

**CHARACTERIZATION AND DEVELOPMENT OF AMYLOID-
REACTIVE PEPTIDES AS TRACERS FOR QUANTITATIVE
MOLECULAR IMAGING**

A Dissertation Presented for the

Doctor of Philosophy

Degree

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DEDICATION

This dissertation is dedicated to:

My wonderfully supportive and loving parents:

W. Michael Martin

&

Sharon Pearson Martin

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As I reflect on my journey as a graduate student, I feel overwhelmed with the need to extend my sincerest gratitude to those who have helped and supported me along the way. I would not have been able to accomplish my goals without each of you.

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ABSTRACT

Amyloidosis is a degenerative protein misfolding disorder that leads to the extracellular accumulation of amyloid deposits containing protein fibrils, heparan sulfate proteoglycans, glycoproteins and apolipoproteins. To date, at least 27 proteins have been identified as components of pathologic amyloid fibrils. In systemic forms of the disease, amyloid deposits can evade the immune system and expand throughout various tissues. As the deposits grow, tissue architecture is disrupted, leading to organ dysfunction and death.

In the US, there are ~3500 newly diagnosed patients with amyloidosis annually. The deposition of light chains as amyloid (AL amyloidosis) is the most common form of visceral disease. The prognoses for patients varies (median survival for AL 12-40 months), but those with cardiac involvement have a considerably less favorable prognosis (~9 months median survival). Many researchers and physicians believe that early detection of amyloidosis could improve patient survival. The current gold standard for diagnosis involves histological examination of biopsy-derived tissue specimens. This method is invasive, subject to sampling error, and can be quite challenging. In an attempt to facilitate diagnosis, molecular imaging techniques using amyloid-reactive tracers have been developed such that a whole body, non-invasive “picture” of amyloid burden in patients may be established. There are several imaging agents used clinically worldwide, but each of these has limitations regarding its adoption in the US (i.e. ^{123}I [^{123}I]-SAP is not FDA-approved and cannot image cardiac amyloid deposits, ^{124}I [^{124}I]-11-1F4 only images ~60% of patients, and $^{99\text{m}}\text{Tc}$ -labeled bone-seeking agents cannot image the most common forms of amyloid disease in patients). Given these limitations, there is an

unmet clinical need for a facile method of quantitatively detecting whole body amyloid burden in patients.

To this end, we have developed synthetic, pan-amyloid-reactive peptides for use as radiotracers by using SPECT/CT or PET/CT molecular imaging to non-invasively detect whole body amyloid load in patients. Through numerous preclinical experiments using *ex vivo* and transgenic murine models of amyloidosis, the structurally related peptides were found to have unique *in vivo* characteristics. Our findings suggest that, if further optimized, certain peptides could have clinical merit for the early detection of multiple types of amyloidosis.

TABLE OF CONTENTS

Chapter 1 Literature Review.....	1
1. Amyloidosis	1
1.1. Overview	1
1.2. Amyloid structure	4
1.2.1 Fibrils and fibrillogenesis	5
1.2.2 Heparan sulfate proteoglycan.....	12
1.2.3 Serum amyloid P component	16
2. Clinical manifestations.....	19
2.1. Overview	19
2.2. Light chain amyloidosis—AL	19
2.3. Transthyretin amyloidosis—ATTR.....	24
2.4. Reactive amyloidosis—AA	28
2.5. Other types of amyloidosis.....	32
2.6. Non-human amyloid-associated diseases.....	41
3. Diagnosis.....	43
3.1. Pathobiology	43
3.2. Imaging	46
3.2.1 Modalities.....	47
CT and MRI	47
PET Imaging.....	51

SPECT Imaging.....	54
Dual Modality Imaging	56
3.2.2 Tracers.....	58
3.2.2.1 Small molecules.....	60
3.2.2.2 Antibodies and proteins	64
3.2.2.3 Visceral amyloid-related agents.....	71
4. Preclinical amyloid targeting	75
4.1. Model Systems.....	75
4.1.1 Synthetic fibrils	75
4.1.2 Tissue extracts	76
4.1.3 Mice.....	77
4.1.4 Naturally occurring disease models.....	83
4.2. Preclinical amyloid-targeting agent: Peptide p5	84
Chapter 2 Project Rationale	93
Chapter 3 Materials and Methods	97
3.1. Peptides and proteins.....	97
3.2. Reagents	97
3.3. Mice.....	99
3.4. AL amyloid extract preparation.....	101
3.5. Radiolabeling peptides with Iodine-125 or Iodine-123.....	102
3.6. Radiolabeling peptides with Iodine-124.....	103
3.7. Radiolabeling peptides with Technetium-99m	105
3.8. Radiochemical purity testing.....	106

3.9. Tissue homogenate preparation	107
3.10. <i>Ex vivo</i> peptide binding assays	108
3.10.1. Tissue homogenates	109
3.10.2. AL extracts and rV λ 6Wil fibrils	110
3.11. Assessment of relative peptide affinity to amyloid using NaCl titration	110
3.12. Imaging acquisitions	111
3.12.1. SPECT imaging	111
3.12.2. Dynamic PET imaging	113
3.12.3. CT imaging	114
3.13. Image analysis	114
3.13.1. SPECT/CT analysis.....	114
3.13.2. Dynamic PET/CT analysis.....	115
3.14. Necropsy	115
3.15. Biodistribution measurements	116
3.16. Autoradiography and Histology	117
3.17. Statistical analyses.....	118
Chapter 4 Peptide characterization studies.....	119
4.1. Overview	119
4.2. AL extract binding studies with p5	120
4.3. Peptide binding to amyloid-laden tissue homogenates	124
4.4. Binding studies in the presence of increasing concentrations of sodium chloride to assess the relative strength of electrostatic binding of peptides with amyloid.....	128

