeCN + 0.1% TFA (20µl/sample), vortexed and centrifuged (10 000 rpm x 5 min). The supernatants transferred to Waters Total Recovery vials for LC-MS/MS on the Velos. A QA sample (six protein mix tryptic digest) should be run before and after the samples; all samples should be followed by a blank run with a 1% MeCN + 0.1% TFA injection. The data was processed using

Proteome Discoverer for MASCOT searching of the NCBI and IPI databases enabling mass spectrometry results to be produced.

Proteomic analysis was performed on the Velos platform and analysed following the method of Rodriguez *et al*.[75, 169] Following extraction into 10mM Tris/1mM EDTA/0.002% Zwittergent buffer (99˚C, 1.5h) and sonication (1h), samples were trypsinised (1.5µg w/v) overnight at 37˚C and then reduced with Dithiothreitol (50µg) at 99˚C for 5 minutes. Digests were run on a nanoACQUITY™ UPLC system (Waters Ltd., Elstree, and Hertfordshire, WD6 3SZ) coupled to an Orbitrap Velos Mass Spectrometer (Thermo Electron, Bremen, Germany). MS data files were analysed using Mascot software. (Matrix Science, London, UK).

Genetic sequencing

Patients with suspected hereditary amyloidosis or clinical features of laryngeal involvement had genotyping performed. Patients at their baseline visit had whole blood taken which was stored in an EDTA tube consequently frozen and stored, with genomic DNA isolated as required.[170] The polymerase chain reaction (PCR) adopting the 'ready to go" tubes (GE Healthcare) were used to amplify the coding regions for the genes and appropriate exons including: 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 2.1.

Table 2.1: Genotyping for Hereditary Amyloidosis illustrating the primers used in this technique

	Primers sequence
FGA exon	
5-5'end	Forward: 5'-GCTCTGTATCTGGTAGTACT-3'
	Reverse: 5'-ATCGGCTTCACTTCCGGC-3'
ApoA1 exon	
3	Forward: 5'-GGCAGAGGCAGCAGGTTTCTCAC-3'
	Reverse: 5'-CCAGACTGGCCGAGTCCTCACCTA-3'
4	Forward: 5'-CACTGCACCTCCGCGGACA-3'
	Reverse: 5'-CTTCCCGGTGCTCAGAATAAACGTT-3'
TTR exon	
2	Forward: 5'-TTTCGCTCCAGATTTCTAATAC-3'
	Reverse: 5'-CAGATGATGTGAGCCTCTCTC-3'
3	Forward: 5'-GGTGGGGGTGTATTACTTTGC-3'
	Reverse: 5'-TAGGACATTTCTGTGGTACAC-3'
4	Forward: 5'- GGTGGTCAGTCATGTGTGTC-3'
	Reverse: 5'-TGGAAGGGACAATAAGGGAAT-3'

Overnight pulse oximetry

All patients recruited to the sleep apnoea study had overnight oximetry performed with simplified instructions, appendix 2. This information was downloaded using the Minolta 300I software and then analysed by Dr Milind Sovani using this program.

Sleep questionnaires

Patients recruited to the sleep apnoea study completed an Epworth Sleepiness Score (ESS) and STOP BANG questionnaires for obstructive sleep apnoea (OSA) to correlate the clinical symptoms in conjunction with the overnight oximetry readings (appendix 3 and 4 respectively).

SAP scintigraphy

This procedure was performed in all patients attending the National Amyloidosis Centre at their first assessment, initially 1988. Each patient having this procedure received approximately 200µg of SAP with 190MBq of 123I which is the equivalent of 3.8mSV of radiation. A dose of 60mg of potassium iodide was immediately given to each patient prior to ^{123}I SAP scintigraphy and a further 5 doses were administered following this over the following 3 days to prevent thyroid uptake. Patients had anterior (AP) or posterior (PA) imaging using an IGE-Starcam gamma-camera (IGE Medical Systems, Slough, UK) at 6 or 24 hours following the injection.

A normal scan was defined upon having no abnormal localisation of the tracer. The quantification of the amyloid load was based on: small – when uptake within one or more organs was visible with the normal intensity in the blood pool; moderate – when abnormal uptake was seen within organs and the blood pool was diminished; and

lastly large – when the blood pool signal was lost with adjustment of the grey scale to include the target organs.

Progression of the amyloid was defined as increase of the tracer within the affected organ(s) and/or decrease in the background blood pool. Regression of the amyloid was defined as reduction of the tracer within the affected organ(s) and/or increase in the background blood pool.

PET/CT imaging

This imaging uses a device combining the use of positron emission tomography (PET) scanner and x-ray computed tomography (CT) scanner to acquire images sequentially and combine to give a superposed image with the use of 18F-flurodeoxyglucose (FDG) PET/CT. An intravenous bolus injection of a dose produced for 2 or 3-FDG is made (doses range from 3.7-7.4 megabecaquerels per kilogram of body weight. The functional aspect depicts the spatial distribution of metabolic or biochemical activity in the body. Quantification of the size of the lesion and location are important. This technique was used to assess patients with localised amyloidosis, specifically related to lymph node, lung nodules or bone lesions.

Quality of life (QoL) questionnaire assessments

The quality of life was assessed at 6 monthly intervals for all patients by the validated Quality Metric SF36v2® Health survey. This is designed to measure functional health and well-being from the patient's perspective. There are eight specified health domains (physical functioning, role physical, bodily pain, general health, social function, role emotional, mental health and vitality) which are assessed are scored individually. The result is expressed in comparison to the American norms. The

average score for healthy controls in each measure is 50, with higher scores representing a better QoL. A change of 10 points or greater in any domain between administrations is considered clinically significant.

Measuring the quality of life (QoL)

Until fairly recently the primary clinical endpoints such a hospitalisation or death were the focus of most clinical trials or disease management programs. Previously patient-reported outcomes (PRO) tended to be in relation to disease specific indicators, such as joint pain in rheumatoid arthritis. However, this does not reflect the overall health-related QoL. As people are living longer, healthcare must focus as much on quality as length of life. Medicine regulatory bodies such as the United States (US) Food and Drug Administration (FDA) and the United Kingdom (UK) Medicines and Healthcare Products Regulatory Agency (MHRA) have become increasingly interested in patient-reported health-related quality of life, and the pharmaceutical industry, in conjunction with academic and clinical researchers, now expected to include validated QoL measures as endpoints in clinical studies[171]

The Quality Metric SF-36v2® and other SF QoL questionnaires are widely recognised as leading PRO measures; used and validated in patients with many different acute and chronic conditions. The SF-36v2 has become invaluable to healthcare professional as a useful utility in evaluating and monitoring disease and assessing the success of therapy.[172-175] The SF-36v2 provides a broad overview of a patient's health status and the effect of the disease/health status on his or her physical, social emotional and mental functioning – i.e. disease burden.[176] In addition to the utility of the survey in measuring disease burden, the SF-36v2 may be administered to a single patient at multiple time-points, offering a baseline comparator for long term monitoring

in chronic conditions and whilst having treatment. It is easy and quick to complete, allowing easy incorporation into clinical practice[177]

The SF-36v2® is composed of 36 questions covering eight health domains or scales:

Physical functioning (PF): The PF domain is composed of 10 items covering distinct aspects of physical functioning including a range of severe and minor physical limitations, such as lifting and carrying groceries; climbing stairs; bending, kneeling, and walking moderate distances. There is also one item dedicated to describing limitations in self-care activities. Low scores indicate significant limitations in performing physical activities, whilst high scores reflect little or no limitations in performing physical activities.

Role-physical (RP): This domain includes four items covering a range of physical role limitations, including limitations in carrying out work or other usual activities and reduction in amount of time spent on work or other usual activities. Low scores indicate interference of physical problems with work or typical usual activities.

Bodily pain (BP*): This domain includes two items: one designed to measure the intensity of bodily pain and one measuring the extent of interference with normal work activities as a result of bodily pain. Low scores indicate high levels of pain significantly affecting normal activities, whilst high scores indicate no pain or impact on normal activities.

General health (GH): The GH scale includes five items, including a rating of health from poor to excellent, and four items addressing the patient's opinion and expectations of his/ her health. Low scores indicate a perception of poor general health, which is expected to deteriorate. High scores indicate a perception of good general health.

Vitality (VT): This scale includes a four-item measure of vitality (i.e. energy level and fatigue). Low scores indicate feeling tired and worn out the majority of the time, whilst high scores reflect feeling energetic most of the time.

Social functioning (SF). This domain includes 2 aspects; designed to assess health-related physical or emotional problems on the quantity and quality of usual social activities.

The low scores indicate extreme or frequent interference of health-related physical or emotional problems with normal social activities.

Role-emotional (RE). This domain includes a three-item scale to assess the impact of mental health-related problems with respect to limitations related to time spent on work or other usual activities. Low scores reflect problems with work or other usual activities as a result of emotional problems and high scores reflect no such limitations due to emotional problems.

Mental health (MH). This five-item MH scale includes anxiety, depression, loss of behavioural and/or emotional control, and psychological wellbeing. Low scores reflect frequent feelings of nervousness and depression all or most of the time, whilst high scores indicate feelings of peace, happiness, and calm.[177]

Each domain or scale is scored individually, with the result expressed in comparison to American norms. Higher scores represent a better QoL and a change of 10 points or more in a domain between administrations is considered clinically significant.

Cardiac assessment

An electrocardiogram (ECG) and echocardiogram were performed as part of the routine baseline assessment to ascertain whether there was cardiac involvement. Cardiac biomarkers including an NT-proBNP and troponin T were performed additionally at baseline and NT-proBNP at consequent follow up visits. A cardiac MRI was also performed either locally or at the Heart Hospital, London for additional assessment to evaluate and discern cardiac involvement and consequent monitoring of improvement/deterioration.

Functional assessment

Patients were evaluated using the Eastern Co-operative Group (ECOG) performance status (Table 2.2).[178]Cardiac heart failure symptoms are also categorised accordingly and were recorded as the New York Heart Association Classification (NYHA) symptoms (table 2.3).[179]

Table.2.2: Definition of the Eastern Co-operative Group Performance Status (ECOG)[178]

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 activity
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 50% of waking hours
4	Immobile	Unable to carry out self-care, completely confined to bed or chair

Table 2.3: Definition of New York Heart Association Classification (NYHA)[179]

NYHA Class	Summary	Description
I	Normal	No limitation of physical activity. Ordinary physical activity does not cause shortness of breath or undue fatigue
II	Mild	Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in fatigue, palpitation or dyspnoea
III	Moderate	Marked limitation of physical activity. Comfortable at rest but less than ordinary activity causes fatigue, palpitation or dyspnoea
IV	Severe	Unable to carry out any physical activity without discomfort. Symptoms of cardiac insufficiency at rest. If any physical activity is undertaken, discomfort is increased

Electrocardiogram

This was performed in all patients for baseline features with some patients having a history of ischemia heart disease, but also assessing for specific features such as low voltage amplitude defined by a mean QRS amplitude less than 0.5mV in leads I,II, III, aVL and aVF. [180]

Electrocardiography

Echocardiography was done on commercially available instruments by GE Healthcare: Vivid E9 (S/N VE94922, VE94921) and Vivid S6 (S/N 4544VS6) using the EchoPac workstation. Standard LV systolic and diastolic function values were collected using 2D and Doppler echocardiography. Determination of the left ventricular wall thickness, left ventricular diastolic function, left ventricular systolic function and atrial diameter were measured according to the guidelines from the British Society of Echocardiography. The tricuspid annular plane systolic excursion (TAPSE) was

determined by using an apical 4-chamber view; measured by the total displacement of the tricuspid annulus (in mm) from end diastole to end systole. There were 3 operators, with intra-observer variability reduced by re-examination of all echocardiograms by one operator. Normal echocardiographic parameters were according to the current guidelines.[181]

Assessment criteria for diagnosis of amyloid and assessment of organ response

The definition of organ involvement in systemic amyloidosis and organ responses were defined according to the Consensus criteria (Table 2.4). Assessment of haematologic response was also assessed and monitored for those patients diagnosed with systemic light chain amyloidosis. (Table 2.5)[87]

Table 2.4: Definition of Organ involvement and organ response in amyloidosis[86]

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) creatinine and creatinine clearance must not worsen by 25% over baseline
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 (>20mmHg fall in systolic BP)	Clinical Assessment

mm – millimetres; CMR – cardiac MRI; g – grams; SAP – Serum Amyloid P; Hg – mercury; BP – blood pressure

Table 2.5: Consensus Haematologic Response in systemic light chain amyloidosis[87]

Haematologic Response	***Criteria***
Complete Response (CR)	Normal serum free light chain ratio with negative serum and urinary immunofixation
Very good partial response (VGPR)	The difference in the free light chains (dFLC) less than 40mg/L
Partial Response (PR)	A reduction in the dFLC greater than 50%
No response	A less than 50% response in dFLC

mg/L – milligrams per litre

Statistical analysis

Statistical analysis was performed using SPSS v20 (IBM SPSS) software. Kaplan Meier estimates were used to calculate the OS and PFS. P values <0.05 were considered statistically significant. Graph Pad Prism (Version 5) was also used. The statistical analysis is described in each methods/results section of each chapter.

Results Section One:

Diagnostic investigations and prognostic implications of amyloidosis

Chapter Three: Comparison of free light chain assays: Freelite™ and N Latex in diagnosis, monitoring and predicting survival in light chain amyloidosis

This chapter is written in context of my article: Comparison of free light chain assays: Freelite™ and N Latex in diagnosis, monitoring and predicting survival in light chain amyloidosis. Shameem Mahmood, Nancy L Wassef, Simon J Salter, Sajitha Sachchithanantham, T Lane, D Foard, Carol J Whelan, Helen J Lachmann, Julian D Gilmore, Philip N Hawkins, Ashutosh D Wechalekar, American Journal of Clinical Pathology, 2016 Jul;146 (1):78-85 (Original article) copyright permission obtained from Oxford University Press, license no.:3917120648467 for use in my thesis.

Introduction

Systemic AL amyloidosis is a rare protein deposition disease. The underlying pathological process driving AL amyloidosis is an underlying clonal proliferation of plasma cells producing an excess of unstable immunoglobulin light chains. There is deposition of immunoglobulin light chains in tissues and organs leading to impairment of the structure and function of the latter.[6, 182] Tracking and monitoring AL amyloidosis is a challenge as many patients have barely a detectable intact monoclonal immunoglobulin. However, the discovery that serum free light chains could be measured in 2000 led to a development that revolutionised this rare disease. The serum Freelite™ assay measures immunoglobulin free light chains using polyclonal

sheep antibodies directed against hidden epitopes on the light chain molecule giving an accurate measurement of serum free kappa and lambda. Our group initially reported the utility of this assay in 2002[183] in this disease, with many studies following since. This assay now forms a standard part of the baseline and serial follow up assessments in systemic AL.[161, 162] It is a part of the revised Mayo staging system and standard international consensus criteria for the disease response assessment.[80, 86, 87]

Polyclonal antibodies to light chain epitopes in the Freelite™ assay continues to be prepared from specifically immunised sheep. However, in any naturally occurring antibody mixture, standardisation remains complex and inter-assay variability is a certainly a possibility. Efforts have been made to develop monoclonal antibodies to the hidden light chain epitopes that would recognise and replicate the success of the polyclonal assay and thus allow better inter-assay standardisation. A group in the Netherlands working with Siemens, Germany, has developed this technology using a mixture to two kappa and two lambda monoclonal antibodies to the hidden epitopes of constant region of the immunoglobulin light chain molecules.[163] The references ranges for both the new N-Latex assay and the polyclonal Freelite™ assay are similar.

With increasing adoption by laboratories of the novel assay, it is important to assess the utility of the new assay compared to the current reference standard polyclonal free light chain assay. We report a comparison of serum free light chains measured by both immunoassays at diagnosis and three further time points in during the initial chemotherapy for patients with systemic AL amyloidosis.

Methods

This study was conducted at the National Amyloidosis Centre, London, UK (NAC), with recruitment of consecutive patients with systemic AL amyloidosis seen at the NAC and under prospective follow up from January 2011 and April 2012 and undergoing chemotherapy for AL amyloidosis. Patients were included if they had a baseline serum sample at initial diagnosis and at least 3 of 4 blood samples available including from baseline 2, 4 and 6 month follow up point intervals following treatment with chemotherapy. Diagnosis of amyloidosis was confirmed in all cases with a tissue biopsy showing the characteristic birefringence on Congo red staining. Typing of AL amyloidosis was confirmed by immunohistochemical staining with appropriate antibodies and exclusion of hereditary amyloidosis, where necessary, by genetic sequencing of the genes implicated. All patients underwent systematic review at presentation and detailed follow up assessments at 6 monthly intervals or dependant on clinical indication. Assessment included clinical examination, detailed blood and urine analysis (including assessment of serum and urine monoclonal immunoglobulin and serum free light chains), serial ^{123}I labelled serum amyloid P component (SAP) scintigraphy to assess whole body amyloid load, electrocardiogram (ECG) and an echocardiogram and routine observations including blood pressure, oxygen saturations and pulse. Organ involvement was defined according to the standard international amyloidosis consensus criteria.(18) Mayo disease stage was defined according to the criteria published by Dispenzieri et al and Kumar et al[80, 184]

Serum samples were separated and stored at -80ºC. All retrieved samples were tested in duplicate for measurement with Freelite™ (The Binding site Ltd, Birmingham, UK) and N-Latex (Siemens Healthcare Diagnostics, Germany) according to the standard manufactures' protocols on a BN™II System nephelometer (Siemens,

Germany). Examination was undertaken to assess haematologic response on results of the free light chain (FLC) assays in available samples at the following points including 2 months; 90 Freelite™ and 90 N Latex, 4 months; 91 Freelite™ and 89 N Latex, 6 months; 61 Freelite™ and 62 N Latex. The discrepancies in the number of available samples for analysis were secondary to the inadequate quantity of serum or error readings in the analysed sample, hence excluded for analysis. In total, serum free light chains were measured in a total of 240 serum samples in duplicate using both the Freelite™ and N Latex assays.

We performed a correlation analysis between the 2 FLC assays of results for kappa, lambda and kappa/lambda ratio. Haematologic response was assessed according to the international amyloidosis consensus criteria.[86, 87] The same criteria were used to assess a patient as a responder or not by both assays; although we recognise that the criteria were developed with results obtained from the Freelite™ assay. This study was not powered or focused to develop new response criteria for the N-latex assay. Statistical analysis was performed using SPSS software using a p value of less than 0.05 considered to be significant. We stated median values with minimum and maximum ranges. We calculated the Pearson's coefficient to evaluate the agreement and concordance between the both FLC assays, with use of scatter plots to illustrate this. Kaplan Meier curves were used to assess and describe the prognostic utility of both assays.

Results

94 patients were identified from the National Amyloidosis database over this period of time. The median age was 64 years (range 55.2 -72.2 years) at diagnosis and 48 (51%) were male. Cardiac involvement was present in 43%; 23% Mayo stage 3. The baseline patient characteristics and organ involvement features are described (table 3.1). Of the patients in this group, 74 (78.7%) had either a monoclonal protein detectable in the serum, urine by immunofixation or the presence of an abnormal free light chain ratio. 50(53.2%) of this group had a measurable monoclonal protein greater than or equal to 1g/L. The revised Mayo disease stage incorporates the dFLC, using FLC measured by Freelite™/N-Latex assays are as follows: Stage 1: 20.2%/25.5%, Stage II: 47.9%/43.6%, Stage III: 29.8%/30.9%, and Stage IV 2.1%/0%, respectively

The FLC in baseline samples showed an abnormal kappa in 41% and lambda in 63% by the Freelite™ assay, and abnormal kappa in 32% and 67% by N Latex assay. The kappa and lambda light chain: 17 (18%) and 75 (82%) respectively. The Freelite™ assay showed a median kappa of 17.3mg/L (range 0.3-1440), median lambda 48.8mg/L (range 7.5-1430) with median difference in involved and uninvolved light chains (dFLC) of 43.2 mg/L (range 55-247.9). The median abnormal kappa was 34.75mg/L (range 0.3-1440) with abnormal lambda 93.85mg/L (range 27-1430). The N latex assay had corresponding concentrations as follows: median kappa 16mg/L (range 2.35-770), median lambda 52.6mg/L (range 3.65-786) and median dFLC 39.1 mg/L (range 58.3-247). The median abnormal kappa by the N-Latex assay was 28.1 (range 2.35-770) with abnormal lambda 77.9 (3.7-786).

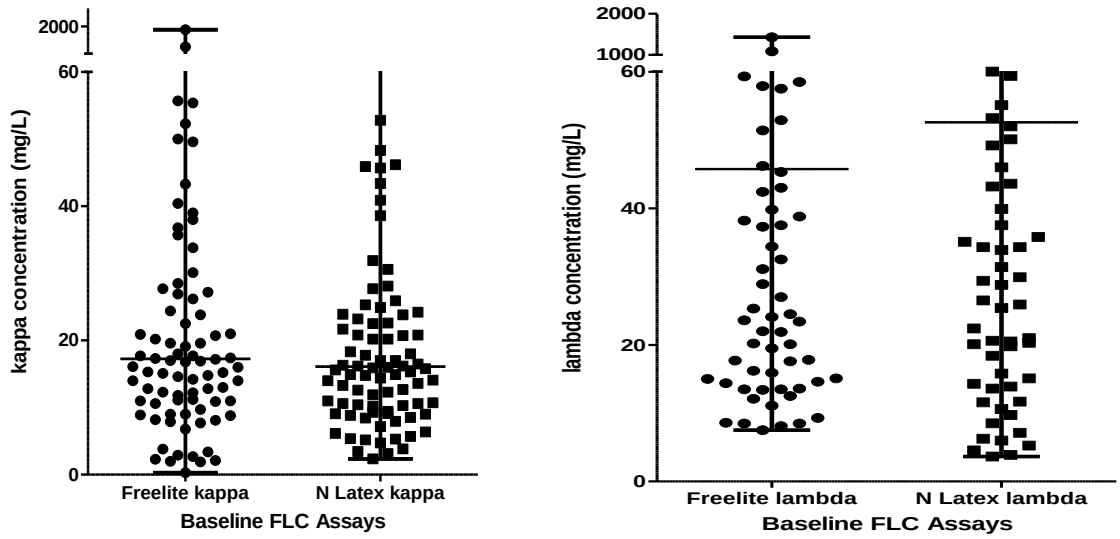
Table 3.1: Patient characteristics

Patient Characteristics	Number (range of percentage)
Age	64 (range 55.2-72.2)
Male	48 (51%)
Kappa clone	17 (18%)
Lambda clone	75 (82%)
Serum paraprotein	50 (53.2%)
Organ involvement	2 (range 1-3)
Cardiac involvement	41 (43%)
Renal involvement	72 (76%)
Liver involvement	10 (10%)
Peripheral neuropathy	13 (14%)
Autonomic Neuropathy	8 (8%)
Soft tissue	16 (17%)
Cardiac Mayo Stage I	25 (26%)
Mayo Stage II	43 (46%)
Mayo Stage III	26 (28%)
Clonal parameter	**Value (range)**
Freelite™ Kappa (mg/L)	17.3 (0.3-1440)
Freelite™ Lambda (mg/L)	48.8 (7.5-1430)
Freelite™ dFLC (mg/L)	43.2 (55-247.9)
N Latex Kappa (mg/L)	16 (2.35-770)
N Latex Lambda (mg/L)	52.6 (3.65-786)
N Latex dFLC (mg/L)	39.1 (58.3-247)

mg/L – milligrams per litre; dFLC – difference between the involved and uninvolved free light chains; reference range for Freelite™ assay – kappa 3.3-19.4mg/L, lambda 5.7-26.3mg/L, kappa/lambda ratio 0.26-1.65; reference range for N-Latex assay – kappa 6.7-22.4mg/L; lambda 8.3-27mg/L; kappa/lambda ratio 0.31-1.56mg/L.

There was not a significant difference in the absolute kappa and lambda values seen by either assay (kappa median 34.8 vs. 28.1 p = 0.64; lambda median 93.85 vs. 77.9; p = 0.41) (Fig 3.1A and B respectively).

Figure 3.1: Comparison of Freelite™ and N Latex assays with respective kappa (A) and lambda (B) values.



The concordance between the two assays was correlated by categorising the results into serum kappa, serum lambda and kappa: lambda ratio as abnormally high, normal and abnormally low as per the normal ranges by each manufacturer (Table 3.2).

Table 3.2A: Kappa at presentation

	Freelite™ kappa (mg/L)			
N Latex Kappa (mg/L)		<3.5	3.5-19.4	>19.4
	<6.7	7		
	6.7-22.4		41	
	>22.4			29

Concordance 85.5%

mg/L – milligrams per litre

Table 3.2B: Lambda at presentation

	Freelite™ lambda (mg/L)			
N Latex Lambda (mg/L)		**<5.7**	**5.7-26.3**	**>26.3**
	<8.3	0		
	8.3-27		17	
	>27			51

Concordance 75.5%

mg/L – milligrams per litre

Table 3.2C: Kappa/Lambda ratio at presentation

	Freelite™ κ/l ratio (mg/L)			
N Latex κ/l ratio (mg/L)		**<0.26**	**0.26-1.65**	**>1.65**
	<0.31	32	37	18
	0.31-1.56	37	27	
	>1.56	12		12

Concordance 78.8%

κ/l – kappa/lambda; mg/L – milligrams per litre

The concordance for the serum kappa light chain was 85% with R^2=0.91 and the Pearson correlation co-efficient r=0.804 (p = 0.0001). The concordance for serum lambda was 75.5% with R^2=0.52 and he Pearson correlation coefficient r=0.50 (p=0.0001). The concordance for the kappa/lambda ratio for the respective values 78.8%, R^2=0.87 and r=0.97 (p=0.0001) (Fig 3.2A, 3.2B and 3.2C respectively and Table 3.4).

Figure 3.2: Scatter plots of N Latex and Freelite™ free light chain assays for (A) kappa N Latex (n=91) and Freelite™ (n=91) and (B) lambda N latex (n=90) and Freelite™ (n=90) and (C) kappa/lambda ratio (n=90) illustrating the concordance of both free light chain assays.

Figure 3.2A

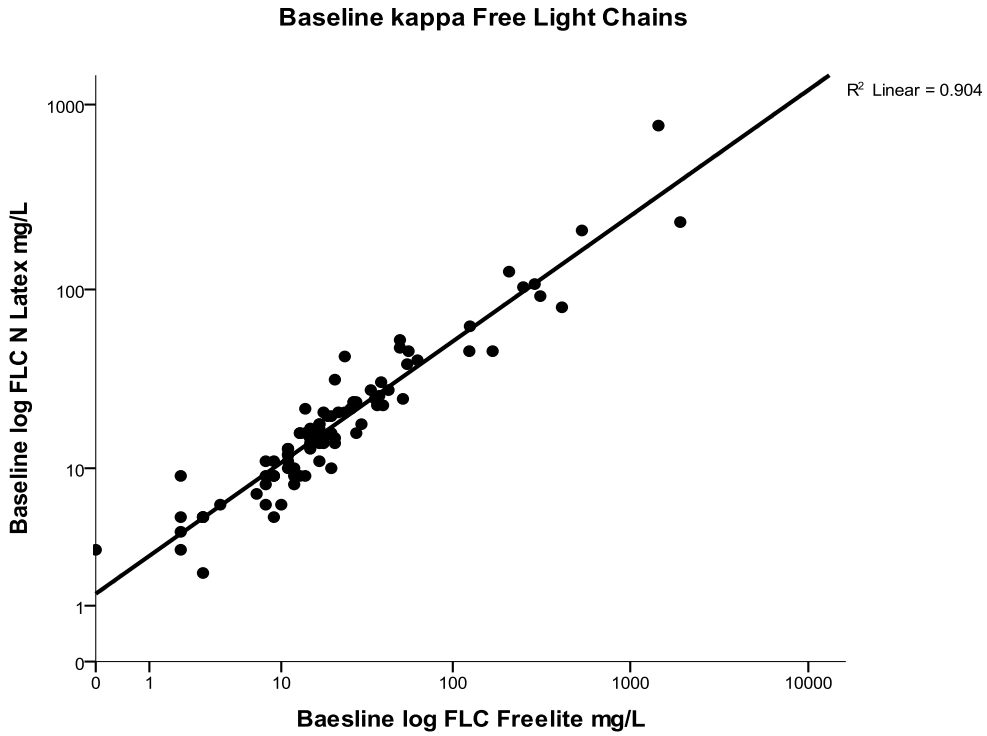


Figure 3.2B

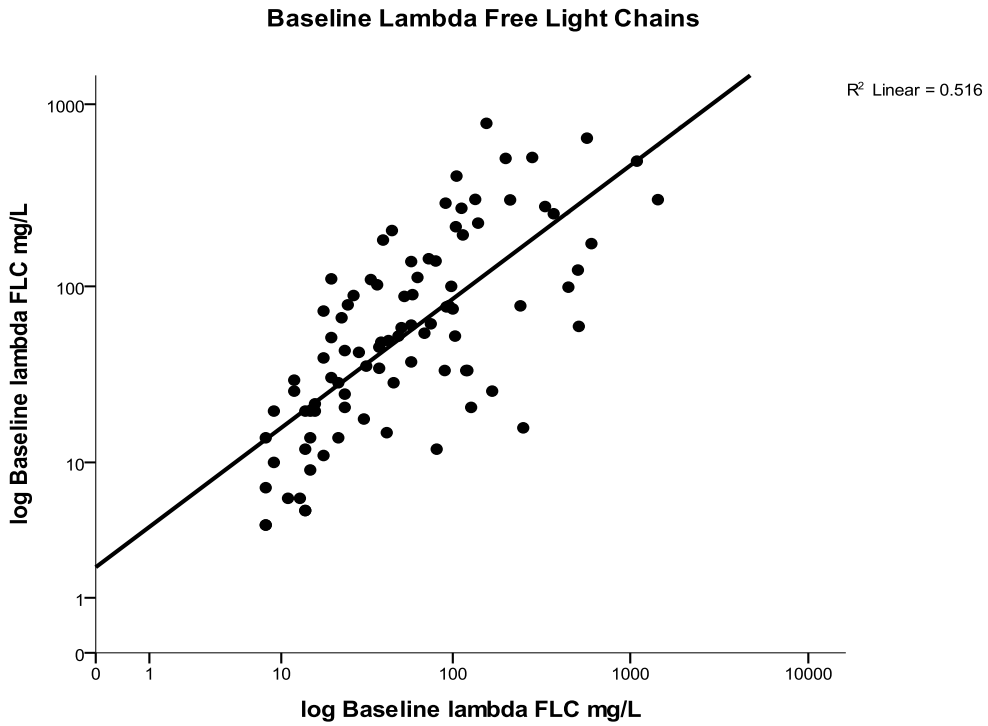


Figure 3.2C

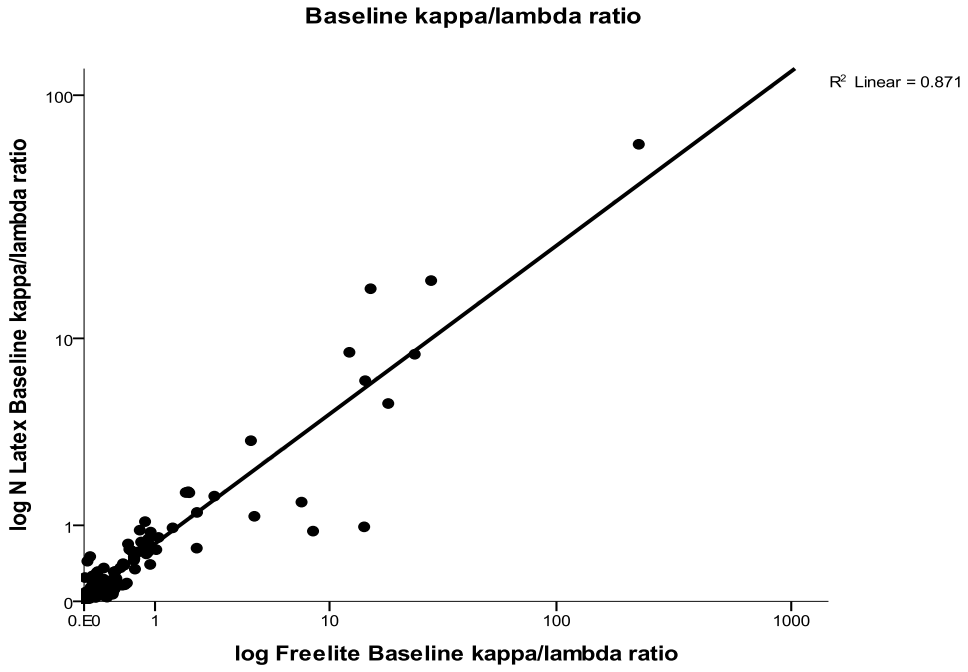


Table 3.3: Kappa/Lambda ratios at presentation

		N Latex Ratio		
		Normal	**Abnormal**	**Total**
Freelite™ Ratio	**Normal**	27	11	38
	Abnormal	10	42	52
	Total	37	53	90

Table 3.4: Concordance analysis for kappa (A), lambda (B) and kappa/lambda ratio (C) for the 2 FLC assays

Group	**Number**	**N Latex FLC (mg/L)**	**Freelite™ FLC (mg/L)**	**Pearson's correlation**
FLC κ	90	16.1 (2.35-770)	17.3 (0.3-1440)	0.804
FLC l	90	52.6 (3.65-786)	48.8 (7.5-1430)	0.493
κ/l ratio	90	0.68 (0.01-63.56)	0.88 (0.0002-224.7)	0.97

FLC – Free light chain; mg/L – milligrams per litre; κ – kappa; l – lambda

There were discordant kappa/lambda ratios at presentation: 10/90 abnormal by Freelite™ but normal by N Latex assay, and 11/90 abnormal by N Latex and normal by Freelite™ (Table 3.3). 10 patients had an abnormal κ/l ratio by Freelite™ assay (normal by N Latex assay): positive IFE by both urine and serum (n=4), positive IFE only by serum (n=2) and positive IFE only by urine (n=3), negative IFE by urine and serum (n=1). There were 11 patients with an abnormal κ/l ratio by N Latex assay (normal by Freelite™ assay): with positive IFE by both urine and serum (n=3), positive IFE by only serum (n=3), positive IFE by only serum (n=4) and negative IFE by urine and serum (n=1).

The diagnostic sensitivity (true positive) and specificity (true negative) with agreement of the κ/l ratios of the FreeliteTM and N Latex assays were calculated and recorded (Table 3.5 and Table 3.6).

Table 3.5: Clinical sensitivity and specificity of the Freelite^{TM} assay and immunofixation electrophoresis (IFE)

	IFE (serum and urine)		
Freelite^{TM} κ/l ratio	Positive	Negative	Total
Abnormal	44	7	51
Normal	37	2	39
Total	81	9	90

Sensitivity 54.3%, Specificity 86.2%

Table 3.6: Clinical sensitivity and specificity of the N Latex assay and immunofixation electrophoresis (IFE)

	IFE (serum and urine)		
N Latex κ/l ratio	Positive	Negative	Total
Abnormal	46	7	53
Normal	35	2	37
Total	81	9	90

Sensitivity 56.7%, Specificity 86.8%

We evaluated the clinical impact of the differences in assessing haematologic response according to the revised Consensus criteria[87] of those with measurable dFLC at 2, 4 and 6 months post chemotherapy treatment with serum samples available at these time points. The number of patients with samples available: at 2 months - 90 Freelite™ and 90 N Latex, at 4 months - 91 Freelite™ and 89 N Latex, at 6 months - 61 Freelite™ and 62 N Latex. At baseline 54 (60%) and 51 (56.7%) had a dFLC >50 mg/L (minimum defined to assess the free light chain response). Correlating responses of Freelite™ and N Latex assays respectively, at 2 months, a complete response (CR) was achieved in 23% and 32%, partial response (PR) in 17% and 14% and no response (NR) in 57% and 49% of the evaluable patients. There was a subtle discrepancy in the proportion classed responders at each time point as depicted in fig 3.3A and 3.3B. Assessing a final response assessment at six months, 11.5%, 4.9% and 0% were discordantly classed PR, VGPR and CR. At 2 months, an abnormal kappa/lambda ratio was present in 45.6% by Freelite™ and 46.7% by the N Latex assays. Monitoring of the kappa/lambda ratio by both assays produced similar results, with subtle differences at 4 months, with 34.4% and 38.2% of patients with available serum samples having an abnormal kappa/lambda ratio by Freelite™ and N Latex assays. At 6 months, 30% and 36.1% of patients had abnormal kappa/lambda ratios by Freelite™ and N Latex assays respectively (figure 3.3).There was an “excess” of patients classed a partial, VGPR or complete response at the following 2 months (21%), 4 months (10.1%) and 6 months (6.5%) by N-Latex assay relative to the Freelite™ assay. This was calculated by adding the total number of patients achieving greater than a PR response by the N Latex assay and subtracting those equivalent responders by the Freelite™ assay. This calculated number was then divided by the N-Latex total at that time point giving an overall percentage.

Figure 3.3A and 3.3B: Difference in involved and uninvolved free light chain (dFLC) response in patients with evaluable disease. (A) Percentage of evaluable patients assessed at 2, 4 and 6 months according to haematologic response including no response (NR), partial response (PR), very good partial response (VGPR) and complete response (CR) with (B) illustrated the differences of the abnormal kappa/lambda ratio at set intervals between the Freelite™ and N Latex assay.

Figure 3.3A

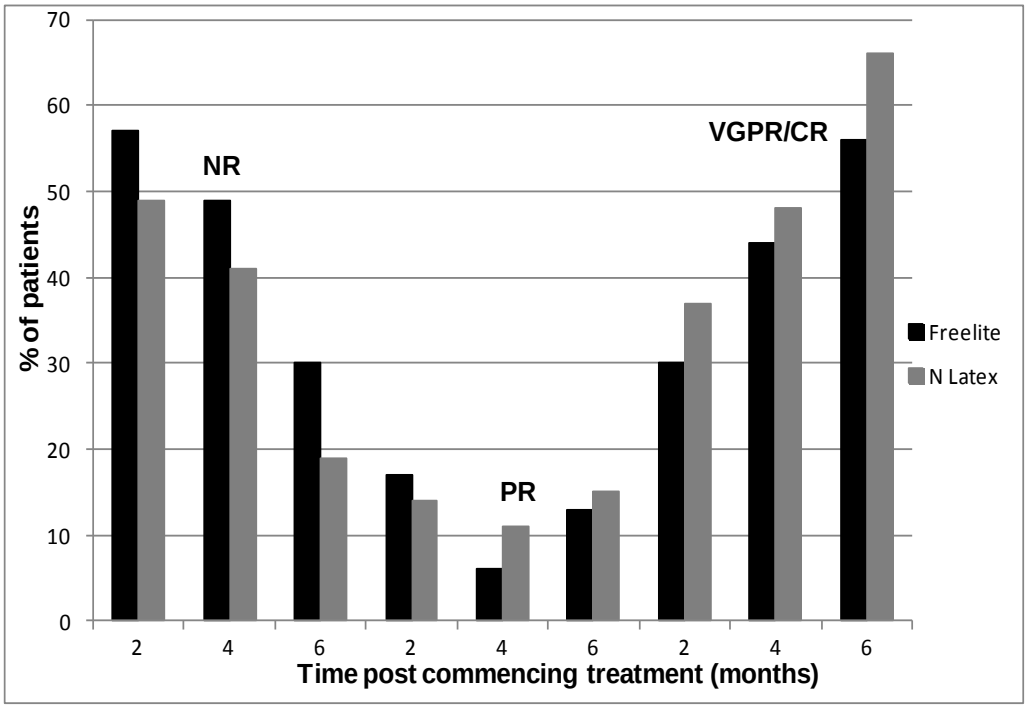
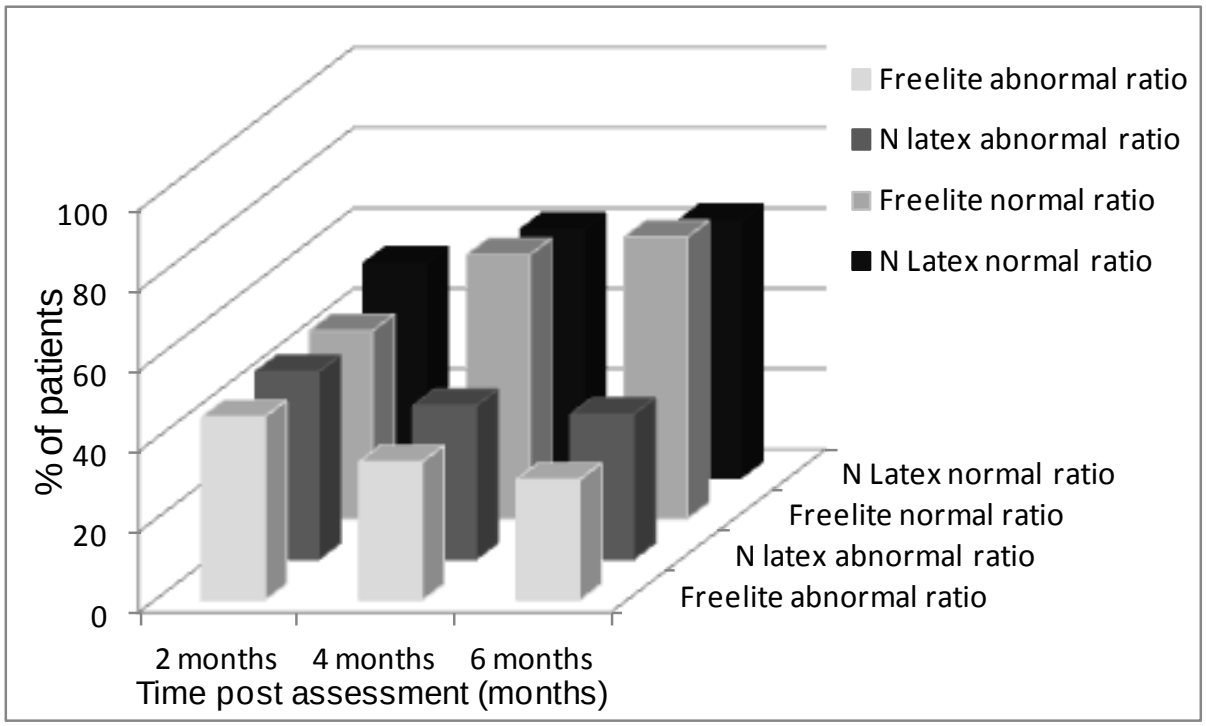


Figure 3.3B



The estimated overall survival (OS) for the 94 patients was 24.1 months with a median follow-up of 8.6 months. Given the differences in the proportion of patients classed as achieving partial response by both assays at 4 months, we evaluated the overall survival of patients achieving a partial response or greater at 4 months by both assays. There was a significant improvement in the overall survival for patients classified as achieving a partial response or better at 4 months by each assay. The median OS was not reached in either assay. The survival benefit for those achieving a PR or better by the Freelite™ at 4 months was HR 7.84, 95% CI (0.94-65.57), log rank p = 0.03 and by the N-Latex assay HR 95.47, 95% CI (0.11-79601), log rank p = 0.004.

Discussion

We report here a comparative utility of using two different assays (one using polyclonal anti-light chain antibodies and other using a combination of monoclonal anti-light chain antibodies) for detection of and serial monitoring of serum free light chains in patients with systemic AL amyloidosis undergoing chemotherapy. This study finds the two assays are broadly comparable for detection of abnormal light chains in the serum with a greater concordance for detection of kappa than for lambda immunoglobulin free light chain. However, there were discrepancies present with the numbers abnormal, the absolute light chain value and time point to reach thresholds for haematological responses and also impact on survival.

The Freelite™ assay was the only assay available for measurement of serum free light chains until development of the N-Latex assay. This is a new and welcome development to allow for a different method to study these complex molecules, but standardisation is needed between the two assays. Both these assays have been studied in myeloma as well as normal controls to assess imprecision, underestimation; antigen excess or non-linear reactivity and overestimation; polymeric forms reacting at multiple antigenic FLC sites of results.[185-187] Our study did not focus on the biochemical or technical aspects of either assay, but designed to assess the clinical utility of each assay in a routine clinical setting in systemic where the serum free light chains are typically abnormal, but low and where FLC measurements forms the cornerstone of patient assessment and monitoring. Previous studies in patients with a monoclonal gammopathy, have the kappa showing the best correlation between both FLC assays, followed by kappa/lambda ratios then lambda, with the Pearson coefficients 0.97, 0.83 and 0.91 respectively.[188] Pretorius et al compared the utility of

both the Freelite™ and N Latex assays in 116 samples showing large variations between both assays, precluding the interchange of these results, with statistically significant non-linearity occurring in approximately half of the monoclonal and polyclonal samples (kappa and lambda) for both assays.[189] The precision of the two assays were further examined in two other studies, showing comparable levels[190] and reproducibility.[191]

The current study confirms that in systemic AL amyloidosis, there is generally excellent concordance between both the assays in detecting an excess of serum free light chains with better concordance of kappa than lambda as previously reported. The overall concordance in our study showed kappa, lambda and kappa/lambda ratios as 85.5%, 75.5% and 78.8% respectively - similar to one study 70-80% [188] but lower than in another study with the concordance quoted as approximately 90%.[163] The diagnostic sensitivity with respect to the serum and urine IFE results was lower than expected by both assays, 54.3% and 56.7% by Freelite™ and N Latex assays respectively. Some patients had lower serum free light chain values at diagnosis, with the presence of serum and/or urine IFE, clinical symptoms and diagnostic biopsy needed to prove the diagnosis in these cases. The serum free light chains are often lower than in Myeloma patients. A concerning result was that ~10% cases with each assay detected an abnormal FLC ratio that was not detected by the other assay. The underlying reason for this discordance is difficult to fully explain or ascertain, given the details of antibodies used to detect the free light chains in both assays are commercially confidential. Given the nature of the monoclonal disease underlying AL amyloidosis, the mutational signature of the plasma cell DNA in each AL light chain is unique. A likely explanation is the antibodies in each assay do not recognise the epitopes on the respective light chains and hence there is a false negative signal. The

Mayo clinic group, has recently demonstrated that such samples also contain a monoclonal light chain using mass spectrometry. Hence suggesting that currently neither method is perfect for detection of all abnormal monoclonal free light chains but as different patients “detected” or “missed” by either assay, there may be role for repeating the FLC measurement by the other assay if an abnormality is not detected in a patient with one assay. This may assist at least partly, overcome the problem in AL amyloidosis of lack of evaluable light chains in a certain proportion of all patients.[183] We compared the κ/l ratios and the IFE results showing that both assays had similar sensitivities (54.3% and 56.7%) and specificities (86.2% and 86.8%) for Freelite™ and N Latex assays respectively.

This is the largest study to examine serial parallel sample assessment to assess the serum free light chain responses by both assays simultaneously. Although there is broad agreement in the response assessment as a percentage of total patients with both assays, important differences are present in a proportion of patients who reach the currently threshold used to define a partial or very good partial response. Eighteen patients reached a PR and VGPR earlier by the N-Latex assay compared to the same class of response by the Freelite™ assay. This is concerning, but in a way not that unexpected. The assays have very different antibodies which measure the free light chain in the serum and hence the actual slopes of the standard curves are not exactly identical. Hence, it is expected that the rate of change will be different. The response criteria in AL amyloidosis have been derived using ROC analysis based on survival of patients using various light chain thresholds attained by the Freelite™ assay. Thus the current thresholds are clinical and are not biochemical end points. Assessing the results, it appears that the N-Latex assay classified patients as responders at an earlier time point than the Freelite™ assay, designing a study to examine a clinically

relevant threshold for defining a response with N-latex is important. Similarly prognostic important thresholds of FLC in AL (dFLC >180 mg/L) may not necessary be the same and will need re-defining for this new era. In this study, 3 patients were misclassified as advanced stage III-IV by the revised classification using the different assays.

It is encouraging that the classification of a patient as a haematological responder (partial response or greater) translates into a survival benefit. However, earlier classification of a greater proportion of patients as responders by the N-Latex assay has risks, as treatment duration in amyloidosis is often shorter than in myeloma with a lower threshold to stop treatment earlier in the responders particularly if there are problems with tolerance to treatment. Unless thresholds are redefined, patients may be classified as responders by the N-Latex assay may get undertreated and hence carry a risk of shorter time to disease progression.

This current study needs to be interpreted cautiously in the context of certain limitations. The numbers are still small and all patients did not have serial samples available. Although the patients recruited in this study are serial AL patients seen at the NAC, there is a selection bias as all patients were required to have serial samples for six months, with only six month survivors are recruited thereby excluding a substantial number of patients with stage III cardiac AL amyloidosis where there is still 30-40% mortality in the first six months.[192] A study of truly unselected serial patients is needed to assess the true correlation of the utility of both these assays. There was higher than expected proportion of patients with non-evaluable light chains, possibly due to selection of the survivors who are typically patients with early stage disease and serum free light chain burden.

In summary, both Freelite™ and N Latex assays are able to detect the abnormal FLC in patients with systemic light chain amyloidosis. Although there is an excellent correlation between these assays for detecting the abnormal light chain subtype the discordance in the absolute values renders cross assay interpolation impossible. Furthermore, each FLC assay appears to have limitations, in missing random yet different patients suggesting a further need by both manufacturers to optimise the anti-light chain antibodies underpinning the respective assays. Studies using clinical end points are important to harmonise the response assessment criteria for each assay so that even though the values may not be comparable, the response categorisation is comparable. This is crucially important, given the emergence of the newer assay becoming more widely adopted and possibly raising problems in the interpretation of clinical and trial results. Designing future clinical trials in AL amyloidosis incorporating FLC measurement by both assays would be ideal to aid in our understanding and harmonisation of the field.