4.5. Conclusions.....	134
Chapter 5 Dual isotope SPECT/CT imaging of peptides in ApoA2c mice	137
5.1. Overview	137
5.2. Dual-isotope SPECT/CT imaging of peptides in ApoA2c mice.....	138
5.3. Conclusions.....	150
Chapter 6 Comparison of <i>in vivo</i> and <i>ex vivo</i> ApoA2c amyloid binding with p5R+14	153
6.1. Overview	153
6.2. Biodistribution measurements of ^{123}I -p5R+14 in ApoA2c and WT mice ...	155
6.3. ^{125}I -p5R+14 binding assay on ApoA2c tissue homogenates	157
6.4. Comparison of <i>in vivo</i> and <i>ex vivo</i> peptide binding data.....	160
6.5. Conclusions.....	163
Chapter 7 Assessment of modified peptides designed to enhance quantitative ApoA2c amyloid detection	165
7.1. Overview	165
7.2. Studies using ^{125}I -p5+14: A peptide addressing amyloid affinity and binding site barrier effects.....	166
7.3. Studies using PEGylated ^{125}I -p5R+14: A peptide to study the effects of longer serum half-life.....	173
7.4. Conclusions.....	180
Chapter 8 A non-dehalogenable, novel peptide for amyloid targeting.....	184
8.1. Overview	184
8.2. <i>Ex vivo</i> tissue binding assays with ^{125}I -p43 and ^{125}I -p50	187

8.3. Time course biodistribution and SPECT/CT studies with ^{125}I -p43 and ^{125}I -p50	192
8.4. Dynamic PET/CT analysis of ^{124}I -p43 in WT mice	205
8.5. Conclusion	211
Chapter 9 Summary and future directions	213
List of References	221
Vita	243

LIST OF FIGURES

Figure 1.1: Schematic of amyloid composition on the cell membrane.....	6
Figure 1.2: Structural characteristics of amyloid.....	7
Figure 1.3: Congo red binding to amyloid.....	9
Figure 1.4: Nucleation-dependent mechanism of fibrillogenesis	10
Figure 1.5: Structures of common glycosaminoglycans	13
Figure 1.6: Crystal structure of serum amyloid P component.....	17
Figure 1.7: Basic mechanism of AL amyloidosis	22
Figure 1.8: Clinical manifestations of AL amyloid in various tissues	23
Figure 1.9: Crystal structure of homotetrameric transthyretin.....	26
Figure 1.10: Mechanism of AA amyloidogenesis	30
Figure 1.11: Possible outcomes of SAA proteolysis leading to fibril formation	31
Figure 1.12: Mechanism of β_2m pathogenicity leading to dialysis-related amyloidosis..	34
Figure 1.13: Proposed methods of AIAPP toxicity leading to β -cell death and amyloid formation associated with type 2 diabetes.....	37
Figure 1.14: Crystal structure of A β (1-42) peptide fragment and demonstration of the fragment within amyloid plaques of a sample of AD brain tissue	38
Figure 1.15: CT image demonstrating visualization of air and bone in a patient	49

Figure 1.16: Types of coincidence events associated with PET photons	53
Figure 1.17: Dual energy SPECT imaging with ^{125}I -SAP and $^{99\text{m}}\text{Tc}$ -p5 in an amyloid-laden mouse	57
Figure 1.18: Dual modality SPECT/CT images	59
Figure 1.19: Co-localization of mAb ^{124}I -11-1F4 with a sc AL amyloid xenograft as evidenced by PET/CT	66
Figure 1.20: The mAb ^{124}I -11-1F4 can image amyloid-laden liver in an AL patient	67
Figure 1.21: SPECT/CT imaging and ARG of both AA and AL amyloid with mAb ^{125}I -2A4	69
Figure 1.22: ^{123}I -SAP planar scintigraphy acquired 24 h pi in an AL patient and a healthy control	73
Figure 1.23: Predicted structure of peptide p5	87
Figure 1.24: Microautoradiography (ARG) of ^{125}I -p5 distribution in various AA mouse tissues and Congo red (CR) histological confirmation of peptide co-localization with amyloid	87
Figure 1.25: Dual energy SPECT/CT images illustrating $^{99\text{m}}\text{Tc}$ -p5 and ^{125}I -SAP distribution in an individual AA mouse	89
Figure 1.26: ^{124}I -p5 PET dynamic time activity curves	91
Figure 1.27: SPECT/CT images revealing the stability of ^{125}I -p5 binding to amyloid	91

Figure 4.1: ^{125}I -p5 binding to AL extracts.....	121
Figure 4.2: Positive correlation of ^{125}I -p5 binding and ThT fluorescence in heparinase-treated samples	123
Figure 4.3: ^{125}I -p5 binds to synthetic recombinant V λ 6Wil fibrils	123
Figure 4.4: Peptide binding to tissue homogenates	126
Figure 4.5: Peptide binding to AA tissues in the presence of NaCl	130
Figure 4.6: Peptide binding to ApoA2c tissues in the presence of NaCl.....	132
Figure 4.7: Peptide affinity for recombinant synthetic V λ 6Wil fibrils measured by NaCl titration.....	133
Figure 4.8: ^{125}I -p5R+14 binding to rV λ 6Wil fibrils in the presence of both NaCl and urea treatments.....	135
Figure 4.9: Urea treatment of rV λ 6Wil fibrils.....	135
Figure 5.1: Quality assurance of radiolabeled p5 and p5R+14	139
Figure 5.2: Dual energy SPECT/CT image representing the distribution of ^{125}I -p5R+14 and $^{99\text{m}}\text{Tc}$ -p5 at 2 h pi	142
Figure 5.3: Dual energy SPECT/CT image demonstrating the distribution of both $^{99\text{m}}\text{Tc}$ -p5 and ^{125}I -p5R+14 in a representative ApoA2c mouse	143
Figure 5.4: Biodistribution measurements of $^{99\text{m}}\text{Tc}$ -p5 in ApoA2c and WT mice at 2 h pi.....	144

Figure 5.5: Biodistribution measurements of ^{125}I -p5R+14 in ApoA2c and WT mice at 2 h pi.....	146
Figure 5.6: Scaled comparisons of $^{99\text{m}}\text{Tc}$ -p5 and ^{125}I -p5R+14 in ApoA2c mice.....	148
Figure 5.7: Image analyses revealed greater ^{125}I -p5R+14 uptake in amyloid-laden organs	149
Figure 5.8: Autoradiographs of ^{125}I -p5R+14 in ApoA2c tissues at 2 h pi	151
Figure 6.1: Autoradiograph of ^{125}I -SAP in ApoA2c amyloid-laden spleen	154
Figure 6.2: Assessment of radiopurity and amyloid-reactivity of ^{123}I -p5R+14.....	156
Figure 6.3: Biodistribution measurements of ^{123}I -p5R+14 in ApoA2c and WT mice at 4 h pi.....	158
Figure 6.4: <i>Ex vivo</i> assessment of ^{125}I -p5R+14 binding to ApoA2c and WT homogenized tissues	159
Figure 6.5: Method for analyzing <i>ex vivo</i> and <i>in vivo</i> data with comparable peptide binding values.....	161
Figure 6.6: Comparative analysis of <i>ex vivo</i> peptide-binding data and <i>in vivo</i> biodistribution data in 3 ApoA2c mice.....	162
Figure 6.7: ApoA2c amyloid deposits which were unidentified by the ^{125}I -p5R+14 radiotracer in various tissues	164

Figure 7.1: Autoradiographs (ARG) indicating disparate AA amyloid targeting with two radiotracers.....	167
Figure 7.2: Assessment of radiopurity and amyloid-laden tissue and synthetic rVλ6Wil fibril reactivity.....	169
Figure 7.3: Biodistribution measurements 2 h pi of either ¹²⁵ I-p5+14 or ¹²⁵ I-p5R+14 in ApoA2c mice	170
Figure 7.4: Autoradiographs demonstrating radiotracer accumulation in amyloid-laden tissues	172
Figure 7.5: Radiopurity and fibril reactivity assays	176
Figure 7.6: Biodistribution of ¹²⁵ I-PEG-p5R+14 and ¹²⁵ I-p5R+14 in WT mice at 15 min and 1 h pi.....	178
Figure 7.7: SPECT/CT images displaying the distribution of ¹²⁵ I-PEG-p5R+14 and ¹²⁵ I-p5R+14 in WT mice at 1 h pi	179
Figure 7.8: Biodistribution of peptides ¹²⁵ I-PEG-p5R+14 and ¹²⁵ I-p5R+14 in ApoA2c mice.....	181
Figure 7.9: Autoradiographs of ¹²⁵ I-PEG-p5R+14 in ApoA2c amyloid-laden tissues...	182
Figure 8.1: Illustration of both the L-type and D-type conformations of valine	185
Figure 8.2: <i>Ex vivo</i> peptide-binding assays with ¹²⁵ I-p43 and ¹²⁵ I-p5	188

Figure 8.3: <i>Ex vivo</i> peptide-binding assay demonstrating ^{125}I -p50 reactivity with AA tissues and WT liver	190
Figure 8.4: NaCl titrations assessing ^{125}I -p43 and ^{125}I -p50 affinity for AA liver and spleen homogenates.....	191
Figure 8.5: Radiopurity and amyloid-reactivity of the radiolabeled peptides.....	193
Figure 8.6: Biodistribution measurements of ^{125}I -p43 in a time course study with AA mice	195
Figure 8.7: Representative SPECT/CT images of ^{125}I -p43 in AA mice at 1 h, 4 h, and 24 h pi.....	196
Figure 8.8: 3D SPECT/CT representation of ^{125}I -p5 in a WT mouse	197
Figure 8.9: Biodistribution of ^{125}I -p43 in WT mice.....	197
Figure 8.10: Representative SPECT/CT images of ^{125}I -p43 in WT mice at 1 h, 4 h and 24 h pi.....	198
Figure 8.11: Co-localization of ^{125}I -p43 to AA amyloid at various time points pi	200
Figure 8.12: Autoradiograph visualization of ^{125}I -p43 binding to healthy kidney and cartilage tissue at various time points pi	202
Figure 8.13: SPECT/CT images of ^{125}I -p50 in WT and AA mice at 1 h, 4 h and 24 h pi.....	204

Figure 8.14: Microautoradiographs displaying ^{125}I -p50 in amyloid-laden tissues representative of various time points pi	206
Figure 8.15: Microautoradiographs demonstrating a lack of ^{125}I -p50 accumulation in various WT tissues	206
Figure 8.16: Time activity curves depicting renal accumulation and clearance of ^{124}I -p43 and ^{124}I -p5	208
Figure 8.17: Time activity curves for ^{124}I -p43 in WT mice.....	210

Chapter 1

Literature Review

1. Amyloidosis

1.1 Overview

Amyloidosis is a protein misfolding disorder which leads to the extracellular deposition of a complex clinical pathology known as amyloid. To date, 27 different native proteins have been identified that, after undergoing a conformational, structural change, have been found associated with the disease in man [1]. Certain types of amyloidosis are localized and affect only the organ in which the precursor protein is produced. These include type II diabetes in which the amyloidotic islet amyloid polypeptide (AIAPP) aggregates and deposits within the pancreas and Alzheimer's disease where amyloid beta ($A\beta$) peptides deposit in the brain. In contrast, the systemic amyloidoses show no limitation to the organs that can be involved. Partial lists of the systemic and localized amyloid diseases, including the associated precursor proteins and the most typically affected organs, are shown in Table 1.1 [1, 2]. Additionally, there are amyloid diseases which have been shown to affect animals. Many of these disorders are systemic in pathology and affect multiple organs much like the systemic diseases seen in humans; others induce localized amyloid aggregates in the brain which can lead to Alzheimer's disease [3]. Animals and humans can also be affected by prion diseases which result in proteinacious aggregates localized to the brain leading to neurological deficits [4].