Chapter Four: Bleeding diathesis, thrombotic tendencies and endothelial dysfunction in amyloidosis patients

This chapter is written with reference to the publication: Utility of factor X concentrate for the treatment of acquired factor X deficiency in systemic light-chain amyloidosis. Shameem Mahmood, Julie Blundell, Anja Drebes, Philip N. Hawkins and Ashutosh D. Wechalekar. Blood. 2014; 123(18):2899-900 (Original article).

Introduction

Systemic light chain (AL) amyloidosis is the most common systemic amyloidosis. Typically the clonal proliferation of a plasma cell clone that consequently results in the synthesis of an excess of light chains and toxic intermediates with specific structural and mechanical effects resulting in systemic fibril toxicity.[193, 194] These fibrils deposit in different organs leading to progressive changes in the affected organ's architecture and function.[6] Severity of this disease depends on the number of organs involved and extent of amyloid deposits, with AL cardiomyopathy carrying a poor prognosis with death arising as a consequence secondary to progressive heart failure, contractility dysfunction, sudden death and associated arrhythmias or refractory untreatable disease.[195, 196] Some studies have explored the AL-light chain cardiotoxicity as an important causal determinant in this disease.[197, 198] Migrino et al demonstrated that AL light chains cause microvascular dysfunction leading to apoptotic and necrotic insults and related oxidative stress,[199, 200] with other studies showing endothelial dysfunction

in small and medium sized vessels, which ultimately may contribute to this cardiotoxicity.[201, 202]

Endothelial dysfunction occurs with reduced bioavailability of vasodilators, especially nitric oxide (NO) and consequent impairment of the endothelium and increased endothelium contracting factors and pro-inflammatory state[203] As such, it is associated with coronary artery disease, hypertension, diabetes mellitus, chronic renal failure and thought to represent a vital early trigger in the event of atherosclerosis[204, 205] with a complex interplay of low NO levels, increased oxidative stress and consequent changes in the V-CAM, I-CAM and e-selectin adhesion molecules in this inflammatory process[205, 206] Of several methods used to assess the endothelium, plasma proteins such as vWF have been examined extensively in cardiovascular disease and atherosclerosis[207-210] and used as a surrogate marker of endothelial dysfunction. Numerous clinical and experimental reports imply that high vWF levels reflect endothelium damage.[211]

Von Willebrand factor is a large glycoprotein synthesised by the vascular endothelial cells through the luminal and abluminal membranes[212] and megakaryocytes. Secretion via the abluminal membrane results in some deposition within the vascular subendothelium to bridge with circulating platelets[212] and forms a non-covalent tightly bound complex with factor VIII. Hence circulating levels of factor VIII are closely linked to circulating levels of vWF, typically 1 molecule of FVIII to 50-100 vWF subunits.[213] Von Willebrand factor has a dual role in haemostasis: promoting platelet adhesion to thrombogenic surfaces and ensuring platelet-platelet cohesion during thrombus formation, and acting as a carrier for FVIII; important in its production, stabilisation, conformation and immunogenicity.[214] Factor VIII serves a role in the intrinsic coagulation pathway as a co-factor to accelerate the activation of factor X by factor

IXa in the presence of calcium ions on a phospholipid surface.[215] Thus the impact of this intricate complex and its equilibrium may give us insight into the endothelial dysfunction of this disease, but also possible thrombogenic and survival risk factors.

Systemic light chain (AL) amyloidosis is also known to be associated with a bleeding diathesis,[164] which may range from small cutaneous bruising, pathgnomic “racoon eyes” to life threatening bleeds. There are a multitude of underlying factors which may be responsible for the coagulation abnormalities in this disease including: factor deficiencies and prothrombotic factors, vessel fragility, amyloid load, amyloid fibrils and light chain burden[216-218]. Factor X deficiency has been extensively described in the literature with an incidence of 6.3-14% in systemic AL patients[164, 218]. There is limited data as to the clinical bleeding manifestations and the underlying coagulation factors which may contribute to this environment.

This prospective study was designed to investigate the haemostasis and endothelial dysfunction in newly diagnosed systemic light chain (AL) amyloidosis patients, with further exploration of light chain toxicity in this disease. This was performed examining the clinical bleeding manifestations using the Royal Free Hospital v4 adapted bleeding questionnaire in conjunction with extensive laboratory investigations such as factor assays, protein S, protein C, Anti-thrombin III, vWF:Ag and VWF:Ricof and ADAMTS13 assays. Endothelial function was explored using vWF antigen (AG) and factor VIII to serve as a surrogate marker and examine its relationship with light chain toxicity. The secondary aim was to assess the prognostic utility of these investigations.

Materials and methods

Patients

The study included 100 patients referred to the National Amyloidosis Centre with probable newly diagnosed AL amyloidosis between May and December 2013. Eligibility was open to all patients, with no exclusions according to age, performance status, co-morbidities and naive to previous treatments No patient had a previous history of an inherited factor deficiency or thrombotic trait. Eight patients were taking warfarin and no patients were taking the newer anticoagulant agents.

Each patient had a detailed baseline assessment of organ function with investigations including a full blood count, renal and liver function tests, serum protein electrophoresis, serum free light chains, and urinary BJP, electrocardiogram and echocardiography and ^{123}I-labelled Serum Amyloid P (SAP) scintigraphy for visceral amyloid deposition quantification. Each patient completed a Royal Free Hospital bleeding questionnaire v4 (appendix 1). Additional blood samples were taken for investigating bleeding and thrombotic tendencies in these patients. The diagnosis of amyloidosis was confirmed on histology by Congo red staining and AL type confirmed by specific immunostaining of amyloidotic tissue by antibodies to kappa or lambda light chains and exclusion of hereditary amyloidosis by gene sequencing, and ^{99m}Tc-dicarboxypropane diphosphonate (^{99m}Tc-DPD) scintigraphy for hereditary and non-hereditary transthyretin based disease. Written consent for retrospective publication of data was obtained from all patients in accordance with the Declaration of Helsinki. Organ involvement was classified according to the updated international amyloidosis consensus criteria.[87]

Haemostasis investigations

16mL of venous blood was drawn from the ante-cubital fossa vein with minimal use of the tourniquet, into 0.106M tri-sodium citrate blood collection tubes [Becton & Dickerson, Poole, United Kingdom (UK)]. All samples were centrifuged at 2000g for 12 minutes, separated into another container then centrifuged again for 12 minutes at 2000g. The platelet poor plasma (PPP) was removed, aliquoted and then stored at -45°C until testing. Unless otherwise stated all assays were carried out using an ACL TOP™ coagulometer [Instrumentation Laboratory (IL), Bedford, USA]. The following investigations were made using each patient's frozen PPP aliquots: Prothrombin time (PT) using HemosIL™ Recombiplastin 2G, activated partial thromboplastin time (APTT) using HemosIL™ SynthAsIL reagent [IL, USA), and to measure Clauss fibrinogen levels using HemosIL™ QFA thrombin reagent [IL, USA]. Thrombin times (TT) were measured using 3 unit/mL bovine thrombin [Diagnostic Reagents Ltd, Thame, UK] titrated to have a normal plasma control time of 14 seconds (s). Reptilase times (RT) were measured using Reptilase® reagent (Pentpharm, Aesch, Switzerland) also titrated so the normal plasma control had a clotting time of 14s. The measurement of extrinsic clotting factors II, V, VII, and X was by one stage PT based clotting assays and intrinsic clotting factors VIII, IX, XI and XII by one stage APTT based clotting assays. All patient clotting factor levels were measured against a laboratory standard with known levels of each clotting factor and reported in IU/dL. Von Willebrand factor antigen (VWF:Ag), antithombin activity (AT:Act), Protein C activity (PC:Act), free Protein S (PS:Free) and activated PC ratios (APC ratio) were measured using methods as previously reported.[219] The measurement of the VWF cleaving protease a disintegrin-like and metalloprotease with thrombospondin type 1 repeats – ADAMTS 13 - activity was by using the Technozym™ ADAMTS 13 activity

assay [Technoclone GmbH, Vienna, Austria] according to the manufacturer's instructions.

Statistical analysis

The primary end point was to evaluate the prognostic utility of vWF Ag and FVIII in AL patients. The median values and ranges are stated as minimum and maximum values. Differences in group characteristics were compared (continuous variables) with use of non-parametric t-test analysis with the Man Whitney test or the one way anova with Post Turkey analysis for comparison of more than one group using Graph pad prism 5 software. Correlation analysis of coagulation factors was performed using scatterplots and the Pearson correlation coefficient determined. ROC curve analysis using SPSS v20 software was used to obtain an appropriate threshold for FVIII and vWF:Ag levels for survival statistics. Overall survival (OS) was calculated from diagnosis until death or last follow-up. Survival endpoints were examined to assess the prognostic utility of the haemostasis investigations. Kaplan Meier estimates were used using SPSS v20 (IBM SPSS) to calculate the OS and PFS. All p values are 2 sided, with significance values based on a $p < 0.05$.

Results

Patient characteristics

Table 4.1 presents the baseline characteristics of all patient groups. There were 55 females and 45 males, with the median age 60.74 years (range 34-87.6). Patients were then subdivided according to their diagnosis: 74 with systemic AL, 9 with no evidence of amyloid but underlying multiple myeloma, 11 with other types of amyloidosis; including transthyretin based disease (ATTR) and localised amyloid, and 6 with no evidence of amyloid. Examining patients with systemic AL: the median age

was 64.7 years (30.9-85.6), and 39.2% were male. 60.8% had lambda light chain predominance, with the median dFLC 143.3mg/L. Cardiac involvement was present in 43.2% with Mayo stage 3 in 29 patients, and greater than 3 organs involved in 15 patients.

Table 4.1: Patient characteristics

Patient characteristics	Systemic AL amyloidosis	Localised and ATTR amyloid	Multiple Myeloma	No evidence of amyloid
Number	74	11	9	6
Male/Female	29/45	8/3	4/5	4/2
Age	64.7 (30.9-85.6)	74.1 (41.3-86.2)	40.3 (34-49.5)	49.6(45.7-76.5)
Light chain type				
Kappa	30	NA	3	NA
Lambda	45	NA	6	NA
dFLC (mg/L)	54.5 (1.8-3182)	2.1 (0.5-12.4)	523 (8.9-8287)	1.8 (0.6-36)
Organ involvement				
Heart	32 (43.2%)			
Kidney	53 (71.6%)			
Liver	12 (16.2%)			
PNS	9 (12.1%)			
ANS	9 (12.1%)			
Soft tissue	14 18.9%)			
Mayo stage 1	12 (17.6%)			
Mayo stage 2	34 (41.9%)			
Mayo stage 3	29 (40.5%)			
Number of organs involved				
1 organ	26 (40.5%)			
2 organs	28 (37.8%)			
≥ 3 organs	15 (21.6%)			
NT-proBNP ng/L	14047 (51-147634)	3645 (25-12956)	178 (33-2012)	736 (135-8067)
Creatinine (µmol/L)	106 (41-1201)	134 (74-232)	83 (57-738)	88 (64-95)
eGFR (mls/min)	53 (7-100)	47 (18-100)	61 (10-100)	83 (54-100)
Albumin (g/L)	34 (16-49)	43 (33-47)	42 (25-50)	48 (34-51)
Proteinuria (g/24hours)	3.45 (0.1-19.6)	0.2 (0.1-6.3)	1.3 (0.1-10.2)	0.1 (0.1-0.2)
CRP (mg/L)	3 (1-49)	3 (1-8)	2 (1-73)	2.5 (1-4)
PT (sec)	11.2 (9.2-28.5)	11.9 (9.6-24.9)	10.7 (10-12.3)	11.4 (10.2-17.9)
APTT (sec)	29.9 (22.6-43)	30.9 (26.6-37.7)	27.8 (23.4-29.4)	28.9 (28.2-32.6)
Fibrinogen (g/L)	4.4 (1.6-9.4)	3.5 (2.3-5.8)	3.5 (2.5-7.7)	3.2 (2.4-3.6)
vWF Ag (IU/dL)	288.5 (89-908)	240 (104-472)	260 (117-732)	130 (81-185)
FVIII (IU/dL)	260 (56-630)	208 (136-272)	226 (176-348)	137 (102-214)
FX (IU/dL)	92 (10-146)	78 (14-126)	122 (92-134)	100 (28-142)
Prot S (IU/dL)	87 (10-216)	87 (52-146)	81 (61-94)	91 (65-189)
Prot C (IU/dL)	133 (53-243)	126 (43-202)	131 (101-176)	114.5 (78-182)
ATIII (IU/dL)	98 (54-128)	97 (86-113)	107 (88-118)	107.5 (85-136)

AL – light chain; dFLC – difference between involved and uninvolved free light chains; mg/L – milligrams; per litre; PNS – peripheral nervous system; ANS – autonomic nervous system; NT-proBNP- N terminal serum brain natriuretic peptide; ng/L – nanograms per litre; µmol/L – micromoles per litre; mls/min – millilitres per litre; g/L – grams per litre; g – grams; mg/L – milligrams per litre; sec – seconds; PT – prothrombin time; APTT – activated partial thromboplastin time; FVIII – factor 8; vWF Ag – von Willebrand antigen; IU/dL – international units per decilitre

Clinical bleeding questionnaire

The clinical bleeding manifestations of all patients were assessed using the validated bleeding questionnaire used in vWF type I disease. Amendments to this questionnaire included denoting the duration of bleeding symptoms and the type of anticoagulation if received. The bleeding questionnaire reported a bleeding score in 24/100 patients (including 1 with multiple myeloma and one with localised gastrointestinal amyloidosis), with a median bleeding score range 0-8 and median symptom duration of 4 months (range 0.5-36). In comparison to other bleeding disorders, a bleeding score greater than 4 was present in only 4 (5.4%) patients and bleeding score of 1 was present in 13 (17.6%) patients. The sites of bleeding included cutaneous (n=17), oral (n=7), epistaxis (n=5), haemarthrosis (n=1), muscle haematoma (n=1) and following surgery (n=2).

Coagulation abnormalities

Prolongation of the PT, APTT and TT occurred in 13 (17.6%), 13 (17.6%) and 35 (47.3%) patients respectively. Prolongation of both the PT and APTT occurred in 13 (17.6%) patients, of which 8 were on warfarin. Thirty one patients had a reptilase time (RT) performed, and was prolonged in 25 (33.8%) patients. An elevated fibrinogen was present in 42 (56.8%) patients.

Factor deficiencies

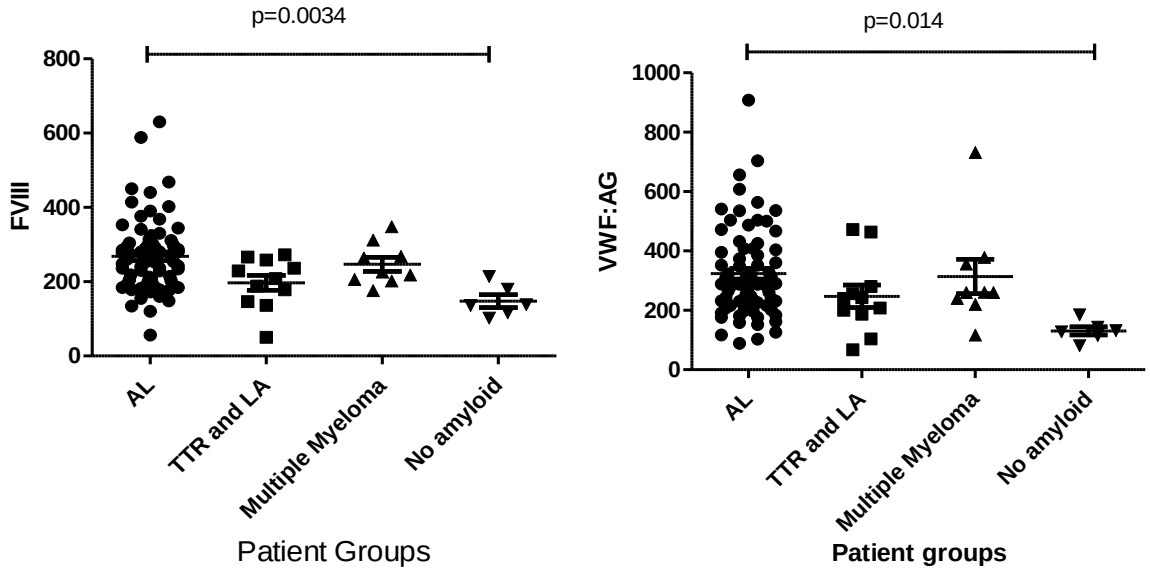
All patients had analysis of all factors including FII, FV, FVII, FVIII, FIX, FX, FXI, FXII performed with a low FII in 5 patients, low FVII in 3 patients, low FVIII in 1 patient, low FX in 8, low FIX in 3 patients and low FXII in 1 patient. The subnormal levels in FII (5), FVII (2), FIX (3), and FX (6) corresponded with patients with concomitant warfarin use.

Interestingly, elevated levels in FII, FV, FVII, FVIII, FIX, FXI, FXII occurred in 1 (1.4%), 42 (57.5%), 5 (6.8%), 67 (90.5%), 20 (27%), 8 (10.8%), 2 (2.7%) patients respectively. An elevated FVIII was a remarkable new finding, and we compared the FVIII levels in the different patients recruited to this study. Figure 4.1A shows a significantly higher FVIII in comparison to patients with no evidence of amyloid and a higher trend relative to other patient groups.

Von Willebrand factor levels, protein S, C and Anti-thrombin results

Von Willebrand collagen binding factor (vWF:CB) and von Willebrand factor antigen (vWF:Ag) were assessed, with elevated levels in 58/74 (78.3%) and 67/74 (90.5%) patients respectively. We compared the different groups recruited into this study for comparison of this surprising elevated VWF:Ag result. Figure 4.1B shows that patients with systemic AL had a higher median vWF:Ag value relative to patients diagnosed other types of amyloid (non-systemic AL), multiple myeloma and patients with no evidence of amyloid. There was a significant difference with patients diagnosed with systemic AL and patients with no evidence of amyloid. Plasma vWF:Ag showed a significant positive correlation with FVIII ($r=0.742$, $p=0.0001$), with the thrombin time ($r=0.634$, $p=0.0001$), and fibrinogen values ($r=0.366$, $r=0.002$) and negatively correlated with Anti-thrombin levels ($r=-0.264$, $p=0.025$) and non-significantly correlated with the other variables. Protein S, C and Anti-thrombin assays were undertaken in these patients, with low levels in 2, 0 and 6 patients respectively, excluding abnormal results on patients taking warfarin. Elevated protein S, C and anti-thrombin levels were seen in 8, 24 and 6 patients respectively.

Figure 4.1A and 1B: Scatter plots comparing FVIII and vWF:Ag levels in all patient groups respectively



ADAMTS13 assays

Plasma ADAMTS13 activity was determined using the Technozym ADAMTS-13 direct assay, with 18 patients having an elevated ADAMTS13 value, and no patients had reduced levels.

Anti-thrombin levels and nephrotic syndrome

Nephrotic syndrome (24 hour proteinuria assessment greater than 3g during this period) was present in 39/74 (52.7%) patients, with a range of albumin levels of 16-36g/L. An albumin less than or equal to 25g/L was present in 16 patients. A low anti-thrombin activity was present in 9 patients, with the patient characteristics illustrated in table 4.2.

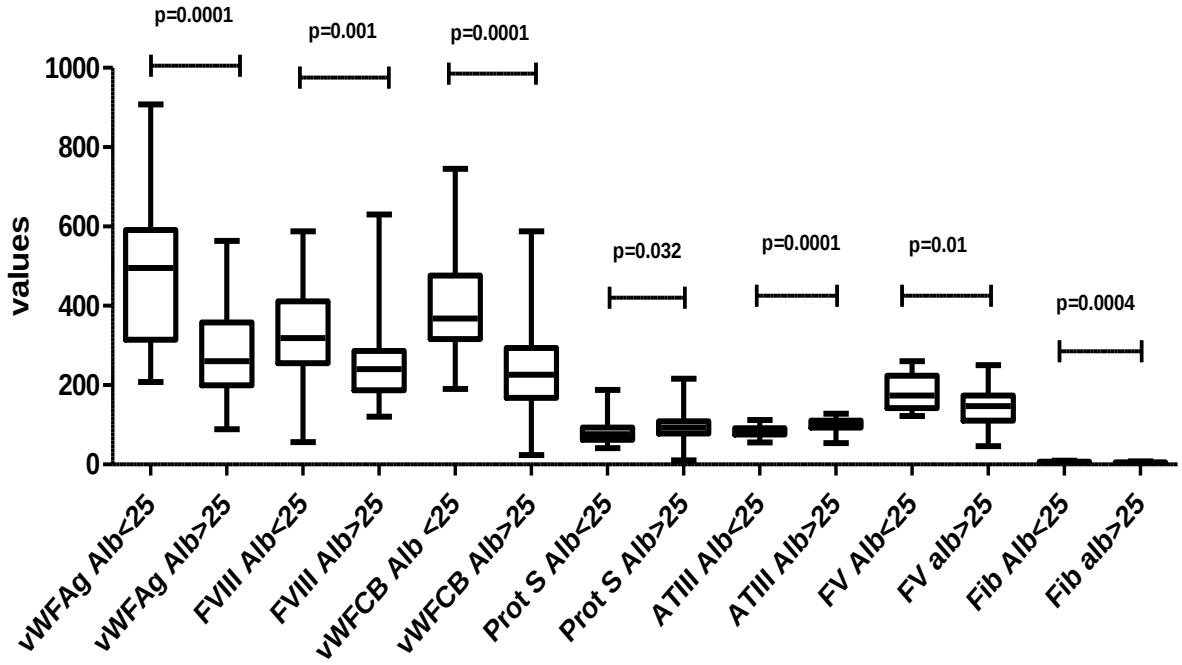
Table 4.2: Low anti-thrombin levels and nephrotic syndrome

Patient	Albumin	Proteinuria	SAP organs	SAP load	dFLC	Protein C	ATIII
1	20	13.9	L,S,K,B	large	126	183	81
2	37	3.0	N/A	N/A	1070	109	72
3	18	5.8	K	small	112	68	75
4	16	19.6	L,S,K	moderate	27	169	62
5	16	13.7	L,S,K	moderate	274	155	55
6	49	0.15	L,S	moderate	25	136	77
7	38	1.25	L,S	large	1160	78	77
8	22	1.42	L,S,K,B	large	76	146	78
9	28	2.00	S,B	moderate	32	90	54

L – liver; S – spleen; K – kidney; B –bones; N/A – not applicable; SAP – Serum Amyloid P; dFLC – difference in the involved and uninvolved free light chains; ATIII – anti-thrombin III.

We performed non-parametric t-tests in all the coagulation factors to assess any significant difference of having a threshold albumin less than or more than or equal to 25g/L, figure 4.1C. An albumin less than 25g/L correlated with a statistically significant rise in vWF:Ag, FVIII, FV and fibrinogen, with a fall in protein S and anti-thrombin III.

Figure 4.1C: Significant coagulation factors dependant on the albumin less than 25g/L in comparison to greater than or equal to 25g/L



Comparison of coagulation factors with albumin levels above and below 25g/L

Prognostic utility of haemostasis investigations

The median overall survival (OS) for the systemic AL cohort was 26.7 months, with 1-year and 2-year OS estimates 74% and 64% respectively, and 26 deaths. Univariate analysis showed cardiac involvement, NT proBNP, a dFLC greater than 180mg/L, FVIII and vWF:Ag thresholds greater than 280IU/dL, ADAMTS13 levels were statistically significant, table 4.3.

Table 4.3: Variables associated with survival

Variable	HR (95% CI)	p
Univariate Analysis		
Heart involvement	3.02 (1.33-6.86)	0.008
Log NT-proBNP	2.69 (1.54-4.70)	0.000
Mayo stage 3	2.37 (1.24-4.50)	0.009
FVIII>280IU/dL	2.77 (1.24-6.19)	0.013
vWF:Ag>280IU/dL	2.44 (1.02-5.84)	0.046
ADAMTS13	0.98 (0.967-0.993)	0.003
Protein C	0.991 (0.981-1.002)	0.097
Anti-thrombin III	0.969 (0.945-0.993)	0.011
dFLC ≥180mg/L	3.85 (1.60-9.24)	0.003
Multivariate analysis		
Log NT-proBNP	2.48 (1.35-4.53)	0.003
FVIII>280 IU/dL	2.53 (1.12-5.74)	0.026
dFLC>180mg/L	3.44 (1.41-8.39)	0.007

FVIII – factor 8; vWF:Ag – von Willebrand antigen; dFLC – difference between involved and uninvolved free light chains; mmol/L – millimoles per litre; mg/L – milligrams per litre; IU/dL – international units per decilitre

In the current study, FVIII and vWF Ag levels greater than 280IU/dL significantly negatively impacted survival with 2-year OS 51% (p=0.01) and 43% (p=0.039) respectively, (figure 4.2A and 4.2B). A Kaplan Meier estimate shows that a dFLC greater than 180mg/L also negatively impacted survival, p=0.001(figure 4.2C). This analysis showed FVIII> 280IU/dL, log NT-proBNP and a dFLC>180mg/L remained significant, with vWF Ag non-significantly linked with survival.

Figure 4.2A and 4.2B: Overall survival stratified according to vWF:Ag and FVIII>280IU/L respectively

Figure 4.2A

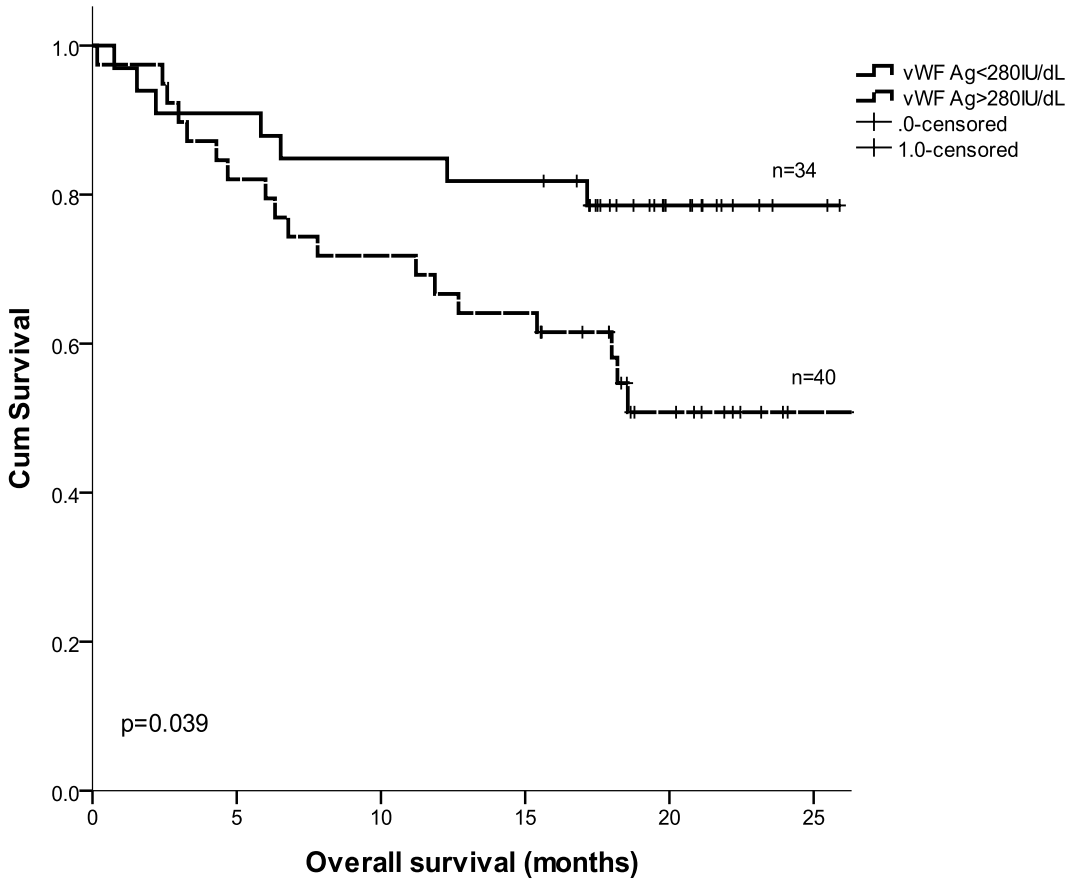


Figure 4.2B

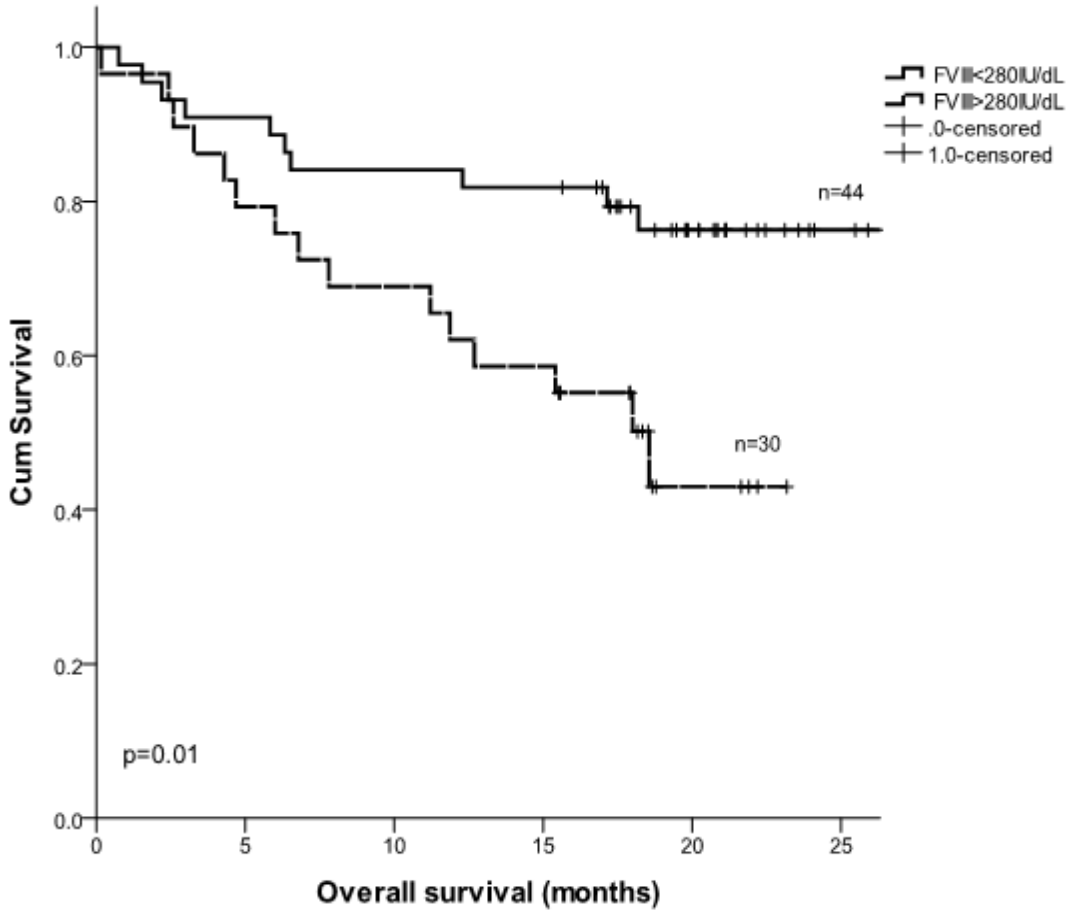
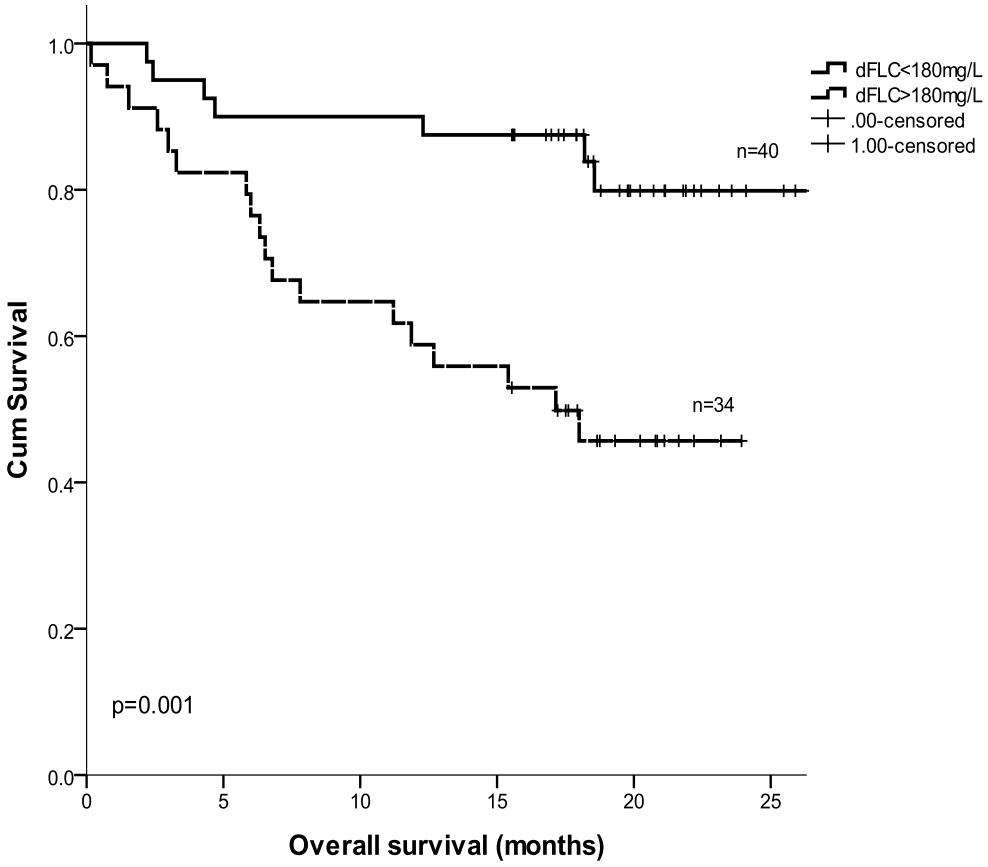

FVIII<280IU/dL
FVIII>280IU/dL
.0-censored
1.0-censored
n=44
n=30
p=0.01
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Overall survival (months)

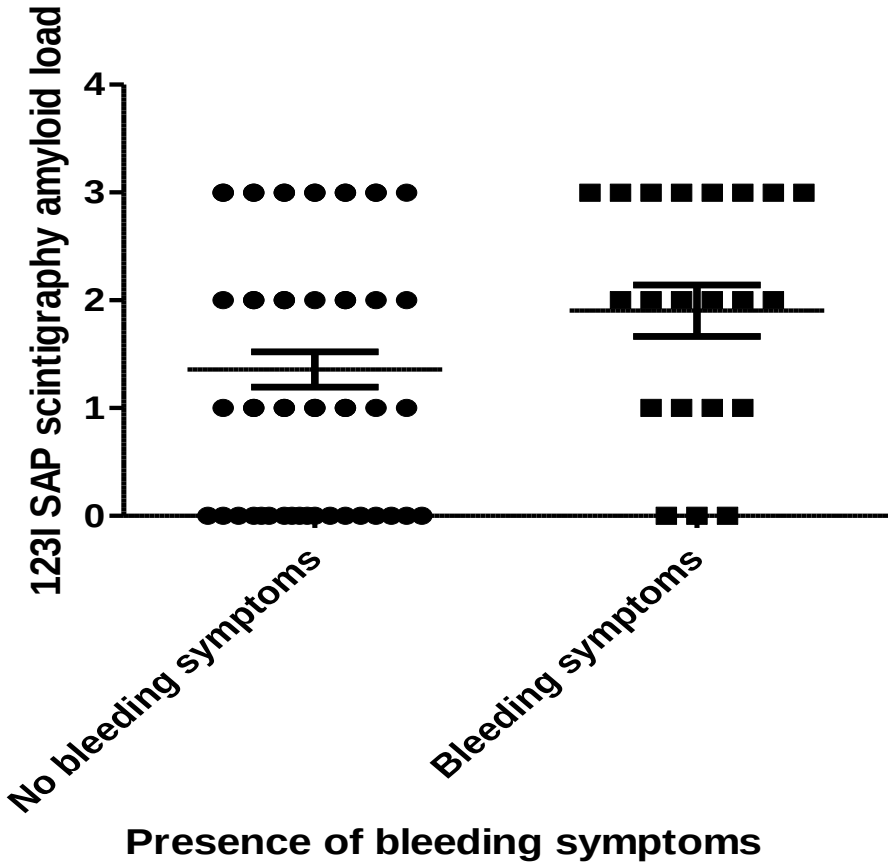
Figure 4.2C: Overall survival stratified according to a dFLC >180mg/L



Bleeding symptoms and association with other variables

We examined the association of bleeding symptoms by amyloid load by ^{123}I SAP scintigraphy. Analysis by scatter plots showed that those patients with bleeding symptoms had a non-significant higher median amyloid load by ^{123}I SAP scintigraphy in comparison to those not describing any bleeding symptoms, figure 4.3.

Figure 4.3: A larger amyloid load by ^{123}I SAP scintigraphy showing a trend to greater bleeding symptoms (Mann Whitney t-test, p=0.076)



VWF:Ag and FVIII levels post chemotherapy

Patients re-attending for their follow up visit 6-12 months (n=22) following chemotherapy had blood samples taken for vWF:Ag, FVIII and fibrinogen analysis. It was not feasible to arrange follow up blood samples in 26 patients due to their appointment arrangements with us in the time frame of this study and 26 patients died. 19 patients exhibited a fall in the vWF:Ag, (figure 4.4A) with a median pre and post chemotherapy 360IU/dL and 277IU/dL respectively. Fewer patients (n=4) had a corresponding fall in the FVIII level, with pre and post chemotherapy levels 272 IU/dL and 277IU/dL, (figure 4.4B). 17 had a starting dFLC greater than 50mg/L and further

analysis was performed on these patients. A complete remission (CR) occurred in 7, partial response (PR) in 6 and no response in 4 patients. There was no significant difference between these individual groups except the notable trend in the fall pre and post treatment, figure 4.4C.

Figure 4.4: Pre and post chemotherapy comparison of vWF:Ag (4.4A) and FVIII (4.4B), and vWF:Ag according to haematologic response (4.4C)

Figure 4.4A

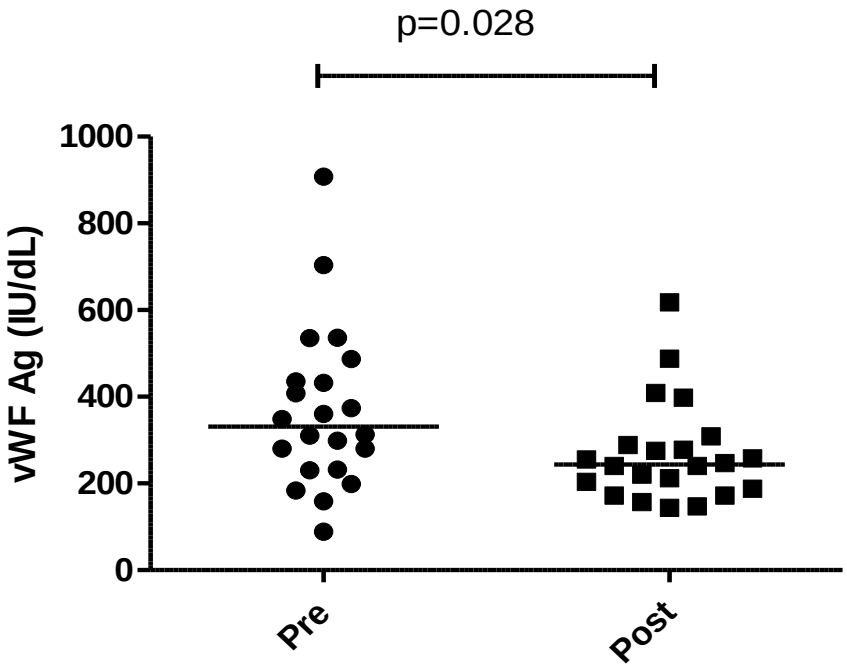


Figure 4.4B

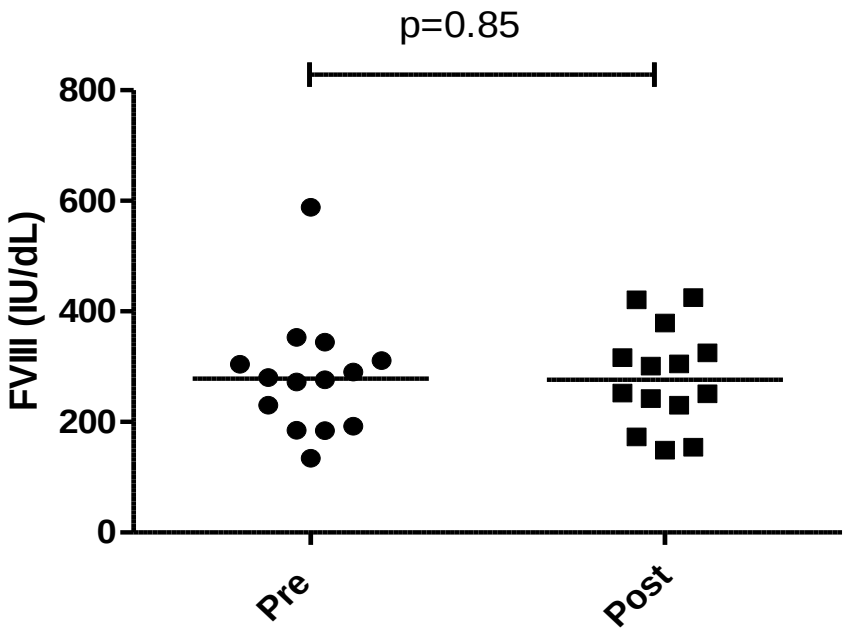
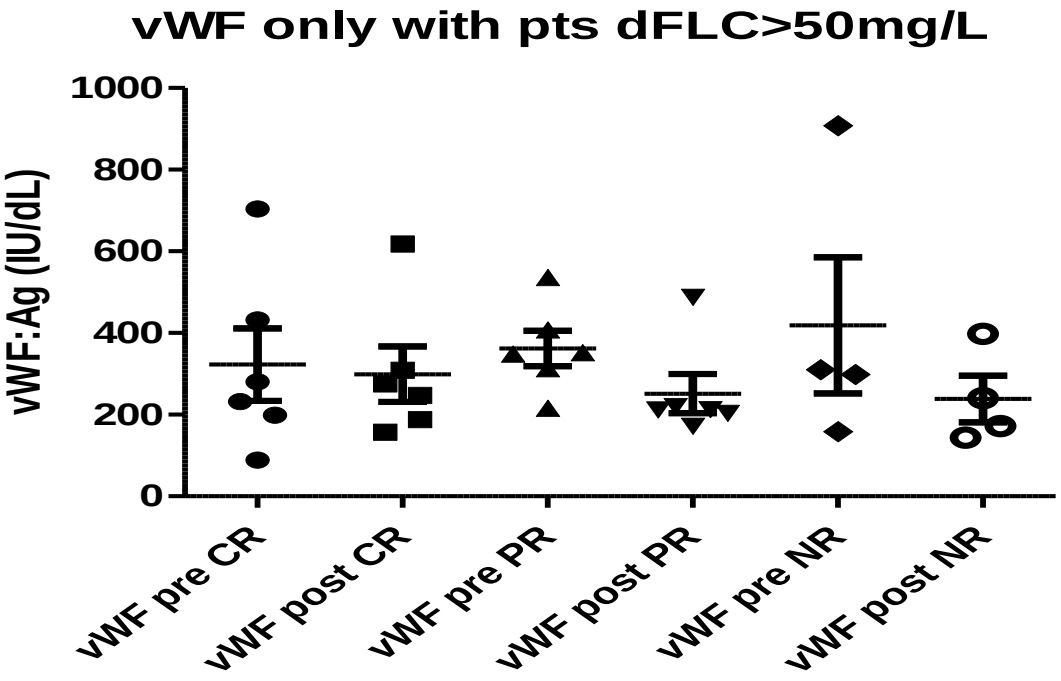


Figure 4.4C



Discussion

To our knowledge, this is the first prospective study investigating the interplay of all the various clotting factors in newly diagnosed systemic AL patients. A novel finding was the elevated vWF:Ag, vWF:CB and factor VIII in these patients. We then examined vWF as a marker of endothelial function and its potential implications in relation to light chain toxicity. This raises the hypothesis of these coagulation factors forming an instigating factor or possibly arising as a consequence of new amyloid deposits, with the thromboembolic potential poorly understood.

We recognise that bleeding complications are also well recognised in AL amyloidosis,[164,220,221] but little known as to the underlying mechanisms of haemostatic factors and co-factors within this environment which may contribute to a bleeding or pro-thrombotic tendency. The clinical bleeding manifestations and laboratory characteristics of our study group, specifically those diagnosed with AL were explored investigating the haemostatic environment with some enlightening findings. In this study, we examined the clinical utility of the only validated bleeding questionnaire, adapted from the vWF bleeding questionnaire to assess the usefulness of the bleeding score and systemic AL amyloidosis. 24 patients reported episodes of bleeding in their history with a median duration of 4 months (range 0.5-36), with 17 patients experiencing cutaneous bleeding. The literature supports that a bleeding score of 4 in other bleeding disorders, namely vWF disease equates to an increased risk of bleeding,[222] with only 4 systemic AL patients having a bleeding score greater than 4. This score in AL patients does not reflect the true nature experienced by many patients, predominantly ecchymosis and other cutaneous bleeding manifestations; experienced in 17 of the 24 patients in our entire cohort. Other studies of amyloidosis

patients have described haemostatic abnormalities with surprisingly fewer bleeding complications, which may be explained by the laboratory investigations.[223]

The fibrinolytic pathway is often affected with prolongation of the PT, APTT, TT and high levels of fibrinogen. In our study, prolongation of the PT, APTT and TT was a predominant coagulation abnormality, concurring with other studies.[164, 224] Previous studies in Myeloma have shown high levels of immunoglobulins having a propensity to increase the blood viscosity and interfering with the fibrin polymerisation[225] Fibrin formation is impaired, consequently interfering with the binding of plasmin and FXIII, ultimately causing ineffectual clot retraction[226] and abnormal clot clumps, with the possible propensity to occlude smaller vessels. In our AL cohort, the TT was prolonged in 35 patients, yet the majority of patients did not describe a bleeding tendency, suggesting other haemostatic defects are needed to result in increased bleeding manifestations such as vessel wall amyloid deposition and vessel fragility,[227] enhanced fibrinolysis or down regulation or consumption of certain co-factors.

Within our study group, 2 of 74 (1%) of patients had a Factor X deficiency, compared to previous studies of 7% [217] or 14%,[164] with the variation clearly dependent upon patient characteristics. Baseline FX levels are not predictive of bleeding risk.[228] Previous studies show that rapid clearance of ^{131}I-labelled FX from the circulation to areas of amyloid deposition particularly within the spleen[216, 229] suggests adsorption on amyloid fibrils as a major cause FX deficiency in AL. Other suggested explanations include synthetic dysfunction due to liver involvement or vitamin K deficiency and discordance between FX activity and FX antigen.[164] These 2 patients with factor X deficiency exhibited prolongation of the PT with a normal APTT, with no bleeding

complications. Further factor analysis was undertaken to assess any other associated factor deficiencies encountered with this disease. Our results reflected a subnormal FVIII in 1 patient and subnormal FXII in another patient, with prolongation of the APTT in the former and no bleeding complications in either patient. The striking feature when analysing the factor levels, was the extent of elevated values for all the factors, predominantly for factor V, VIII and IX. Little data exists as to bleeding or thrombotic complications related to FV, with only one patient having an activated protein C resistance. Interestingly, FVIII results were elevated in 91%. High factor VIII levels are thought to be an independent risk factor for venous thrombosis, with levels greater than 150% associated with a five-fold increase risk of venous thrombosis.[230] Heikal et al explored elevated factor IX levels retrospectively in 81 patients, showing an association with arterial and venous thrombosis[231] The consequence of these elevated factors remains undetermined but may provide a more prothrombotic environment.

The FVIII/vWF glycoprotein complex may serve as important platform from which endothelial dysfunction can be assessed in this disease. Clearly intertwined in equilibrium, vWF is a vital partner for FVIII's function, stability, structure and immunogenicity.[214] Electrostatic interactions have been proposed as one driving force for the FVIII-vWF complex,[232] with Dimitrov et al showing binding of FVIII to vWF demonstrated a high sensitivity to acidic pH and sensitivity to ionic strength.[233] One study showed that 50% high FVIII levels were associated with high vWF Ag levels, implying that there are other factors which affect this interaction. Other studies have shown that factors including BMI, diabetes mellitus, insulin, fibrinogen and triglycerides are associated with elevated FVIII levels.[234] Typically a rise in FVIII will lead to a

concordant rise in vWF:Ag levels. Our study showed a concordant rise in both vWF and FVIII levels.