Table 1.1: Partial list of human amyloidoses

Amyloid disease (abbreviation)	Precursor protein	Affected tissue(s)
<i>Systemic</i>		
Light chain amyloidosis (AL)	Immunoglobulin light chain	Heart, kidney, liver
Heavy chain amyloidosis (AH)	Immunoglobulin heavy chain	Kidney, liver
Senile systemic amyloidosis (SSA) [#] now known as wild type transthyretin (wt-TTR)	Transthyretin	Heart, vessels, soft tissues
Familial amyloidotic polyneuropathy (FAP; also known as ATTR)	Transthyretin	Heart, kidney, central nervous system
Secondary (reactive) amyloidosis (AA)	Serum amyloid A protein	Kidney, liver, spleen
Dialysis-related amyloidosis (A β_2 M)	β_2 -microglobulin	Joints, heart, gastrointestinal tract, lung
Lysozyme amyloidosis (Alys)	Lysozyme	Kidney, liver, spleen
Apo AI amyloidosis (AApoAI)	Fragments of apolipoprotein AI	Kidney, heart, liver
Apo AII amyloidosis (AApoAII)	Fragments of apolipoprotein AII	Kidney
Fibrinogen amyloidosis (Afib)	Fibrinogen α chain mutants	Kidney
Finnish hereditary amyloidosis (AGel)	Fragments of gelsolin mutants	Cornea, facial and peripheral nerves, skin
Cystatin amyloidosis (ACys)	Cystatin C	Cerebral blood vessels

Table 1.1. Continued

Amyloid disease (abbreviation)	Precursor protein	Affected tissue(s)
<i>Localized</i>		
Alzheimer's disease (A β)	β -amyloid precursor protein	Brain
Diabetes mellitus type II (AIAPP)	Islet amyloid polypeptide	Islets of Langerhans (pancreas)
Spongiform encephalopathies (APrP)	Prion protein	Brain
Pituitary prolactinoma (APro)	Prolactin	Pituitary
Senile aortic, median amyloidosis (AMed)	Lactadherin	Heart
Familial corneal amyloidosis (ALac)	Lactoferrin	Eyes

[#] The amyloidosis nomenclature committee made this announcement at the International Symposium on Amyloidosis 2014.

The underlying biochemical and biophysical causes of amyloidosis are likely myriad and vary widely, but they include hereditary conditions which lead to the production of proteins with mutations (e.g. ATTR), acquired diseases due to altered physiological conditions resulting in an increase in the production of improperly folded proteins (e.g. AL, AA, AIAPP) and acquired diseases associated with aging (e.g. AMed, wild type TTR).

While there are numerous structurally and functionally different precursor proteins associated with amyloidosis, the amyloid deposits themselves share certain remarkably similar characteristics. The following sections will discuss many of these characteristics, a possible mechanism of pathogenesis and a description of the most prevalent components of the pathological amyloid deposit.

1.2. Amyloid Structure

The term “amyloid” was derived from the Greek word *amylon* which means starch; however, the term was initially a descriptor for plant composition. Amyloid was introduced into the field of medicine in the mid-1800’s when Virchow and others used it to describe lardaceous tissue deposits that stained positively, specified by a bluish color, with iodine solution, indicating the presence of a starch-like substance [5, 6]. Amyloid has since been used as a designation for the extracellular, proteinaceous deposits which primarily consist of protein fibrils. However, these deposits also ubiquitously include other associated secondary components, whose role in the pathogenesis of the disease remains enigmatic, such as proteoglycans, namely

heparan sulfate proteoglycan (HSPG), serum amyloid P component (SAP), and apolipoproteins. (See Figure 1.1) [5, 7-9].

1.2.1. Fibrils and fibrillogenesis

The initial stage of amyloid fibril formation involves the transition of normally soluble, amyloidogenic precursor proteins, which are natively folded, into insoluble, linear fibrils. Researchers have slightly different perspectives on this process; one possible mechanism of fibrillogenesis that is commonly seen and has been referred to as “widely accepted” [10] will be presented later. The structure of amyloid fibrils has also been a topic of intense discussion amongst amyloid researchers for years, but three traits common to all types of amyloid fibril have been identified (see Figure 1.2). Firstly, analysis of data generated from electron microscopy studies suggests that fibrils are “self-assembling”, unbranching filaments that are approximately ~10 μm in length, ~10 nanometers wide and display crossovers at regular distances (seen as white arrowheads in Figure 1.2A) [11, 12]. In addition to these features, a second defining characteristic of amyloid fibrils is the presence of a cross- β -sheet secondary structural motif as evidenced in X-ray diffraction studies [13-15]. Characteristic deflections in the X-ray diffraction pattern are ever-present in amyloid fibrils, regardless of the precursor protein from which the fibrils are formed [16]. These deflections are indicative of interstrand and intersheet distances at 4.7 and 10-13 Å, respectively (Figure 1.2B) [17, 18]. In addition, these data suggest that the β -sheet motifs are arranged perpendicular to the long axis of the fibril (see Figure 1.2C). The third common feature associated with all amyloid fibrils is the ability to give rise to green-birefringence upon staining with Congo red (CR) dye and viewing the sample microscopically with cross-polarized

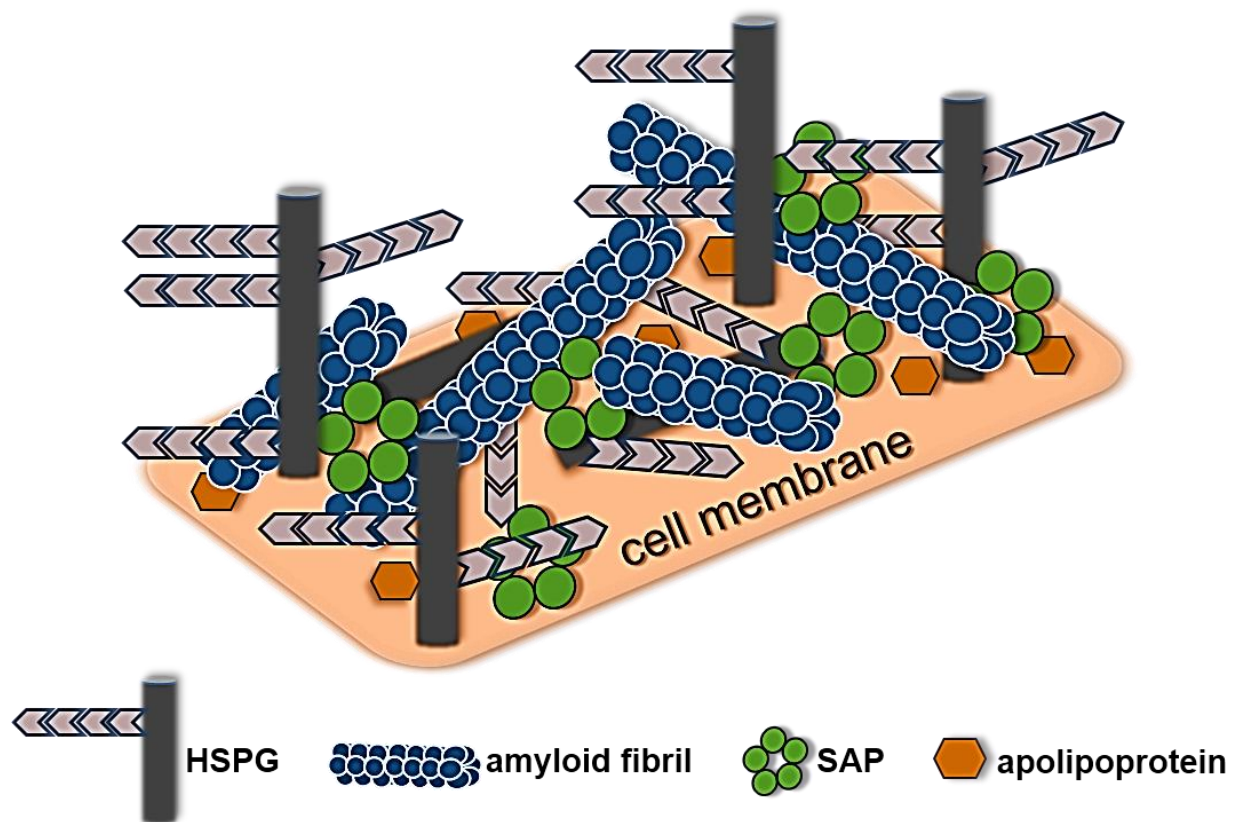


Figure 1.1: Schematic of amyloid composition on the cell membrane. This model demonstrates the complexity of amyloid where HSPG is heparan sulfate proteoglycan and SAP is serum amyloid P component. The various components that are depicted have all been found associated ubiquitously with amyloid deposits [5, 7-9, 19].