In the present study, elevated plasma vWF was shown to be associated with patients diagnosed with systemic AL amyloidosis, with 90.5% of patients having elevated vWF activity, supporting previous studies examining vWF[235] Kastritis et al, examined 81 patients with systemic AL with a serum vWF level of 181 IU/dL (range 20-557) with vWF levels greater than 230 IU/dL were associated with a poor survival (median 4 months versus 47 months, p=0.001). In our study, the vWF activity was significantly higher in AL patients in comparison with patients with no amyloid, p=0.014 in comparison with other patient cohort groups. We found a significant survival disadvantage with a factor VIII level and vWF antigen level greater than 280IU/dL in comparison to the Greek group,[235] although FVIII levels were not explored by the latter group. The differences in the survival differences between the Greek group and our study most likely reflect the patient cohorts recruited. In comparing all the patient groups in our study, patients with systemic AL and Multiple myeloma (with the latter not significantly different from controls) had higher median values of FVIII and vWF:Ag, suggesting the underling plasma cell or light chains as the probable instigating factor.

Von Willebrand factor is a large multimeric glycoprotein produced in endothelial cells and megakaryocytes playing an important role in haemostasis by promoting platelet adhesion. A number of clinical and experimental studies have shown that elevated vWF levels reflect endothelial damage and dysfunction.[209] Previous studies have proposed vWF as a marker of endothelial damage or dysfunction; which is secondary to a pro-inflammatory and pro-coagulant state. The increased vWF:Ag and vWF:CB

are likely to result in increased platelet plug formation. In systemic AL amyloidosis, light chains misfold and aggregate as amyloid fibrils in vital organs. In vitro fibril studies show that there are various factors which may play a part in the kinetics and morphology of the fibrils: thermodynamic stability, low pH and oxidative stress.[236-238] Earlier studies have shown that amyloid deposits occur earlier within the blood vessels, with these patients presenting with early microcirculatory dysfunction.[202] Thus systemic AL patients with higher levels of vWF signify a unique marker for endothelial dysfunction and may lead to a higher risk of thromboembolic disease and cardiovascular disease.

Typically, large VWF multimers are produced in and released from vascular endothelial cells, predominantly biologically active whilst interacting with platelets under a high shear stress. The large multimers are cleaved and degraded into smaller fragments by A Disintegrin And Metalloprotease with ThromboSpondin 1 repeats (ADAMTS13); an enzyme produced by the liver. To investigate the significantly high levels of vWF:Ag and vWF:CB levels, we examined the plasma ADAMTS13 activity in these patients. The most remarkable observation of our study was the higher plasma vWF Ag levels and normal and elevated levels of ADAMTS13 activity, with the biological significance of this uncertain. Low levels of ADAMTS13 activity may be secondary to high levels of substrate or sequestration of this protease to the vascular endothelial cells. In AL patients, the vWF cleaving protease activity was normal and elevated in 18 patients, likely to represent the increased levels of vWF:Ag and hence increased need to ensure these multimers are cleaved.

Proteins C and S are two vitamin K-dependent plasma proteins acting as natural anticoagulants. Activated protein C acts as a proteolytic component of the complex, whilst protein S is used as an activated protein C binding protein; both needed for assembly of the anticoagulant complex on cell surfaces. This is primarily expressed through the selective inactivation of Factors Va and VIIIa. Many patients deficient in proteins C and S have been described with an associated thrombotic tendency. There are various mechanisms and/or drugs which can lead to acquired deficiencies of these proteins including oral anticoagulation, liver disease, DIC and in the case of protein S, nephrotic syndrome, lupus erythematosus and certain hormones. Protein S plays an important role as a cofactor in the inactivation of FVa and FVIIIa.

Protein C deficiency is known to be associated with thromboembolic phenomenon, with little data as to increased protein C levels. In this study, 24 patients (32.4%) had high levels of protein C with only 1 patient with activated protein C resistance. This highlights the complex interplay of the various coagulation factors and bystanders that contribute to proteinuria, not solely a low anti-thrombin level.

In our study nephrotic syndrome was present in 53% of the AL cohort, with prolongation in the thrombin time and reptilase time in all these patients, suggesting a close link between prolongation of the TT and RT and proteinuria, typically secondary to the abnormal fibrin polymerisation that occurs in both cases.[239, 240] Low anti-thrombin levels in nephrotic syndrome have been described in many studies,[241-243] likely secondary to urinary loss and increased consumption. Anti-thrombin acts as an important inhibitor of serine protease clotting factors, with heparin and Glycosaminoglycans (GAGS) accentuating its inhibitory effect; which bind to amyloid

fibrils.[244] Gamba et al described increased plasma values of thrombin-antithombin (TAT) complexes and enhanced thrombin activation present, chronic consumption of AT, accounting for differences in AT activity and AT:Ag levels. Thrombin generation may be additionally enhanced by delayed fibrin formation.[243] Of the nephrotic patients, only 7 had reduced anti-thrombin values, with 5 patients having corresponding albumin levels less than or equal to 25g/L. Interestingly the other 2 patients with albumin levels of 28 and 38g/L had raised protein C levels and a low quantitative level of proteinuria. There is very likely a change in the haemostatic factors including protein C which occur prior to lower albumin levels and hence more extensive proteinuria.

Amyloid deposits in the kidneys typically occur within the glomerulus, and consequently results in pathological damage, with proteinuria often seen which may be nephrotic or sub-nephrotic. The renal tubules function to reabsorb the plasma proteins (specifically in the proximal convoluted tubule) which pass through the glomerular filtration barrier, which provides a charge and size selective barrier to albumin. Podocytes are terminally differentiated glomerular epithelial cells, with endothelium activation and loss of selectivity resulting in prolonged exposure of the podocytes to these proteins.[245] This overall damage leads to reduced vascular endothelial growth factors (VEGF).[246] Previous studies have explored the risk of thromboembolic disease in nephrotic syndrome.[247-249] In our study, comparison of patients with an albumin less than 25g/L and those with an albumin greater than or equal to 25g/L for the different coagulation factors showed definite differences. The former group showed differences to the latter, with significantly higher vWF:Ag, FVIII, FV, and fibrinogen levels and lower protein S and ATIII levels. This is the first study to compare all coagulation factors to assess the possible aetiology of thrombotic

phenomena or bleeding risks in patients diagnosed with systemic AL. Understanding of the coagulation cascade has shown that elevated levels of FVIII and fibrinogen, lower levels of protein S and anti-thrombin contribute to a prothrombotic tendency. The latter findings were found in patients with an albumin less than 25g/L. Although these factors work as part of a cog wheel together, this study highlights the potent risk of prothrombotic tendency in patients with an albumin less than 25g/L, and hence the awareness and need for prophylaxis or full anticoagulation consideration.

There is limited data as to managing patients given the paradox of bleeding and thrombotic risks. A common finding in our study and previous studies[164, 224, 250] was the prolongation of TT and RT. Typically, hyperfibrinolysis may be related to lower levels of α2-antiplasmin or increased urokinase type plasminogen activity. As such, ε-aminocaproic acid are effective in controlling some bleeding complications.[251] Until recently, Factor X deficiency treatment deficiency therapeutic options included fresh frozen plasma (FFP), prothrombin complex concentrates (PCCs), activated prothrombin complex concentrates (aPCC) or recombinant factor VIIa (rFVIIa)[252] – each with significant risks in this fragile patient population. A larger series reported 44% of patients had complications when treated with rFVIIa preoperatively including bleeding, thrombosis, or death.[228]. In addition to FFP, PCC, aPCC or rFVIIa, tranexamic acid, plasma exchange[253] and splenectomy[217] were anecdotally reported as treatments for FX deficiency in AL; the latter targeted at removing the splenic amyloid fibril burden. As a result of a variable amount of FX in FFP, large volumes are needed to achieve a haemostatic effect with the true clinical risk of fluid overload in patients with cardiac involvement, especially those with advanced cardiac involvement or elderly population. A serious concern arises regarding thrombotic risks

of FVIIa or aPCC in patients with AL amyloidosis who are elderly with cardiovascular risk factors and often nephrotic due to renal involvement (hence inherently prothrombotic). BPL (Bio Products Laboratory Ltd, Elstree, UK) have also developed a high purity plasma derived factor X concentrate and currently undergoing phase III clinical trials used in patients with hereditary factor X deficiency. In one analysis, one patient with inherited Factor X deficiency presenting with a shoulder haemarthrosis, achieved good haemostatic response following FX dose of 25IU/kg daily[254] with a biological half-life 24 to 48 hours.

Our Centre also reported an initial report of using high purity factor X (HP-FX) concentrate in two patients with systemic AL amyloidosis and acquired FX deficiency.[255] This involved 2 patients with biopsy proven AL amyloidosis, with evidence of liver involvement and a large amyloid load by ^{123}I serum amyloid P component scintigraphy, one patient presenting with a forearm haematoma and the second patient presenting with a ruptured spleen and knee haemarthrosis due to acquired FX deficiency (both with FX levels 8 IU/dL). HP-FX concentrate was used to treat each bleeding episode in each case given as a single dose of 40IU/kg (with the FX activity assessed by one stage clotting prothrombin time (PT) based assays) and haemostasis achieved in conjunction with supportive measures. There was an obvious difference between the FX recovery times between patients with inherited FX deficiency in comparison with our AL patients; the latter showed less predictable kinetics, and a more rapid decline to baseline in 2-4 hours, consistent with the theory of FX adsorption on amyloid fibrils. Each patient with AL amyloidosis has a different amyloid load and a unique fibril sequence with a potentially different avidity for binding FX – both likely to lead to large differences in the half life and dose needed as in the second patient with a larger load and earlier decrease in FX levels. We tried to

ascertain whether there was a correlation between bleeding symptoms and amyloid load by ^{123}I SAP scintigraphy, not showing a statistically significant difference.[255] High purity factor X concentrate is useful to treat bleeding due to acquired factor X deficiency in systemic AL amyloidosis. Higher and/or more frequent dosing is likely to be required to achieve adequate FX levels for haemostasis with frequent monitoring of FX levels given the unpredictable kinetics; target FX thresholds similar to patients with inherited FX deficiency: doses of 10-15 IU/dl for minor bleeding and >50 IU/dl for major bleeding, trauma or surgery. Thus HP-FX has the advantage that the haemostatic response can be monitored and treatment tailored to the patient individual needs.[255]

Light chain toxicity may also serve as a further insult in this process of AL deposits. Various experiments have attempted to explore the underlying mechanisms of light chain toxicity, with no correlative factor for endothelial dysfunction. Previous studies have proposed vWF as a marker of endothelial damage or dysfunction; which is secondary to a pro-inflammatory and pro-coagulant state. The increased vWF:Ag and vWF:CB are likely to result in increased platelet plug formation. Bauer et al described the concept of vWF fibres laid down within the lumen of the cancer associated vessel wall, with tumour derived VEGF inducing angiogenesis, thus affecting the pathophysiology and activation of the endothelium.[256] In systemic AL amyloidosis, light chains misfold and aggregate as amyloid fibrils in vital organs. In vitro fibril studies show that there are various factors which may play a part in the kinetics and morphology of the fibrils: thermodynamic stability, low pH and oxidative stress.[236-238] Earlier studies have shown that amyloid deposits occur earlier within the blood vessels, with these patients presenting with early microcirculatory dysfunction. [202] As such, it seems feasible that systemic AL patients have higher levels of vWF signifying

a unique marker for endothelial dysfunction and may lead to a higher risk of thromboembolic disease and cardiovascular disease. In comparing all the patient groups in our study, patients with systemic AL and Multiple myeloma (with the latter not significantly different from controls) had higher median values of FVIII and vWF:Ag, suggesting the underling plasma cell or light chains as the probable instigating factor.

The underlying mechanism of light chain toxicity remains unknown, with some reports showing that the prefibrillar light chain proteins, consequent endothelial dysfunction and ischaemic vascular injury occurs prior to amyloid deposition. [200, 202] Exposure to light chains and oxidative stress and microvascular dysfunction has been proposed in the AL pathophysiology.[199] Recently further exploration to examine the dysregulation of an autophagy flux in the setting of proteotoxicity of the light chains has shown to be important in those with cardiac AL. Guan et al found that inhibition of the autophagy flux, specifically lysosomal dysfunction is important in the AL-light chain cardiotoxicity and hence development of AL cardiomyopathy.[257] In vitro experiments showed that lysosomal dysfunction is an early point in the cascade of events that occur in cardiac AL, specifically the light chain toxicity, followed by mitochondrial dysfunction, ROS dysfunction and consequent cell death and dysfunction. This also highlights the potential for a possible autophagy related targets, replicated in vivo halting cardiac AL mortality using rapamycin and transient overexpression of transcription factor EB (TFEB).[257]

This resultant rise in vWF and FVIII are likely to create a consequential pro-coagulant and pro-inflammatory environment. Several studies have attempted to explore whether FVIII or vWF are the causative stimulant in arterial or venous thrombogenesis.

The ARIC study examined strong associations of FVIII and vWF as risk factors for hypertension, diabetes mellitus, body mass index or triglycerides. Some of the latter variables are associated with endothelial and vascular inflammation.[211, 258] The consequent high shear forces especially within narrowed vessels lead to increased vWF secretion by the vascular endothelium, which stimulates platelet adhesion and aggregation at the damaged arterial walls, with the high risk leading to thrombus formation.[259]

Previous studies have shown that high levels of factor FVIII over time are present in patients with thrombosis[260], and not reflective of an acute phase reaction[261] We compared the C-reactive proteins (CRP) in each group to ascertain whether the elevated vWF Ag and FVIII were secondary to an inflammatory or infective pathology; with the former showing relatively low levels except for occasional patients with infection (table 4.1). The latter finding negates underlying inflammation as a trigger for the vWF rise.

In an attempt to explore the relationship between FVIII and vWF Ag with light chains, we repeated blood samples for these coagulation factors post chemotherapy. This has not been explored before, with the results showing that 86% (19/22 patients) with these investigations undertaken exhibited a fall in the vWF:Ag level following chemotherapy, suggesting a likely correlative relationship. Interestingly the FVIII levels did not fall in the majority of patients. The type of haematological response did not correlate with the level of fall in the vWF:Ag implying there is a much more complex interplay of the light chains, the vascular endothelium and formation of amyloid deposits. It is difficult to interpret this, with small numbers and no experimental or

electron-microscopy to aid with understanding. One possible hypothesis would suggest that treatment with chemotherapy in an attempt to reduce the light chains, also serves to improve the function of the vascular endothelium, but may still provide a thrombotic environment.

Our study highlights the prothrombotic potential in patients with newly diagnosed systemic AL, irrespective of the presence of nephrotic syndrome or type of chemotherapy regimen used. This raises the question as to what the optimum treatment of thrombotic risk should involve. Ordinarily thromboprophylaxis would suffice, but given the additional elevated risk factors of vWF:Ag, FVIII, fibrinogen and lower anti-thrombin and protein S levels, further analysis is needed to explore whether full anticoagulation is necessary.

In summary, this is the largest series prospectively examining all the underlying coagulation factors, protein C and S, anti-thrombin in newly diagnosed AL amyloidosis. Little is known in the setting of AL amyloidosis with the paradox of a bleeding diathesis and thromboembolic disease. Haemostatic dysfunction likely results from different interacting pathogenic pathways and coagulation factors. From our data, we showed that prolongation of the thrombin time was a common finding and low levels of anti-thrombin activity and proteinuria were associated with albumin levels less than 25g/L. In addition these patients had elevated pro-thrombotic factors and reduced anticoagulant factors which will contribute to the pro-thrombotic status of these patients. An elevated FVIII and vWF:Ag level greater than 280IU/L carried a significant survival disadvantage. Thromboprophylaxis or treatment anticoagulation is an important consideration in all these patients not having bleeding complications.

Our study points to underlying vascular endothelial damage that occurs in systemic AL, and possibly contributing to the light chain toxicity environment. The correlative fall in the vWF levels post chemotherapy may reflect the underlying vascular endothelial changes which occur. Further studies to better understand the pathophysiology of these findings are needed to explore the endothelium by electron microscopy, and hence the utility of the prognostic findings as part of patient risk stratification.

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Chapter Five: Frequent occurrence of recurrent nocturnal desaturations in systemic AL amyloidosis

This chapter is written in the context of my publication: High prevalence of recurrent nocturnal desaturations in systemic AL Amyloidosis: a cross-sectional study. S Mahmood, M Sovani, P Smith, L George, C Quarta, S Sachchithanantham, M Fontana, CJ Whelan, HJ Lachmann, JD Gillmore, PN Hawkins, AD Wechalekar. Sleep Medicine. Accepted for publication and available online December 21st 2016. (Original article). Copyright permission obtained from Elsevier publishers, license no.: 4025341342092 for use in my thesis.

Introduction

Systemic AL amyloidosis is a rare disorder caused by deposition of misfolded immunoglobulin light chains in organs or tissues. Outcomes of patients with amyloidosis depend predominantly on the extent of cardiac disease, although involvement of other organs and, particularly, soft tissues of the oropharynx contribute to significant symptoms and morbidity. It is well recognised that early and unexpected cardiac deaths account for 20-40% of all deaths within a few months of diagnosis in AL amyloidosis. The exact terminal event and its triggers remain unclear. Bradyarrhythmias[262] or other arrhythmias may be the cause. Central sleep apnoea (CSA), characterised by the faulty respiratory drive during sleep, is a well-recognised complication of heart failure and can lead to recurrent episodes of nocturnal hypoxemia worsening symptomatic heart failure,[263] causing increased morbidity and mortality. Obstructive sleep apnoea (OSA) is now a well-recognised cause of acute and chronic adverse cardiovascular effects. In addition to cardiovascular effects, both

CSA and OSA cause marked day time fatigue and/or sleepiness contributing to morbidity.

Systemic AL amyloidosis is one of the few disorders which cause acquired progressive heart failure and can also be associated with marked infiltration of the oropharyngeal soft tissues – both potential risk factors for CSA or OSA, respectively, or CSA and OSA together in patients with soft tissue and cardiac disease. The recurrent hypoxic episodes could have a profound effect on the cardiac function in patients with advanced amyloidosis.

We report here the results of a pilot study of overnight continuous pulse oximetry in patients with systemic light chain (AL) amyloidosis, based on the hypothesis that recurrent nocturnal hypoxaemia (likely due to sleep disordered breathing OSA and/or CSA) could occur in patients with amyloidosis reporting a high incidence of recurrent nocturnal oxygen desaturations and raise a question whether these desaturations may be the trigger for sudden cardiac mortality.

Methods

Study population

This study included consecutive patients seen at the UK National Amyloidosis Centre with amyloidosis between July 2013 and June 2014 who underwent overnight pulse oximetry, using the Minolta 300I pulse oximeter. The main inclusion criteria were: the presence of clinical macroglossia and/or cardiac amyloidosis. Written consent for retrospective publication of data was obtained from all patients in accordance with the Declaration of Helsinki. Amyloid deposition was confirmed on a tissue biopsy by the presence demonstration of Congo red positivity under cross polarise light and fibril

typing was done by immunohistochemistry or mass spectrometry. All patients had a detailed assessment for organ involvement as per standard protocol at the National Amyloidosis Centre. Blood tests included a full blood count, renal, liver and bone profiles, cardiac biomarkers including N terminal fragment of the prohormone brain natriuretic peptide (NT-proBNP) and troponin T. Other investigations included an electrocardiograph (ECG), echocardiography and ^{123}I serum amyloid P component (SAP) scintigraphy. Organ involvement, haematologic and organ responses were classified according to the updated international amyloidosis consensus criteria.[87] All patients underwent overnight pulse oximetry, with instructions given to each patient (appendix 2). An episode of significant desaturation was defined as per the standard definition as 4% or greater decrease in oxygen saturations from the average oxygen saturations in the preceding 120 seconds lasting for more than 10 seconds. The oxygen desaturation index (ODI), the hourly average number of desaturation episodes over the whole night, was calculated. ODI is a standard measure used to score oxygen desaturations, but may not always show evidence of hypopnoea.[264] Heart rate (HR) variability was defined as HR change of >6 beats per minute – tachycardia is a normal physiological response to desaturation.[181]

All patients completed the Epworth Sleepiness Score (ESS) (appendix 3) and STOP BANG (appendix 4) questionnaires for obstructive sleep apnoea (OSA). The STOPBANG (Snoring, Tiredness during the daytime, Observed apnoea, high blood Pressure, Body mass index, Age, Neck circumference and Gender) and ESS (Epworth Sleepiness Scale) (included as supplementary data) are validated screening tools of OSA with a high sensitivity[265] but low specificity. Patients were classified as high risk of OSA if the STOP-BANG score was greater than and equal to 3 and low risk if the score was less than 3. The ESS (Epworth Sleepiness Scale) questionnaire is based

on the probability to fall asleep during different situations[266] and patients were classified as being high risk if the ESS score was greater than or equal to 10.

Analytical plan

Baseline characteristics and clinical investigations are presented as medians with minimum and maximum values for continuous variables and percentages with proportions for categorical variables. Statistical significance for comparison between groups was analysed with the one way anova variance for continuous variables and Turkey post-test analysis using the Graph pad prism version 5 software. Correlation statistics were performed using linear scatter plots and Pearson coefficients. A two-sided P value of less than 0.05 was considered as statistically significant. A univariate model was used to assess the poor prognostic features of those diagnosed with cardiac AL. Multivariate models are not presented due to instability from small patient numbers. Survival was assessed by the method of Kaplan-Meier analysis and patients with cardiac AL amyloidosis and those with transthyretin amyloidosis were analysed separately due to a different disease natural history.

Results

Baseline characteristics

A total of 72 patients were included in this study. The median age of all patients was 68.8 years (47-83), with 80.5% with cardiac involvement, median NT-proBNP 2568ng/L (136-146203) and median 4%ODI 7.9 (0.9-59). Patients were stratified as systemic AL amyloidosis with cardiac involvement, AL amyloidosis with macroglossia,

AL amyloidosis with both macroglossia and cardiac involvement, and wild type transthyretin amyloidosis (ATTR), (Figure 5.1A). Table 5.1 illustrates the baseline presenting characteristics in each group.

Figure 5.1 Study Population. A total of 72 patients initially recruited, with proven cardiac and/or macroglossia amyloid. Specifically transthyretin (n=17), cardiac light chain amyloidosis (n=25), cardiac and macroglossia light chain amyloidosis (n=16) and patients with light chain amyloidosis with solely macroglossia (n=14).

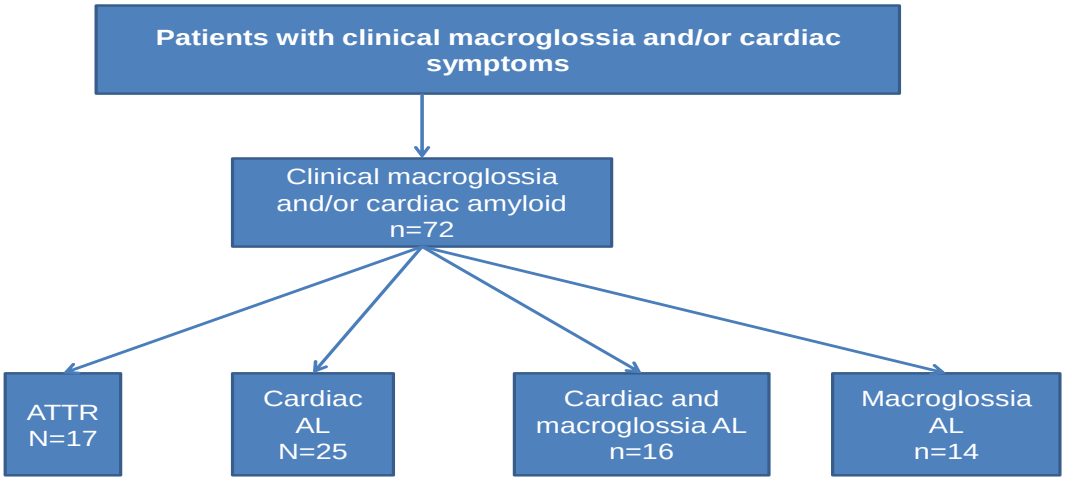


Table 5.1: Patient characteristics

Type Number	Cardiac AL n=25	Macroglossia AL n=14	Cardiac & Macroglossia AL n=16	ATTR n=17	Patients Who died n=12
Age (years)	66 (49-80)	71 (47.4-83.8)	65 (39.3-81)	75 (61-83)	69 (59-83)
Organ Involvement					
Heart	25	0	16	17	12
Kidneys	14	4	5	0	7
Liver	8	0	0	0	3
Macroglossia	0	14	16	0	3
Mayo Disease Stage					
1	4	7	2	2	1
2	9	6	6	5	1
3	12	1	8	10	10
NT-proBNP (ng/L)	5585 (136-146203)	186 (34-11788)	1314 (136-13288)	3703 (847-12712)	6619 (424-146203)
Troponin T (ng/L)	0.1 (0-0.8)	0.02 (0.01-0.1)	0.07 (0.01-0.15)	0.083 (0.04-0.63)	0.09 (0.01-0.8)
Albumin (g/L)	42 (24-49)	40.5 (30-46)	40.5 (30-52)	46 (43-50)	36 (25-46)
Alk Phos (IU/L)	81 (37-636)	76 (59-123)	82 (49-135)	109 (69-187)	83 (64-315)
Creatinine (µmol/L)	105 (58-582)	75 (46-472)	88 (45-385)	126 (70-188)	109 (71-226)
eGFR (mls/min)	59 (10-100)	73 (10-100)	71 (10-100)	52 (31-100)	60 (26-77)
GGT (U/L)	49 (12-621)	34 (7-213)	21 (9-153)	114 (42-310)	39.5 (9-621)
Urinary protein (g/24 hours)	0.2 (0.1-6.8)	0.2 (0.1-4.8)		0.2 (0.1-0.4)	0.9 (0.1-6.8)
TDI (cm/s)	0.12 (0.08-0.18)	0.15 (0.09-0.21)	0.12 (0.06-0.2)	0.09 (0.05-0.17)	0.11 (0.06-0.19)
TAPSE (mm)	15 (6-27)	22 (12-31)	17.5 (7-31)	12 (7-20)	12 (6-22)

AL – light chain; BMI – body mass index; ATTR – transthyretin; BP – blood pressure; 4%ODI - hourly average number of desaturation episodes defined as a 4% decrease in saturations from the average saturations in the preceding 120seconds and lasting for more than 10seconds; NT-proBNP – N terminal of the prohormone brain natriuretic peptide; eGFR – estimated glomerular filtration rate; GGT – gamma-glutamyltransferase; RV TDI – right ventricle tissue Doppler imaging; TAPSE – tricuspid annular pulmonary systolic excursion; cm – centimetres; bpm – beats per minute;; pMol/L – picomoles per litre; ng/L – nanograms per litre; g/L – grams per litre; IU/L – international units per litre; µmol/L – micromoles per litre; mls/min – millilitres per minute; U/L – units per litre; g – grams; cm/s – centimetres per second; mm – millilitres.

Overnight oximetry

Overnight oximetry tracings were recorded and collected in all 72 patients. Table 5.2 shows findings in systemic AL amyloidosis with cardiac involvement, AL amyloidosis with macroglossia, AL amyloidosis with both macroglossia and cardiac involvement, and wild type transthyretin amyloidosis (ATTR) and those patients who died. The mean oxygen saturation of all patients in the study was 93% (±SD 1.99, 87-96). The mean oxygen saturations were similar in all groups. The ATTR group had the lowest number of abnormal oximetry tracings. Figures 5.2A and 5.2B illustrate a normal and abnormal overnight oximetry tracing in 2 patients with cardiac amyloidosis respectively.

Table 5.2: Oximetry data in the different amyloid groups

Type Number	Cardiac AL n=25	Macroglossia AL n=14	Cardiac & Macroglossia AL n=16	ATTR n=17	Patients Who died n=12
BMI	25 (18-30)	25.5 (19-45)	25.3 (19-32)	26 (21-37)	23.4 (19-26)
Neck circ (cm)	39 (31-44)	39 (34.3-43)	38 (33-42)	41 (34-45)	40 (34-43)
Mean pulse (bpm)	77 (60-108)	68 (55-93)	72 (56-89)	69 (53-80)	80 (61-108)
Mean systolic BP (mmHg)	109 (91-150)	117 (102-154)	120 (102-166)	123 (106-151)	102 (96-150)
Mean oxygen saturations	93 (88.7-96)	93 (87-96)	94 (91-96)	94 (91-96)	93 (88-96)
Abnormal Oximetry	21/25 (84%)	11/14 (79%)	10/16 (63%)	9/17 (53%)	9/12 (75%)
4% ODI	11 (1-48)	6 (3-41)	6 (0.9-52.1)	8 (0.57-59)	6 (3.8-26)
Heart rate change >6bpm	12 (1.9-113)	13 (4.3-51.5)	20 (2.3-69.7)	24 (0.3-80)	10 (0.3-113)
ESS score	8 (0-15)	6 (2-13)	6 (1-12)	5 (2-17)	8 (0-15)
ESS>10	7/25 (28%)	3/14 (21%)	3/16 (19%)	4/17 (24%)	4/12 (33%)
STOP BANG score	3 (1-5)	3 (2-5)	3 (1-6)	4 (1-6)	3 (1-4)
STOP BANG>3	16/25 (64%)	10/14 (71%)	11/16 (69%)	12/17 (71%)	8/12 (67%)

AL – light chain; ATTR – transthyretin based disease; circ – circumference; n – number; BMI – body mass index; cm – centimetres; bpm – beats per minute; 4%ODI – hourly average number of desaturation episodes defined as a 4% decrease in saturations from the average saturations in the preceding 120seconds and lasting for more than 10 seconds; STOP BANG - Snoring, Tiredness during the daytime, Observed apnoea, high blood Pressure, Body mass index, Age, Neck circumference and Gender; ESS – Epworth Sleepiness Score; mmHg – millimetres of mercury

The number of significant nocturnal desaturations (4% ODI) was non significantly higher in patients with cardiac AL (median 11 episodes (range 1-48)) compared to patients with macroglossia, cardiac and macroglossia and ATTR amyloidosis (6.02 (p=0.94), 6.03 (p=0.17) and 8.39 (p=0.34) episodes respectively, (Figure 5.3A). Figure 5.1B illustrates a normal tracing of overnight oxygen desaturations and pulse in a patient with cardiac amyloidosis showing repeated desaturations, with an abnormal tracing of these parameters illustrated in Figure 5.1C.

Figure 5.2A and 5.2B: Overnight oximetry tracing of 2 patient with cardiac amyloidosis showing oxygen saturations (red tracing) and pulse variability (blue tracing). A normal oximetry tracing is illustrated in Figure 5.2A, with the mean SpO2 of 94.6% and 3% ODI of 3.6 events per hour. Figure 5.2B illustrates grossly abnormal oximetry findings with the mean SpO2 of 96% and 3% ODI of 46 events per hour.

Figure 5.1A

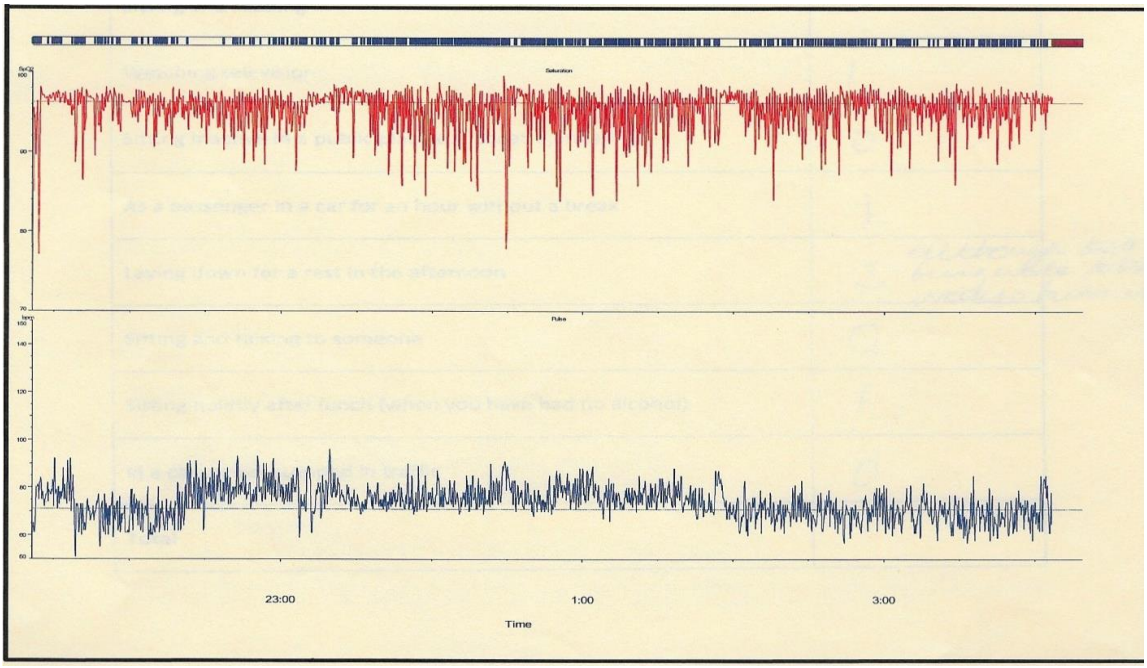
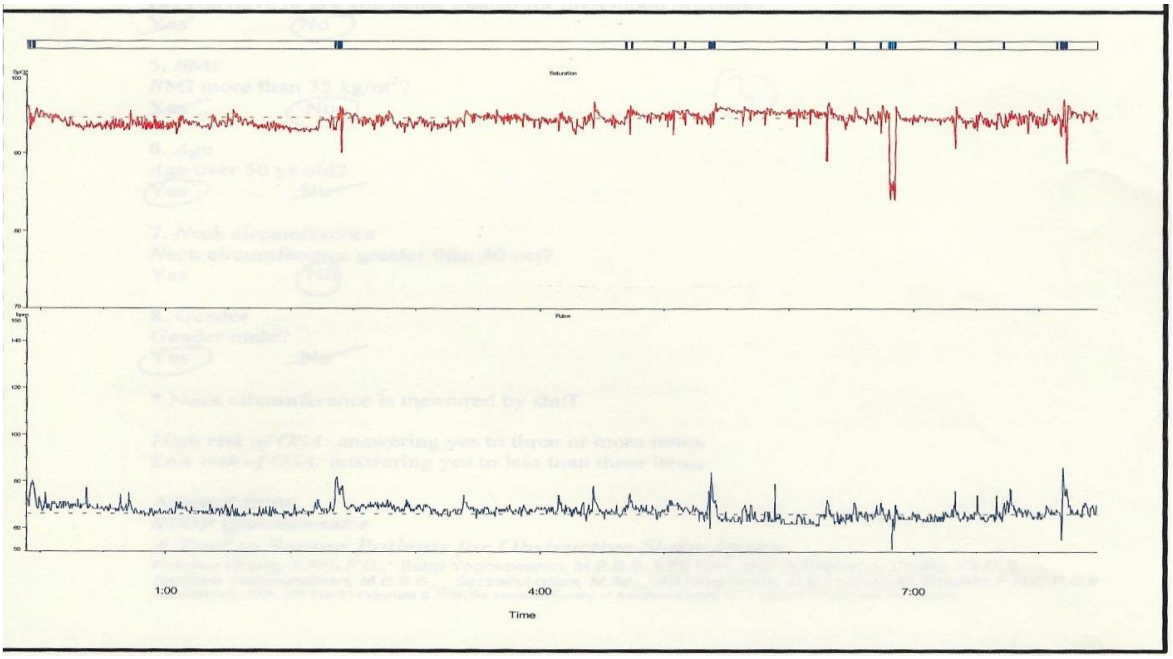


Figure 5.1B



Repeated desaturations were frequent and the total number of episodes of 4% ODI in one night in patients with cardiac AL, AL macroglossia, AL cardiac and macroglossia and ATTR were: more than 15 per night – 32%, 14%, 18% and 29% respectively; and between 10-15 episodes per night in 20%, 21%, 6% and 12% respectively. Heart rate variability (a HR change of >6 beats per minute), a normal physiological response to desaturation, was seen in: cardiac AL amyloidosis - 17/25(68%), patients with macroglossia - 10/14(71%), ATTR - 15/17(88%), and cardiac and macroglossia patients - 13/16 (81%), and in the patients who died - 8/12 (67%), (Figure 5.3B). The patients who died, as a group, had a lower blood pressure, more abnormal oxygen oximetry readings, a high ESS score (including proportion with ESS >10), in addition to markers of poorer cardiac function like a higher NT-proBNP and lower TAPSE.

Figure 5.3A and 5.3B: Relationship of 4%ODI and heart rate change greater than 6bpm in different types of amyloid respectively. This illustrates that cardiac AL patients experience the highest number of oxygen desaturations and have reduced heart rate variability.

Figure 5.3A

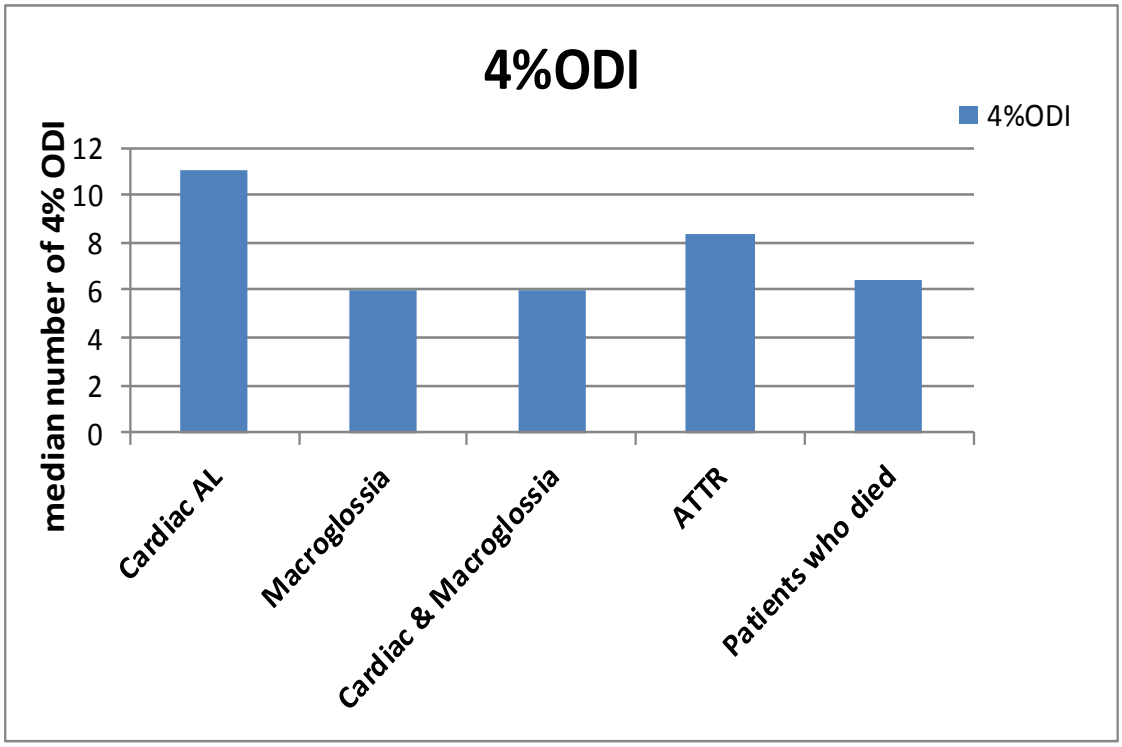
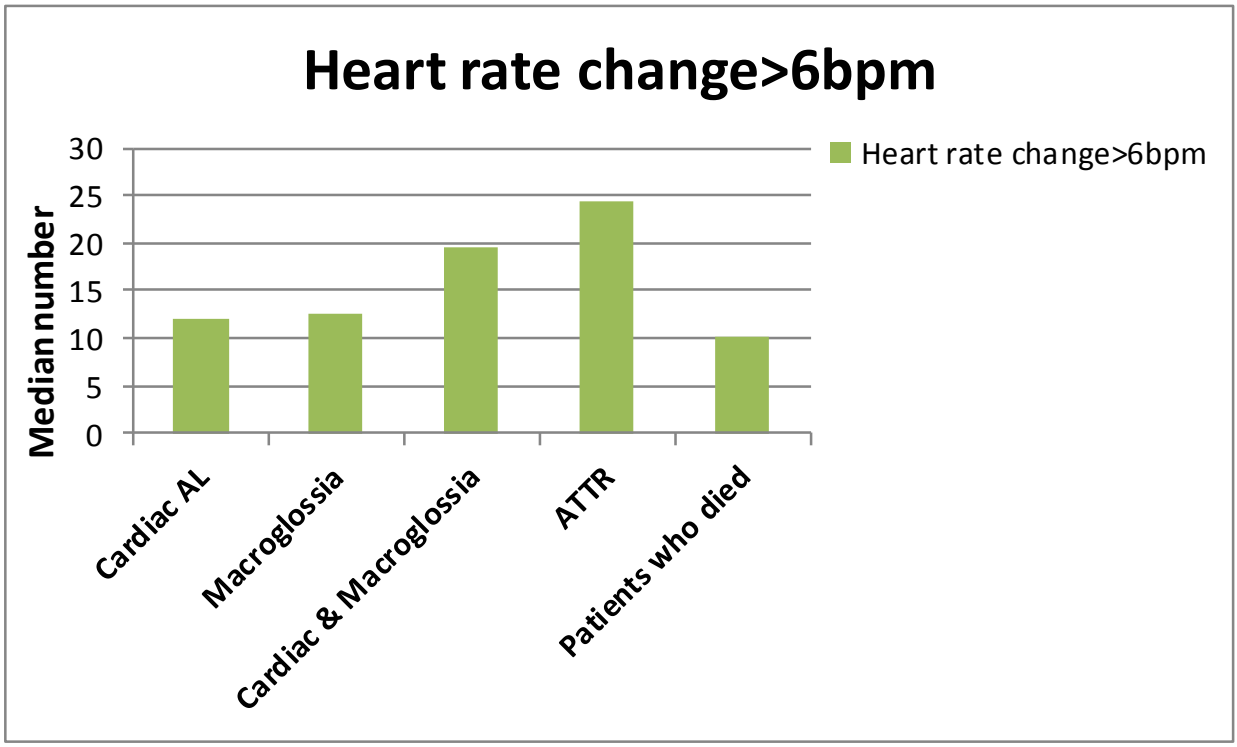


Figure 5.3B



The STOP-BANG and ESS questionnaires were completed by all 72 patients. The STOP-BANG score all four groups were high (71%, 69%, 71% and 64%) and would suggest a high risk of OSA, (Figure 5.3C). An abnormally high ESS score (>10) was seen in 28%, 21%, 19%, 24% in patients in the four groups, (Figure 5.3D). It was higher in those with cardiac AL (28%) and those who died (33%).

Figure 5.3C and 5.3D: STOP BANG questionnaire and ESS questionnaires in different amyloid groups respectively, showing evident elements of obstructive sleep apnoea and central sleep apnoea in these different groups. There is a relative lower risk of obstructive sleep apnoea and high risk of central sleep apnoea in cardiac AL patients.

Figure 5.3C

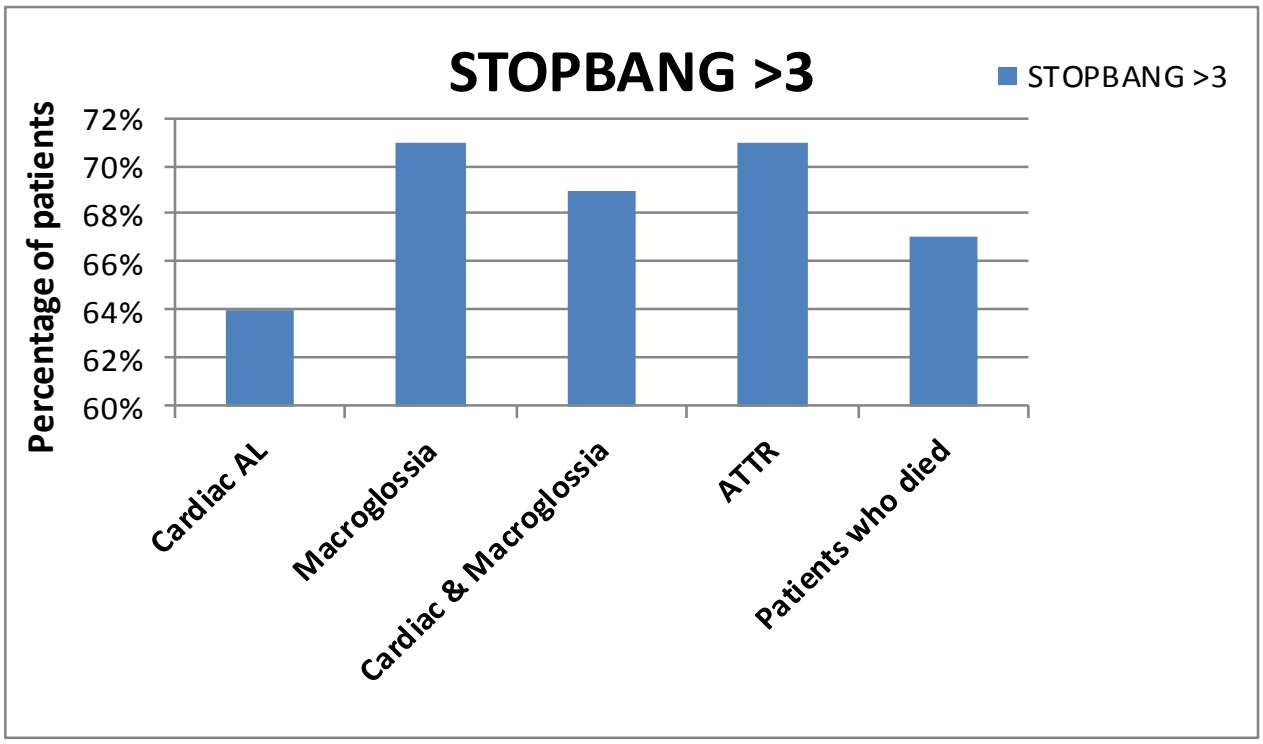
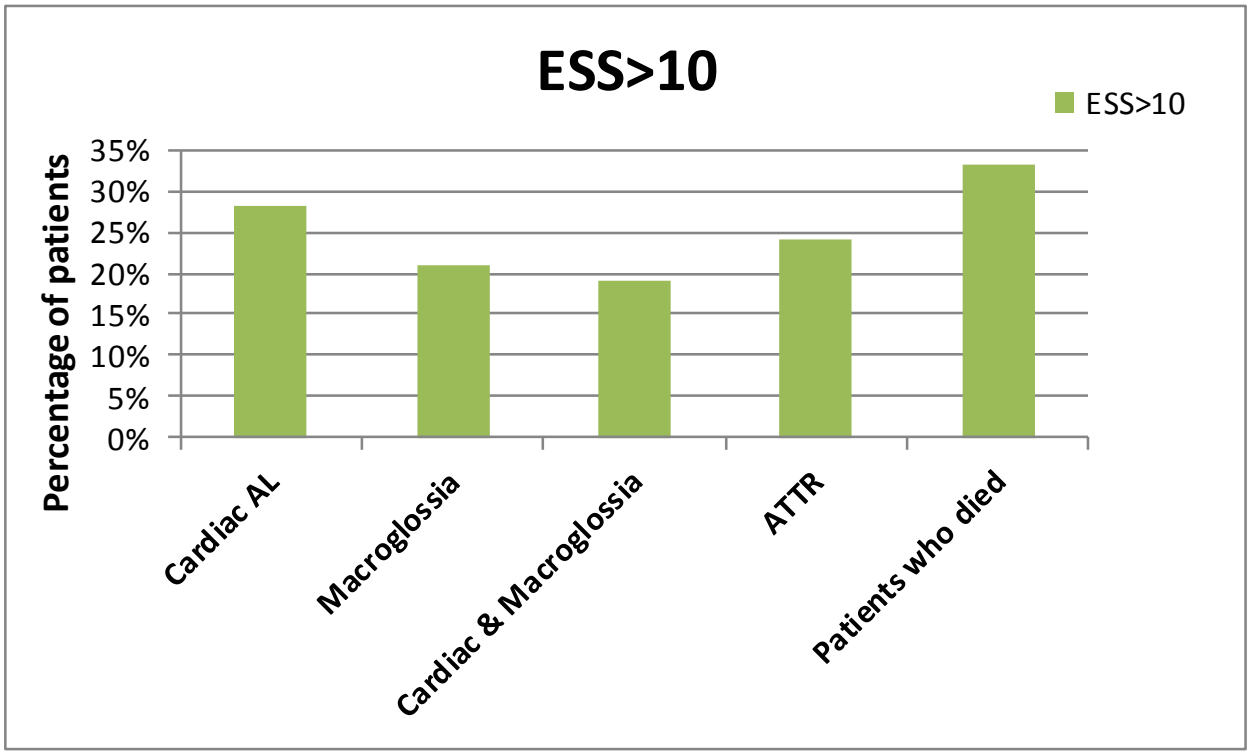


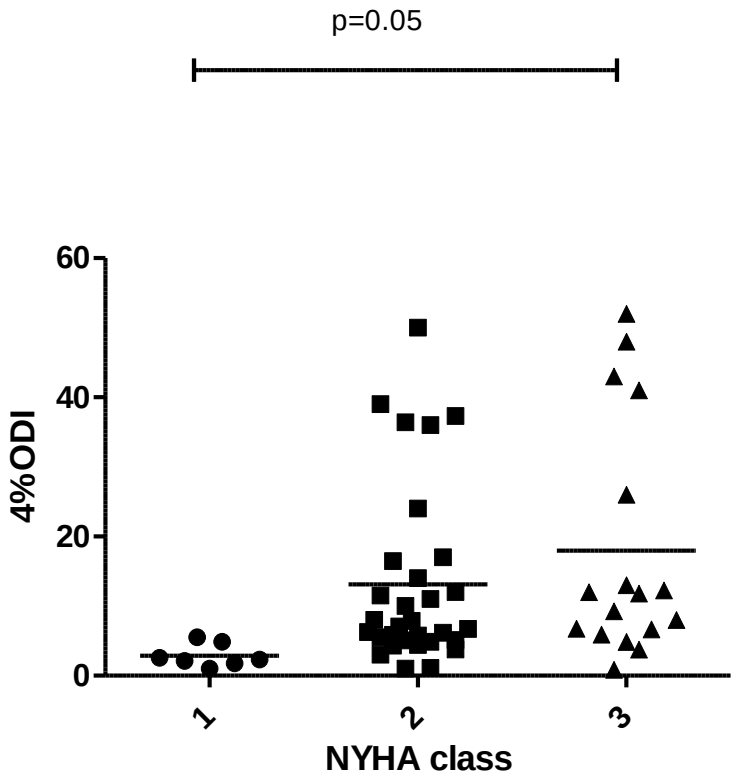
Figure 5.3D



Relationship between overnight oximetry and cardiac amyloidosis

In all patients with AL amyloidosis, increasing NYHA class directly correlated with higher incidence of 4% ODI p=0.05, figure 5.4. A higher NT-proBNP significantly correlated with reduced heart rate variability (correlation coefficient r^2 0.185, p=0.0002) There was no correlation of 4% ODI with right ventricle Doppler imaging (RVS TDI) (r^2=0.02, p=0.4) or tricuspid annular plane systolic excursion (TAPSE) (r^2=0.02, p=0.4). There was no correlation between TAPSE and heart rate variability (r^2=0.030; p = 0.30).