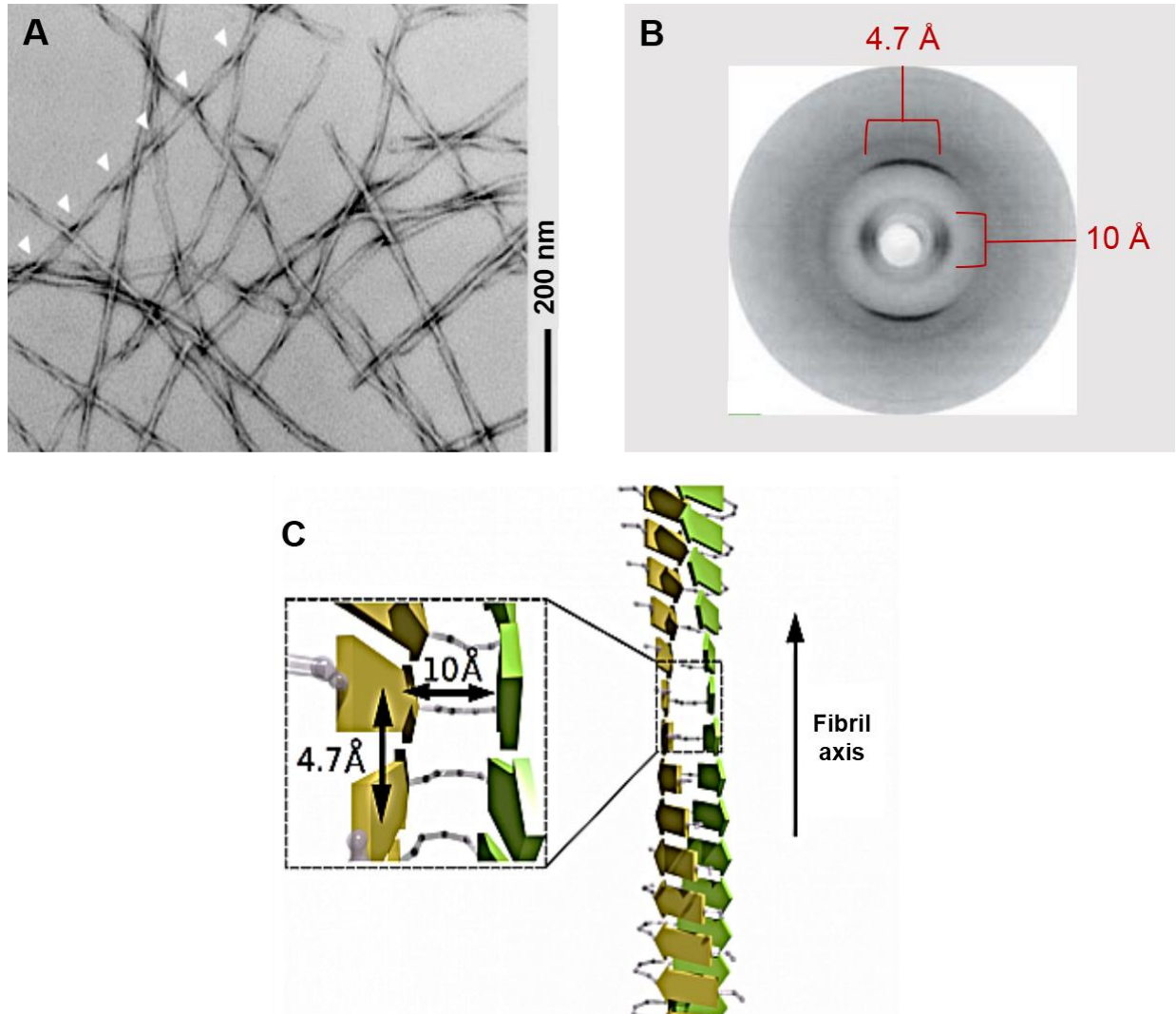


Figure 1.2: Structural characteristics of amyloid. (A) *Electron micrograph of amyloid fibrils.* The image demonstrates the unbranched structure of amyloid fibrils; arrowheads pinpoint to the regularly-spaced crossover positions throughout the length of the fibril. (Image taken from [11]). (B) *X-ray diffraction pattern of cross-beta sheet associated with fibrils* (Image adapted from [17]). (C) *Demonstration of cross-beta sheet positioning along fibril axis.* The beta sheets are indicated as yellow or green broad arrows. As shown, they point in opposing directions which creates the cross-beta pattern. This image additionally reveals that the beta sheets align in a perpendicular fashion along the length of the fibril axis (Image taken from [20]).

illumination [11, 21]. An example of CR-stained heart tissue and the localized green birefringence indicating amyloid deposition is shown in Figure 1.3A. The molecular rationale underlying the birefringent properties of fibrils is suggested to be due to the intercalation of several CR molecules amidst the cross- β sheet secondary structure of the amyloid fibril (Figure 1.3B).

While these three characteristics are associated with all types of amyloid fibril, they do not give insight into the mechanism by which fibrils form. Researchers agree that there must be a protein conformational change prior to fibrillogenesis – a shift in the structure of the native state of the precursor protein that renders it in a pro-amyloid state [10, 22-24]. Once misfolded, (and perhaps following the dissociation of multimeric complexes, as in the case of TTR) monomeric precursor proteins self-assemble forming dimeric and oligomeric “nuclei” which then leads to fibril formation. This process has been called ‘nucleation-dependent’ fibrillogenesis because it requires the association of monomers to “seed” the transition, analogous to the formation of salt crystals by the addition of a pre-formed “seed” or nucleating crystal. As Kumar and Walter’s proposed mechanism illustrates (see Figure 1.4), the misfolding of the native protein and the formation of intermediary species are reversible processes and not thermodynamically favorable; thus, the progression to fibrils is not favored, and this phase of fibrillogenesis is termed the lag phase or nucleation phase [10, 22-24]. Once a sufficient population of oligomeric species have been established, likely within affected tissues, they progress toward protofibrils, assemblies of protofilaments intertwined to form a pre-fibrillar state, and mature fibrils proceeds much more rapidly—this step is considered to be auto-catalytic (associated with exponential kinetics) and is known as the ‘elongation

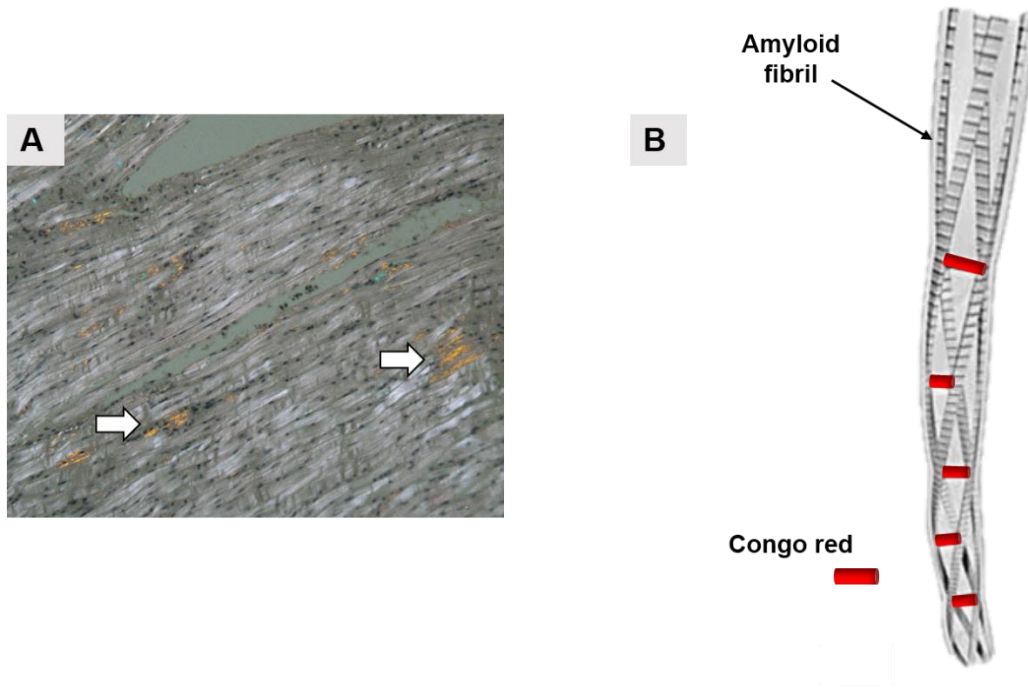


Figure 1.3: Congo red binding to amyloid. (A) *Congo red (CR) birefringence in amyloid laden tissue.* When viewed with cross-polarized light, the golden regions, indicated by white arrows, reveal amyloid deposits in cardiac tissue from a murine model of amyloidosis. (B) *Proposed incorporation of CR onto amyloid fibrils.* The cross-linking of CR on amyloid fibrils yields birefringent properties which are used for amyloid detection (Image adapted from [17]).

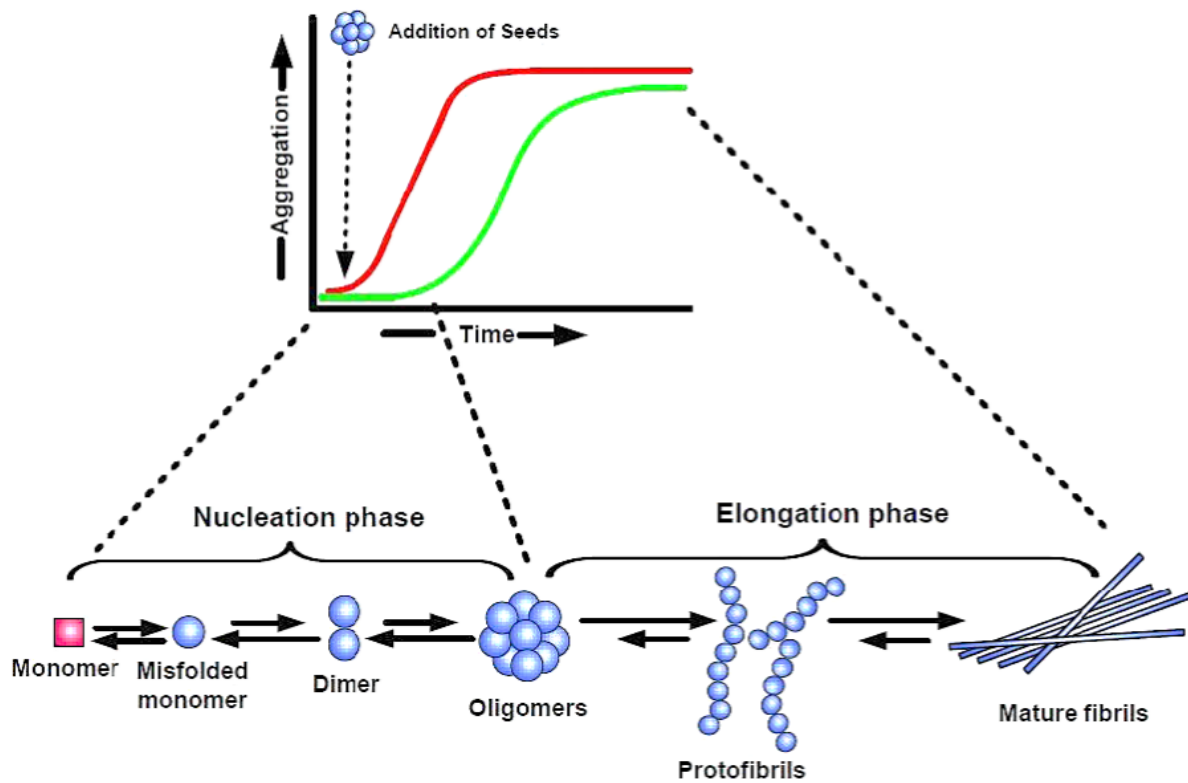


Figure 1.4: Nucleation-dependent mechanism of fibrillogenesis. Misfolded monomeric proteins self-assemble as dimeric and oligomeric species which “nucleate” fibrillogenesis (nucleation phase). This phase is thermodynamically unfavorable, but once a nucleus is formed, the progression to protofibrils and mature fibrils proceeds rapidly (elongation phase). (Image taken from [10]).

phase (Figure 1.4). The sigmoidal curve (green) indicates the different kinetic properties of each of these phases. Furthermore, the schematic shows what happens following the addition of pre-formed nuclei to the lag phase of the reaction. As noted above, this is analogous to seeding a crystallization reaction, and in the case of amyloid fibrils has been shown to induce the formation of prefibrillar assemblies and increase the rate of fibril formation, thus shortening (or even completely eliminating) the lag phase (Figure 1.4 red curve). Many researchers take advantage of this phenomenon in preclinical animal studies of amyloidogenesis by administering, intravenously, a tissue homogenate containing preformed oligomeric species and amyloid fibrils, known as amyloid enhancing factor (AEF), to expedite amyloid deposition *in vivo* [25-27]. This process shortens the time necessary for amyloid formation in animal models of the disease, and may be the underlying phenomenon governing the transmissibility of amyloid-associated diseases such as the spongiform encephalopathies and prion diseases [17, 28-30].

Fibrillogenesis and the pathogenesis at each phase of the transformation is not fully understood, however. Some researchers hypothesize that it is the oligomeric species that form during the lag phase that may be cytotoxic and result in the death of neurons in patients with Alzheimer's disease ($A\beta$) or pancreatic β -cells in patients with type 2 diabetes (AIAPP) — not the fibril, itself [31-35]. In Alzheimer's disease, the monomeric and fibrillar $A\beta$ peptides are biologically inert, but the oligomeric $A\beta$ species have been shown to be cytotoxic and show the best correlation with synapse loss and dementia in neuro-psychiatric analyses [36-40]. However, the systemic amyloidoses that involve multiple, if not all, visceral organs will be the focus of this thesis, and it is

widely accepted that in these patients, the deposition of fibrils and amyloid in the organs is responsible for the pathologic affects.

1.2.2. Heparan Sulfate Proteoglycan

In addition to the protein fibrils within amyloid, glycosaminoglycans (GAGs), namely heparan sulfate, are major secondary (non-fibrillar) components of the deposit. GAGs are unbranched heteropolysaccharides that are composed of repeating disaccharide units [5, 41, 42]. There are five types of GAGs which include heparin/heparan sulfate, chondroitin sulfate, dermatan sulfate, hyaluronan and keratan sulfate (See Figure 1.5; Keratan sulfate is not pictured because, unlike the others, it is not made with uronate). Of these, heparan sulfate GAG, which is structurally similar to heparin, is ubiquitously associated with amyloid deposits in the form of the perlecan proteoglycan. The role of heparan sulfate (HS) in amyloidogenesis has been widely investigated. In the case of AA amyloidosis, serum amyloid A protein, the precursor protein to the disease, has a HS binding-domain within its sequence which allows for direct interaction with GAGs on cell surfaces. In addition, as the deposition of AA amyloid increases the quantity of HS increases in parallel in a mouse model of the disease [5].