Figure 5.4: Relationship between 4%ODI and NYHA class symptoms in AL patients, showing a statistical trend in NYHA class I and III patients (p=0.05).



The median follow up for all cardiac amyloidosis patients was 10 months (range 2-15), with 12 deaths: 11 with cardiac AL and 1 with TTR. Higher NYHA class and NT-proBNP, well recognised markers of poorer prognosis in cardiac amyloidosis, were also markers of poorer prognosis in this study. The median OS of patients with AL amyloidosis with cardiac involvement was 12.7 months, macroglossia was 14.6 months and ATTR was 12.3 months (Figure 5.5A). The OS of patients with newly diagnosed AL was 10.4 months compared to those with AL previously treated 14.4 months (log rank p=0.004) from entry into the study, (Figure 5.5B). The recurrent oxygen desaturations may influence prognosis in cardiac AL patients within the initial phase of chemotherapy; number of 4%ODIs showing subtle changes in this period,

(Figure 5.5C). 4% ODI did not impact survival in the patients with ATTR amyloidosis or AL amyloidosis with just macroglossia.

Figure 5.5: Kaplan Meier curves illustrating the **(A)** overall survival categorised by the type of amyloidosis: including cardiac AL (blue line), soft tissue involvement with macroglossia (green line) and ATTR (yellow line); **(B)** overall survival comparing newly diagnosed cardiac AL (green line) and previously treated cardiac AL patients (blue line) **(C)** overall survival risk stratified on the 4%ODI frequency, with 4%ODI<10 (blue line), 4%ODI 10-15 (green line) and 4%ODI >15 (yellow line) in patients diagnosed cardiac AL patients.

Figure 5.5A

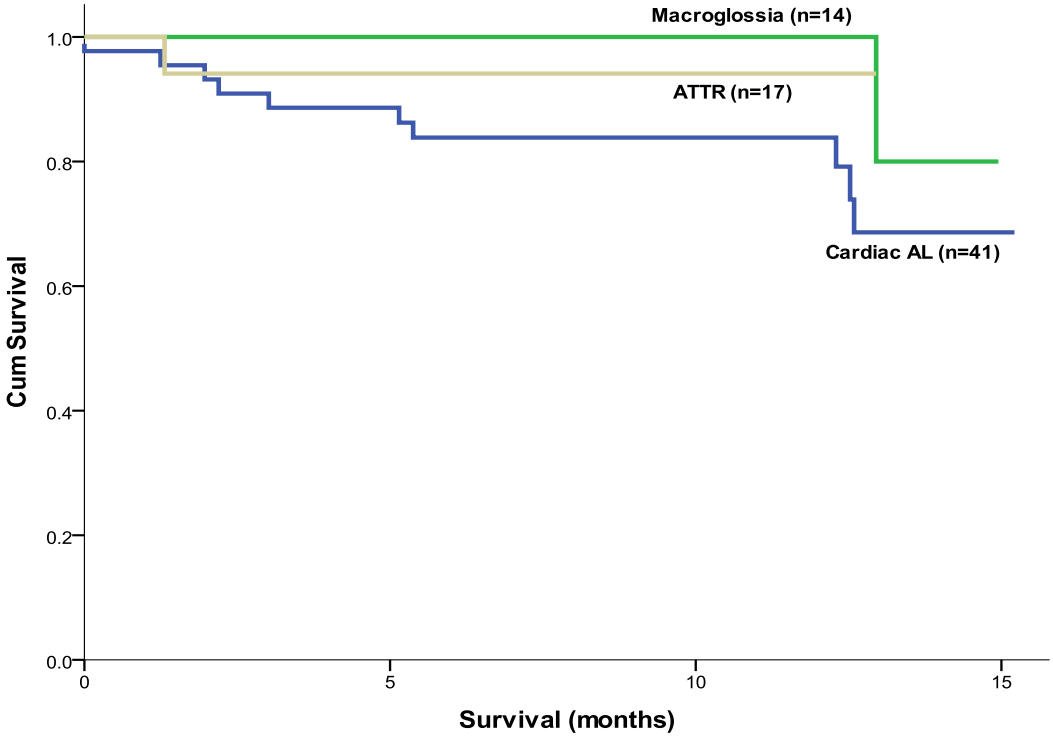


Figure 5.5B

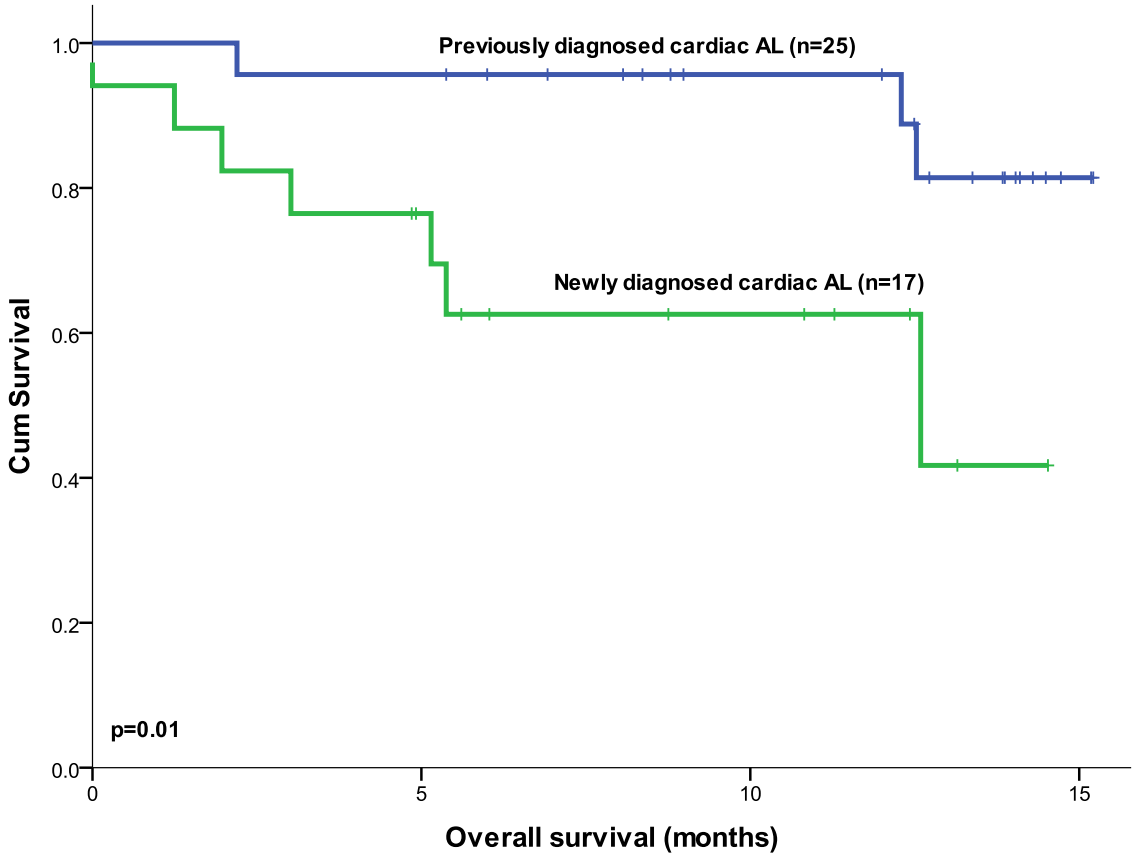
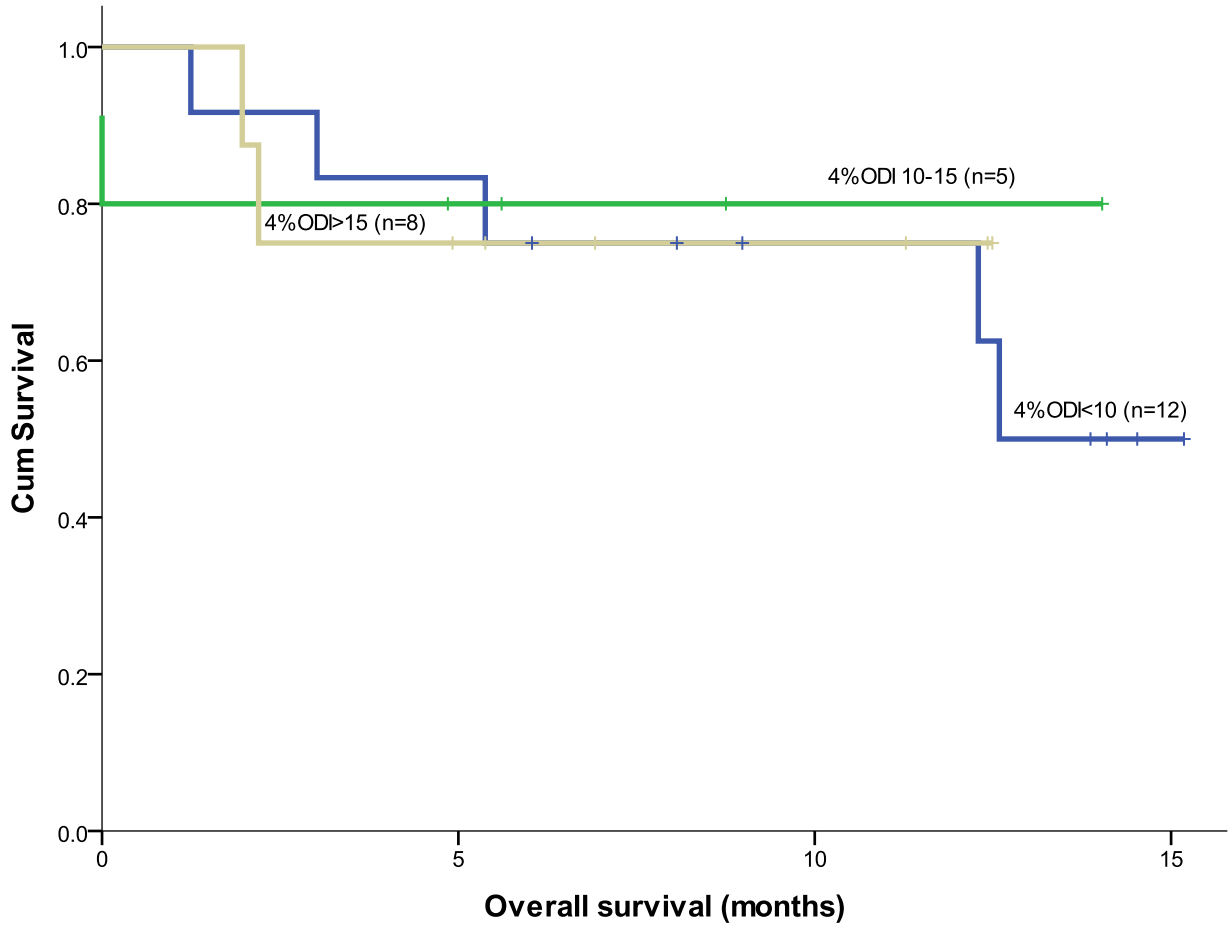

Previously diagnosed cardiac AL (n=25)
Newly diagnosed cardiac AL (n=17)
p=0.01
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
Overall survival (months)

Figure 5.5C


1.0
0.8
0.6
0.4
0.2
0.0
Cum Survival
4%ODI 10-15 (n=5)
4%ODI>15 (n=8)
4%ODI<10 (n=12)
0
5
10
15
Overall survival (months)

On univariate analysis of newly diagnosed cardiac AL patients (table 5.3), the factor significantly impacting survival was: TAPSE (HR 0.69, p=0.01). Small patient numbers make a multivariate model unreliable.

Table 5.3: New cardiac AL

Variable	HR (95% CI)	p
Univariate Analysis		
Neck circumference*	1.37 (0.31-6.13)	0.68
Body mass index*	0.81 (0.63-1.04)	0.10
Mean oximetry*	1.03 (0.7-1.52)	0.88
Mean pulse*	1.05 (1.0-1.11)	0.12
Systolic blood pressure*	0.96 (0.92-1.01)	0.13
Heart rate variability>6bpm*	1.03 (0.97-1.07)	0.08
4%ODI*	0.95 (0.87-1.04)	0.28
TAPSE*	0.69 (0.51-0.92)	0.01
dFLC >180mg/L	0.14 (0.02-1.18)	0.07
Log NT-proBNP*	2.79 (0.54-14.38)	0.22

bpm – beats per minute; TAPSE – tricuspid annular pulmonary systolic excursion; dFLC – difference between involved and uninvolved free light chains; 4%ODI – hourly average number of desaturation episodes defined as a 4% decrease in saturations from the average saturations in the preceding 120seconds and lasting for more than 10seconds; AL – light chain.

* denotes continuous variables

Discussion

Treatment of patients with systemic AL amyloidosis with cardiac involvement remains a challenge. There is a complex interaction between tissue deposition of the amyloid fibrils leading to multi-organ dysfunction causing often unexpected problems in cardiac patients which have the potential to worsen or complicate the clinical picture. In this study, we report the occurrence of repeated and significant nocturnal hypoxaemia by overnight pulse oximetry. This pattern is strongly suggestive of sleep disordered breathing which is likely to be central sleep apnoea in patients with cardiac amyloidosis, obstructive sleep apnoea in those with soft tissue amyloidosis and

possibly a combination of the two in patients with simultaneous occurrence of both cardiac/soft tissue amyloidosis. The nocturnal desaturations correlated with the worsening grade of heart failure and worse right ventricular function – both markers of poor prognosis. The impact of these repeated oxygen desaturations on the function of an already fragile myocardium is unclear but may potentially cause worsening of function or predispose to arrhythmic events. The observation of repeated nocturnal hypoxia opens the possibility of simple and easy to use oxygen supplementation for such patients. It also raises the possibility of using overnight pulse oximetry to provide objective data which could potentially be used to monitor disease progression or to assess response to treatment. A particularly interesting finding in patients who died of cardiac amyloidosis was the loss of ability to mount an appropriate heart rate response to hypoxia – possibly a marker of involvement of the sympathetic nerves.

Overnight pulse oximetry is an easy screening method for sleep apnoea, with the sensitivity ranging from 31 to 98% and specificity 41-100%.[267-269] It gives a continuous recording of oxygen desaturations with a characteristic pattern of overnight oxygen saturations. Clinical prediction models are useful with sensitivities between 76-96% and specificities 13-54% and useful in excluding a diagnosis,[270] but in conjunction with pulse oximetry can confirm the presence of sleep apnoea.[271]

The striking finding in our study was presence of significant overnight oxygen desaturations in all groups of patients. Our initial supposition was that we may find marked hypoxemic episodes in patients with macroglossia or soft tissue amyloid deposits due to the severe anatomical alterations. Although such patients also had 4% ODIs, these were less frequent than in the cardiac AL group and were not seen in all cases. In obstructive sleep apnoea which occurs in absence of amyloidosis, the laxity of the oropharyngeal soft tissues is an important component in causing the

collapse of the tissues during inspiration leading to obstruction of the upper airways. The current findings of less than expected severe hypoxia in patients with significant soft tissue amyloid deposits raises an interesting question whether increased stiffness of the soft tissue, which is a hallmark of amyloid deposition, actually protects against the “traditional” cause of OSA and true OSA in amyloidosis only occurs when there enough amyloid deposition to alter the anatomy towards obstruction a supine position.

Central sleep apnoea (CSA) is a well-recognised complication of systolic heart failure and leads to repeated episodes of nocturnal hypoxia. CSA is worse in patients with an ejection fraction of <40% and has been reported to be associated with increase in non-sustained ventricular tachycardia as well as reduction in heart rate variability.[272] The occurrence of repeated severe nocturnal oxygen desaturations seen in patients with cardiac amyloidosis strongly suggest CSA also occurs in AL amyloidosis – a finding that will need formal confirmation by polysomnography. In our study, the oxygen desaturations were greatest in those with cardiac AL and ATTR amyloid, again with the median number of desaturations higher in those with cardiac AL. Moreover, 4% ODI correlated with NYHA class and NT-proBNP – suggesting a direct correlation with worsening amyloid burden/heart failure with ODI. Although there was no direct impact of the number of 4% ODIs on overall survival in patients with newly diagnosed AL amyloidosis, these repeated insults with other factors are likely to influence these patients. This is a strikingly different finding from that in systolic heart failure where the cardiovascular impact of CSA is universal. In AL amyloidosis, newly diagnosed patients have the most unstable heart disease with 30-40% patients dying of cardiovascular complication within 6 months of the initial diagnosis. The impact of nocturnal hypoxia correlating with increased deaths raises important questions – do the repeated hypoxemic changes increase the vulnerability of an already fragile

myocardium to arrhythmias? Ventricular arrhythmias have been proposed as a causal mechanism for the peak of sudden cardiac death during sleeping hours in OSA patients.[273] The risk of cardiac arrhythmias with OSA appears to be related to disease severity - majority of OSA patients presenting significant arrhythmias have moderate or severe forms of the disease.[274-276] The amyloidogenic light chains are directly toxic to the myocardium in AL amyloidosis and additional hypoxemic insults may compound this toxicity. Lastly, patients receive chemotherapy soon after diagnosis and hypoxaemia may influence the toxicity of chemotherapy. Repeated nocturnal hypoxia beyond the newly diagnosed setting may have different consequences. In CSA, the hypoxaemia has consequences in addition to worse mortality, which include increase in day time sleepiness, worsening fatigue and poorer quality of life.[277, 278] Although the latter two were not studied in our cohort, a very high proportion of patients with cardiac AL amyloidosis and ATTR had abnormal ESS questionnaires – a measure of day time sleepiness. Persistent and profound fatigue is a symptom in AL amyloidosis which has never been adequately explained. It is always vaguely assumed to be multifactorial from the wide range of organ involvement. The current findings suggest that CSA/OSA may be a substantial contributor to this symptom. Recurrent hypoxemia was seen in both AL and ATTR cardiac patients but were more marked in the AL cohort – in keeping with the worse cardiac profile of AL patients compared to ATTR in general.

An interesting finding in this study was the lack of or markedly reduced heart rate variability in patients with cardiac AL amyloidosis. This lack of heart rate response to hypoxia was greater in AL amyloidosis than those with ATTR cardiac amyloidosis. Autonomic dysfunction is a known phenomenon in AL amyloidosis but the current finding had no correlation with clinical autonomic neuropathy. Cardiac autonomic

denervation is well recognised feature of systolic heart failure[279, 280] and ^{123}I-MIBG – Metaiodobenzylguanidine (MIBG) scintigraphy is a useful tool for its identification.[279, 281] MIBG scans have been reported to be abnormal in cardiac amyloidosis both AL and ATTR type.[281-284] The most marked lack of heart rate variability was seen in the patients who died. Due to a restrictive left ventricle in cardiac AL, any needed increases in cardiac output are largely dependent on appropriate increase in the heart rate. The lack of physiologic tachycardia in response to hypoxia could make the systemic consequences more profound. This finding suggests that further assessment of cardiac autonomic denervation may be provide useful information in AL amyloidosis and open a potential therapeutic avenue for consideration of device therapy to counter a lack of the autonomic drive.

The markedly abnormal findings in this pilot study, if confirmed by polysomnography, suggests possible avenues for intervention. For many years, there was a suggestion that correction of CSA in systolic heart failure would improve outcomes. A randomised study (SERV-HF) in systolic heart failure and moderate to severe heart disordered breathing showed that assisted servo ventilation (ASV) controlled sleep disordered breathing but it did not improve survival; in fact there was increased cardiac mortality in the ASV group.[285] The restrictive cardiomyopathy in AL is very different from the patients in reported in that study and those findings cannot be extrapolated to cardiac amyloidosis. In addition, newly diagnosed AL is a disease of very high mortality not just chronic morbidity, in which SDB may impact. Just as drugs which treat systolic heart failure effectively, like beta-blockers and ACE inhibitors, often have profoundly negative consequences in AL - impact of intervention on sleep disordered breathing in AL may have different consequences and will need a separate study of formal polysomnography sleep study to confirm occurrence of SDB (CSA and/or OSA) as

well as impacts of intervention with simple oxygen supplementation or indeed more complex intervention for SDB.

We recognise the limitations of this pilot study – a small study cohort and the use of overnight pulse oximetery only. The group of patients was mixed with newly diagnosed AL, those on longer term follow up as well as ATTR amyloidosis limiting ability to assess survival impact in subgroups. There was no polysomnography to confirm the cause of the recurrent nocturnal hypoxia or clarify type of SDB.

In conclusion, recurrent nocturnal oxygen desaturations are very common in patients with cardiac amyloidosis (both AL and ATTR type) as well as in patients with soft tissue amyloid deposits affecting the oropharyngeal tract. A high proportion of patient score 'high risk' for sleep disordered breathing by questionnaires designed to screen for such patients. Increased number and frequency of nocturnal desaturations may be associated with poorer survival in patients with newly diagnosed cardiac AL amyloidosis. Lack of heart rate variability (suggesting cardiac autonomic neuropathy) is a frequent occurrence on cardiac AL and particularly in those patients who died – findings which need further clarification. The role of hypoxia in precipitation of cardiac arrhythmias or sudden death in AL needs to be clarified. Nocturnal hypoxia is a simple target for intervention in cardiac AL amyloidosis and could potentially help to reduce early mortality in AL which has remained an unmet medical need for over 25 years.

Results Section Two:

Localised amyloidosis and subtypes

Chapter Six: Natural history and outcomes in localised immunoglobulin light chain (AL) amyloidosis: a long-term observational study

This chapter is in context of my publication: Natural history and outcomes in localised immunoglobulin light-chain amyloidosis: a long-term observational study. Shameem Mahmood, Frank Bridoux, Christopher P Venner, Sajitha Sachchithanantham, Janet A Gilbertson, Dorota Rowczenio, Thomas Wagner, Rabya Sayed, Ketna Patel, Marianna Fontana, Carol J Whelan, Helen J Lachmann, Philip N Hawkins, Julian D Gillmore, Ashutosh D Wechalekar. Lancet Haematology. 2015; 2(6):e241-50. (Original article)

Introduction

Amyloidosis comprises a heterogeneous group of disorders produced by the extracellular deposition of misfolded proteins in an insoluble fibrillar form.[286] Systemic light chain (AL) amyloidosis is the most common and serious type, where the amyloid fibrils are derived from circulating monoclonal immunoglobulin light chains produced by an underlying plasma cell or B-cell clone. Hereditary systemic amyloidosis is associated with mutations in numerous variant proteins including transthyretin (ATTR amyloidosis) and apolipoprotein AI (AApoA1 amyloidosis).[287] In systemic types of amyloidosis, the respective amyloid fibril precursor protein is present and circulating

in the bloodstream and results in relentless amyloid deposition throughout the body, progressive vital organ dysfunction and early death.[288]

Localised deposits of amyloid can occur in various tissues in the body and are usually presumed to be of AL type, with the consequent presence of a focal monoclonal B cell dyscrasia within the affected tissue. As such, the clinical effects of the localised amyloid deposits depend on their precise anatomical location, and can result in substantial morbidity.

Localised amyloidosis is much rarer than systemic types, and consequently remains very poorly studied; with most knowledge arising from individual case reports or small series of less than 20 patients. There are commonly reported sites include the urinary tract, respiratory tract, larynx, skin and eyelids.[165] Currently, data on long term outcomes and progression to systemic disease is lacking with the need for further exploration in this field. We have examined the clinical features and outcomes of a large series of patients with localised AL amyloidosis highlighting the striking differences from systemic AL amyloidosis with respect to the lack of progression, benefit from debulking procedures, limited need for cytotoxic chemotherapy therapy and excellent overall long term outcomes.

Methods

Study design

All the patients with a specified disease setting of localised amyloidosis diagnosed, assessed and followed up at the UK National Amyloidosis Centre (NAC) between January 2, 1980 and December 15, 2011 were identified using the NAC database and written medical records. There was no exclusion criteria dependant on age or

performance status, with all comorbidities permitted. All the patients were newly diagnosed and treatment naïve. Localised amyloidosis was typed with biopsy proven amyloid deposition confined to a single site or tissue (with the relevant histology of the tissue examined), and no evidence of vital organ involvement, the latter defined as cardiac, renal, liver involvement or peripheral or autonomic neuropathy. Soft tissue involvement was defined as clinical evidence of a single deposit of amyloid in the tongue or one site of deposition in other soft tissues. Standard tests of vital organ function were assessed and absence of visceral uptake was documented by ^{123}I serum amyloid P component (SAP) scintigraphy.[289] All patients had SAP scintigraphy performed at their first visit except those diagnosed before 1988 (when the technique was introduced into clinical practice), and had this following availability of the procedure, which was invaluable in assessing for any visceral organ uptake. A detailed protocolised assessment was followed by all patients at baseline and follow up visits to assess disease evaluation including a full blood count, renal and liver function tests, serum and urine protein electrophoresis and immunofixation, electrocardiogram (ECG), echocardiography and ^{123}I SAP scintigraphy. Serum free light chains (FLC) were also measured prospectively on all patients diagnosed after 2002 on blood samples at presentation and consequent follow up visits; and also retrospectively on stored serum samples, where available, for patients prior to 2002. A bone marrow assessment was performed in patients with a proven plasma cell dyscrasia for further investigation. Histological confirmation of amyloid deposition of the affected tissue was conducted by showing Congo red staining and demonstration of typical birefringence under cross polarised light. Fat biopsies were not performed in addition to the affected tissue biopsy. The AL fibril type was determined by immunohistochemical (IHC) staining using a panel of antibodies and defined as

staining with antibodies kappa and lambda immunoglobulin light chains and/or no staining with antibodies to transthyretin, serum amyloid A protein (SAA) and ApoA1, with AL the likely type. Hereditary amyloidosis was excluded by genetic sequencing in patients in whom the clinical features could be in keeping with hereditary types – particularly all patients with laryngeal symptoms underwent Apo A1 gene sequencing; with laryngeal amyloid deposits a recognised feature of patients with Apo A1 mutations. Transthyretin gene sequencing was performed if any biopsies had transthyretin staining to exclude ATTR. F18-flurodeoxyglucose positron emission tomography (FDG-PET/CT) was performed in 18 patients following this technique becoming an investigative tool at our centre.

Local disease progression was defined as progression or recurrence of the amyloid deposit within the original localised site. Progression to systemic AL amyloidosis was clarified as development of a new vital organ involvement or new evidence of visceral amyloid deposits by ^{123}I SAP scintigraphy, and stated as a number and percentage of the total localised amyloidosis cohort. Statistical analysis in assessing survival endpoints was performed using SPSS version 20. The overall survival (OS) was determined from the date of diagnosis defined from the date at which a biopsy proof of amyloidosis was obtained. The overall survival was estimated by the Kaplan Meier method, calculated from the date of diagnosis until death or last follow-up, with the causes of death known in those who died. All the patients consented to their details being used anonymously and the study received approval from the Royal Free Hospital ethics committee.

Results

Study population

The recruitment to this study included a total of 606 patients with localised amyloidosis identified. Overall, this accounted for 12% (606/5050) of 5050 newly diagnosed patients with all types of amyloidosis evaluated at our Centre during the study period. 97 patients with laryngeal symptoms had Apo A1 gene sequencing, and interestingly transthyretin staining was present on 3 bladder biopsies, with transthyretin gene sequencing performed to exclude ATTR. For the purposes of survival statistics, 11 patients were excluded as three patients had ATTR amyloid deposition on bladder biopsy (with ATTR was not seen at any other localised site), 4 with lichen amyloidosis, 3 with insulin amyloidosis and 1 patient had AApoA1 amyloid deposition in a laryngeal biopsy and shown to be heterozygous for ApoA1 Ala164Ser mutation by ApoA1 gene sequencing. These patients were excluded from only survival analysis with few numbers and little known as to underlying prognosis and complications of this group. All remaining patients were included in analyses of survival and progression to systemic disease illustrated in Figure 6.1A.

The survival outcomes are available for all patients with data obtained from the Office of National Statistics in the UK.[290] Baseline characteristics are illustrated in Table 6.1, with the median age 59.5 years (range 48.8-87 yrs, IQR 50.2-74.5) and 51% (307/606) were male. The median symptom duration was 7 months (range 0.5-360 months, IQR 4-24) prior to diagnosis. There were many sites of localised amyloidosis including: bladder 95/606 patients (15.7%); laryngeal/tonsillar 92/606 (15.2%); cutaneous 84/606 (13.9%); pulmonary nodular 47/606 (7.8%); gastrointestinal 36/606 (5.9%); oral 36/606 (5.9%); tracheobronchial 35/606 (5.8%); lymph node 31/606 (5.1%);

conjunctival 27/606 (4.5%); bone 24/606 (4%); eyelid 22/606 (3.6%); orbital 21/606 (3.5%); breast 13/606 (2.1%); ureteric 10/606 (1.7%); urethral 10/606 (1.7%); soft tissue 10/606 (1.7%); prostate 9/606 (1.3%); cerebral 3/606 (0.5%) and aortic valve 1/606 (0.1%), (Figure 6.1B). Some patients had amyloid deposits at more than one localised site including cutaneous (9 patients) and within the gastrointestinal tract (2 patients). The multiple deposits within the skin included - 5 patients with deposits in both lower limbs, and 3 patients having 2 separate skin sites involved (right calf and right elbow, face and skin over the breast, right hand and left elbow). Patients with gastrointestinal involvement included (gastric involvement in 7 patients, small bowel involvement in 10, 17 with large bowel involvement and 2 with small and large bowel involvement).

Figure 6.1A: Progression to systemic AL amyloidosis or need for therapy. A flow chart illustrating the patient flow, outcomes, treatment and progression to systemic AL in all 606 patients described in the series. Light blue denotes patients in the study, with the dark blue showing additional information regarding the immunohistochemistry.

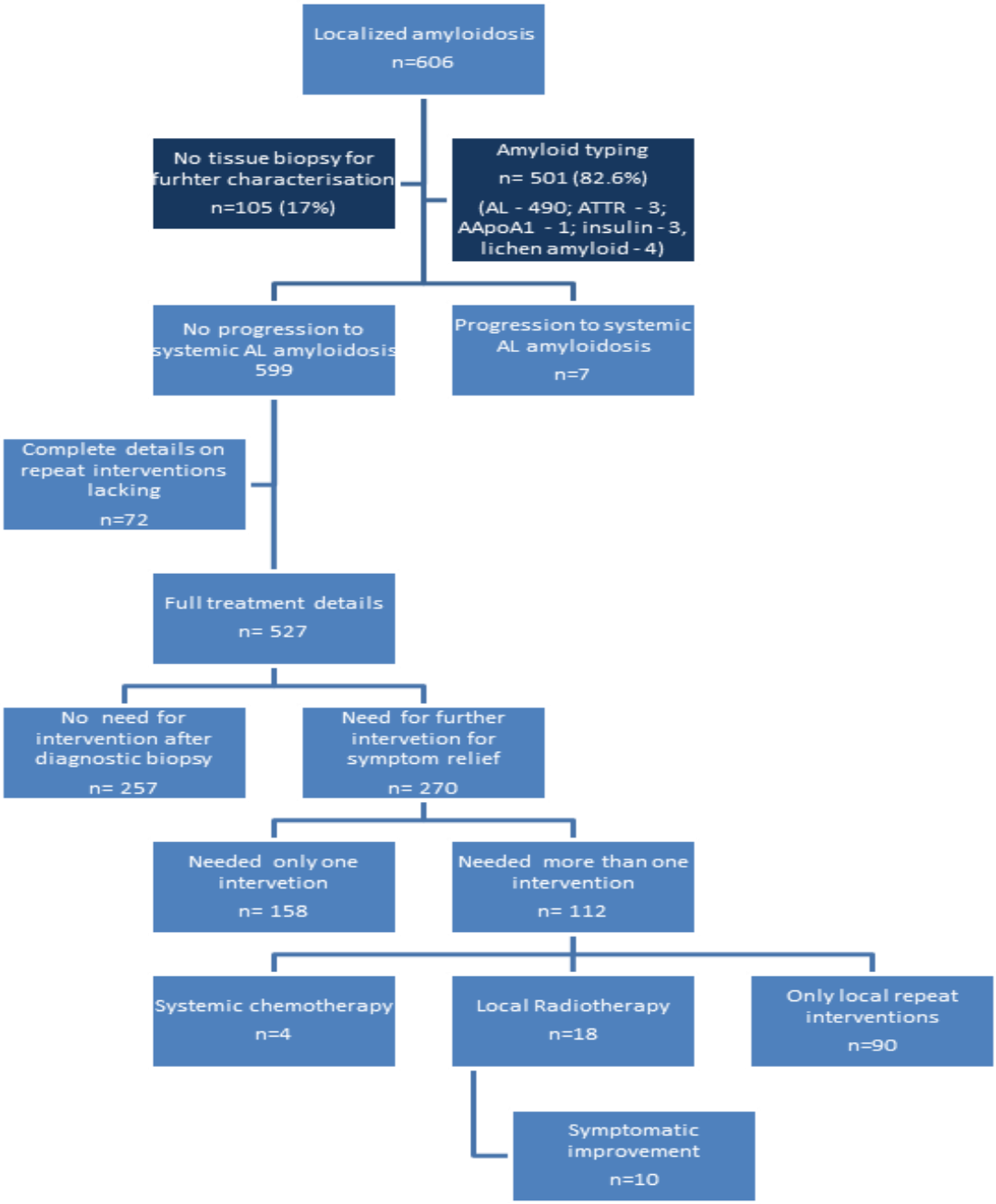


Figure 6.1B: Overall distribution of localised amyloidosis by site of amyloid deposition

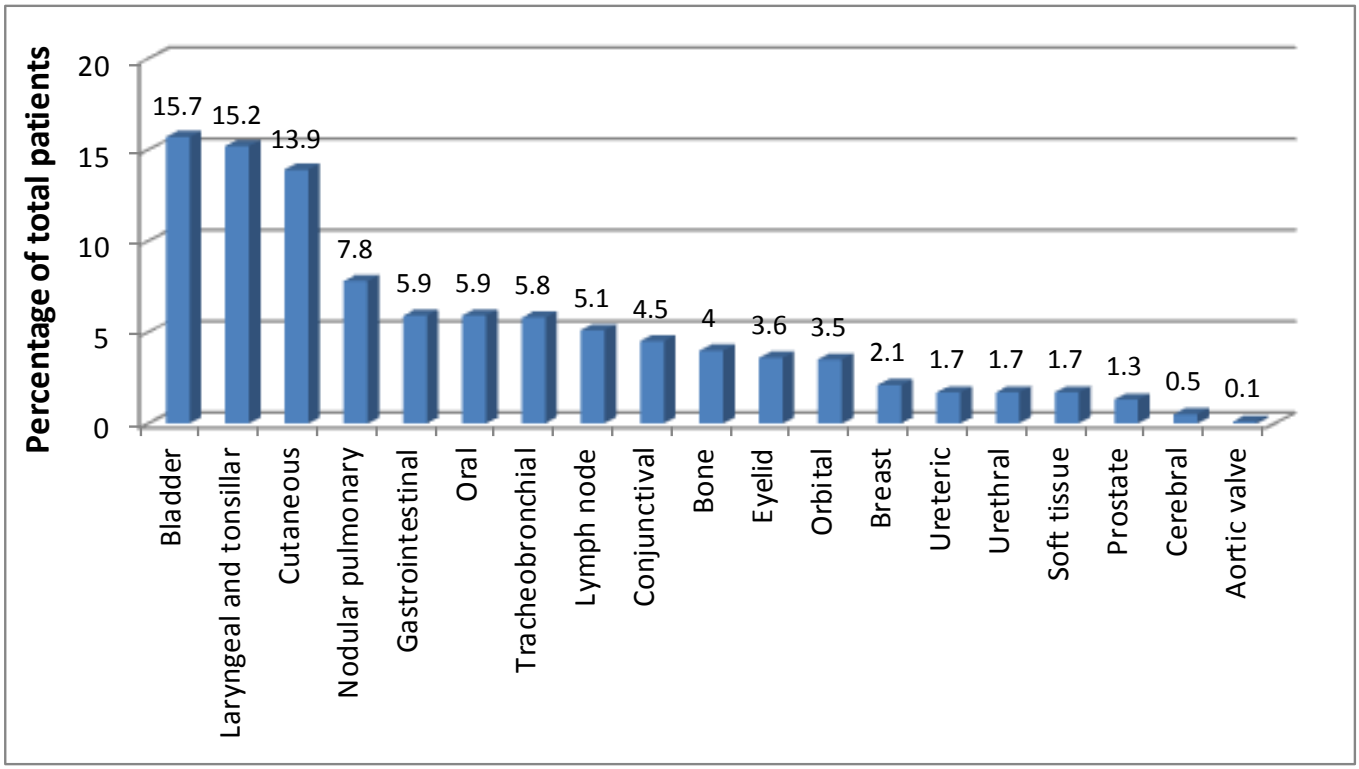


Table 6.1: Patient characteristics

Patient Characteristics	Number (%)/Median(range)
Age	59.5 years (48.8-87, IQR 50.2-74.5)
Male	307 (51%)
Duration of symptoms	7 months (0.5-360, IQR 4-24)
Monoclonal protein	76 (12.5%)
Monoclonal protein level	6.5 g/L (1-35, IQR 3-12)
Monoclonal protein type	
IgG	38 (6.2%)
IgA	8 (1.3%)
IgM	29 (4.7%)
IgD	1 (0.2%)
Serum free light chains	
Abnormal kappa/lambda ratio	79 (13.0%)
Abnormal lambda	28 (4.6%)
Abnormal kappa	51 (8.4%)
Involved light chain (iFLC)	56.2 mg/L (range 5.6-2100, IQR 5.6-59.3)
Difference between involved and uninvolved FLC (dFLC)	43.75 mg/L (range 3.2-2023, IQR 3.2-44.2)
dFLC >50mg/L	37 (6.1%)
Creatinine	79 µmol/L (range 69-91, IQR 69-90.5)
Estimated glomerular filtration rate	81ml/min (range 70-100, IQR 70-100)
Alkaline phosphatase	72 U/L (range 59-89, IQR 60-89)
Albumin	44 g/L (range 42-46, IQR 42-46)
24 hour urinary proteinuria	<0.1 g (range 0.1-3.2, IQR 0.1-0.2)
Haemoglobin	13.5 g/dL (range 12.5-14.3, IQR 12.5-14.4)
Patients diagnosed in time intervals	
1980-1985	24 (4%)
1986-1990	23 (3.8%)
1991-1995	40 (6.5%)
1996-2000	78 (12.9%)
2001-2005	132 (21.8%)
2006-2011	309 (51%)

g/L – grams per litre; U/L – units per litre; µmol/L – micromoles per litre; g/dL – grams per decilitre. Normal ranges for kappa: 3.30-19.4 mg/L, lambda: 5.71-26.3 mg/L, kappa/lambda ratio: 0.26-1

The presenting symptoms were determined by the site of amyloid deposition and Table 6.2 summarises the clinical features for each site of amyloid deposition, median age and duration of symptoms and accompanying ranges stated as minimum and maximum values. Figure 2 shows different types of localised amyloidosis. 67 of 606 patients (11.0%) were known to have an autoimmune disorder including 17/606 (2.8%) with Sjögren's syndrome, 18/606 (3.0%) with hypothyroidism, 8/606 (1.3%) having rheumatoid arthritis and 6/606 (1.0%) diagnosed with systemic lupus erythematosus (SLE). Taking into account the 17 patients with Sjögren's syndrome, amyloid deposition was present in the lungs in 5, breast in 3, conjunctiva in 2 - accounting for 10.6% (5/47), 23.1% (3/13), 7.4% (2/27) of all lung, breast and conjunctival amyloidosis patients respectively- and four in the skin. The remainder had amyloid deposition at a variety of other localised sites. An unrelated history of non-haematological malignancies was present in 37 patients (6.1%); cervical and ovarian cancer 3, carcinoma of the prostate 10, sigmoid cancer 6, transitional cell carcinoma 4, Essential Thrombocythaemia/Polycythaemia Rubra Vera 3, breast carcinoma 7, renal cell carcinoma 2, brain tumour 1, and lung cancer 1. All these malignancies had occurred at a site remote from the presenting site of localised amyloid deposition.

Figure 6.2: A clinical spectrum of showing localised amyloidosis: (A) ^{123}I labelled SPECT/CT showing a localised amyloidoma in the parieto-temporal region in a patient presenting with unexplained visual problems; (B) F-18 PET/CT scan in a patient with cutaneous amyloidosis showing multiple subcutaneous areas of FDG avidity at sites of amyloid deposition; (C) Bronchoscopy in a patient with tracheobronchial amyloidosis showing nodular amyloid deposits in the tracheo-bronchial tree; (D) A large cutaneous amyloid deposit in the scalp; (E) An enlarged axillary node in an elderly patient with localised lymph node amyloidosis; (F) and (G) A left iliosacral lesion in a patient with isolated localised bone amyloidosis showing marked uptake the lesion with ^{123}I labelled SPECT-CT (F) which has completely disappeared at six months after completion of local radiotherapy with no ^{123}I uptake at the site of the lesion and some sclerosis (G).

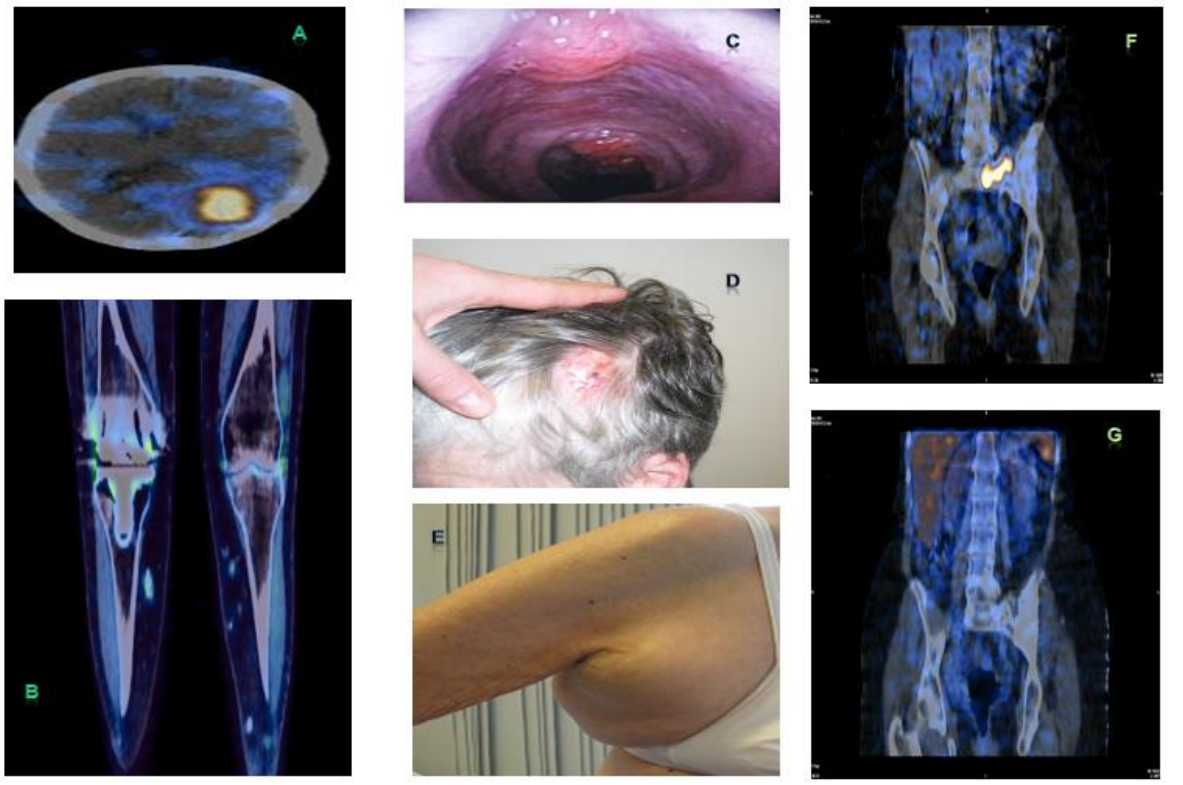


Table 6.2: Patient characteristics according to amyloid type

Amyloid Type (n)	Gender	Age at diagnosis Median yrs. (range)	Predominant Symptom	Symptom Duration Median months (range)	Associated underlying clonal disorder	Paraprotein (n)	Abnormal κ/λ ratio (n)	Surgical Intervention (n)	Progression at the primary site (n)	Non-surgical treatment	PET Positive (positive/n with total PET scan)
Laryngeal and Pulmonary System											
Laryngeal+ Tonsillar (92)	46M 46F	57.4 (13-83.3)	Hoarse voice	10.5 (1-360)		3	7	51	63	**Chemotx (1) *Radiotx (1)	
Tracheobronchial (35)	14M 21F	47.6 (31-81.7)	Shortness of breath	4.3 (1-78)		2	5	18	22	**Chemotx (1) *Radiotx (3)	
Nodular Pulmonary (47)	13M 34F	65.5 (36-80)	CXR abnormality Haemoptysis	6 (1-180)	MALT (2)	9	10	4	4	*Chemotx (2)	7/18
Urothelial System											
Bladder (95)	35F 60M	64 (37.1-87)	Haematuria	6 (1-120)		6	4	29	32	*DMSO (7)	
Urethral (10)	10M	39.8 (27.1-78.6)	Urinary tract infections	12 (2-36)			2	5	5		
Ureteric (10)	6M 4F	59.6 (43.1-78.4)	Haematuria	8 (2-112)		1	1	14	8		
Cutaneous and Soft tissue System											
Cutaneous (84)	44M 40F	56.1 (24-80.9)	Rash/lump	12 (3-240)	MM (1) NHL (1)	12	15	25	26 ***	**Chemotx (2) *Radiotx (1)	1/18
Soft tissue (10)	3M 6F	65 (35-78.6)	lump	12 (4-72)	MM (1) LPL (1)	1		6	6	**Chemotx (2) *Radiotx (2)	2/18

Oral and Gastrointestinal System											
GI (36)	29M 7F	61.4 (37-83.9)	PR bleeding Abdominal pain	4 (1-37)	MM (1) MALT (1)	8	9	8	8 ****	*Chemotx (2)	
Oral (36)	17M 14F	58.2 (24-78)	Persistent lump	6 (1-55.3)	NHL (1) MALT (1)	2	1	23	15	*Radiotx (1)	1/18
Eye Related Systems											
Orbital (21)	7M 15F	42.44 (25.3-84.2)	Ptosis	7.5 (1-62)		2	5	6	6	*Radiotx(2)	
Eyelid (22)	8M 13F	60.03 (26.7-81)	Lump	12 (4-83)		4	3	20	18		
Conjunctiva (27)	11M 16F	51.1 (24.6-76)	Swelling	12 (1-79)		1	0	20	22		
Lymph node, Bone and Sexual Organ Systems											
Lymph Node (31)	17M 14F	62.1 (34-77.8)	Persistent lymph node	7 (1-57.1)	MM (3) LPL (1)	10	9	15	9	*Chemotx (2) **Chemotx (1) *Radiotx (2)	6/18
Bone (24)	12M 12F	63.4 (47-82.2)	Bone pain	6 (1-132)	MM (1)	7	8	7	7	*Radiotx (5)	1/18
Breast (13)	13F	59.9 (36-78.3)	Breast lump	3.5 (2-4.75)	MALT (1)	1		11	9		
Prostate (9)	9M	68.1 (57.6-73.5)	Haematuria	8 (5-16.5)	MM (1)			7	3	**Chemotx (1)	

Head and Cardiac Systems							
Cerebral (3)	2M 1F	62 (30-68)	Weakness	4 (2-6)		2	**Chemotx (1) *Radiotx (1)
Aortic Valve (1)	1M	71.6	asymptomatic	0.5	1	0	

PET - positron emission tomography; M – male; F – female; PR – per rectum; GI – gastrointestinal; DMSO – Dimethylsulpoxide; CXR – chest radiograph; Radiotx – radiotherapy; Chemotx – chemotherapy; MM – multiple myeloma; LPL – lymphoplasmacytic lymphoma; NHL – non-Hodgkin lymphoma; κ/λ – kappa/lambda; * - Cytotoxic treatment for amyloidosis; ** - Cytotoxic treatment for underlying plasma cell dyscrasia; *** - 9 patients with cutaneous involvement had multiple amyloid deposits;2 patients with gastrointestinal involvement had multiple amyloid deposits.

A circulating monoclonal immunoglobulin or an abnormal serum free light chain (FLC) excess was present in 121/606 (20%) of patients, with Seventy six 76/606 (12.5%) having a monoclonal protein in the serum, and 79/606 (13.0%) having an abnormal FLC. A certain proportion of patients; 34/606 (5.6%) had the presence of a monoclonal protein and an abnormal FLC. The monoclonal immunoglobulin was measurable in 52/606 (8.6%), with a median concentration of 6.5 g/L (range 1-35 g/L, IQR 3-12), and a kappa or lambda serum free light chain (FLC) excess was present in 51/606 (8.4%) and 28/606 (4.6%) patients respectively. The 79/606 patients (13.0%) with a monoclonal light chain had IHC confirming amyloid deposits of AL lambda and AL kappa type in 16 and 5 patients respectively, with the monoclonal light chain in the serum and IHC staining of the same isotype in all these cases. The presence of scattered lymphocytes and/or plasma cells was frequently seen at the site of amyloid deposition but these were too scanty in number to demonstrate clonality by IHC. Interestingly one patient with an involved FLC (iFLC) of 2100mg/L with cutaneous amyloidosis had no vital organ involvement and a normal bone marrow assessment. Seventeen patients showed evidence of a clonal plasma/B-cell disorder by bone marrow assessment. The bone marrow examination was otherwise normal and did not reveal a clonal plasma cell or B lymphoid dyscrasia. In 490/501 (97.8%) patients, amyloid deposits were in keeping with AL type. Immunohistochemical staining confirmed 67/501 (13.4%) consistent with AL kappa and 24/501 (4.8%) of the AL lambda type. However in the 410/501 (81.8%) remaining patients, with no significant staining with antibodies to kappa, lambda, and no staining with SAA, transthyretin and ApoA1 and thus were more in keeping with a diagnosis of AL type. No patients had visceral uptake on ^{123}I SAP scintigraphy at presentation. The 18 patients who had a FDG-PET/CT showed FDG avidity at the site of amyloid deposition with a median

standardised uptake values (SUV) of 2.7 (2.5-10) in 14 of the 18 patients with reported SUV values (Appendix 5, Supplemental table 1).

Treatment

Complete details regarding interventions at the local disease site were available in 527/606 (87%) cases, and incomplete treatment details in 72/606 (11.9%), with survival details also present in these patients. In total, 90/606 (14.9%) patients opted for local follow up preferably, with the open option of re-attending the National Amyloidosis Centre should they developed progressive symptoms or want to be seen at the NAC. A certain proportion of patients, 257/527 (48.8%) required no interventional treatment following the diagnostic biopsy or excision. 270/527 (51.2%) proceeded to at least one interventional procedure for symptom relief and 112/527 (21.3%) patients required more than one repeat procedure. The symptom triggering the need for intervention was often the predominant/presenting symptoms based on site of deposits (table 6.2 illustrates these symptoms). The symptom of increasing size of the lesions usually prompted the need for intervention in cutaneous, oral, soft tissue, lymph node, nodular pulmonary and breast lesions. Pain was the trigger for in urethral and bone amyloid deposits. Interventional procedures included surgical treatment in 44% and laser therapy in 7% (Table 6.2). The surgical removal (n=4) or radiotherapy treatment (n=4) of the amyloid deposits in 8 patients led to disappearance of the circulating clonal markers, (Appendix 5, Supplemental table 2).

Local radiotherapy has been used to treat patients more recently, and 18 patients were treated with this modality in our cohort (table 2). Symptomatic improvement was evident in 10/18 patients - 5 with bone lesions, 4 laryngeal/tracheobronchial and 1 patient with a localised amyloidoma in the femoral nerve/lumbar plexus. Three

patients received radiotherapy for tracheobronchial amyloidosis and one diagnosed with laryngeal amyloidosis following failure of prior surgical resections and stent insertions. With a median follow up of 11 months (range 2-14 months), all patients noticed a significant improvement in their symptoms.

Chemotherapy was used in four patients: 2 having symptomatic pulmonary and 2 with lymph node amyloid deposits with stabilisation of symptoms but no significant improvement. There were a proportion of patients in whom corticosteroids alone were used; 12/606 (2%) cases (in 7 patients with tracheobronchial amyloidosis). Topical dimethylsulfoxide (DMSO) was used in 7 cases of bladder amyloidosis with 5 cases noticing a symptomatic benefit. Eleven other patients were treated with chemotherapy for progression of an underlying haematological disorder, not particularly for amyloid progression; with no significant impact on the local amyloid deposits.

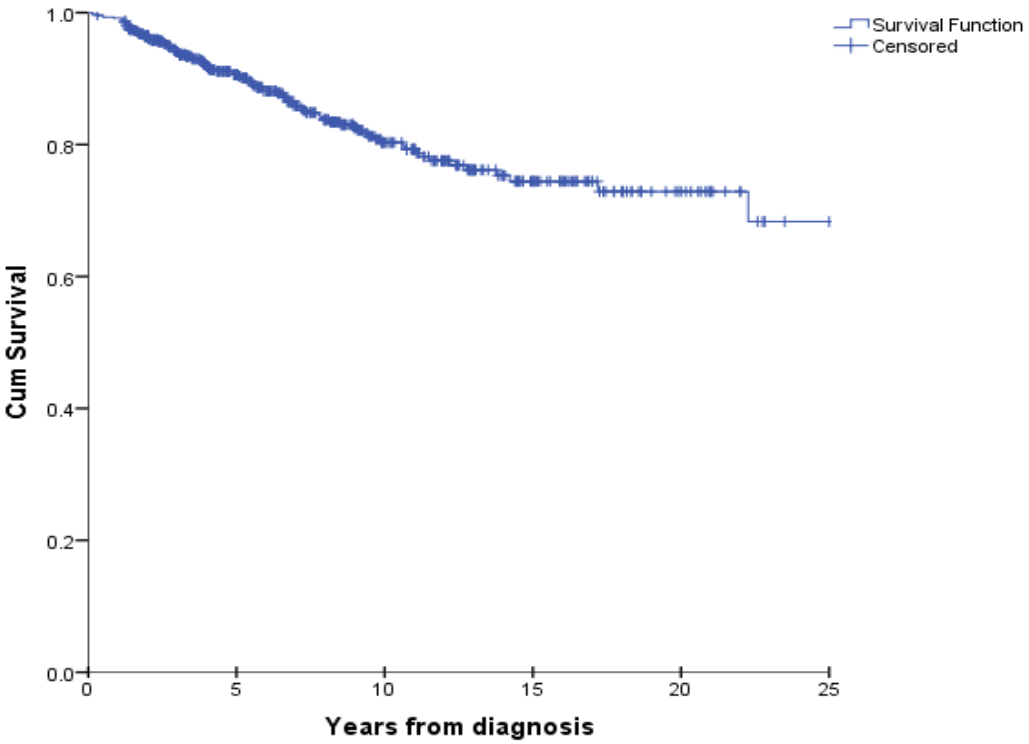
Progression and survival

The median follow up of the study cohort is 74.4 months (range 3.7-349.2 months, IQR 37.2-132), and 51% (309/606) of patients were diagnosed after 2006 and 27.2% (87/606) before 2000 (illustrated in table 6.1). Progression at the primary site was present in 264/606 (43.5%) patients (table 6.2). Progression to systemic amyloidosis was rare, occurring in only 7/606 cases (1.2%); (five had a circulating monoclonal protein and one had a with detectable plasma cell clone in the marrow). The latter 7 patients included 5 patients with lymph node (LN) involvement, one had eyelid and another bone involvement, progressing at a median on 51 months (20, 51, 60, 77, 84, 48 and 51 months in each case respectively) from the diagnosis point. The site of progression to systemic amyloidosis included: three patients with soft tissue progression, two developed renal amyloidosis (one with additional soft tissue) and one

each having new lymph node or bone lesions (Appendix 5, Supplemental table 3). One patient developed asymptomatic uptake within the spleen by SAP scintigraphy.

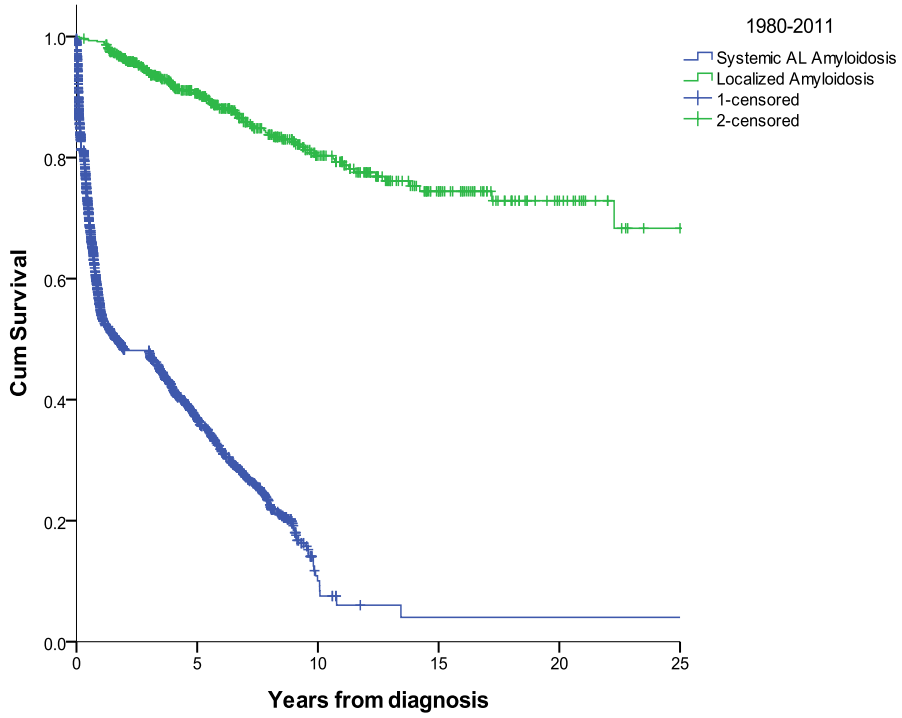
During the follow up period, there were a total of 94 deaths, and only three deaths directly attributed due to progression to systemic AL. The cause of death was determined in 84 cases from the death certificate data from the Office for National Statistics.[290] In 10 patients, the patient's general practice doctor was contacted to ascertain the cause of death. The majority of patients had other co-morbidities including chronic obstructive airways disease, sepsis, ischaemic heart disease, autoimmune disease and cancer of other aetiology. The 5 and 10 year estimated survival was 90.6% (95%CI; 87.7-92.9) and 80.3% (95%CI; 75.7-84.1) respectively (illustrated in Figure 6.3A); with the median survival not reached for the entire cohort. The median survival for patients aged 70 years or older was 12.1 years (95% CI; 10.5-13.7). We compared our localised AL cohort to systemic AL patients diagnosed during this same time frame, (Figure 6.3B) showing the estimated survival for systemic AL amyloidosis over this time period was significantly shorter, 1.6 years (95%CI; 1.1-2.1) and 5 and 10 year estimated survival 37.0% (95%CI;0.37-0.43) and 26% (95%CI;0.23-0.28)% respectively. We examined the OS of patients diagnosed over different time periods of this 30 year study when assessed as 5-yearly cohorts (except 1985-1995; included as one group due to smaller patient numbers) (Figure 6.3C), showing no significant difference and the numbers diagnosed showing an increasing trend. The 5 year survivals were as follows: 1980-1995 [(97%(95%CI;0.82-0.97) and (87%(95%CI;0.75-0.94)], 1996-2000 [(88%(95%CI;0.79-0.93)and (77%(95%CI;0.67-0.85)], 2001-2005 [(91%(95%CI;0.84-0.94) and (79%(95%CI;0.68-0.85)], 2006-2011 [(91%(95%CI;0.86-0.94) and (85%(95%CI;0.76-0.91)].

Figure 6.3A: Kaplan-Meier curve with overall survival in all patients with localised amyloidosis



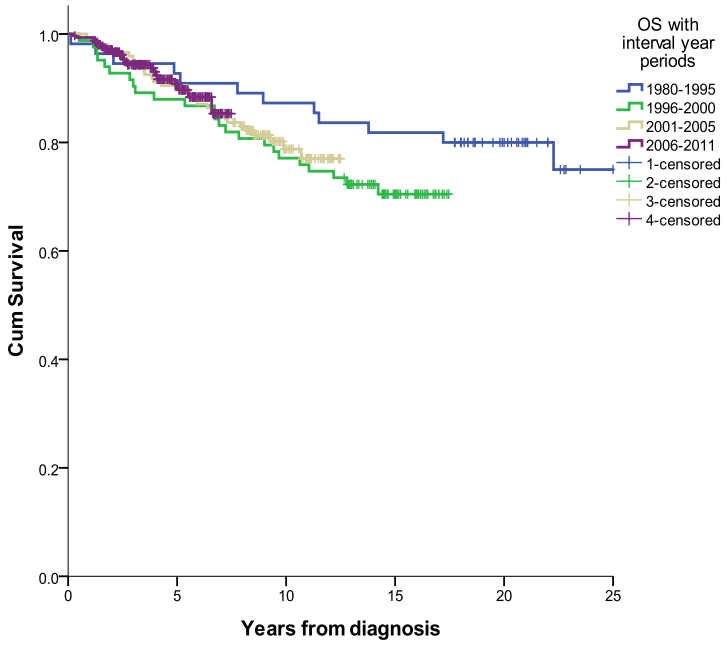
Numbers of patients at risk	**595**	**346**	**164**	**74**	**29**	**10**

Figure 6.3B: Kaplan Meier curve illustrating difference between patients diagnosed with systemic AL amyloidosis (blue) and localised AL amyloidosis (green). The median survival of patients with localised AL amyloidosis has not been reached compared to a median of 1.6 years for patients with systemic AL diagnosed over the same time period.



Numbers of localized patients at risk	**595**	**346**	**164**	**74**	**29**	**10**
Numbers of AL patients at risk	**2703**	**443**	**12**	**2**	**1**	**1**

Figure 6.3C: Kaplan-Meier curve illustrating the overall survival in all patients with localised Amyloidosis divided into 5 year intervals (except 1980-1995 (as there were smaller patient numbers)). The number of patients in each group was 1980-1995 – 4% (24/606) during1980-1985, 3.8% (23/606) during 1986-1990, 6.5% (40/606) during 1991-1995, 12.9% (78/606) during 1996-2000, 21.8% (132/606) during 2001-2005 and 51% (309/606) during 2006-2011.



Numbers of patients at risk	**1980-1995**	**87**	**80**	**62**	**45**	**29**	**10**
	1996-2000	**78**	**73**	**63**	**29**	**0**	
	2001-2005	**132**	**128**	**52**	**0**		
	2006-2011	**309**	**89**	**0**			

Discussion

This study describes the largest cohort of patients with localised AL amyloidosis with clinical features and natural disease course showing stark differences from systemic AL amyloidosis. Localised AL amyloidosis is classified as having a relatively indolent disease course that rarely evolves systemically. Most patients can be managed adequately with local interventions, which is the primary treatment modality. In this series, in striking contrast to systemic AL amyloidosis, localised amyloidosis does not appear to impact survival and, with only 3 deaths directly attributed to progression to systemic AL amyloidosis.

Defining localised amyloidosis is critically important in assessing isolated deposits and the also in determining progression. We defined localised amyloidosis as a patient with amyloid deposition at one single non-vital organ site with no evidence of amyloidotic end organ damage by the diagnostic criteria of the international society of amyloidosis of systemic organ involvement (in relation to no cardiac, renal, liver, nerve or macroglossia). We had a unique modality to rule out visceral amyloid deposition with a very high level of confidence, due to the availability of SAP scintigraphy (a very sensitive and highly specific method for imaging visceral amyloid deposits) at our centre. Hence, in absence of SAP scintigraphy, this diagnosis has to be approached with greater caution especially with those with a detectable FLC excess or M-protein and follow up would reveal the true nature of the diagnosis.

Systemic AL amyloidosis typically affects the heart, kidney, liver, peripheral and autonomic nerves.[86] In contrast, localised AL amyloidosis occurs mainly in mucosal sites (affecting the airways, bladder, gastrointestinal tract, and conjunctiva), skin or glandular sites (like breast) as in the current series and also previously reported.[291-293]

The presenting symptoms are site specific and may range from a localised lump (e.g. under the skin or breast lump), bleeding due to mucosal erosion (symptoms of haematuria or haemoptysis or melena) to obstructive symptoms when deposits are located at a luminal site (bronchial, ureter or GI tract obstruction). Multiple sites of involvement were present in certain patients with cutaneous and gastrointestinal amyloidosis.

Localised amyloidosis has generally been reported to be of AL type. In the current series, of a total of 606 patients with localised amyloidosis, we considered 98% (490/501) patients to be in keeping with the AL type. However, we do recognise one of the limitations of our study was typing amyloid deposits with a high proportion of cases in the present series having staining with antibodies to both kappa and lambda light chains (although lacking staining with antibodies to SAA, transthyretin and ApoA1). In the era before access to laser capture followed by mass spectrometry (the current gold standard), AL amyloidosis was a diagnosis of exclusion in cases where specific staining with light chain antibodies could not be demonstrate by excluding the presence of amyloid deposits of other types by appropriate staining. Due to the long duration of this study, the vast majority of the patients were diagnosed using this algorithm. However, it was notable that the staining with antibodies to kappa or lambda was much lower than generally seen in patients with systemic AL amyloidosis, and forward planning with the use of proteomic analysis will assist in this clarification. A recent case series of nodular pulmonary amyloidosis from the Mayo group, using laser capture micro dissection followed by tandem mass spectrometry (LC-MS) for fibril typing, suggested that amyloid fibrils are a mixture of heavy (AH) and light chain (AL) type;[294] a potential explanation for difficulties in accurate staining by anti-light chain antibodies. The retrospective nature of this study over a long period limited the

scope of LC-MS being undertaken for the current series and does remain a limitation of this series.

Generally, over 95% of patients diagnosed with systemic AL amyloidosis have a detectable monoclonal protein or abnormal FLC[295] (which directly impacts on prognosis[296]) and a detectable bone marrow plasma cell clone; in contrast with the present series in which a normal bone marrow was seen in those with an abnormal FLC or monoclonal protein (except 17 patients with an underlying plasma cell dyscrasia/B-cell clone) and 80% with no evidence of a circulating M-protein or abnormal FLC. In our study, 20% (121/606) had a monoclonal protein or abnormal serum free light chain excess, with the presence of a monoclonal protein in 76/606 (12.5%) and abnormal serum free light chain excess in 79/606 (13.0%), and some overlap of these groups. We recognise this figure may be higher than other centres, and this difference predominantly due to referral bias and age of the referred populations in different respective centres. Previous studies have shown that 5-10% of the population have an incidental monoclonal protein in those older than 70 years of age.[297] Patients with lymph node involvement had a higher chance of having a detectable monoclonal protein, with one study showing this group as having a high risk of progressing to systemic AL.[298] Of seven patients who progressed to systemic AL, 5 had evidence of a detectable clonal marker in the serum. Patients with systemic AL amyloidosis having abnormal light chains for many years prior to diagnosis,[299] localised AL patients with an abnormal FLC still warrant long term follow up for progression. Lack of clonality by bone marrow assessment in localised amyloidosis supports previous reports of limited clonal proliferation at the site of amyloid deposition as documented by highly sensitive methods like IgH-PCR[300, 301] with only few patients of the present series having an obvious local clonal cell infiltrate. The location of skin

and mucosal sites raise the possibility of chronic antigenic stimulation or autoimmunity as drivers for local clonality[302] particularly as Mucosa-associated lymphoid tissue (MALT) lymphoma is a reported cause of lung and breast amyloidosis.[303, 304] 23% of all cases of breast amyloidosis in this series were associated with Sjögren's syndrome (an association in 17 cases in total), also a previously reported association of localised amyloidosis with MALT.[305] The lack of detectable clonal cells remains an enigma in most cases. Oligomeric light chains may be toxic to cells and tissues – with one theory suggesting that plasma cells in localised AL are part of a "suicide" neoplasm and die to due to light chain toxicity locally.[306] Hence, this is an interesting hypothesis to explain lack of cells as well as lack of systemic progression.

Serial monitoring of patients with localised amyloidosis is a difficult hurdle since the standard serum or urine markers are frequently normal or not contributory with ^{123}I labelled SAP scintigraphy rarely abnormal. Surprisingly, all 18 patients who had a FDG-PET/CT performed in the current series (Supplementary table 1) showed presence of FDG avidity at the site of amyloid deposition, in keeping with previous reports of high proportion of FDG-PET/CT positivity in localised amyloidosis.[307] This potentially useful finding is a metabolic conundrum given the lack of identifiable clonal cell infiltration in the deposits. Inflammatory or giant cells within the amyloid deposits have been suggested as a potential cause of the metabolic activity detected by FDG-PET/CT.[308] Further studies are needed to investigate the utility FDG PET/CT in both diagnosis and monitoring of patients with Localised amyloidosis.

In contrast to systemic AL amyloidosis, approximately 50% of the patients in the current series did not require any intervention following the diagnostic biopsy/excision. Patients with obstructive/pressure symptoms or bleeding needed endoscopic or

surgical resection. Half of all patients needed one such procedure and a fifth of all patients required repeated procedures for symptom control highlighting the need for long term monitoring. Four patients in the current study needed chemotherapy for localised amyloidosis. Radiotherapy (targeting the presumed clonal proliferation) appears to be a useful, and possibly underutilised, treatment modality in localised amyloidosis with all patients in the current series treated with radiotherapy showing lack of progression and over half achieving good symptomatic improvement following radiotherapy. Tracheo-bronchial deposits can be very troublesome and symptomatic with marked morbidity and are a particular challenge amongst Localised AL. In this group, radiotherapy has been reported to give symptomatic benefit (in 8/10 [309] and 7/7 [310] patients treated) and this was also evident in all three treated patients in the current cohort. The dose and scheduling of radiotherapy require further systematic study.

Systemic AL amyloidosis typically remains a disease with a poor prognosis with a median overall survival of about four years and, despite much progress, nearly a third of all patients die in the first few months following diagnosis.[192] We compared our localised cohort with patients diagnosed with systemic AL during this same time frame, with an explicitly better prognosis in the former. This large series reassuringly examines the natural disease course of Localised amyloidosis, showing it has an excellent prognosis with a median survival not reached in our cohort compared with an overall surviall of 1.6 yrs for patients with systemic AL amyloidosis seen during the same period. Most of the patients died of other causes and only three deaths attributable directly to localised AL. The suvival of the patients compared with that expected in the British population (medain survival of the over 70 yrs patients in the current series 12.1 yrs which was similar to that for the British person at the age of 70 years) (date from Office of National Statistics UK).[290] Another cruciaal point was that

only seven patients or 1.2% progressed to systemic AL amyloidosis. We specifically ruled out visceral deposition by serum amyloid P (SAP) scintigraphy and cardiac involvement by echocardiography plus normal cardiac biomarkers. There are two groups of patients with localised AL which pose some problems – those with lymph node involvement and those with a detectable circulating light chain/M-protein having the same isotype as the amyloid type. It is contentious whether we can confidently say that some patients with a localised lymph node amyloid deposit have localised disease or "systemic" disease. Patients with no vital organ involvement and a localised lymph node were categorised as localised amyloidosis, however this group is very different from the other types of localised amyloidosis; with the increased risk of "systemic disease" and spread through the lymphatic system, akin to other types of lymphoma. In our series, 5/31 patients with lymph node involvement progressed to systemic AL and 6/79 patients with a clonal light chain excess progressed (5 of these having lymph node patients), with only one patient with no obvious light chain excess progressed. We recognise that patients with isolated lymph node amyloidosis are deemed at high risk of progression, and similarly those with an isotypic excess of FLC also need careful monitoring. The very low chance of systemic progression in other patients with localised amyloidosis implies that these patients should not be subjected to frequent invasive monitoring.

There are a number of gaps in our knowledge of localised amyloidosis, with the retrospective nature of this study. A longer median follow up is always important in following and understanding a disease entity, recognising that 51% (309/606) of patients were diagnosed after 2006. Accurate identification of the amyloid fibril type by LC-MS is important with immunohistochemistry sometimes inadequate for fibril typing in this setting. Novel approaches to clarify the nature of the underlying clonal

disorder(s) is required to guide therapy and unravel the prediliction for specific sites. International colloborative studies are needed to prospectively evaluate the utility of radiotherapy and other treatment modalities.

In summary, localised AL amyloidosis is a disease different from systemic AL amyloidosis. Local surgical resection is adequate in most cases when treatment is needed. Radiotherapy appears to have a useful role in patients not controlled by local measures. Progression to systemic AL amyloidosis is extremely rare except in patients with lymph node involvement. Patients with lymph node involvement and those with a isotypic specific circulating free light chains warrant closer follow up for development of systemic AL. The majority with localised AL have excellent long term outcomes. Most die with the disease rather than as a consequence of it.

Chapter Seven: Laryngeal tracheobronchial amyloidosis: clinical and proteomic analysis showing an association with Apo A1 and insulin-like growth factor binding protein complex

Introduction

Laryngo-tracheobronchial amyloidosis is a rare type of localised amyloidosis, characterised by insoluble fibrillar proteins deposited within the upper and lower airway tract.[311, 312] The underlying amino acid sequences and building block structure of this and other types of amyloidoses share a common β-sheet conformation of the polypeptide backbone,[313] and thus forming a fibrillar, insoluble, proteolytic resistant structure in all types of amyloid. Exploring the mechanism of the amyloid fibril formation is vital in gaining insight into the mechanisms in the underlying polymerisation of the soluble, monomeric peptide into the insoluble β-pleated sheet. Earlier studies have shown that this conversion of a soluble peptide to an insoluble fibril often includes the formation of a partially unfolded intermediate.[314, 315] Some studies propose a nucleation dependant polymerisation model to explain the fibril formation.[316] The underlying pathogenic factors are unknown, with the hypothesis including a population of clonal plasma cells or B cells instigating this process in a localised area. This entity is clearly different from systemic light chain (AL) amyloidosis, in which the clonal plasma cells arise from the bone marrow and secrete excessive quantities of monoclonal immunoglobulins which then circulate to target organs.[317]

The aims of this study were to (a) report our experience of patients presenting with laryngeal and tracheobronchial amyloidosis, examining the clinical symptoms,

treatment strategies and overall survival from a clinical perspective, (b) analyse the laryngeal /tracheobronchial biopsies and undertaking proteomic analysis of these patients and ascertain any potential underlying pathogenic instigators in this process from a laboratory perspective.

Patients and methods

Patients

Patients with laryngeal and tracheobronchial amyloidosis between January 1980 and December 2011 were identified from the database of the UK National Amyloidosis Centre. Written consent was obtained from all patients in accordance with the Declaration of Helsinki. Exclusion of systemic AL and vital organ involvement (including cardiac, renal, liver, peripheral or autonomic neuropathy) was performed on review of a detailed baseline assessment of organ function and no uptake by ^{123}I serum amyloid P component (SAP) scintigraphy, with patients before 1988 (availability of ^{123}I SAP scintigraphy) having this procedure later in their follow up course. Baseline investigations included a full blood count, renal and liver function tests, serum protein electrophoresis, serum free light chains (prospectively in samples after 2002 and retrospectively on stored serum samples where available), and urinary BJP, electrocardiogram and echocardiography and ^{123}I SAP scintigraphy. Histological analysis was performed on all biopsies with Congo red staining and apple green birefringence and immunohistochemical staining for kappa, lambda, transthyretin or serum amyloid A (SAA) antibodies undertaken in the majority. Genetic sequencing was undertaken dependent upon clinical details and immunohistochemical staining, such as Apolipoprotein (Apo) AI in all patients with available blood samples. During this time frame, 63 patients with laryngeal involvement and 34 patients with

tracheobronchial amyloidosis with biopsy proven amyloid deposition were recruited. The pathology slides were requested and reviewed at our centre and laser capture of the slides undertaken to then perform proteomic analysis, with a total of 60 biopsies retrieved. A total of 37 biopsies were not able to be analysed due to inaccessibility of the original samples requested from the local hospitals/referring centres.

Patient questionnaire with quality of life assessment

A patient questionnaire was sent to all patients to ascertain the following data with 52 responses and 46 responses concerning quality of life. The questionnaire comprised of 2 questions related to symptoms and duration and treatment options, and quality of life with a grading score 0-10; score of 0 depicting a poor quality of life and 10 describing an excellent quality of life. The median overall score and ranges were gained to reflect an understanding in the clinical impact of this disease from a patent's perspective.

Protocol for laser capture

Laser capture micro dissection and mass spectrometry (LDMS) is an invaluable tool for identifying proteins from formalin-fixed, paraffin embedded tissues, with more in depth procedure described in the methods chapter.[169] Visualisation and locating the amyloid was done using bright field (Figure 7.1A) and Rhodamine filter sets (TRITC) on the Zeis Palm Microbeam Laser capture microscope, with amyloid typically yellow on a "red" background. (Figure 7.1B) Areas of interest were drawn around, appearing yellow under a Tritc florescent filter (Figure 7.1C) or under bright field (Figure 1D) and "cut" out using laser capturing into 0.5ml micro-centrifuge adhesive cap tubes (Zeiss) and checking the cap to ensure that some tissue had been captured. Figure 7.1D illustrates the amyloid tissue post dissecting the tissue of interest and the figure 7.1E

shows the dissected amyloid tissue for proteomic analysis, with figure 7.1F showing the dissected tissue. Figure 7.1G shows congo-red staining of the tissue and apple-green birefringence illustrated in figure 7.1H.

Figure 7.1A:. Isolation of amyloid under bright field and (B) the TRITC filter

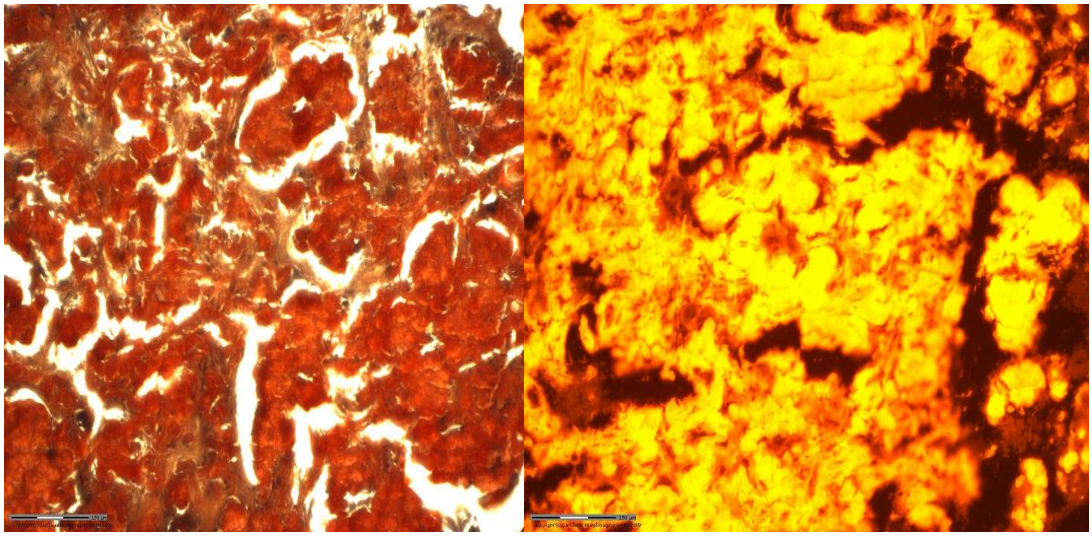


Figure 7.1C and 7.1D: Demarcation of the areas of amyloid to be dissected under TRITC filter (C) and (D) under bright field

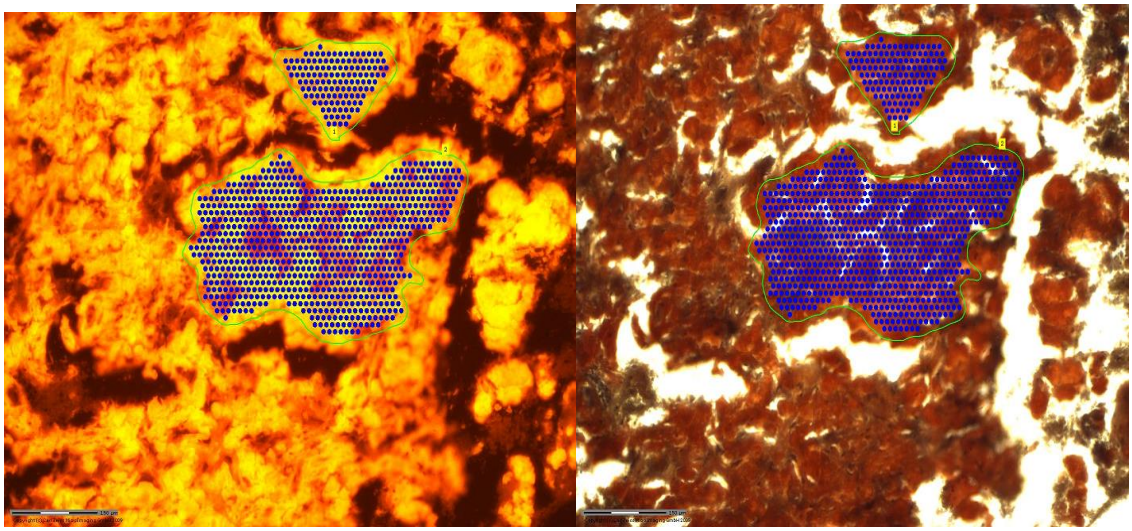


Figure 7.1E: Tissue under bright field post dissecting the amyloid tissue

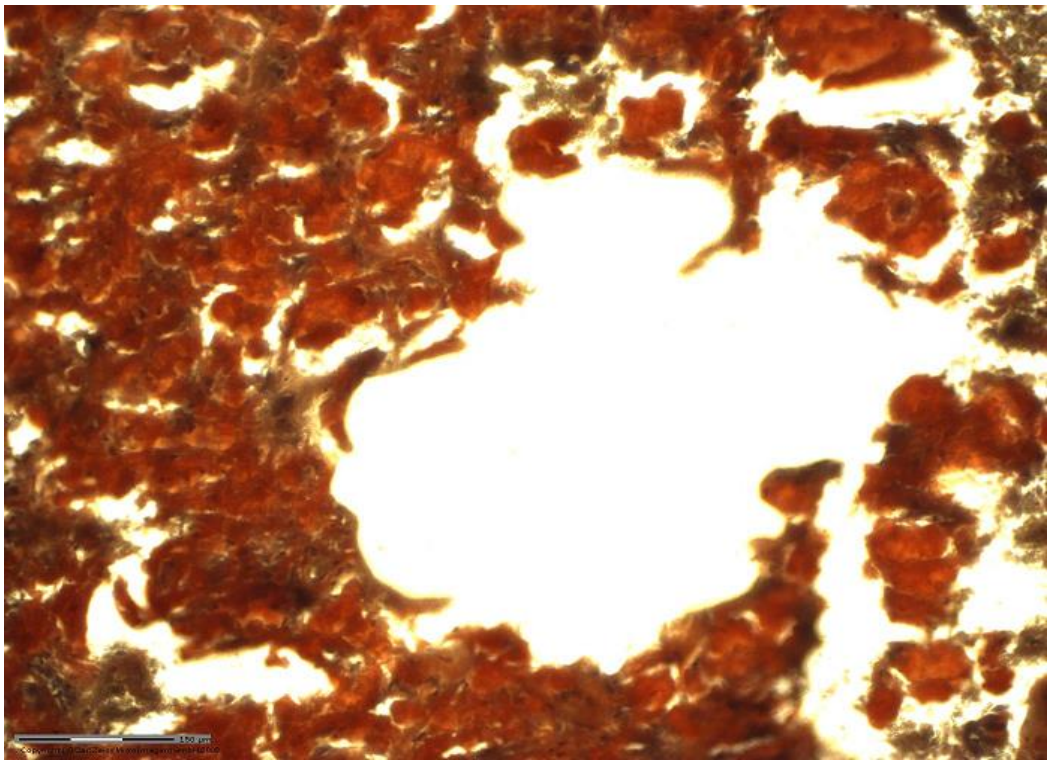


Figure 7.1F: The dissected piece of tissue for proteomic analysis

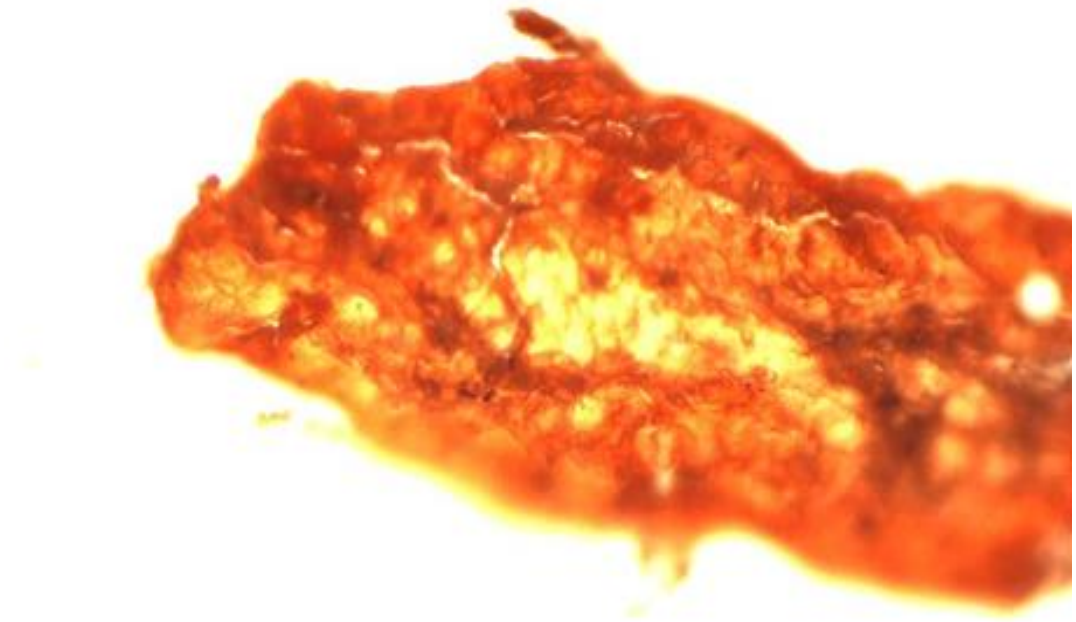


Figure 7.1G: Congo red staining of tissue

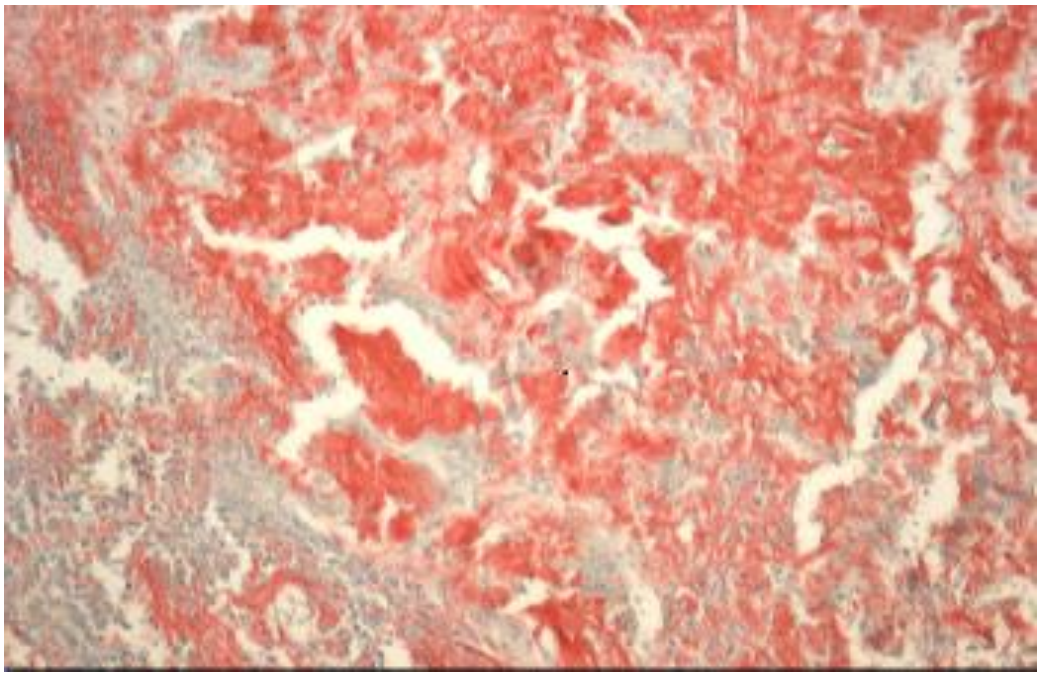
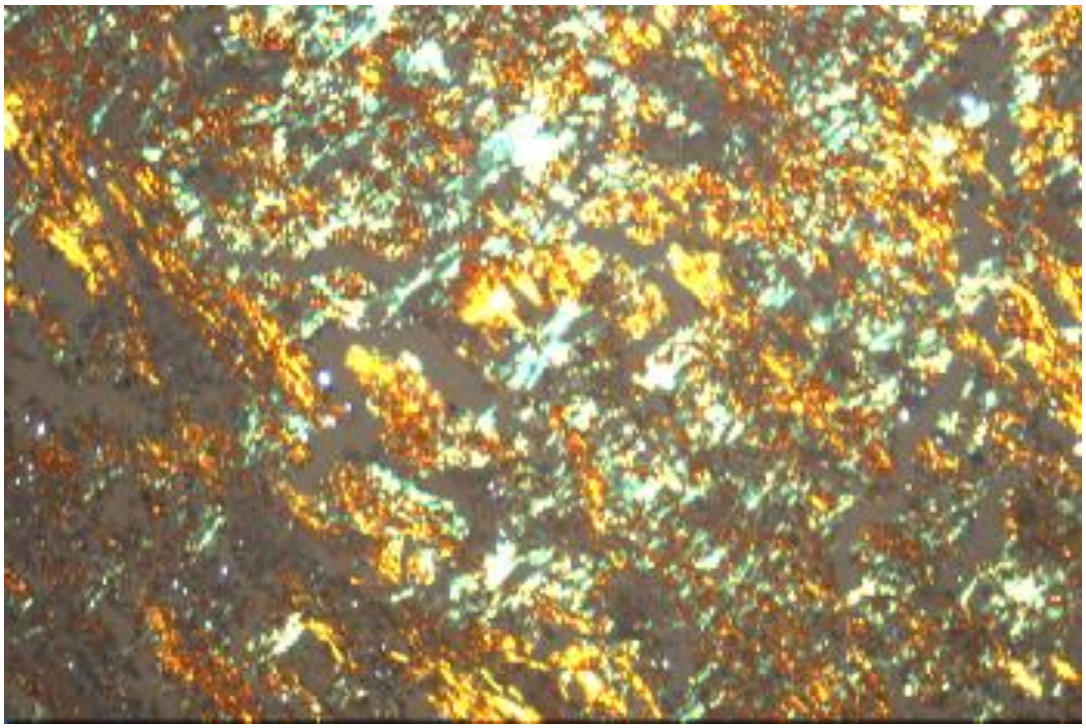


Figure 7.1H: Apple green birefringence of the tissue



The data was processed using Proteome Discoverer for MASCOT searching of the NCBI and IPI databases enabling mass spectrometry results to be produced.

Statistical analysis and overall survival

Data was analysed and summarised using the number (percentage) and median (minimum and maximum range). Quality of life assessments were based upon a score 0-10, with 0 and 10 equating to no quality of life and an excellent quality of life respectively. Kaplan Meier curves were used to estimate the overall survival (OS); calculated from the start of symptoms until death or last follow-up for the laryngeal and tracheobronchial cohorts. Kaplan Meier estimates were used using SPSS v20 (IBM SPSS) to calculate the OS and PFS. All p values are 2 sided, with significance values based on a $p < 0.05$.

Results

Patient population

The study was conducted between January 1980 and December 2011 and clinical characteristics are summarised in table 7.1 and 7.2 with the symptoms within each group illustrated in figure 2A and 2B. No patient developed systemic AL amyloidosis. Genetic sequencing was performed in 59 patients, with only 1 patient diagnosed with laryngeal involvement heterozygote for the Apolipoprotein A1 Ala160Ser mutation and no evidence of other organ dysfunction.

Table 7.1: Patient characteristics in laryngeal group (n=63)

Patient Characteristics	Number (%)
Male	29 (46%)
Age	57.4 (44.7-66) years
Symptom duration	10.5 (6-24) months
Personal history of cancer	8/56
Smoking history	16/56
Autoimmune disorders	7/56
GORD	6/56
Hypertension	10/56
Location	
Supra-glottic	6
Vocal cord/tonsil	33
Subglottic	16
Not specified	8
Investigations	
Creatinine	78 (54-128) µmol/L
Estimated glomerular filtration rate	87 (49-100) ml/min
Albumin	45 (33-52) g/L
Alkaline phosphatase	70 (30-131) U/L
24 hour urinary protein	0.1 (0.1-0.5) g
Haemoglobin	13.5 (10-17.3) g/dL
Positive serologic clonality	10
Immunoparesis	0
Quality of life score	8 (3-10)

Table 7.2: Patient characteristics in tracheobronchial group (n=34)

Patient Characteristics	Number (%)
Male	12 (35%)
Age	47.6 (54-62.5) years
Symptom duration	4.3 (8-24) months
Personal history of cancer	4/28
Smoking history	22/28
Autoimmune disorders	3/28
GORD	2/28
Hypertension	7/28
Location	
Tracheal	4
Right sided bronchial	11
Left sided bronchial	3
Bilateral bronchial	12
Not specified	4
Investigations	
Creatinine	80.5 (40-358) µmol/L
Estimated glomerular filtration rate	84 (10-100) ml/min
Albumin	43 (32-50) g/L
Alkaline phosphatase	70 (39-207) U/L
24 hour urinary protein	0.1 (0.1-3.2) g
Haemoglobin	14 (10-16.6) g/dL
Positive serologic clonality	10
Immunoparesis	0
Quality of life score	5 (1-9)

Figure 7.2A: Presenting symptoms in the laryngeal cohort

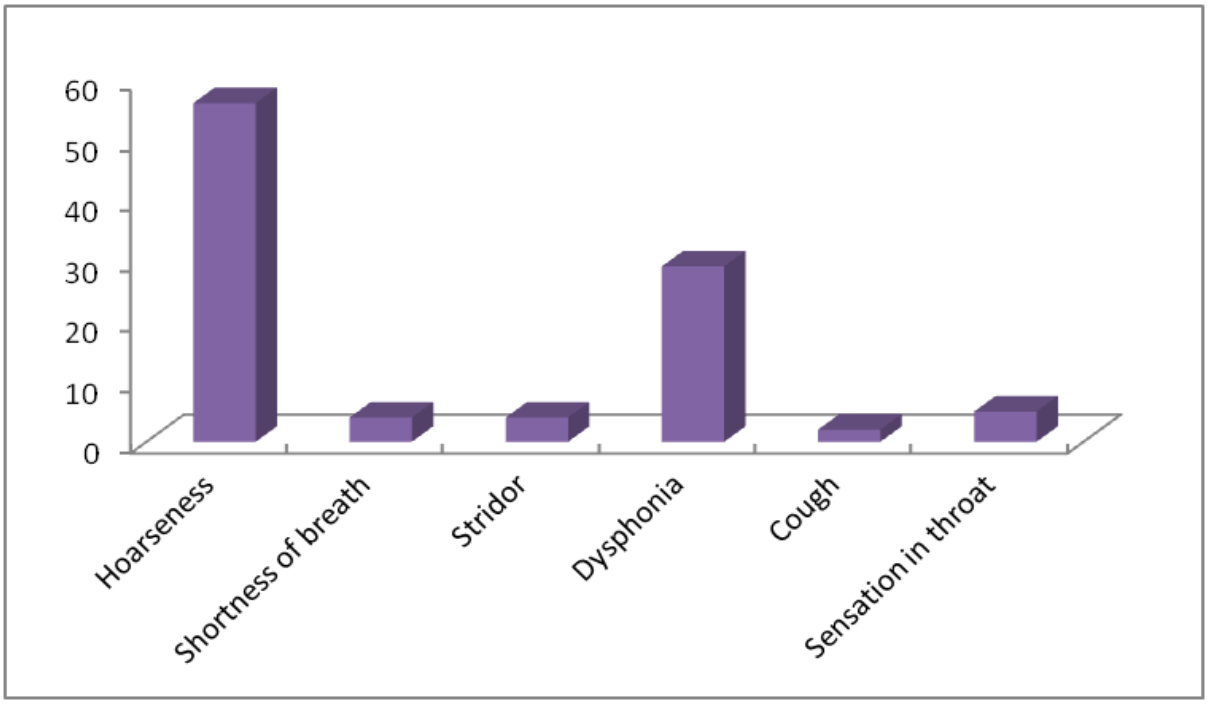
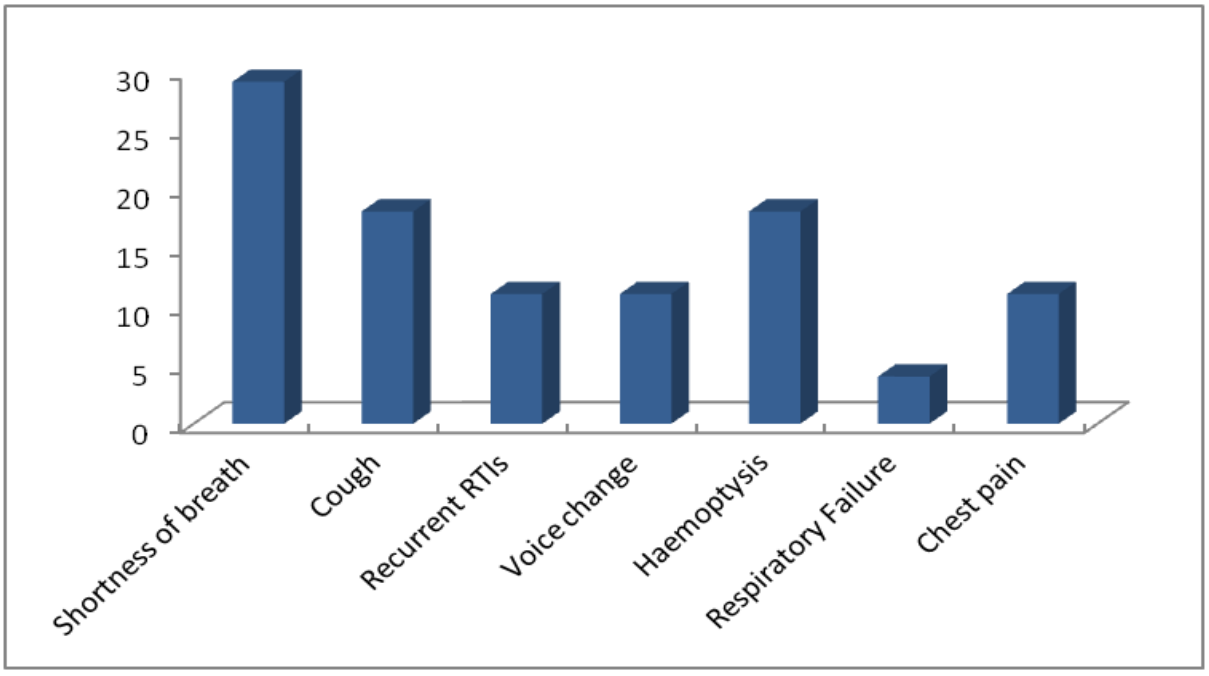


Figure 7.2B: Presenting clinical symptoms in the tracheobronchial cohort



Treatment strategies

Surgery

Surgical and laser therapeutic options were pursued in 39/63 (62%) and 18/34 (52.9%) patients with laryngeal and tracheobronchial amyloidosis with 29% (n=18) and 24% (n=8) requiring repeated procedures respectively.

Radiotherapy

A total of 4 patients with symptomatic airway amyloidosis involvement (1 laryngeal and 3 tracheobronchial) received radiotherapy with a median dose of 20 Gy in 10 fractions. Following a median follow up of 18.7 months range (6-47), all patients had experienced symptomatic local control.

Chemotherapy

One patient with laryngeal involvement and one patient with tracheobronchial amyloidosis received chemotherapy; each with a lambda light chain excess and no evidence of an excess of plasma cells by bone marrow assessment. Both patients achieved a fall in the lambda light chains to less than 40mg/L with minimal improvement in their symptoms. It is difficult to ascertain or comment on whether their symptoms would have progressed with no treatment.

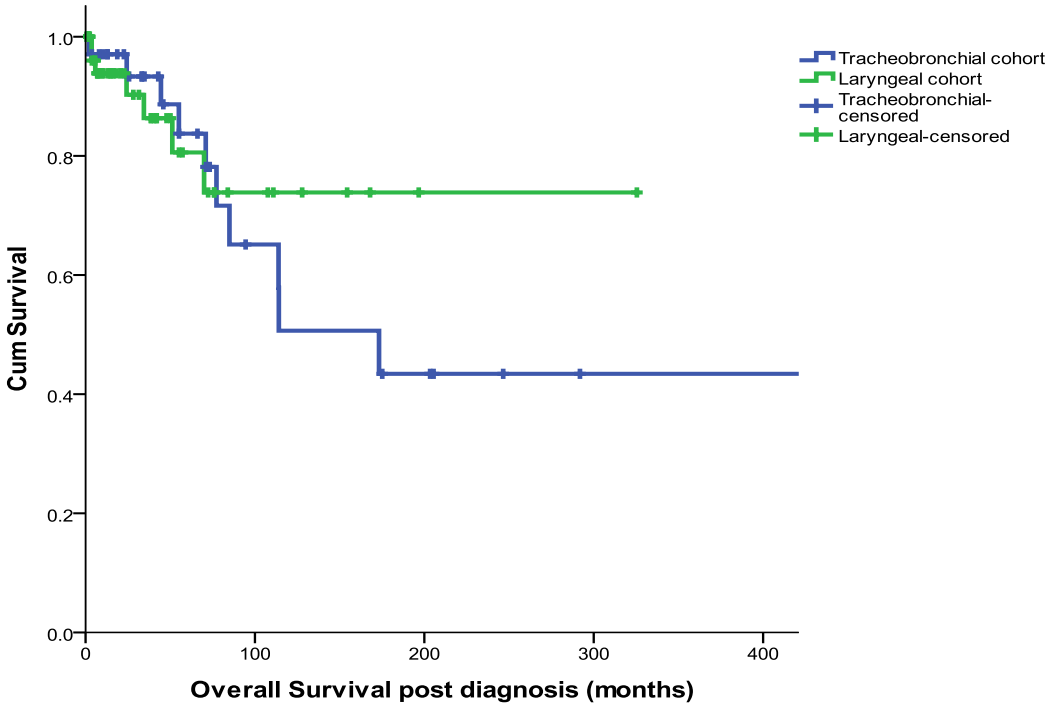
Quality of life assessment

A patient questionnaire was sent to all patients, with a response in 46 patients. The median quality of life score in 33 laryngeal patients and 13 tracheobronchial patients was 8 (range 3-10) and 5 (range 1-9) respectively. This is illustrated in table 7.1 and 7.2.

Overall survival

The 2 and 5 year OS for the laryngeal and tracheobronchial cohort were 90% and 81%, and 93% and 83% respectively, figure 7.3. The majority of patients had other co-morbidities with the median age of death in the laryngeal cohort 69.5 years (45.2-82.5) and tracheobronchial cohort 65.3 years (55.1-79.2).

Figure 7.3: Kaplan Meier curve comparing overall survival in patients diagnosed with laryngeal amyloidosis with tracheobronchial amyloidosis, although not statistically significant (p 0.66), the curves show a clear difference in the time course in each disease following approximately 75 months.

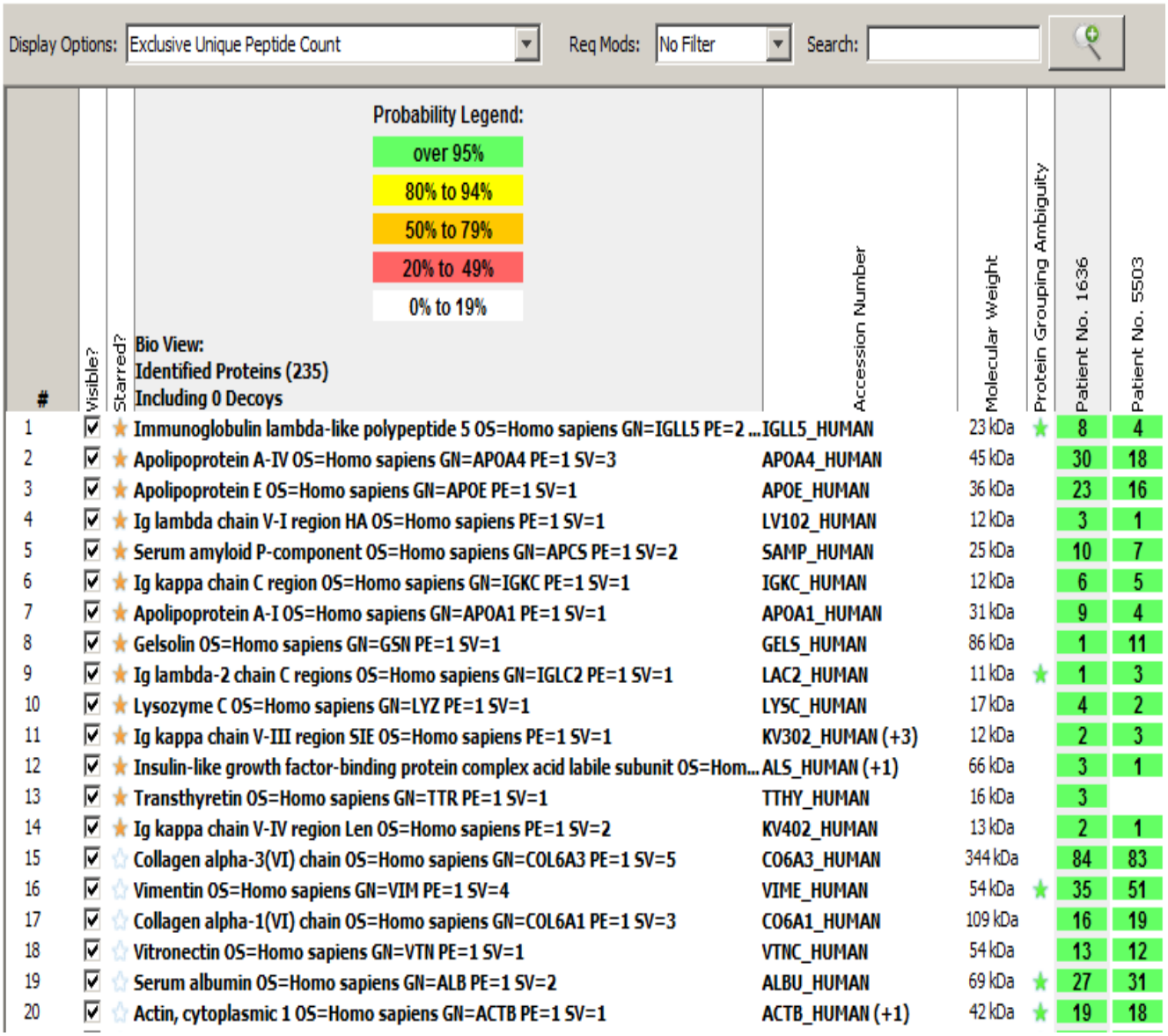


Proteomic analysis

The proteomic analysis on 60 patients diagnosed with laryngeal and tracheobronchial amyloidosis showed evidence of the 3 signature amyloid proteins – Serum amyloid P (SAP), Apo A4 and Apo E. Interestingly, these patients all had evidence of insulin-like growth factor binding protein complex and Apo A1. Comparison was made between these samples and in those patients diagnosed with systemic immunoglobulin light chain (AL) and transthyretin based disease, with patients with the systemic disease showing no proteomic evidence of insulin-like growth factor binding protein complex and a much lower ApoA1 peptide quantification than patients with laryngo-tracheobronchial disease.

We analysed laryngeal and tracheobronchial normal tissue in 5 samples; acting as normal controls. The results showed that insulin-like growth factor binding protein complex and Apo A1 were not normal constituents of this tissue.

Figure 7.4: Proteomic analysis of a micro-dissected amyloidotic area showing the presence of the amyloid signature proteins, along with light chains, 3 insulin-like growth factor binding protein complex and other proteins.



Display Options: Exclusive Unique Peptide Count | Req Mods: No Filter | Search:

Probability Legend: over 95% | 80% to 94% | 50% to 79% | 20% to 49% | 0% to 19%

Bio View: Identified Proteins (235) Including 0 Decoys

#	Visible?	Starred?		Accession Number	Molecular Weight	Protein Grouping Ambiguity	Patient No. 1636	Patient No. 5503
1	☑	★	Immunoglobulin lambda-like polypeptide 5 OS=Homo sapiens GN=IGLL5 PE=2 ...	IGLL5_HUMAN	23 kDa	★	8	4
2	☑	★	Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3	APOA4_HUMAN	45 kDa		30	18
3	☑	★	Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1	APOE_HUMAN	36 kDa		23	16
4	☑	★	Ig lambda chain V-I region HA OS=Homo sapiens PE=1 SV=1	LV102_HUMAN	12 kDa		3	1
5	☑	★	Serum amyloid P-component OS=Homo sapiens GN=APCS PE=1 SV=2	SAMP_HUMAN	25 kDa		10	7
6	☑	★	Ig kappa chain C region OS=Homo sapiens GN=IGKC PE=1 SV=1	IGKC_HUMAN	12 kDa		6	5
7	☑	★	Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1	APOA1_HUMAN	31 kDa		9	4
8	☑	★	Gelsolin OS=Homo sapiens GN=GSN PE=1 SV=1	GELS_HUMAN	86 kDa		1	11
9	☑	★	Ig lambda-2 chain C regions OS=Homo sapiens GN=IGLC2 PE=1 SV=1	LAC2_HUMAN	11 kDa	★	1	3
10	☑	★	Lysozyme C OS=Homo sapiens GN=LYZ PE=1 SV=1	LYSC_HUMAN	17 kDa		4	2
11	☑	★	Ig kappa chain V-III region SIE OS=Homo sapiens PE=1 SV=1	KV302_HUMAN (+3)	12 kDa		2	3
12	☑	★	Insulin-like growth factor-binding protein complex acid labile subunit OS=Hom...	ALS_HUMAN (+1)	66 kDa		3	1
13	☑	★	Transthyretin OS=Homo sapiens GN=TTR PE=1 SV=1	TTHY_HUMAN	16 kDa		3	
14	☑	★	Ig kappa chain V-IV region Len OS=Homo sapiens PE=1 SV=2	KV402_HUMAN	13 kDa		2	1
15	☑	☆	Collagen alpha-3(VI) chain OS=Homo sapiens GN=COL6A3 PE=1 SV=5	CO6A3_HUMAN	344 kDa		84	83
16	☑	☆	Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4	VIME_HUMAN	54 kDa	★	35	51
17	☑	☆	Collagen alpha-1(VI) chain OS=Homo sapiens GN=COL6A1 PE=1 SV=3	CO6A1_HUMAN	109 kDa		16	19
18	☑	☆	Vitronectin OS=Homo sapiens GN=VTN PE=1 SV=1	VTNC_HUMAN	54 kDa		13	12
19	☑	☆	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2	ALBU_HUMAN	69 kDa	★	27	31
20	☑	☆	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1	ACTB_HUMAN (+1)	42 kDa	★	19	18

Figure 7.5. Protein sequence showing a repeating peptide sequence (red) in patients with localised laryngeal and tracheobronchial amyloidosis by proteomic analysis.

Protein sequence coverage: 7%

Matched peptides shown in ***bold red***.

```
  1 MALRKGGLAL ALLLLSWVAL GPRSLEGADP GTPGEAEGPA CPAACVCSYD
 51 DDADELSVFC SSRNLTRLPD GVPGGTQALW LDGNNLSSVP PAAFQNLSSL
101 GFLNLQGGQL GSLEPQALLG LENLCHLHLE RNQLRSLALG TFAHTPALAS
151 LGLSNNRLSR LEDGLFEGLG SLWDLNLGWN SLAVLPDAAF RGLGSLRELV
201 LAGNRLAYLQ PALFSGLAEL RELDLSRNAL RAIKANVFVQ LPRLQKLYLD
251 RNLIAAVAPG AFLGLKALRW LDLSHNRVAG LLEDTFPGLL GLRVLRLSHN
301 AIASLRPRTF KDLHFLEELQ LGHNRIRQLA ERSFEGLGQL EVLTLDHNQL
351 QEVKAGAFLG LTNVAVMNLS GNCLRNLPEQ VFRGLGKLHS LHLEGSCLGR
401 IRPHTFTGLS GLRRLFLKDN GLVGIEEQSL WGLAELLELD LTSNQLTHLP
451 HRLFQGLGKL EYLLLSRNRL AELPADALGP LQRAFWLDVS HNRLEALPNS
501 LLAPLGRLRY LSLRNNSLRT FTPQPPGLER LWLEGNPWDC GCPLKALRDF
551 ALQNPSAVPR FVQAICEGDD CQPPAYTYNN ITCASPPEVV GLDLRDLSEA
601 HFAPC
```

Genetic sequencing

Genetic sequencing was undertaken in all patients for Apo A1, with only 1 patient being heterozygote for the Ala160Ser mutation.

Discussion

This is the first and largest study examining patients diagnosed with laryngeal and tracheobronchial amyloidosis, from a clinical and laboratory perspective. The clinical symptoms differed between these groups, with some overlap. Treatment options are limited and typically directed toward the site of amyloid deposition including surgical, laser techniques and in limited cases radiotherapy and chemotherapy. Proteomic analysis in 60 samples demonstrated the presence of all 3 amyloid signature proteins

in all samples as expected, and interestingly the presence of Apo A1 and insulin-like growth factor binding protein complex, a feature not present in patients with systemic disease. This finding may serve as a platform to better understanding of patients with localised amyloidosis of the airway tract.

Strengths and limitations of the study

This study reports the largest patient series of the upper and lower airway tract; invaluable in gaining insight into a disease where little is known about the clinical outcomes, treatments and underlying aetiology. Clinical characteristics were collated using medical records, computerised database of the UK National Amyloidosis Centre and patient questionnaires to enable a more extensive and complete data collection. The main limitation was the retrospective nature of this study, with some incomplete data as to the clinical symptoms and although a large number, only 60 histological samples of the 97 patients available for mass spectrometry analysis. Proteomic analysis of these 60 samples enabled us to hypothesise a relationship with 2 underlying proteins Apo A1 and insulin-like growth factor binding protein complex.

Clinical presenting features

Amyloidosis affecting the upper and lower airway tract present with respiratory symptoms like many other respiratory tract conditions, with a prior history of a laryngitis and asthma/bronchitis in 4 and 7 patients respectively. Analysis of our cohort show that the median age is 57.4 (range 44.7-66) and 47.6 (54-62.5) in both laryngeal and tracheobronchial groups with a greater female predominance. Figure 7.2A and 7.2B illustrate the symptoms experienced. The median symptom duration was shorter in the tracheobronchial group 4.3 months (range 8-24) versus 10.5 months (range 6-24) the upper airway tract, corroborating another large case series.[318] Triggering factors have

not been defined in these diseases [318, 319]. In our series, smoking was present in 16/56 28.5% (laryngeal) and 22/28 78.6% (tracheobronchial). Within the laryngeal group, the location of the lesion was situated in the supra-glottic region, vocal cords, subglottic and not stated in 6, 33, 16 and 8 patients respectively. The predilection for bilateral and right sided bronchial deposits may arise secondary to the anatomy of these lower airway tracts, with the right bronchial tract wider, shorter and more vertical than the left. Previous studies have reported the location of the lesion has having a major bearing upon the risk to respiratory failure, specifically higher risk patients with proximal and mid-bronchial lesions. [320]

Treatment strategies

Excisional therapeutic options are the primary modality in managing these patients.[321-323] Here the focus is to remove the tissue containing amyloid deposits and associated plasma/B cells. Systemic chemotherapy is not used frequently due to the low clonal plasma/B cell burden, making the risks and toxicity of this treatment outweighing the potential gain, despite the possible associated serum light chain excess or monoclonal protein[311]

Radiotherapy has been described in some cases with LBTA[309, 311, 318, 324] with the basis that plasma cells are radiosensitive, with the majority of patients being treated with 20 Gy in 10 fractions.[309] Similarly, in our series one patient with laryngeal involvement and three patients with tracheobronchial amyloidosis received radiotherapy, following failure with prior surgical resections and stent insertions to achieve local control. With a median follow up of 18.7 (6-47) months, these patients have had notable improvements in their quality of life, consistent with previously published literature.

Truong *et al* described outcomes in 10 patients post-radiotherapy with symptomatic airway amyloidosis patients (3 laryngeal and 7 tracheobronchial). With a median follow up of 6.7 years (1.5-10.3) 8 of 10 patients had symptomatic improvement.[309]

Overall survival

The OS of localised laryngeal and tracheobronchial amyloidosis is excellent with the 2 and 5 year survival 90% and 81%, and 93% and 83% respectively. Figure 3 illustrates the latter with comparison of both groups. Although not statistically significant (p 0.66), the OS following 75 months shows a sharper decline in the tracheobronchial group. These differences are most likely due to the underlying pathogenic factors, possibly the extent of the localised deposits, smaller bronchial anatomy and likeliness to occlude the airway tract.

Laser capture and proteomic analysis

Laryngeal and tracheobronchial amyloid deposition was characterised further with laser capture and proteomic analysis. All 60 cases exhibited evidence of 3 amyloid protein signature proteins serum amyloid P (SAP), Apo E and Apo AIV in conjunction with Apo A1 and insulin-like growth factor binding protein complex. Proteomic analysis was also performed in a total of 60 patients with systemic AL amyloidosis (renal, cardiac and liver biopsies) or transthyretin based disease (cardiac, bone marrow) as a control, with none of these samples showing evidence insulin-like growth factor binding protein complex and a much lower peptide content of Apo A1.

Apo A1 has been described in hereditary and non-hereditary cases. In hereditary ApoA1, deposition of amyloid occurs in different organs and tissue caused by the germline mutation in the ApoA1 gene. Non-hereditary ApoA1 is characterised by the

non-variant protein and has been described in atherosclerotic intima[325] and within the respiratory tract; thought to be reduced in patients with chronic pulmonary disease (COPD) resulting in airway limitation.[326]

Insulin growth factor (IGF) and insulin growth factor binding proteins (IGFBP) proteases are involved in somatic growth, cell proliferation, cell transformation and apoptosis.[327-329] IGFBPs and IGF may form binary compounds and allowing selective transport into tissues[327] Insulin growth factor (IGF) is thought to be a key modulator of lung fibroblast proliferation and is found at sites of inflammation[330, 331] Further studies have shown the regulation of IGF signalling via expression/down regulation of ligands, receptors and/or regulatory binding proteins in previous malignancies.[332] IGF-1 genetic polymorphisms may represent the long term exposure of IGF-1 on the cellular and circulatory sites.

The literature supports increasing evidence that amyloid fibril formation and aggregation arises from a partially folded conformation of the aggregating protein, [333] with the initial lag in fibril formation thought to be secondary to the slow assembly of a critical nucleus in a nucleation-polymerisation mechanism.[334] Although aggregation occurs when high concentrations of the key partially folded intermediate is present, other factors including the pH, temperature, amino acid sequence and concentration of the intermediate play a role. The presence of ApoA1 and insulin-like growth factor binding protein complex in the LTBA proteomic analysis is a new finding and further work is needed to elucidate the significance in localised amyloid in the laryngeal and tracheobronchial cases. Further studies are needed to elucidate the function of these factors before a significant pathogenesis is attributed with exploration in other types of localised amyloidosis.

Future directions

Further studies are needed to ascertain whether Apo A1 and insulin-like growth factor binding protein complex assist in instigating an underlying inflammatory process, bystanders or involved in maintaining the self-limited localised nature of this disease.

In summary, we believe that LTBA is a type of localised amyloidosis with a more troublesome clinical and hence problematic therapeutic quandary than other types of localised amyloidosis.

Results Section Three:

Management therapeutic options for systemic amyloidosis

Chapter Eight: Lenalidomide and dexamethasone for systemic AL amyloidosis following prior treatment with thalidomide or Bortezomib regimens

This chapter is written in the context of the publication: Lenalidomide and dexamethasone for systemic AL amyloidosis following prior treatment with Thalidomide or Bortezomib regimens. Shameem Mahmood, Christopher P. Venner, Sajitha Sachchithanantham, Thirusha Lane, Lisa Rannigan, Darren Foard, Jenny H. Pinney, Simon D. J. Gibbs, Carol J. Whelan, Helen J. Lachmann, Julian D. Gillmore, Philip N. Hawkins and Ashutosh D. Wechalekar. British Journal of Haematology. 2014; 166(6):842-8.

Introduction

Amyloidosis is a disorder characterised by protein misfolding leading to formation and deposition of amyloid fibrils in different tissues with change in structure and consequent dysfunction of the affected organs. Systemic immunoglobulin light chain (AL) amyloidosis is the most common type[286] resulting from an underlying plasma cell dyscrasia and treatment targeted toward suppressing this underlying clone. Treatments are directed against the underlying clone and hence have been adapted from those developed for treatment of myeloma. Immunomodulatory drugs (IMiDs) are emerging as an important backbone for treatment of AL amyloidosis in both the relapsed/refractory (Dispenzieri*, et al* 2007 Sanchorawala*, et al* 2010, Wechalekar*, et al* 2007 Palladini*, et al* 2012) and upfront setting.[85, 105, 108, 335] Small phase II studies

have shown good clonal responses using lenalidomide-dexamethasone in 67% [104, 105] and similar clonal response rates of 50%-62% with the addition of alkylating agents. [89, 106-108] Very few series have reported organ responses following lenalidomide treatment. It is well recognised that organ responses are usually generally delayed in AL amyloidosis with the exception of cardiac responses based on biomarkers, with the latter difficult to interpret in patients in IMiD based therapies.[136] Premature assessment of an organ response may lead to not recognising the full clinical impact of any treatment in this disease.

We report a substantial cohort of patients treated with lenalidomide in the relapsed/refractory setting, following the use of Bortezomib and/or thalidomide with long term follow up assessing the impact of previous therapies on haematologic responses. This study reports an unexpectedly high rate of organ responses on long term follow up in patients treated with Lenalidomide based treatment.

Patients and methods

This patient cohort comprised of all patients with systemic AL amyloidosis followed at the UK National Amyloidosis Centre from July 2007 to August 2013 who received treatment with lenalidomide for relapsed (n=62) or refractory (n=22) clonal disease, with at least one prior line of therapy. Confirmation of amyloidosis was confirmed by histology with Congo red staining and AL type confirmed by specific immunostaining of amyloidotic tissue by antibodies to kappa or lambda light chains with exclusion of hereditary amyloidosis by gene sequencing depending on the clinical details. All the patients had evidence of either an abnormal serum free light chain (FLC) ratio or a monoclonal immunoglobulin component by serum or urinary immunofixation by electrophoresis. Written consent for retrospective publication of anonymous data was

obtained from all patients in accordance with the Declaration of Helsinki. Organ involvement, haematologic and organ responses were classified according to the updated international amyloidosis consensus criteria, and a renal response was described as a 50% decrease and at least a decrease of ≥ 0.5g/24 hours of 24 hour urinary protein in patients with baseline urinary protein >0.5g/L, with no worsening of the creatinine/creatinine clearance by 25% above the baseline. Patients with no urine or urine quantification less than 0.5g/24 hours had assessment of their renal outcome based according to the estimated glomerular filtration rate and are reported separately. FLC and monoclonal protein response criteria were according to the updated Consensus guidelines.[87] Patients received 21 days of a 28 day cycle of lenalidomide with the addition of variable doses of dexamethasone 10-40mg once weekly, (Table 8.1). Lenalidomide treatment was given until there was disease progression or unacceptable toxicities.

The overall survival (OS) was assessed from the start of lenalidomide treatment until death or last follow-up. Progression-free survival (PFS) was calculated in responding patients from the commencement of treatment with lenalidomide until relapse, death or last follow-up. Survival endpoints were also examined to assess the impact of previous treatment lines. Statistical analysis was performed using SPSS v20 (IBM SPSS) software. Kaplan Meier estimates were used to calculate the OS and PFS. P values <0.05 were considered statistically significant.

Table 8.1: Patient Characteristics[336]

Patient Characteristics (n=84)	**n (%)/median(range)**
Male/Female	43 (51%)/41(49 %)
Age (yrs)	64.5 (45.5-79.1)
Detectable Monoclonal protein	35 (41.7%)
Light chain type Kappa Lambda	 31 (37%) 53 (63%)
Involved FLC (mg/l)	109.5 (19.5-1480)
dFLC (mg/L)	92.6 (6.4-1478)
Number of previous therapies	2 (1-6)
Prior Thalidomide	64 (76.1%)
Prior Bortezomib	58 (69%)
Treatment strategies	
Prior ASCT	13
Lenalidomide dose	
5 mg	10 (11%)
10 mg	8 (10%)
15 mg	21 (25%)
25 mg	45 (54%)
Dexamethasone	
10 mg	17 (20%)
20mg	48 (57%)
40 mg	19 (23%)
Organ involvement	
Heart	42 (50%)
Kidney	52 (61.9%)
Liver	18 (21.4%)
PNS	23 (27.3%)
ANS	17 (20.2%)
Soft tissue	32 (38%)
Number of organs involved	
1 organ	20 (23.8%)
2 organ	29 (34.5%)
≥ 3 organs	36 (42.9%)
Mayo Stage	
Stage I	24 (28.6%)
Stage II	47 (56%)
Stage III	10 (11.9%)
mean LV wall thickness (mm)	12 (10-18)
NT-proBNP (ng/L)	1034 (110-79,576)
Serum creatinine (µmol/L)	110 (47-976)
eGFR (mls/min)	51 (10-100)
CKD stage 4 and 5	17 (20.2%)
24 hour proteinuria (g)	0.7 (0.1-16)

n, number; k, kappa; l, lambda; FLC, free light chain; mg/L, milligrams per litre; dFLC, difference in involved and uninvolved free light chains; NT-proBNP, N-terminal fragment of brain natriuretic peptide; ng/L, nanograms per litre; mm, millilitres; µmol/L, micromoles per litre; mls/min, millilitres per minute; eGFR, estimated glomerular filtration rate; CKD, chronic kidney disease; LV left ventricular; ASCT, autologous stem cell transplantation; mg, milligrams

Results

A total of 84 patients were identified from our database listing all registered patients. The baseline clinical characteristics are illustrated in table 8.1. The median age was 64.5 years (range 45.5-79.1) and 52% were male. 37 (44%) had 3 or more organs involved. 48 (56%) and 17 (20%) patients had a recorded pre-treatment estimated glomerular filtration rate (eGFR) < 60 ml/min and < 30 ml/min respectively. The median pre-treatment N-terminal fragment of brain natriuretic peptide (NT-proBNP) was 1034 ng/L with 11 patients having an NT-proBNP >8500ng/L, and a median threshold of 1881ng/L at 6 months. The median number of lines of treatment was 2 (range 1-6). Seventy six percent and 68%, respectively received prior treatment with thalidomide and Bortezomib based regimens. The median time from diagnosis to the commencement of lenalidomide based treatment was 26.4 months (range 6-394). The median duration of lenalidomide treatment was 6.5 months (1-52), with 31 (37%) patients continuing following 12 cycles.

Haematologic response and survival

Lenalidomide regimens were used in the relapsed setting for 62 patients, with the 2 year OS and PFS 82% and 73%, respectively, versus 84% and 74% in the refractory setting (n=22). On an intention-to-treat (ITT) basis, 51 (61%) achieved a haematological response with a complete response (CR) in 17 (20%), very good partial response (VGPR) in 6 (8%), partial response (PR) in 28 (33%) and no response (NR) in 33 (39%) at 6 months (range 1-13) when the haematological response was assessed. The median time to haematologic response was 3 months (range 1-19), with haematological responses seen in 36 (56%) (VGPR or better in 14 (22%)) patients

treated with prior thalidomide and in 32 (55%) (VGPR or better in 13 (22%)) patients treated with prior Bortezomib.

The median follow-up for this study was 21 months (range 1.2-80.6) and 21 deaths occurred during this time. The estimated 1 and 2 year overall survival (OS) was 90% and 84% respectively (Figure 8.1A) with the estimated 1 and 2 year PFS 82% and 73% respectively (Figure 8.1B). Previous exposure to thalidomide did not confer a worse survival outcome and the estimated 2-year OS with and without thalidomide treatment was 82% versus 83% ((HR 1.45 (0.48-4.38); p = 0.51), respectively. The estimated 2-year PFS for those receiving prior thalidomide therapy was 80% in comparison to 36%; those not receiving thalidomide ((HR 0.82 (0.36-1.85); p=0.623)). Similarly, previous exposure to Bortezomib did not confer a worse survival (estimated 2-year OS 80% versus 85%, (HR 1.56 (0.6-4.06); p=0.36)) and the PFS ((estimated 2 year PFS 78% vs. 57%; HR 1.32 (0.60-2.91); p=0.492).

Patients who achieved a partial haematological response or better conferred an estimated 2 year survival of 85% in comparison to 66% for those not responding (HR 0.45 (0.19-1.08); p 0.67). Achieving a light chain response but not necessarily a paraprotein response was important in translating to a survival advantage with patients achieving at least a partial dFLC response (PR or greater) having a median estimated 2 years OS of 79% in comparison to 65% for non-responders (HR 0.25 (0.08-0.72); p=0.014)) (Fig. 8.1C). A paraprotein response in isolation, did not confer a similar survival advantage (HR 0.71 (0.16-3.22); p=0.66)) (Fig. 8.1D). Multivariate analysis showed that lack of a dFLC response and NT-proBNP >8500 ng/L were the sole independent factors adversely impacting on survival (table 8.2).

Figure 8.1A: Kaplan Meier estimated overall survival on an intention to treat basis. The median overall survival has not been reached[336]

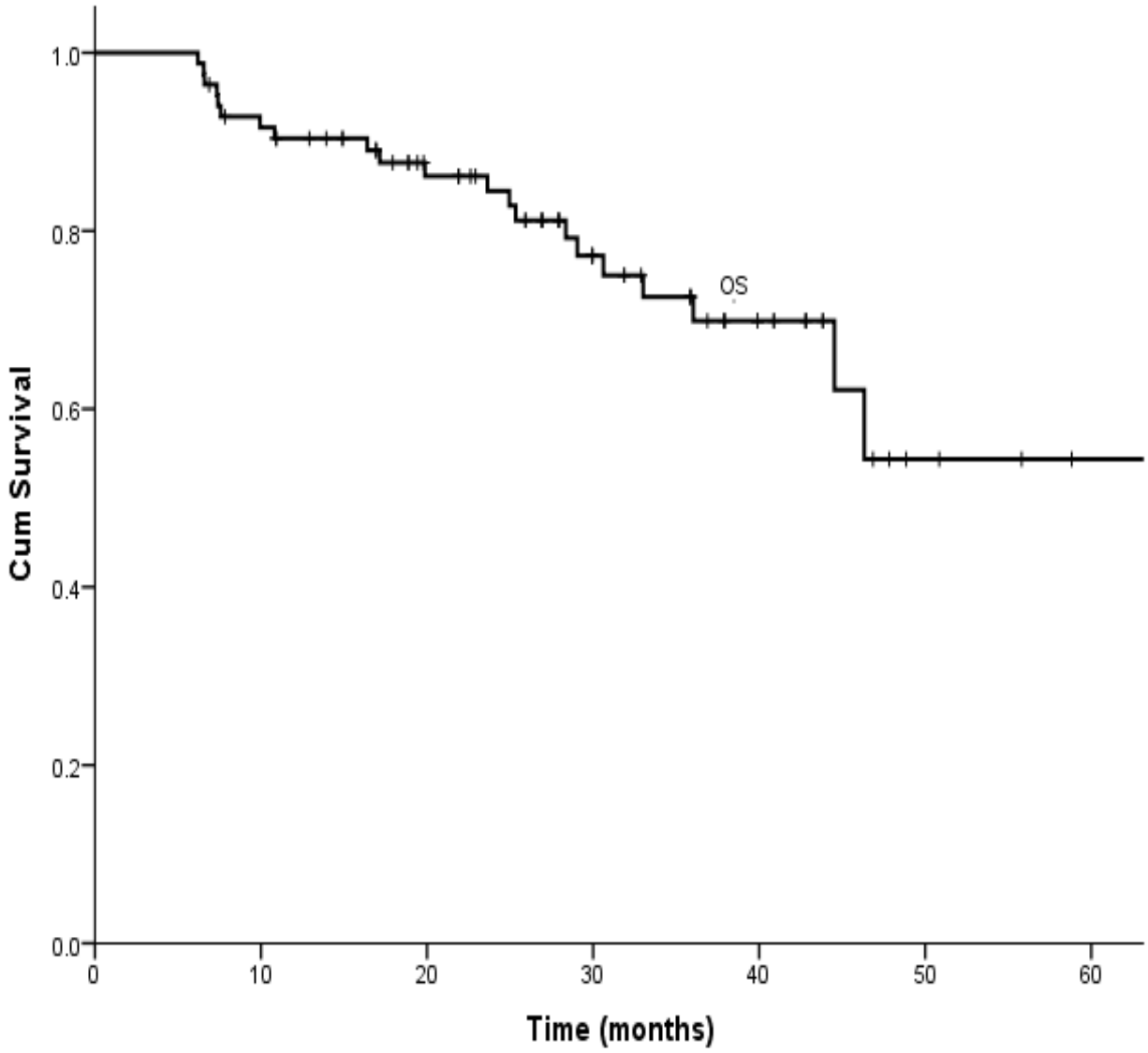


Figure 8.1B: Kaplan Meier estimates of progression free survival. The median progression free survival was 44.5 months[336]

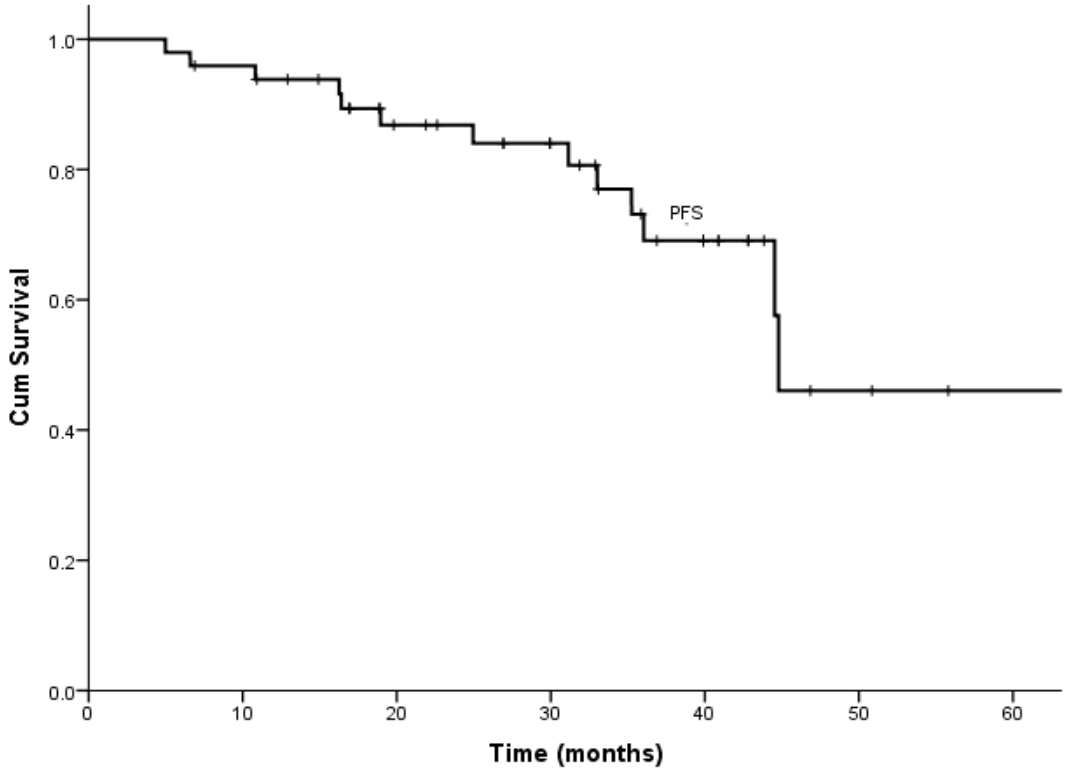


Figure 8.1C: Overall Survival stratified by the dFLC response achieved. Patients achieving a partial dFLC response or greater (solid line) had a significantly better overall survival than those who did not achieve a dFLC response (dashed line)[336]

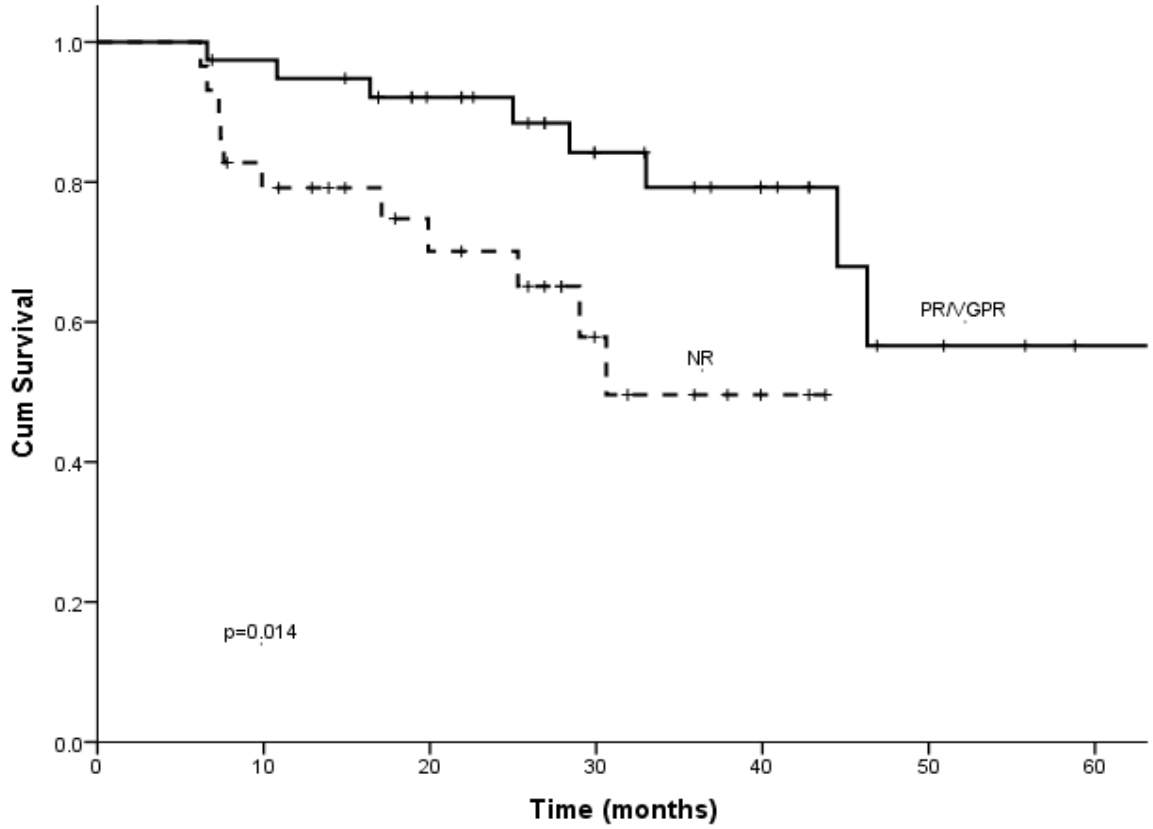


Figure 8.1D: Overall Survival stratified by the monoclonal protein response. There was no significant impact of an isolated monoclonal protein response on survival (patients achieving a partial haematological response or greater (solid line) and no response (dashed line))[336]

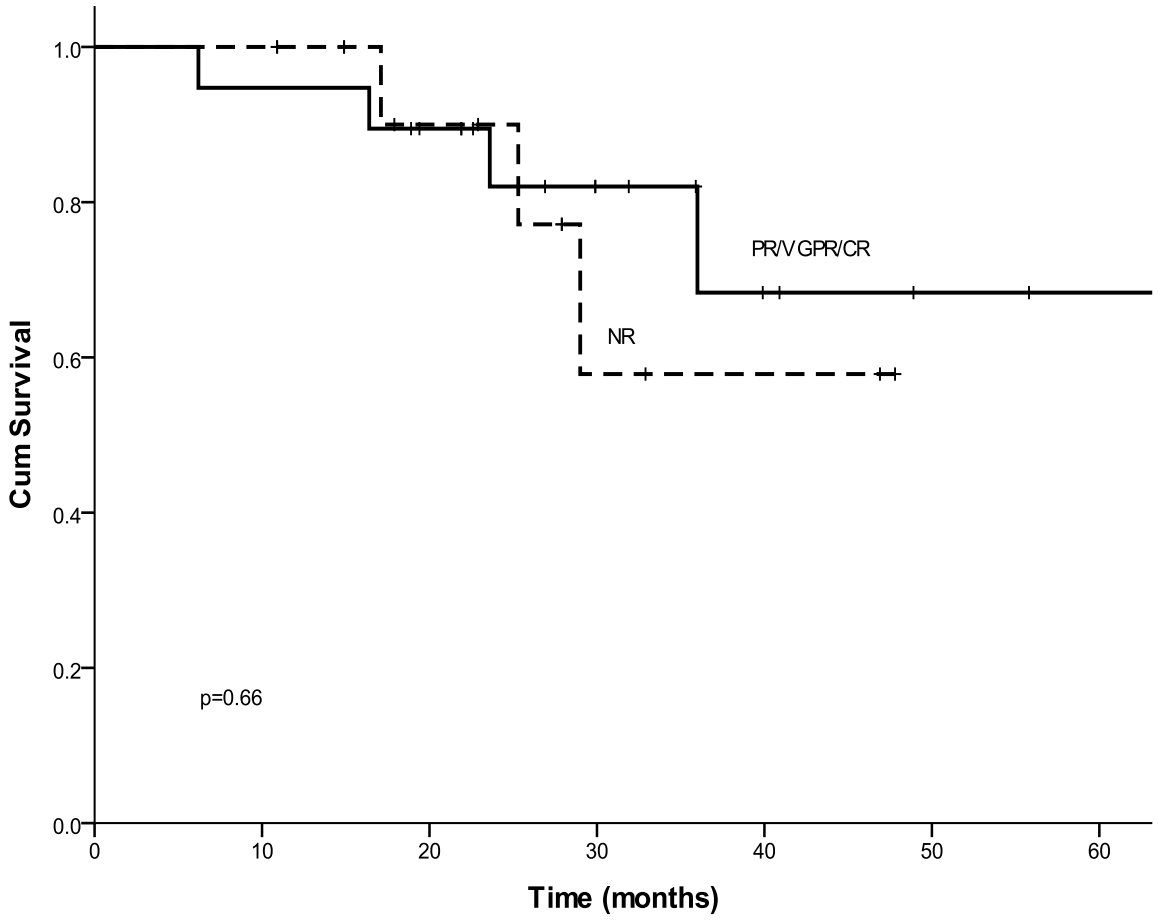


Table 8.2: Variables associated with survival[336]

Variable	HR (95% CI)	p
Univariate Analysis		
Age >65 years	1.73 (0.72-4.13)	0.45
Heart involvement	2.93 (1.17-7.36)	0.02
NT-proBNP≥8500ng/L	4.23 (1.47-12.1)	0.001
Paraprotein haematologic response	0.71 (0.16-3.22)	0.659
dFLC haematologic response	0.25 (0.08-0.72)	0.014
Performance status ≥3	2.96 (0.39-22.66)	0.296
dFLC ≥180mg/L	1.52 (0.51-4.52)	0.45
Organ involvement ≥3	1.16 (0.49-2.73)	0.74
Multivariate analysis		
dFLC response	0.34 (0.12-0.95)	0.039
NT-proBNP≥8500ng/L	2.75 (0.94-8.04)	0.065

IVSd, interventricular septal dimension; mm, millimetres; NT-proBNP, N-terminal fragment of brain natriuretic peptide; ng/L, nanograms per litre; LVEF, left ventricular ejection fraction; dFLC, difference in involved and uninvolved free light chains; eGFR, estimated glomerular filtration rate

We undertook a subgroup analysis taking into account the dexamethasone dose (10, 20, 40mg) with a 2 year OS (HR when compared to dose of 40 mg) of 76% (HR 2.32 (093-5.78 p 0.07)), 84% (HR 1.1 (0.46-2.61 p 0.84)) and 93% (reference) respectively and 2 year PFS 57% (HR 2.44 (1.13-5.27) p 0.023)) 73% (HR 1.08 (0.51-2.26) p 0.85)) and 89% (reference) respectively.

Organ responses

We assessed the median time to first organ response assessment as 6 months (range 6-24). On an ITT basis, at six months, organ responses were observed in 14/84 (16%) of patients. 5/42 evaluable (12%) patients achieved a cardiac response and 2/18 (11%) achieved liver responses. Twenty one out of 38 (55%) evaluable patients achieved a renal response (40% on an ITT basis) – 7 (18%) at 6 months, 7 (18%) at 12 months and an additional 7 (18%) patients at 18 months by long term follow up. The median duration of lenalidomide in these 38 patients was 11 months in total (range

6-52 months). An eGFR <15 ml/min was present in 5 patients and could not be included in any renal response analysis. Two patients with proteinuria <0.5g had stable eGFR values following treatment and 1 patient experienced a decline in eGFR from 41ml/min to 15 ml/min. Fifty seven patients had evaluable visceral amyloid deposition by ^{123}I serum amyloid P component scintigraphy with regression evident in 17 (30%) patients at 6 months.

Toxicity

Twenty three patients experienced grade 3-4 toxicity (27%): neutropenia in 14 patients, sepsis in 4 patients, skin rash in 3 patients, thrombosis in 1 patient and diarrhoea in 3 patients. 14 (16%) required dose reduction (from starting doses of 25mg) to 5-15mg primarily due to neutropenia. One patient developed repeated thrombosis of a dialysis fistula which resulted in cessation of treatment but there were no other thrombotic complications (including in 8 patients with a prior personal history of thrombosis who received full intensity anticoagulation during their treatment). No significant change in the eGFR was evident following treatment (baseline median 51 (range 10-100) mls/min to 47 (range 10-100) ml/min post therapy). No patients developed secondary malignancies during this follow up period.

Discussion

There has been significant evolution of treatment of AL amyloidosis during the last decade. Although oral melphalan-dexamethasone remains widely considered as a standard of care, currently most patients receive a novel agent based regime, usually as induction chemotherapy and again frequently at relapse. The optimal sequence for using novel agent based therapies and the outcomes of relapsed patients have been studied to a lesser degree. The current treatment of AL amyloidosis is directed against

the underlying clonal disorder; with the ultimate goal to achieve amyloid regression in the affected organs. Modern treatments are increasingly successful in achieving the former but the latter goal seems less tangible and also poorly studied and reported less in studies of patients treated by chemotherapy based approaches. Ultimately it is the organ response that will impact on patient survival and quality of life.

The present study reports the largest cohort of patients treated with lenalidomide-dexamethasone (LD) currently, showing overall good haematological responses, in systemic AL amyloidosis specifically in the relapsed/refractory setting. The overall haematological response rate of 58% (CR -20%) achieved is reflective similarly to earlier phase II studies of LD.[104,105] The haematological response, particularly the dFLC response translated into a significant survival advantage (HR 0.25 (0.08-0.72); p=0.014)). The dFLC-responses were rapid and the median time to respond was 3 months – comparatively better than that reported in earlier LD studies [104, 105] and also similar to those reported for LD-alkylator combinations, often in those treatment naïve and previously treated [89, 106, 107] hence the benefit of an additional alkylator in treatment of patients with systemic AL.

Data is limited data regarding the impact of prior Bortezomib or thalidomide based treatments on lenalidomide responses. Myeloma studies analysis seems to suggest a poorer response in previously thalidomide treated patients.[337] Although retrospective, this patient cohort had two thirds of all patients treated in a relatively uniform manner with oral cyclophosphamide-thalidomide-dexamethasone [85] as the standard first line therapy followed by Bortezomib at first clonal relapse and lenalidomide dexamethasone at second clonal relapse - a treatment line consequence as a result of treatment directed by funding availability clearly, in the UK, which is

regulated by the National Institute of Clinical Excellence (NICE). Italian colleagues in a study showed that a median 2 year OS following lenalidomide dexamethasone with Bortezomib pre-treatment was 47%.[338] In the current study, treatment with thalidomide or Bortezomib did not impact on either the haematological response, overall or progression free survival (2 year OS was ~ 80% in those with and without previous exposure to either chemotherapeutic agent). Our study showed that the 2 year OS was 76%, which was better than that reported in the Italian study – very likely due to baseline disease characteristics which dominate survival outcomes in AL amyloidosis. Typically cardiac involvement, specifically Mayo stage 3 carries a poorer prognosis. The OS in this study is very different in compared to the reported outcomes of newly diagnosed patient outcomes as reported for prospectively followed up patients in our Alchemy study cohort[339] predominantly due to a very high proportion (29% early deaths in 714 patients (44% with Mayo stage 3)) in newly diagnosed patients with AL amyloidosis. We performed a subgroup analysis determined by the dexamethasone dose which showed a trend for an improved OS and PFS in those having a higher dose but is difficult to interpret due to patient selection and the retrospective nature of the cohort. This was also shown in the recent study illustrating better outcomes in patients administered higher dose dexamethasone in the upfront setting[340] and may reflect rapid disease control in AL which may be different from myeloma. Lack of impact of prior therapy on outcomes is encouraging and may be partly explained by the treatment strategy. It is important to consider that apparent poorer outcomes in patients who did not receive prior thalidomide may reflect patient selection with significant neuropathic disease in which case tolerance may be poorer. Due to significant risk of morbidity, it has become practice in the UK for patients to discontinue chemotherapy following one or two cycles following best haematological

response. However, this may prevent selection of resistant plasma cell clones, and thus allowing for responsive disease at relapse. There was a non-significant trend to poorer progression free and overall survival in patients in those who had not received prior thalidomide or prior Bortezomib, accounting for a third of the patients in the current series (mainly comprising of patients with significant peripheral and/or autonomic neuropathy); limiting tolerance and availability of therapies, likely, leading to poorer overall outcomes. Therapeutic options for treatment of patients with relapsed disease are always changing. Given the high upfront responses to Bortezomib, re-challenge may be useful but data is limited at this stage; trials incorporating drugs such as MLN9708 or Carfilzomib in this setting are currently ongoing.

Organ response following chemotherapy treatment are generally fewer in number than haematological responses, and remain less well studied or explored. Generally long term follow up of chemotherapy patients is scarce with most organ responses reported at six months – and likely to underestimate the true (delayed) advantages of these. Our colleagues from Italy recently reported the long term follow up of patients treated with oral Melphalan-dexamethasone with renal responses reported in 24% of patients.[341] We have previously reported a renal response rate of 32.6% in 429 patients seen from our centre until May 2008.[342] The organ response rates in the current series at six months, seen in a fifth of the patients, is similar to the previous studies of patients treated with lenalidomide-dexamethasone (17% and 35% of patients from early phase II studies[104, 105] respectively) and cyclophosphamide-lenalidomide-dexamethasone (22% [89] and 31% [107]). Assessment of cardiac response post chemotherapy using the standard NT-proBNP assay may not reflect the true benefit of lenalidomide in the current series due to potential for the paradoxical

increase as previously reported,[136] with a rise in the median NT-proBNP 1881ng/L at 6 months from baseline 1034ng/L in the current cohort. The striking result was the fact that 55% of evaluable patients achieved a renal response by 18 months (with the median duration on lenalidomide - 11 months). Interestingly, a select cohort of patients treated with long term lenalidomide (median duration - 9 months) reported by colleagues from Boston also showed an amyloidotic organ response in 44% of evaluable patients.[343] The renal responses in both these series appear to be significantly higher than the previous reports of chemotherapy treated patients (25% in long term follow up of MDex treated patients in comparison to 55% in the current cohort) which appears to be similar to those treated with autologous stem cell transplantation.[344] The amyloidotic organ responses are important and in LD treated patients are especially striking as the complete response rate is not particularly high (approximately 20%). Lenalidomide is thought to increase the lymphoid immune function by increasing the natural killer (NK) cell numbers and antibody-dependent cell-mediated cytotoxicity and thus enhancing T-cell cytokine production.[345] Lenalidomide also induces NK cells to stimulate production of granulocyte-macrophage colony-stimulating factor, TNF-α, and various other immune recruiting chemokines including RANTES, IL-8, MCP-1, and MIP-1α/β in response to antibody-coated tumour cell lines, which contributes to a more effective cellular (including macrophage) immune response.[346] Previous studies from our group have shown a natural slow clearance of amyloid from organs is affected by a macrophage type mediated mechanism, which can be substantially enhanced with therapeutic antibodies that target the deposits.[15] Unexpectedly high percentage of renal responses raises the possibility that lenalidomide may directly enhance the natural regression of amyloid through its immunomodulatory effects. Supposing this was the

case, lenalidomide may play an adjuvant role in anti-amyloid immunotherapies which are currently in development, as seen in its similar use in myeloma immunotherapy with elotuzumab. There are a number of caveats which remain in bearing in mind interpretations of these results: this is a retrospective series with limited patient numbers, the possible selection of patients who are long term survivors and have the resilience to tolerate long term therapy; with possible underestimation of organ responses in other studies due to the early assessment time points.

Approximately a third of all patients in the current series developed grade 3 or greater toxicity. Although worsening renal function[347] or high incidence of skin toxicity has previously been reported with lenalidomide; not seen in the current cohort. The major grade 3 toxicity experienced was cytopenias which consequently led to dose reductions. The grade 3 myelosuppression which developed improved following a reduction of the lenalidomide dose, with a lenalidomide dose of 15 mg better tolerated in our study, similar to previous reports,[104, 106, 108] suggesting that this is an appropriate starting dose. A few patients needed dose reduction after the first few cycles with the stable dose achieved with good tolerance and considered for longer term therapy. Although longer follow up is needed, there were no secondary malignancies in this patient series. The toxicity profile reported in this series appears to better than that of reported in prospective trials, very likely due to underestimation of toxicity due the retrospective nature of this study and should be interpreted with caution accordingly.

Thus in summary, lenalidomide/dexamethasone is an effective regimen in treating patients with systemic AL amyloidosis with relapsed and/or refractory clonal disease with prior Bortezomib or thalidomide based regimens. Lower commencing doses compared to that routinely used in treatment of patients with myeloma should be

considered to allow better tolerance. There was an encouragingly high rate of organ responses recorded in this study in those patients receiving long term lenalidomide is intriguing and needs further study in a larger patient cohort, with consideration of evaluating this concept in experimental models to evaluate the potential of lenalidomide in directly modulating the clearance of amyloid deposits.

Chapter Nine: Use of targeted therapy for reducing circulating SAP in patients with hereditary fibrinogen amyloidosis and dialysis related amyloidosis

Introduction

Systemic amyloidosis can be a fatal condition which is either acquired or hereditary, leading to the accumulation of amyloid deposits within different tissues and organs, resulting in dysfunction in the latter, with a high risk of mortality depending on the organ involved.[194] Hereditary fibrinogen amyloidosis was initially described in a Peruvian kindred in 1993.[45] Fibrinogen related amyloidosis has predominant renal involvement with end stage renal failure within 5 years of presentation with proteinuria.[47] There are 4 novel mutations reported with the main mutation E526V variant.[45] Currently there are no definitive therapeutic cures for this condition, with renal transplantation performed in a selected cohort with a median graft survival of 6.7 years, with a combined liver and renal transplantation considered only for very young, fit patients but also associated with a high mortality.[47]

Beta-2-microglobulin (β2M) amyloidosis is a condition that arises in patients receiving long term haemodialysis or continuous ambulatory peritoneal dialysis (CAPD).[348, 349] Studies have shown that β2M is a major constituent of amyloid fibril formation, with deposition within the synovial membranes and osteo-articular sites and hence clinically destructive osteo-arthropathies, carpal tunnel syndrome, flexor tenosynovitis, subchondral bone cysts, erosions and fractures.[350]

Serum amyloid P component (SAP) is a normal constituent plasma glycoprotein previously shown to bind calcium-dependently to amyloid fibrils.[351, 352] and an earlier

study using ((R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2 carboxylic acid (CPHPC)) to target SAP as a treatment for systemic amyloidosis[1] with an excellent safety profile and variable SAP depletion in the various patient cohort. We therefore investigated the clinical, safety, biochemical results with the quality of life effects of CPHPC in patients with hereditary fibrinogen amyloidosis (n=10) and dialysis related amyloidosis (n=3) in a pilot study.

Patients and methods

Patients

The primary cohort consisted of 10 patients diagnosed with hereditary fibrinogen A α-chain amyloidosis (AFib) and 3 patients with dialysis related amyloid ($A\beta_2M$) attending the National Amyloidosis Centre initially recruited from August 2012, with patient characteristics illustrated in table 9.1. We sought permission from the Royal Free Hospital's Drugs and Therapeutics Committee and all patients consented for compassionate use of CPHPC, with written consent for retrospective publication of data obtained from all patients in accordance with the Declaration of Helsinki.

Table 9.1: Patient characteristics

Number of patients	13
Sex	
Male	10
Female	3
Age (years)	
Median	66.7
Range	48.9-74.1
Amyloid type	
Hereditary fibrinogen (AFib)	10
Dialysis related ($A\beta_2M$)	3
ECOG	
0	11
2	2
Organ involvement	
Renal	13
CKD stage 1-2	3
CKD stage 3-4	7
End stage renal failure (dialysis dependant)	3
Renal allograft	1 (with CKD stage 3-4)
Baseline PET/CT involvement in ($A\beta_2M$) patients	3

CKD – chronic kidney disease; PET/CT – positron emission tomography – computed tomography.

Study design and dosing

We conducted a single centre study with compassionate use of CPHPC approved by Glaxo Group Ltd and the Royal Free Hospital in patients with hereditary fibrinogen A α-chain amyloidosis and dialysis related amyloidosis. The CPHPC administration was typically given as subcutaneous injections for patients with hereditary fibrinogen amyloidosis and via intravenous route for those with dialysis related amyloidosis. The CPHPC dose was dependent upon the estimated glomerular filtration rate (eGFR). Patients with an eGFR greater than 25mls/min received 60mg subcutaneously three times a day, and those with an eGFR less than 25mls/min received 60mg subcutaneously twice a day. For those on haemodialysis patients ($A\beta_2M$) a dose of

30mg CPHPC was given intravenously three times a week at the end of each dialysis session. The clinical status of the patients were assessed at baseline and serial monitoring performed with clinical assessment and investigations including haematological, biochemical renal parameters and urinary quantification of proteinuria, creatinine clearance, quality of life questionnaire 6 monthly, visual analog scale (VAS) scores for any joint pain and whole body ^{123}I-labelled SAP scintigraphy (with a 4 week washout period to ensure no residual effect of the CPHPC effect). The VAS score is a continuous scale comprised of a horizontal (10cm) line with the extremes of pain 0; no pain and 10; maximum pain marked. It was self-completed by each patient. Patients with dialysis related amyloid had pre-treatment 2-deoxy-2-[fluorine-18] fluoro–D-glucose positron emission tomography- computed tomography (18F FDG PET-CT) with reassessment of this imaging at 6 months post-treatment.

Quality of life (QoL) questionnaire assessments

The quality of life was assessed at 6 monthly intervals for all patients with the use of the validated Quality Metric SF36v2® Health survey. This is designed to measure functional health and well-being from the patient's perspective. There are eight specified health domains (physical functioning, role physical, bodily pain, general health, social function, role emotional, mental health and vitality) which are assessed are scored individually.[177] The result is expressed in comparison to the American norms. The average score for healthy controls in each measure is 50, with higher scores representing a better QoL. A change of 10 points or greater in any domain between administrations is considered clinically significant. This is explained in detail in the methods chapter.

Results

Depletion of circulating SAP

The median (range) SAP concentration in our patient cohort was 25 mg/L (range 8-38), with the normal range 8-55 mg/L.[353] The first follow up appointment was arranged for 6-8 weeks following the initial administration of CPHPC, showing the SAP concentration had fallen to undetectable limits, <4mg/L at the second visit and consequent visits, aside from visits which co-incided with an SAP scan; which the CPHPC had been stopped 2 weeks previously. The quantification of the visceral amyloid deposits by ^{123}I labelled SAP scintigraphy performed yearly did not show any significant difference in the amyloid load.

Clinical results

Among the 10 patients administered with CPHPC, 3 patients progressed to dialysis (one patient post renal transplantation and renal function deteriorating secondary to a further renal insult). The median follow up for the whole cohort was 28.9 months (range 9.1-38). The pre-treatment median eGFR was 31mls/minute (19-78) with pre-treatment quantitative proteinuria 3g (range 1.1-18.6). Following treatment with CPHPC 7 patients have remained dialysis independent. Comparison was made with 9 matched historical (eGFR matched) controls with reference made to the renal parameters as end points, table 9.2. Monitoring of the natural course of the biochemical parameters including the estimated glomerular filtration rate (eGFR) and creatinine are illustrated in figure 9.1A and figure 9.1B respectively, showing a more rapid decline in eGFR and corresponding rise in creatinine in the historical controls in comparison to patients treated with CPHPC.

Table 9.2: Comparison of clinical and laboratory features of AFib patients treated with CPHPC and historical controls

	CPHPC	**No CPHPC**
Number of patients	10	9
Baseline creatinine clearance (mls/minute)		
Median	39.8	41
Range	8.2-144	28-138
Age (years)		
Median	66.7	51.7
Range	48.9-74.1	46.8-67.9
Proteinuria (g of protein/24 hours)		
Pre	3 (1.1-18.6)	4.8 (0.2-8.2)
Range	2.96 (1.6-10.8)	4.9 (0.2-7.6)
Creatinine pre	177.5 (84-289)	173 (77-276)
Creatinine post	257.5 (77-595)	452 (91-848)
eGFR pre	31 (19-78)	28 (20-90)
eGFR post	22 (9-71)	10 (10-59)
Systolic blood pressure		
Median	132	140
Range	98-186	129-170
Diastolic blood pressure		
Median	73	89
Range	56-97	70-105
ACE inhibitor or ARB		
Number of patients taking 1 agent	7	6
Number of patients taking 2 agents	2	0
Dialysis dependant	3	7

Figure 9.1A: The natural history of estimated glomerular filtration rate (eGFR) in hereditary fibrinogen amyloidosis patients treated with CPHPC (solid lines) and CKD matched controls (dashed lines)

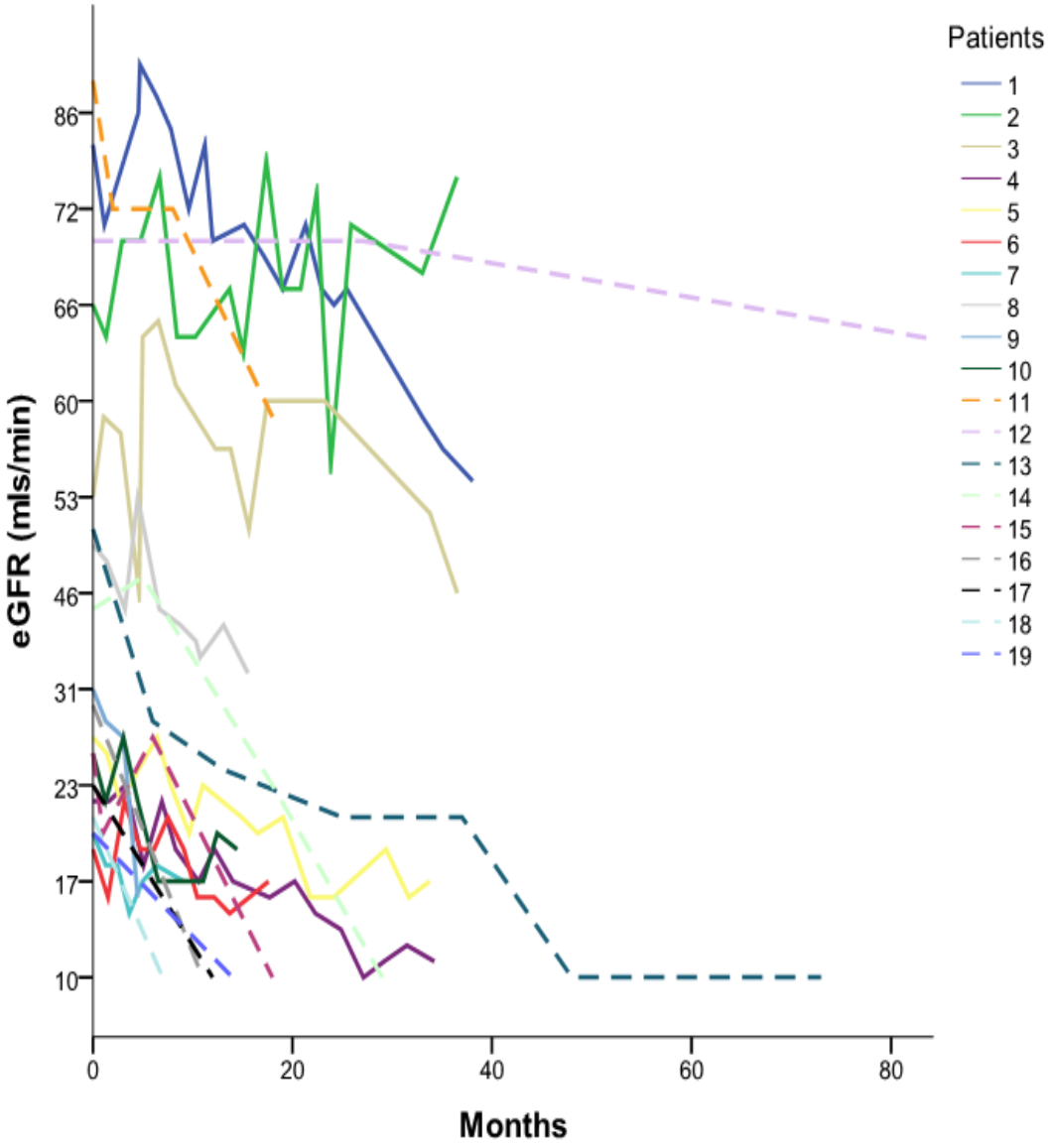
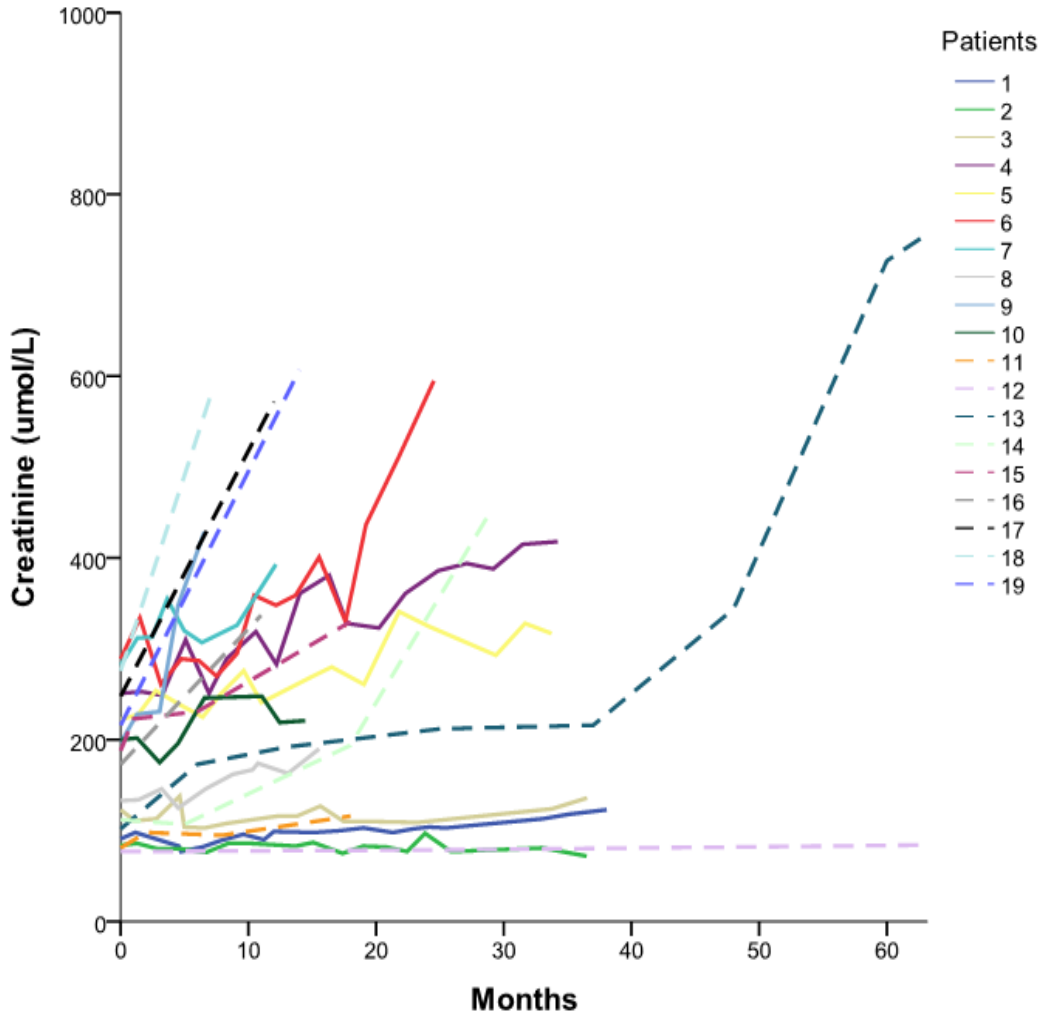
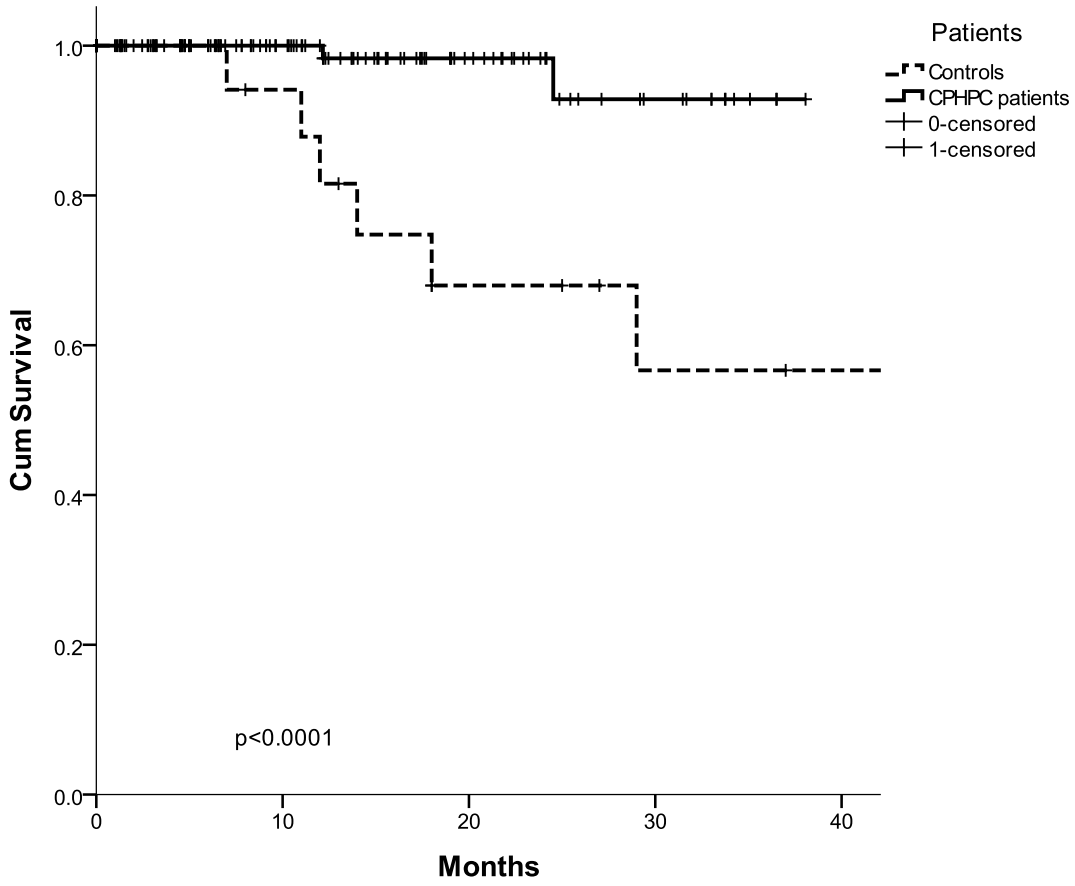


Figure 9.1B: Natural history of creatinine in hereditary fibrinogen amyloidosis patients treated with CPHPC (solid lines) and CKD matched controls (dashed lines)



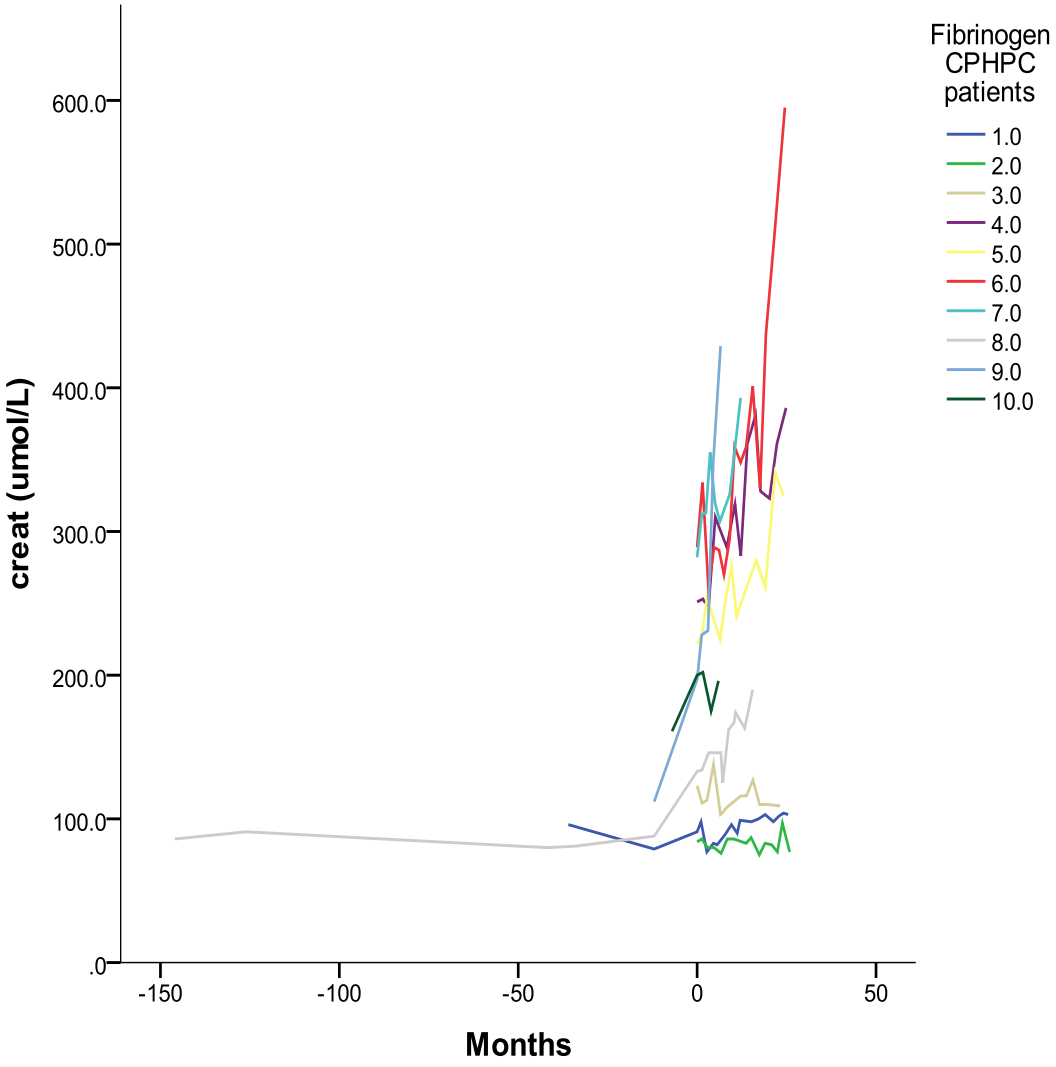
We assessed the time to dialysis as an end point showing a statistically significant difference between patients receiving CPHPC (n=10) and historical controls (n=9), with the median time to dialysis for the latter 60 months and not reached in those patients receiving CPHPC, figure 9.1D. Cox regression analysis also showed a HR of 10.86 (2.17-54.45), p=0.004.

Figure 9.1D: Renal survival in patients receiving CPHPC (solid line) was significantly better (p<0.0001) than CKD matched controls (dashed line)



We contacted the local doctors caring for these patients for prior renal function results to assess the natural history and time course of this disease, to assess the natural renal course in this disease, figure 9.1E. Unfortunately many patients had a limited history of serial biochemical monitoring, with the majority of patients presenting with renal impairment and proteinuria, and shortly followed by assessment at our Centre.

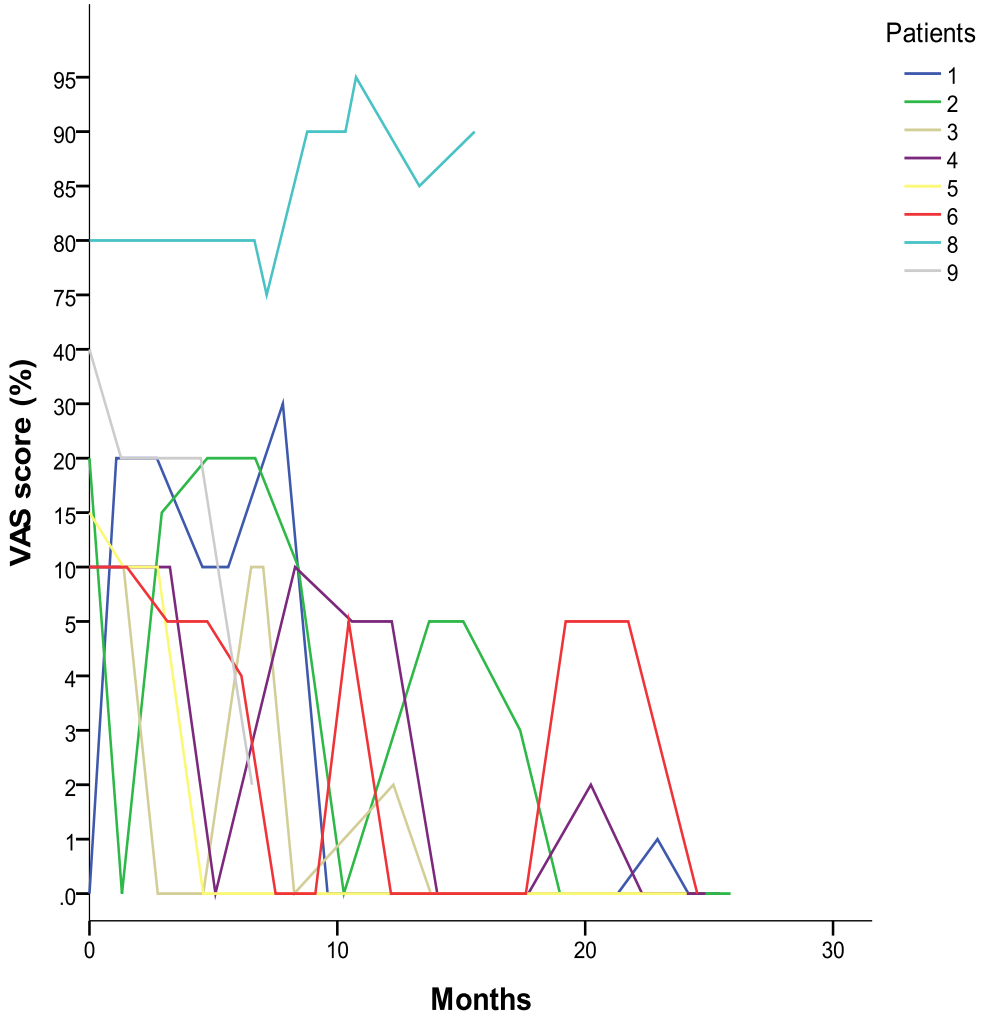
Fig 9.1E: Natural history of the creatinine pre CPHPC treatment in a select group of patients



VAS scores in hereditary fibrinogen and dialysis related amyloidosis patients

VAS scores were recorded at each visit for a follow up of 6-12 months, recorded in figure 9.2. Patients with hereditary fibrinogen amyloidosis recorded an improvement in their joint symptoms, with 1 patient not describing any joint pain.

Figure 9.2: Recordings of VAS scores in 9 patients treated with CPHPC showing a progressive decline in joint pain whilst patients were on treatment



VAS scores were also recorded in the dialysis related amyloidosis patients, but multiple joints were described as painful at baseline, with the pictorial images in figure 9.3 illustrating the chronological change in their symptoms following treatment.

Figure 9.3A: Patient 1 with (β2M) amyloidosis illustrating a pictorial image of the recorded VAS scores at baseline and following 12 months of treatment with CPHPC

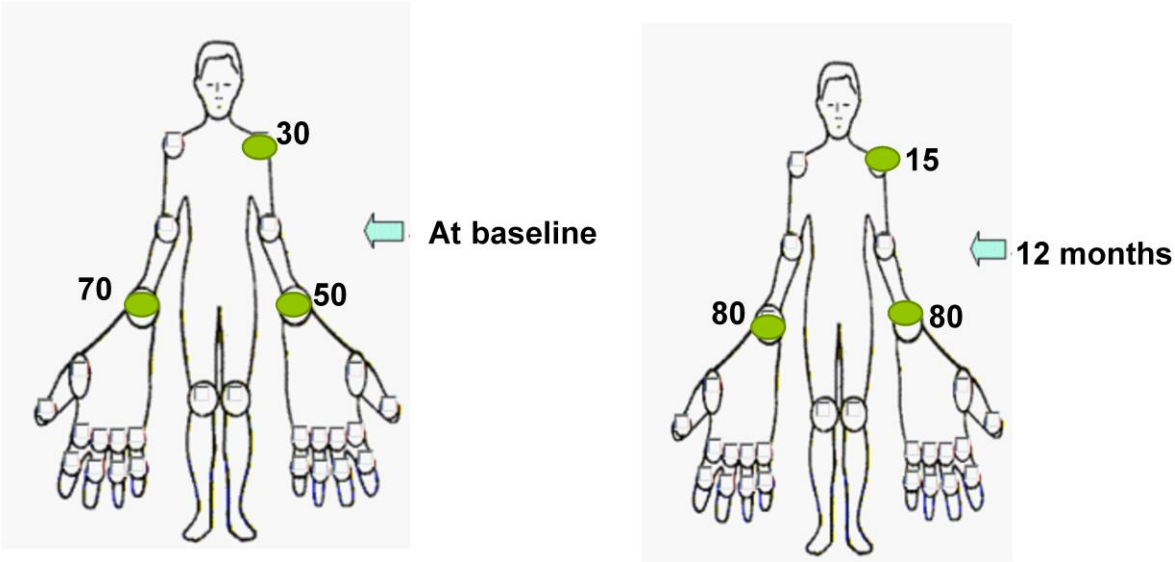


Figure 9.3B: Patient 2 with (β2M) amyloidosis illustrating a pictorial image of the recorded VAS scores at baseline and following 12 months of treatment with CPHPC

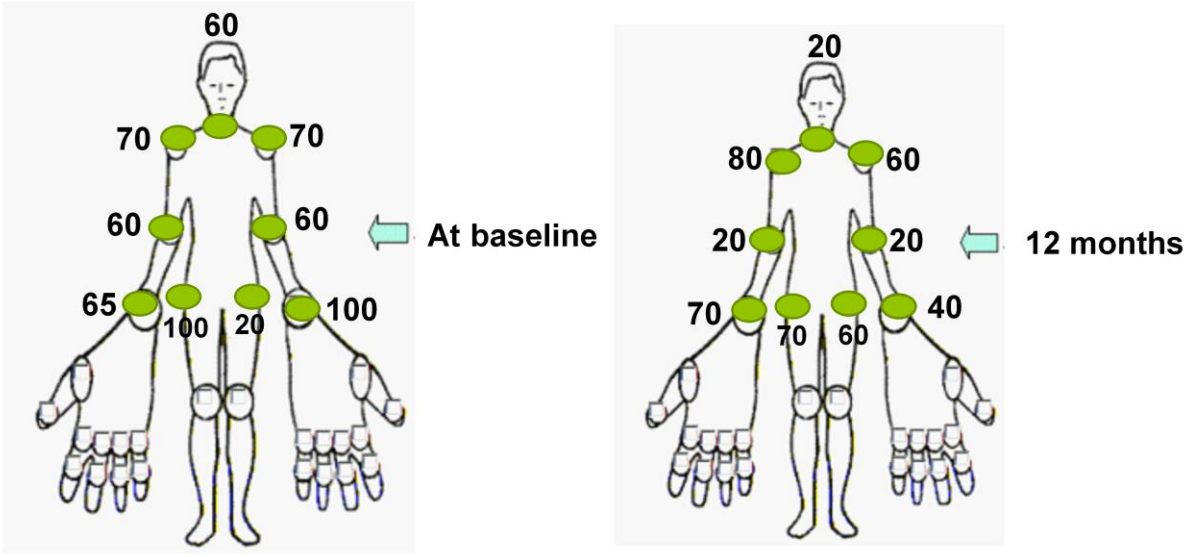
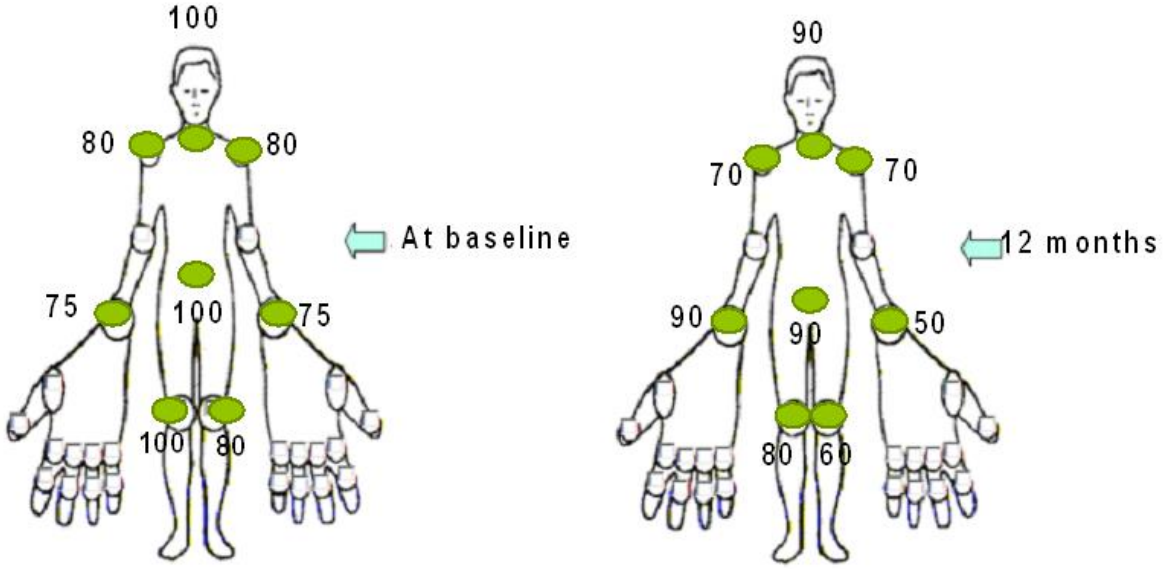


Figure 9.3C: Patient 3 with (β2M) amyloidosis illustrating a pictorial image of the recorded VAS scores at baseline and following 12 months of treatment with CPHPC



18F FDG PET-CTs in DRA patients

18F FDG PET-CTs were performed in the 3 patients with dialysis related amyloidosis, typically showing increased metabolic activity in the peri-articular soft tissue and bones. Clinically figure 9.3A, 9.3B and 9.3C illustrates the pattern of joint pain by each patient, with the typical joints showing uptake by ^{18}F-FDG PET-CT the hip, shoulders and sacroiliac joints, figure 9.4A and 9.4B. Comparison of these images from baseline to 12 months following CPHPC treatment showed stable appearances, a result which is difficult to interpret particularly in light of not fully understanding why there is increased metabolic uptake in this disease.

Figure 9.4A and 9.4B: illustrate the coronal images of a PET/CT with increased metabolic activity in the thickened soft tissue surrounding both shoulders and hip joints at baseline (A) and 12 months post treatment respectively (B)

Figure 9.4A

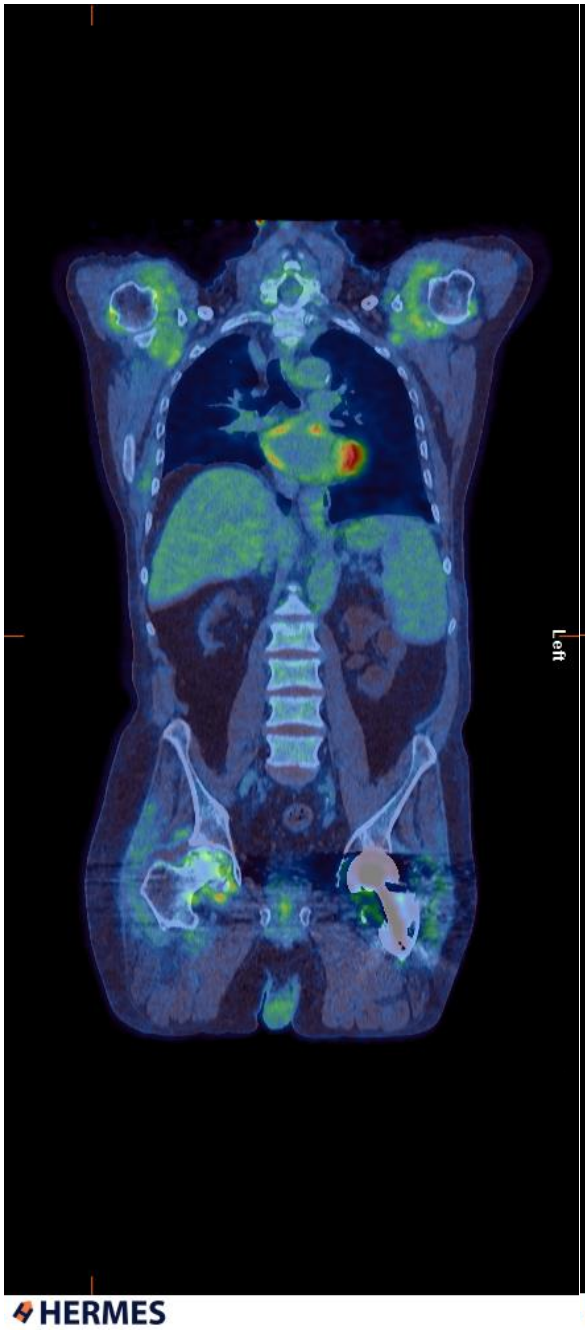
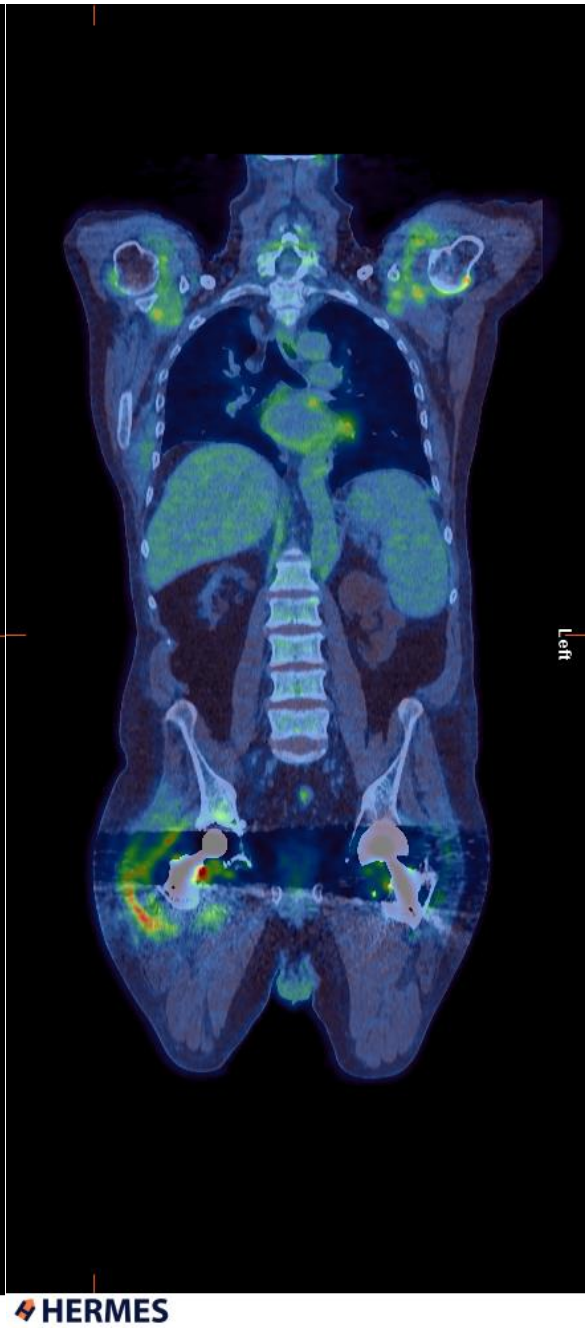


Figure 9.4B



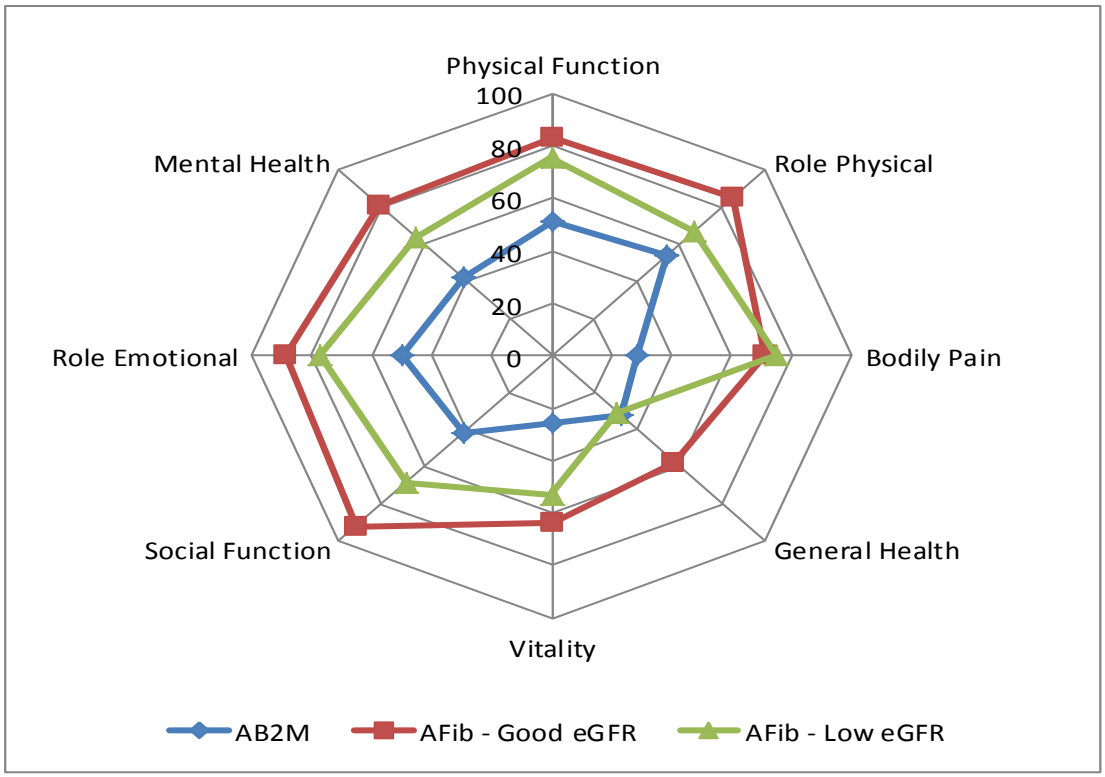
Safety and adverse side effects

No serious adverse incidents occurred. All hereditary fibrinogen patients administered the CPHPC subcutaneously either twice or three times per day, governed by the underlying eGFR. Initially patients reported momentary local stinging at the injection site. The formulation was diluted slightly at 12 months post commencement of treatment resulting in less stinging. No patients withdrew from the study. During the winter months, a few patients reported crystallisation of the CPHPC which improved following warming the vial by placing it in a container containing warm water. All 3 DRA patients received CPHPC following each session of haemodialysis with no complications.

Quality of life

The SFv36 data was available in all patients recruited, with results subcategorised into AFib patients; good eGFR (eGFR>30mls/min) and low eGFR (eGFR<30mls/min), and DRA patients showing a declining trend of results from the former to latter groups, pictorially in figure 9.5.

Figure 9.5: Baseline QoL for AFib and DRA patients



The QoL assessments were performed 6 monthly in DRA patients, with figure 9.6A and figure 9.6B showing 6 month and 12 month assessments from baseline. Interestingly, the QoL assessments showed an improvement in mental health, physical role activities and social function the latter by 12 months, despite no solid improvement in their joint symptoms.

Figure 9.6A and 9.6B: QoL at 6 and 12 months in DRA patients

Figure 9.6A

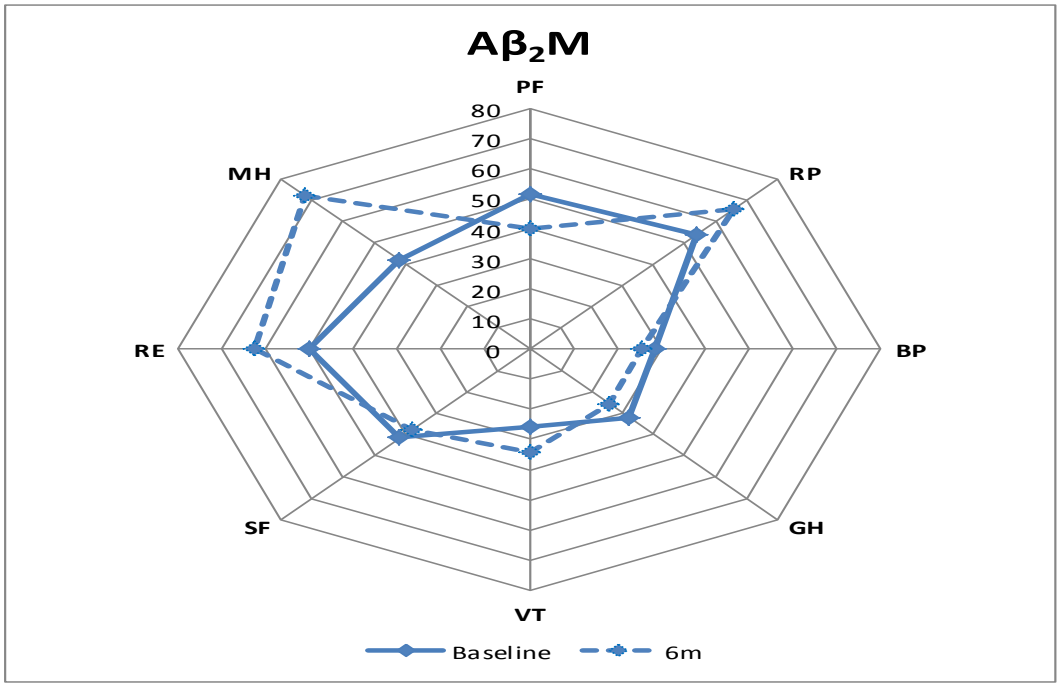
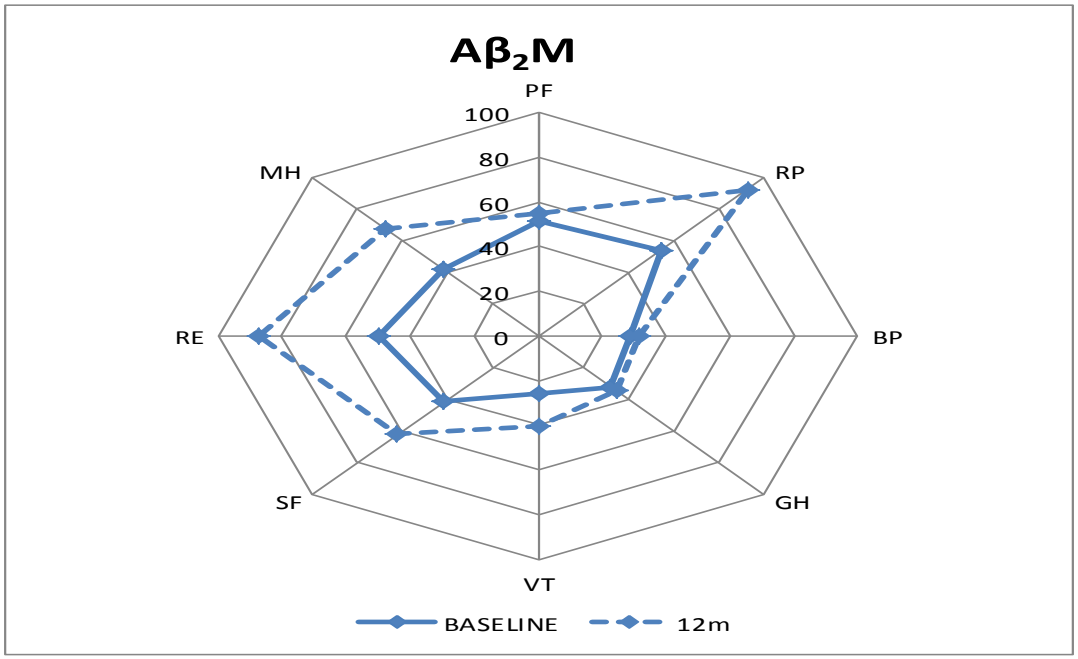


Figure 9.6B



The QoL at 6months, 12 months, and 24 months illustrate a slight overall decline in all parameters at 6 months, near baseline parameters by 12 months and general improvement at 24 months in physical function, physical role, bodily pain and general health, figure 9.7A, 9.7B and 9.7C.

Figure 9.7A, 9.7B and 9.7C: QoL at 6, 12 and 24 months in AFib patients with a good eGFR

Figure 9.7A

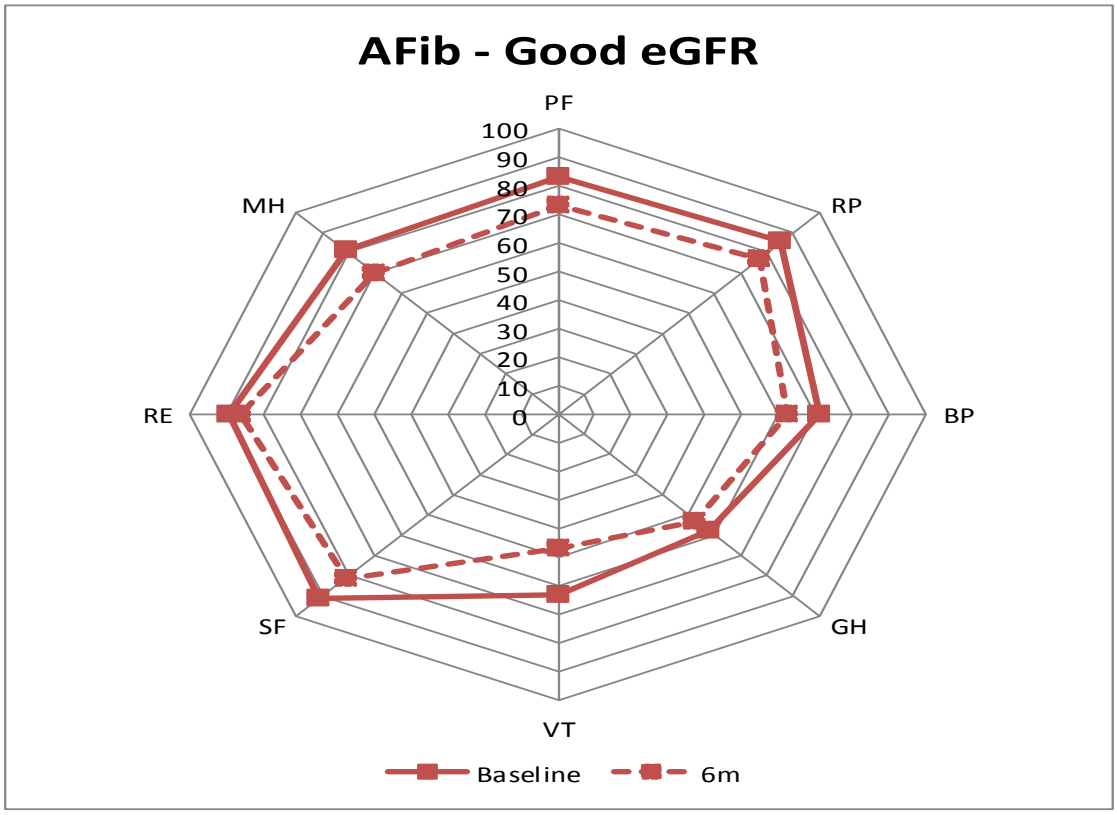


Figure 9.7B

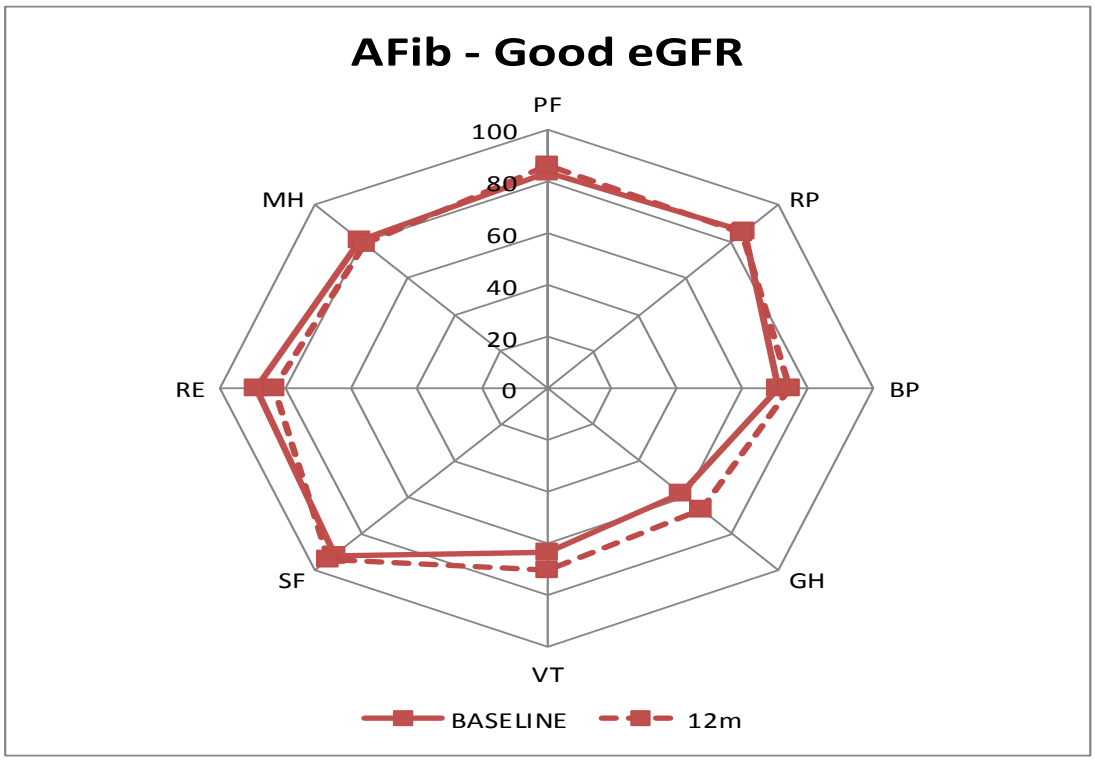


Figure 9.7C

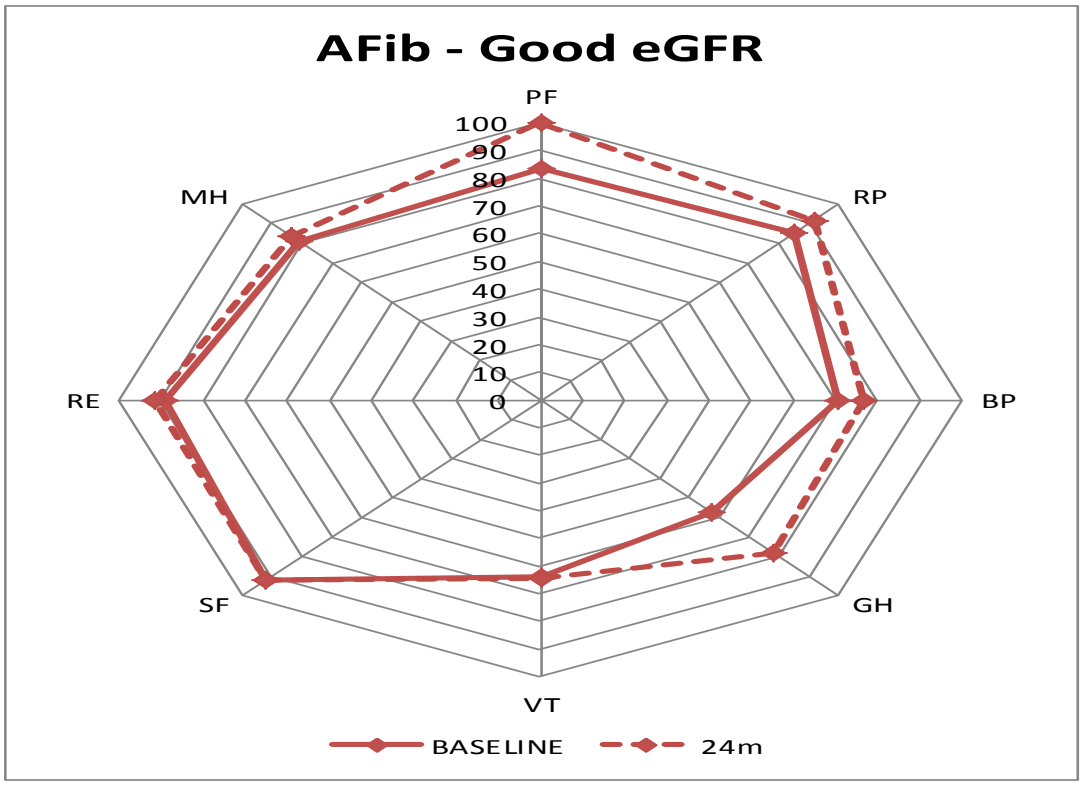


Figure 9.8A, 9.8B and 9.8C illustrates the QoL at 6months, 12 months, 24 months illustrate a slight improvement in mental health, emotional role, and social function at 6 months, with a slight decrease in all parameters by 12 months and general improvement in all parameters by 24 months.

Figure 9.8A, 9.8B and 9.8C: QoL at 6, 12 and 24 months in AFib patients with a poor eGFR

Figure 9.8A

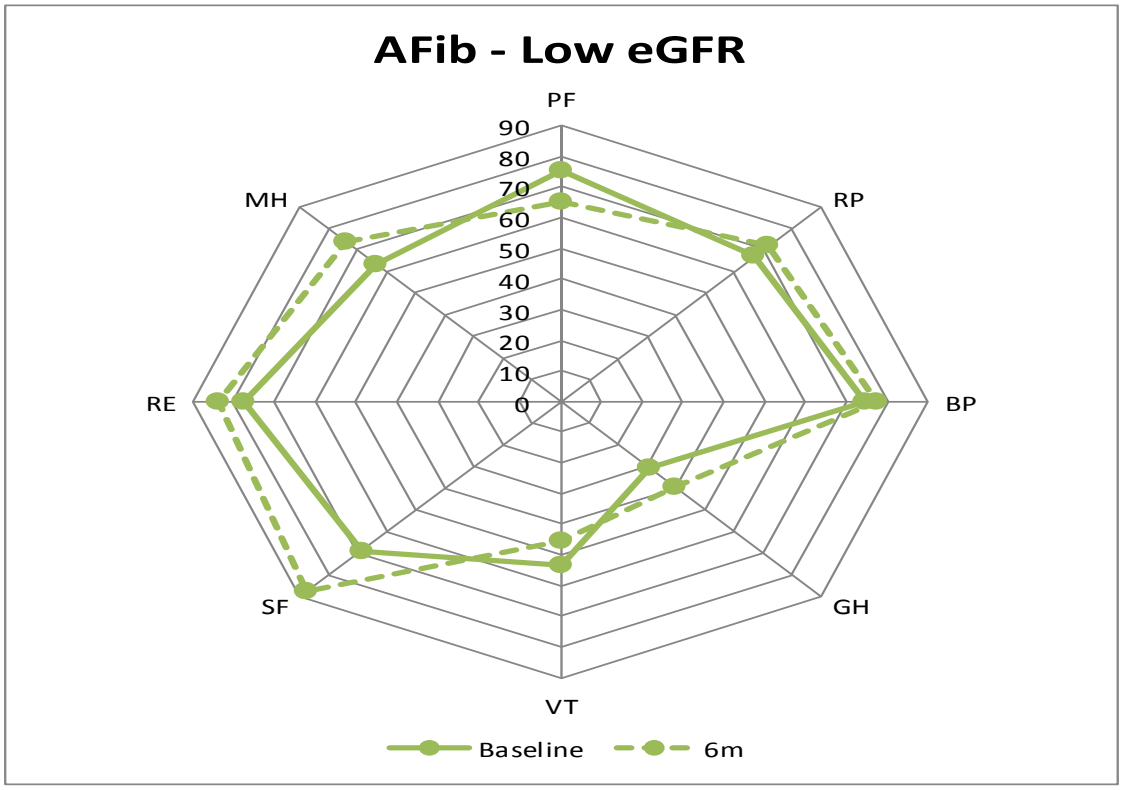


Figure 9.8B

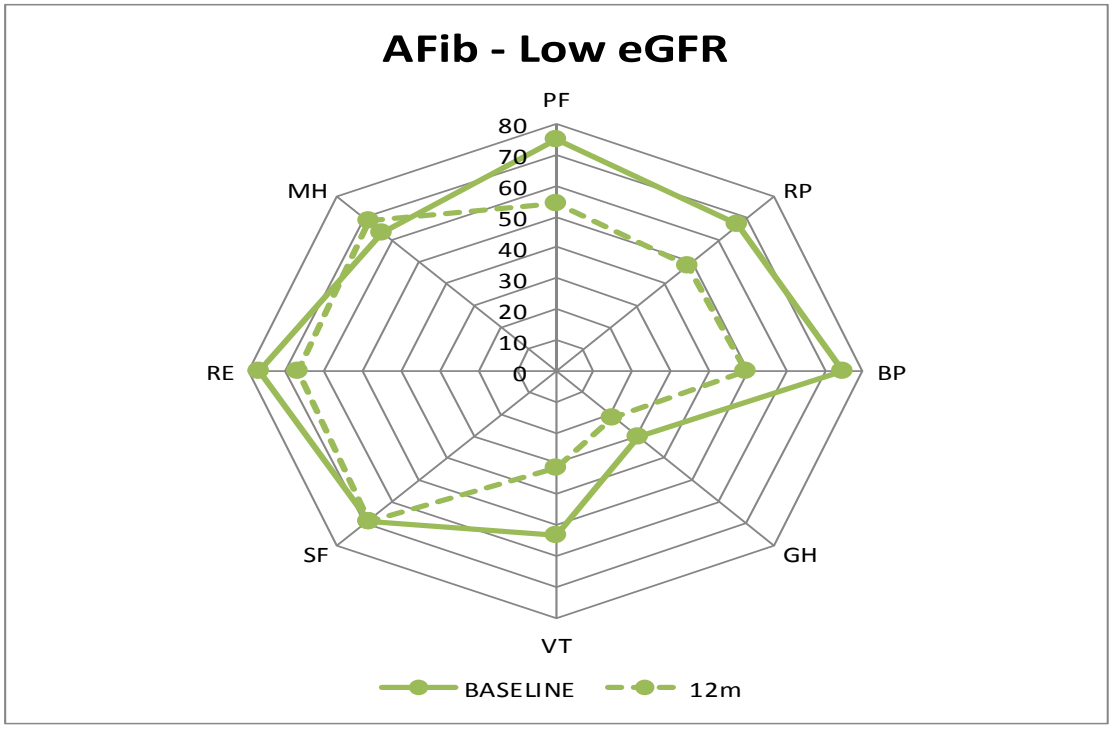
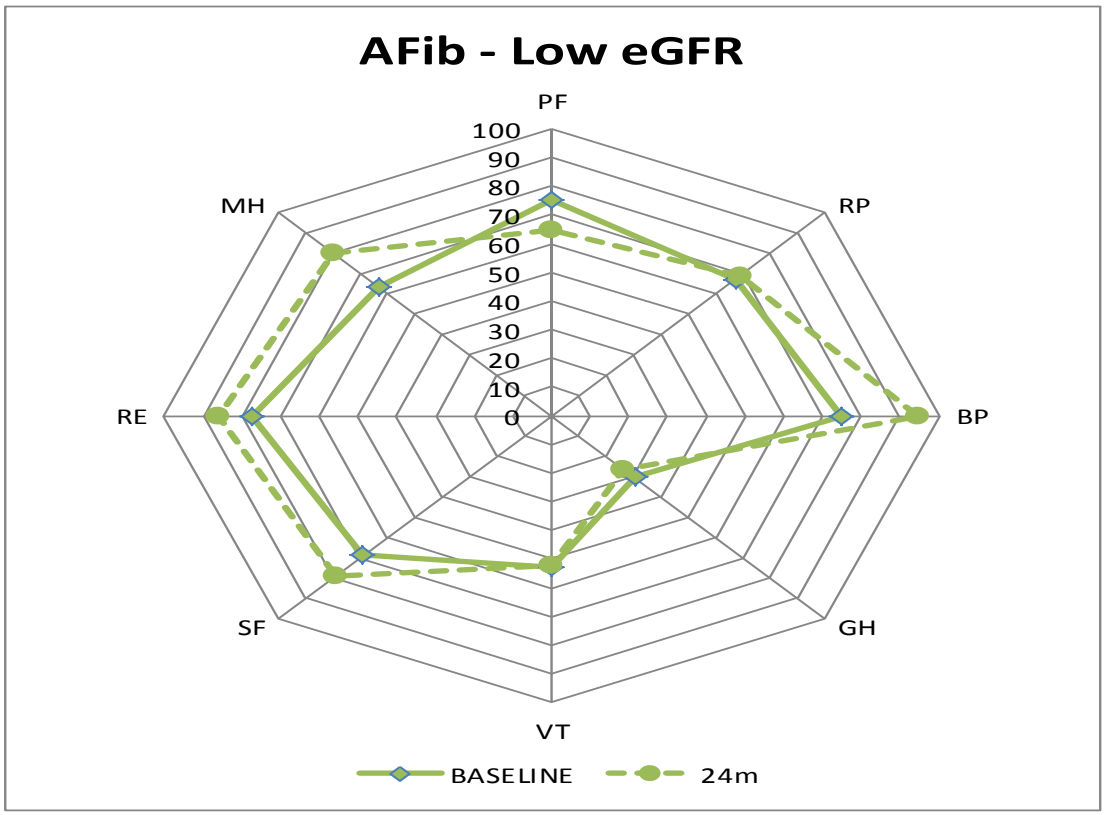


Figure 9.8C



Discussion

In systemic amyloidosis, amyloid deposits may occur in many tissues and hence result in impairment of the mechanical, structural and functional capacity of the tissue affected. Fibrinogen amyloidosis typically affects the renal function and beta-2-microglobulin amyloidosis is recognised to affect the osteo-articular surfaces, bone and Carpal Tunnel Syndrome, with limited therapeutic options for both.[160] Thus using this principle of reducing the serum amyloid P, and possible removal of the amyloid deposits, restoration of the organ function was the ultimate goal. Earlier studies have explored the use of CPHPC to deplete circulating CPHPC, and we wanted to analyse its use in these 2 groups of systemic amyloidosis.

The predominant finding was that the regular administration of CPHPC resulted in sustained depletion of circulating serum amyloid P, and hence the most probable reason for a slower decline in the renal function in patients with fibrinogen related amyloidosis, in comparison to controls. This finding was variable with the various factors influencing the latter including the estimated glomerular filtration rate, degree of quantitative proteinuria and other incipient renal insults. The resulting improved renal survival was clearly apparent with the renal decline in controls described as 13mls/min (6-21) per year, and previously described 25.8 (7.1-49.2) mls/min per year.[160] In our study, the median renal decline of CPHPC treated patients was 4.5mls/min per year (range 2.3-26.6), with 1 patient having an improvement of 3.9mls/min per year. We also demonstrated a significantly improved renal survival in patients receiving CPHPC ($p<0.0001$) in comparison to CKD matched historical controls.

As in the earlier study by Gillmore et al, the current data supports the slowing of the natural decline in renal function seen in patients with fibrinogen amyloidosis, with early use of this drug likely to result in later need for dialysis. Studies with CPHPC and IgG antibody anti-SAP show promising results; with rapid clearance of the amyloid from the liver and other tissues.[354]

During the study we recorded patients joint symptoms with a VAS score. Although these patients did not have confirmed osteoporosis or a diagnosis of joint disease, (with non-specific joint pain in a particular joint), they acknowledged that their pain score improved over the course of time, and can only describe this as a possible consequence secondary to CPHPC use with formal studies needed for its use in patients with a diagnosis of osteoporosis. There were minimal complications with an excellent safety profile.

Given the profound joint symptoms that patients with beta-2-microglobulin amyloidosis have, a suitable hypothesis arises that CPHPC may deplete the circulating SAP and hence improvement in their joint symptoms. The latter finding was seen in patients on haemodialysis who then had a renal transplant, resulting in a marked reduction in their joint symptoms.[355] In our study, all three patients did not report a noticeable difference in their joint symptoms; as evidence by no significant change in their VAS scores.

Earlier studies have described multiple myeloma patients with arthropathy,[356] with MRI imaging showing a homogenous low-to-intermediate signal intensity by both T1 and T2 weighted images. Peri-articular amyloid may demonstrate mild enhancement following gadolinium administration.[357, 358] A case report of a patient with multiple myeloma and amyloid deposition within the peri-articular joints was also described[359] hence we thought to explore the utility of this investigation in this cohort of patients

and also in assessing their response following CPHPC treatment. ^{18}F FDG PET-CT is a useful non-invasive imaging modality which demonstrates the amyloid arthropathy when other imaging such as x-rays do not delineate these changes. Our study also confirmed this finding and their potential use in diagnosing and assessing patients with dialysis related amyloidosis, or indeed in patients with systemic amyloidosis.

The QoL serves as an important assessment in patients concerns and satisfaction with different facets affecting their lifestyle. In our study, we used this to assess whether this played an impact in their treatment. Given there were different groups, we subcategorised the groups into DRA, good and poor eGFR (the latter dependent upon the eGFR ≥30mls/min or <30mls/min respectively). The QoL showed a subtle improvement in different areas including mental health, physical role activities and social function but documented no overall improvement in their joint symptoms. All patients with AFib noticed an improving trend by 24 months, with a slight decline in parameters prior to this. Interestingly the decline in their parameters was earlier in the good eGFR group at 6 months compared to the poor eGFR group at 12 months. This may be partly due to the concern for the overall outcome of this treatment and the effectiveness over time.

In summary, our pilot study shows the slower renal decline and hence effects of amyloid accumulation following commencement of CPHPC administration and clinical efficacy in patients with hereditary fibrinogen amyloidosis. Our study showed a significant improvement in the natural progression of renal decline with improved renal survival in the latter group with an excellent safety profile. There was some anecdotal improvement in joint symptoms, which will need further exploration in prospective

controlled studies for osteoporosis. The efficacy of this drug remains undetermined in patients with beta-2-microglobulin amyloidosis, with minimal clinical benefit seen.

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Chapter Ten: General conclusions

The studies undertaken in this thesis illustrate important unique findings in the diagnostic investigations, prognosis and management of systemic amyloidosis, and inform a greater understanding of localised amyloidosis and subtypes.

Serum free light chain measurements have a crucial role in diagnosing and monitoring patients with systemic AL amyloidosis, now forming a standard part of the baseline and serial follow up clinical assessments.[161, 162] Inclusion of free light chain concentration has lately been incorporated into the revised Mayo staging system and the international consensus criteria for assessment of disease response to treatment assessment.[80,86,87] Our study is the largest study to date exploring the two commercially available free light chain assays and comparing the results in the clinical setting. The serum Freelite™ assay measures immunoglobulin free light chains using polyclonal sheep antibodies directed against hidden epitopes on the light chain molecule producing an estimate of serum free kappa and lambda light chains. The N Latex assay uses a mixture to two kappa and two lambda monoclonal antibodies to the hidden epitopes of constant region of the immunoglobulin light chain molecules. We showed that there is an excellent correlation between the different assays for detecting the abnormal light chain subtype, but with the discordance in the absolute values of the FLC; crucially, this renders cross assay interpolation impossible. This is critically important as the newer N Latex assay becomes more widely adopted, with considerable potential for causing misinterpretation of patients' responses to chemotherapy.

Coagulation abnormalities are often present in newly diagnosed systemic AL patients. There is a well described associated bleeding diathesis,[164] which may range from small cutaneous bruising, pathgnomic “racoon eyes” or life threatening bleeds, with little known as to the underlying coagulation factor abnormalities aside from factor X deficiency and dysfibrinogenaemia. We described the novel finding of elevated factor VIII and vWF antigen levels in these patients, with normal ADAMTS13 results; thus excluding autoantibodies. This finding was not evident in normal controls. An albumin threshold less than 25g/L corresponded with a statistically significant rise in vWF:Ag, FVIII, FV and fibrinogen, with a fall in protein S and anti-thrombin III; the latter factors previously shown in prothrombotic states. Thus this finding emphasises the increasing importance of consideration of thromboprophylaxis or full anticoagulation in these patients. We also explored light chain toxicity using vWF Ag as a surrogate marker, with a FVIII and vWF Ag >280IU/L negatively impacting survival. Our study hypothesises that underlying vascular endothelial damage occurs in systemic AL, and possibly contributes to the light chain toxicity environment. The correlative fall in the vWF levels post chemotherapy may reflect the underlying vascular endothelial changes which occur. Further studies to better understand the pathophysiology of these findings are needed to explore the endothelium by electron microscopy, and hence the utility of the prognostic findings as part of patient risk stratification.

Fatigue is most universal in systemic amyloidosis and sleep disordered breathing patterns were shown to be frequent and a potential contributor to fatigue symptoms. Recurrent nocturnal oxygen desaturations are very common in patients with cardiac amyloidosis (both AL and ATTR type), and also in patients with soft tissue amyloid deposits affecting the oropharyngeal tract. Sleep disordered breathing questionnaires such as STOPBANG and ESS questionnaires report a high proportion of patients

scoring ‘high risk’ for sleep disordered breathing. Increased number and frequency of nocturnal desaturations may be associated with poorer survival in patients with cardiac AL amyloidosis. Lack of heart rate variability (suggesting cardiac autonomic neuropathy) is a frequent occurrence in cardiac AL and also noted in those patients who died – findings which need further clarification. The role of hypoxia in precipitation of cardiac arrhythmias or sudden death in AL needs to be clarified. Nocturnal hypoxia is a simple target for intervention in cardiac AL amyloidosis and could potentially help to reduce early mortality in AL which has remained an unmet medical need for over 25 years.

Localised amyloidosis is a very rare type of amyloidosis, with commonly reported sites including the urinary tract, respiratory tract, larynx, skin and eyelids.[165] This has been poorly studied and our study describes the largest cohort of patients with localised AL amyloidosis with clinical features and natural disease course showing stark differences from systemic AL amyloidosis. Patients with obstructive/pressure symptoms or bleeding needed endoscopic or surgical resection. Our results show that half of all patients needed one such procedure and a fifth of all patients required repeated procedures for symptom control highlighting the need for long term monitoring. Four patients in the current study needed chemotherapy for localised amyloidosis. Radiotherapy (targeting the presumed clonal proliferation) appears to be a useful, and possibly underutilised, treatment modality in localised amyloidosis with all patients in the current series treated with radiotherapy showing lack of progression and over half achieving good symptomatic improvement following radiotherapy. Surprisingly, all 18 patients who had a FDG-PET/CT study performed in the current series showed presence of FDG avidity at the site of amyloid deposition, in keeping with previous reports of high proportion of FDG-PET/CT positivity in localised amyloidosis.[307]

Progression to systemic AL amyloidosis is extremely rare except in patients with lymph node involvement. Patients with lymph node involvement and those with a isotypic specific circulating free light chains warrant closer follow up for development of systemic AL. The majority with localised AL have excellent long term outcomes. Most die with the disease rather than as a consequence of it.

Laryngo-tracheobronchial amyloidosis is a rare type of localised amyloidosis, characterised by insoluble fibrillar proteins deposited within the upper and lower airway tract.[311, 312] and we describe the largest series to date. In our study, we describe the laryngeal and tracheobronchial amyloid deposition was characterised further with laser capture and proteomic analysis. All 60 cases exhibited evidence of 3 amyloid protein signature proteins serum amyloid P (SAP), Apo E and Apo AIV in conjunction with Apo A1 and insulin-like growth factor binding protein complex. Proteomic analysis was also performed in a total of 60 patients with systemic AL amyloidosis (renal, cardiac and liver biopsies) or transthyretin based disease (cardiac, bone marrow) as a control, with none of these samples showing evidence insulin-like growth factor binding protein complex and a much lower peptide content of Apo A1. Excisional therapeutic options are the primary modality in managing these patients.[321-323] Radiotherapy has been described in some cases with LBTA[309, 311, 318, 324] with the basis that plasma cells are radiosensitive, with the majority of patients being treated with 20 Gy in 10 fractions.[309] Similarly, in our series one patient with laryngeal involvement and three patients with tracheobronchial amyloidosis received radiotherapy, following failure with prior surgical resections and stent insertions to achieve local control. With a median follow up of 18.7 (6-47) months, these patients have had notable improvements in their quality of life, consistent with previously published literature.

Lenalidomide based treatment following prior novel agent based therapy has been little studied, which we endeavoured to do so in 84 systemic AL amyloidosis patients with relapsed/refractory clonal disease following prior treatment with thalidomide (76%) and/or Bortezomib (68%). On an intention to treat (ITT) basis, overall haematologic response rate was 61%, including 20% complete responses. The median overall survival (OS) has not been reached; 2 year OS and progression free survival (PFS) was 84% and 73% respectively. Achieving a free light chain (FLC) response was an independent good prognostic factor for OS in multivariate analysis. 16% achieved an organ response at 6-months, with a marked improvement in organ responses in patients on long term therapy (median duration 11 months), 55% achieving renal responses by 18 months. Lower commencing doses compared to that routinely used in treatment of patients with myeloma should be considered to allow better tolerance. Lenalidomide/dexamethasone therapy achieves good haematological responses in patients with AL amyloidosis with relapsed/refractory clonal disease. The rate of renal responses among patients who received prolonged treatment was unexpectedly high, raising the possibility that immunomodulatory effects of lenalidomide therapy might enhance the otherwise slow natural regression of amyloid deposits.

((R)-1-[6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2 carboxylic acid (CPHPC)) has emerged as a potential therapeutic target to deplete SAP as a treatment for systemic amyloidosis.[1] We explored this treatment in patients with hereditary fibrinogen amyloidosis (AFib) and in patients with dialysis related amyloidosis. Our study showed a significant improvement in the natural progression of renal decline with improved renal survival in the latter group with an excellent safety profile. The resulting improved renal survival was clearly apparent with the renal

decline in controls described as 13mls/min (6-21) per year, and previously described 25.8 (7.1-49.2) mls/min per year.[160] In our study, the median renal decline of CPHPC treated patients was 4.5mls/min per year (range 2.3-26.6), with 1 patient having an improvement of 3.9mls/min per year. We also demonstrated a significantly improved renal survival in patients receiving CPHPC ($p<0.0001$) in comparison to CKD matched historical controls. There was some anecdotal improvement in joint symptoms, which will need further exploration in prospective controlled studies for osteoporosis. The QoL questionnaires were a useful adjunct in assessing patient's treatment, showing a general improvement in several parameters from baseline. The efficacy of this drug remains undetermined in patients with beta-2-microglobulin amyloidosis, with minimal clinical benefit seen.

Thus this thesis highlights the important techniques of FLC assays, coagulation assays, sleep disordered breathing important in the setting of diagnostic, monitoring and prognostic tests in systemic AL. Our knowledge of localised disease and a subtype – laryngeal and tracheobronchial amyloidosis are enriched following this study, showing this disease a separate entity to systemic AL, with the potential repeating peptide sequence in these patients a possible insight to this disease. Management is dependent on the type of systemic amyloidosis, with chemotherapy for systemic AL and treatments targeted against depletion of SAP for hereditary fibrinogen amyloidosis.

Future studies planned

Results from chapter 4 describe endothelial dysfunction that occurs in systemic AL. Von Willebrand factor has been used in other diseases as a surrogate marker of endothelial dysfunction and preliminary results show likely light chain toxicity implications. There are ongoing plans to initiate a new study examining this relationship further by electron microscopy.

The results from chapter 5 describe sleep disordered breathing and oxygen desaturations in systemic amyloidosis. This is a large study outlining the latter issues in patients with systemic amyloidosis, with the hypothesis that these oxygen desaturations may increase morbidity and mortality in this group. Initial plans are underway to study this prospectively including the use of supplemental oxygen in newly diagnosed systemic AL amyloidosis patients with Mayo stage 3 cardiac biomarkers.

Chapter 6 outlines the largest detailed characteristics and natural history and survival features in localised amyloidosis. There are initial plans to form a collaborative group for this disease, enabling the patient details, treatment and outcome measures to be recorded prospectively.

The results of chapter 7 describe a large series of patients diagnosed with laryngeal and tracheo-bronchial amyloidosis, elaborating on the clinical and proteomic analysis of these patients. The interesting finding of increased quantification of Apo A1 and presence of IGBF in these patients and not found in those diagnosed with systemic AL or transthyretin based disease, has initiated plans to study this further in another proteomic based study.

Chapter 8 outlines the use of lenalidomide based treatment in systemic AL. This was a large series of 84 patients, but retrospective in nature, and we are planning on confirming these results in a larger patient series treated with long term lenalidomide. We aim to also use a matched series of patients treated with other chemotherapy regimens. There was an encouragingly but surprisingly high rate of organ responses recorded in this study for those patients receiving long term lenalidomide. This is intriguing and needs further study in a larger patient cohort, with plans for consideration of evaluating this concept in experimental models to evaluate the potential of lenalidomide in directly modulating the clearance of amyloid deposits.

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Appendices

Appendix 1

Royal Free Hospital Bleeding Questionnaire v4 (Adapted)

Name of Patient: **Date of Clinic:**
Hospital Number: **Name of Assessor:**
Date of Birth: **Bleeding Score:**

Grades of bleeding severity used to compute the bleeding score						
Symptom	**- 1**	**0**	**1**	**2**	**3**	**4**
Epistaxis	-	No or trivial (<5 episodes / year)	>5 episodes or >10 min duration	Consultation only	Packing or cauterisation or TXA	Blood products / DDAVP / concentrate
Cutaneous	-	No or trivial (<1 cm)	>1cm and no trauma	Consultation only		
Bleeding from minor wounds	-	No or trivial (<5 episodes / year)	>5 episodes or > 5 min	Consultation only	Surgical haemostasis	Blood products / DDAVP / concentrate
Oral cavity	-	None	Referred at least once	Consultation only	Surgical haemostasis or TXA	Blood products / DDAVP / concentrate
Tooth extraction	No bleeding in at least 2 extractions	No extractions or no bleeding in 1 extraction	Reported, no consultation	Consultation only	Resuturing or packing	Blood products / DDAVP / concentrate
Surgery	No bleeding in at least 2 surgeries	None performed or no bleeding in one Surgery	Reported, no consultation	Consultation only	Surgical haemostasis or TXA	Blood products / DDAVP / concentrate
Menorrhagia	-	No	Consultation only	TXA or pill use	Dilatation & Curettage, iron therapy	Blood products / DDAVP / concentrate / hysterectomy
Postpartum haemorrhage	No bleeding in at least 2 deliveries	No deliveries or no bleeding in one delivery	Consultation only	Dilatation & Curettage, iron therapy, TXA	Blood products / DDAVP / concentrate	Hysterectomy
Muscle Haematomas	-	Never	Post-trauma no therapy	Spontaneous, no therapy	Spontaneous or traumatic, treated with DDAVP / concentrate	Surgical intervention or blood transfusion
Haemarthrosis	-	Never	Consultation only	Spontaneous, no therapy	Spontaneous or traumatic, treated with DDAVP / concentrate	Surgical intervention or blood transfusion
CNS Bleeding	-	Never	-	-	Subdural, any intervention	Intra cerebral, any intervention
GI Bleeding	-	No	Associated with GI pathology e.g. ulcer, varices, haemorrhoids, angiodysplasia	Spontaneous	Surgical haemostasis Blood products / DDAVP / concentrate/ TXA	

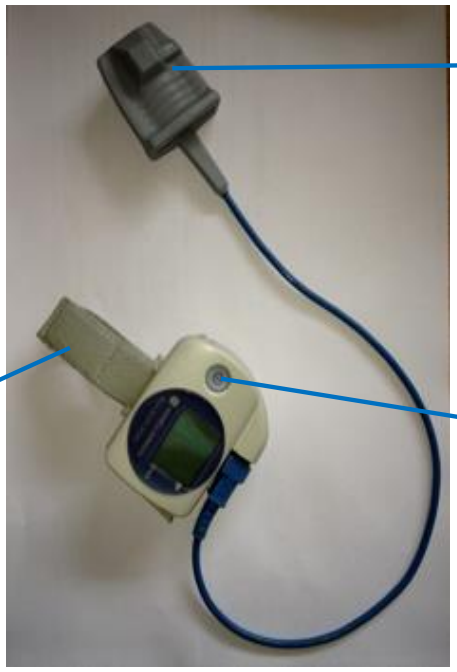
Duration of bleeding symptoms _____ **Aspirin Yes/No (please circle)**
Site_____ **Warfarin Yes/No (please circle**

Appendix 2

To help understand whether your oxygen saturations fall while you sleep.

Before you go to sleep, please place pulse oximeter on.

Instructions for overnight pulse oximeter



2. Place this on your finger. It should fit snugly.

1. Place Velcro strap band on your **wrist.**

3. Press power switch on. Press firmly and display screen should light up.

4. Sleep with pulse oximeter on.

5. When you awake:

- switch power button off.

- remove Velcro wrist strap and pulse oximeter.

- give back to nurses at National Amyloidosis Centre for Shameem

Please fill in the STOPBANG and EPWORTH questionnaire.

Thank you.

Appendix 3

EPWORTH SLEEPINESS SCORE

How to work out your score

How likely are you to doze off or fall asleep during the following situations, in contrast to just feeling tired?

For each of the situations listed below, give yourself a score of 0 to 3 where:
0 = would never doze
1 = slight chance
2 = moderate chance
3 = high chance

Work out your total score by adding up your individual scores for situations 1 to 8

(If you have not been in the following situations recently, think about how you would have been affected).

Situation	Score (0 – 3)
Sitting and reading	
Watching television	
Sitting inactive in a public place e.g. theatre, meeting	
As a passenger in a car for an hour without a break	
Laying down for a rest in the afternoon	
Sitting and talking to someone	
Sitting quietly after lunch (when you have had no alcohol)	
In a car, whilst stopped in traffic	
Total	

Appendix 4

STOP BANG QUESTIONAIRRE

Height inches/cm Weight lb/kg

Age Male/Female BMI

Collar size of shirt: S, M, L or XL Neck Circumference * cm

1. Snoring.

   Do you snore loudly (louder than talking or loud enough to be heard through closed doors)?

   Yes No

2. Tired.

   Do you often feel tired, fatigued or sleepy during daytime?

   Yes No

3. Observed.

   Has anyone observed you stop breathing during your sleep?

   Yes No

4. Blood pressure.

   Do you have or are you being treated for high blood pressure?

   Yes No

5. BMI.

   BMI more than 35 kg/m^2?

   Yes No

6. Age

   Age greater than 50?

   Yes No

7. Neck circumference.

   Neck circumference greater than 40cm?

   Yes No

8. Gender.

   Gender male?

   Yes No

* Neck circumference measured by staff

High risk of OSA: answering yes to 3 or more items.

Low risk of OSA: answering yes to less than 3 items

Appendix 5

Supplemental Table 1: PET/CT in localised amyloidosis

Patient	Age/Sex	Organ Involved	Positive FDG PET/CT positive	FDG uptake	SUV
1	74/F	LN	Yes	Cervical and retroperitoneal LN, L ischium	3.5 3.9
2	62/M	LN	Yes	R paratracheal, Subcarinal, R supraclavicular	10 5.1 9.4
3	62/M	LN	Yes	L supraclavicular, R lung base, Para-aortic, aortocaval and mesenteric LN	2.3 2.7 3-6
4	53/M	LN	Yes	L hilum, R para-aortic LN	2.5
5	77/F	LN	Yes	Cervical, axillary, para-aortic and inguinal lymphadenopathy	2.5
6	81/F	LN	Yes	Mediastinal lymph nodes, soft tissue in the pericardiac region of left upper lobe	3.8-4.2
7	66/F	Pulmonary	Yes	L upper lobe nodule	4.1
8	74/F	Pulmonary	Yes	Multiple lung nodules, peribronchial and subpleural	6.3
9	66/M	Pulmonary	Yes	R upper lobe soft tissue mass	2.5
10	59/F	Pulmonary	Yes	Right pulmonary nodule	2
11	74/F	Pulmonary	Yes	Right apical lung nodule	NA
12	77/M	Pulmonary	Yes	Right pleural lesion in R upper lobe	2.6
13	79/M	Pulmonary	Yes	R lower lobe lesion	NA
14	57/M	Subcutaneous nodules	Yes	Soft tissue along iliac vessels, bilateral pleural masses. Calcification in both breasts	2.5
15	51/F	Bone	Yes	T9 vertebral lesion	NA
16	72/F	Skin nodules	Yes	Subcutaneous nodules within the legs	2.7
17	80/F	Abdominal mass	Yes	Mass extending from anterior rectum, enveloping bladder anteriorly, and posteriorly encasing the uterus and ovaries	NA
18	67/M	tongue	Yes	Right paramedian tongue base lesion	4.5

PET/CT – positron emission tomography; FDG – flurodeoxyglucose; SUV – standardised uptake values; F – female; M – male; R – right; L – left; LN – lymph node; NA – not available.

Supplemental Table 2: Characteristics of patients with disappearance of clonal markers following intervention

Gender	Site of Localised Amyloid	Intervention	Pre-treatment Abnormal FLC or Paraprotein	Post-treatment FLC or Paraprotein	Time duration post-treatment
F	L ptygeropalatine fossa (bone)	Radiotherapy	IgGl 4g/L	0	4 months
M	R ileum	Radiotherapy	IgGk 5g/L	0	6 months
F	T10 bone lesion	Radiotherapy	IgGk 2g/L	0	Not available
M	R axilla LN	Radiotherapy	IgGk 2g/L	0	4 months
M	R inguinal LN	Surgery	IgGl 2g/L	0	2 months
M	R eyelid lesion	Surgery	IgMl 1g/L	0	2 months
M	Cutaneous leg deposit	Surgery	IgGk 3g/L	0	2 months
M	Right supraclavicular LN	Surgery	Kappa 200mg/L	Kappa 30mg/L	6 months

FLC - free light chain; F - female; M - male; LN -lymph node; R – right; L – left; T – thoracic; k – kappa; l – lambda; mg/L – milligrams per litre; g/L; grams per litre

Supplemental Table 3: Characteristics of patients with localised amyloidosis progressing to systemic disease

Gender	Site of Localised Amyloid	Site of progression to Systemic AL	Time to progression (months)	Abnormal FLC (mg/L)	Paraprotein (g/L)	Urine BJP
M	LN	Renal	84	Lambda 500 mg/L	IgAl by IF	Lambda
M	LN	Macroglossia	20	Kappa 610 mg/L	IgMk by IF	none
M	LN	Splenic uptake by ^{123}SAP scintigraphy	60	none	none	none
F	LN	Bone	77	Lambda 300 mg/L	IgAl 5	none
M	LN	Macroglossia and renal	51	Lambda 341 mg/L	IgMl 13	Lambda
F	Eyelid	Macroglossia	48	Kappa 330 mg/L	IgGk 18	none
F	Bone	LN	51	Kappa 124 mg/L	none	none

AL – Light chain; FLC – free light chain; BJP – Bence Jones Protein; mg/L – milligrams per litre; g/L – grams per litre; M – male; F – female; LN – lymph node; SAP – Serum Amyloid P; IF - immunofixation