Circular dichroism analysis of amyloid precursor proteins, A β and SAA, in the presence of HS revealed an increase in the β -sheet content within these proteins, indicating that the interaction with HS rendered them more likely to become fibrillogenic [5, 43-46]. This enhanced β -sheet content was only induced by HS; no other GAG that was examined possessed this property [5]. Studies conducted in transgenic mice that overexpress human heparanase further suggested the importance of HS in the

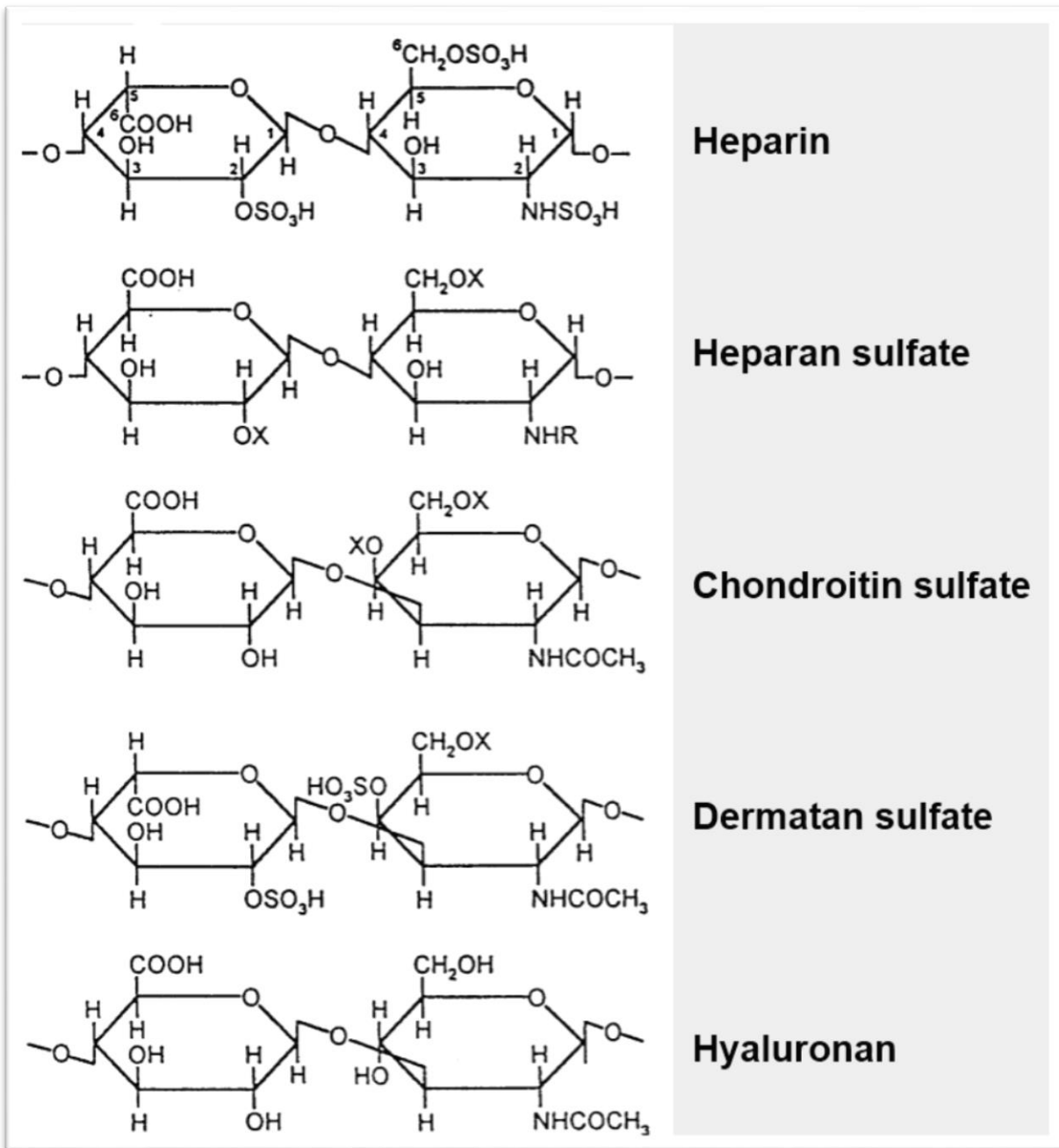


Figure 1.5: Structures of common glycosaminoglycans (Image adapted from [5]).

development of AA amyloid [47]. In this chimeric mouse, organs known to produce high quantities of HS, such as the liver and the kidneys, generated drastically shortened HS chains. Due to the improper HS production, these organs failed to develop AA amyloid following induction of the disease. Organs that were capable of producing full chain HS, e.g. the spleen, remained susceptible to AA amyloid as evidenced by extensive CR birefringence. These studies unequivocally demonstrated the critical importance of HS in amyloid formation [47].

It is noteworthy that Lindahl and Lindahl demonstrated, by using chromatographic techniques, that the HS found associated with perlecan proteoglycan [48] in amyloid-laden liver, spleen and kidney was structurally similar, but this type of HS differed from that found in amyloid-free tissues [7]. This observation suggested that amyloid-associated HS is structurally different from the HS found in healthy tissues. This finding was confirmed by Smits et al. in which a phage display-derived small chain variable fragment (scFv), designated NS4F5, was used to show the HS heterogeneity between healthy and diseased tissues [49]. The anti-HS scFv specifically bound to a region of highly sulfated disaccharides that is commonly found in heparin but is rarely seen in HS [49, 50]. When NS4F5 was introduced into a murine model of AA amyloidosis [27], the antibody bound to the HS affiliated with amyloid-deposits and did not bind to the normally distributed HS throughout healthy tissue as indicated by microautoradiography and histological examination of the co-localization of radiolabeled NS4F5 in regions observed to be rich in Congo red birefringence [49]. Thus, the major difference between amyloid-associated HS and normal HS may be the enhanced sulfation pattern found in the amyloid-associated HS; the greater abundance of negative

charges generated by the excessive sulfation may enable amyloid-associated HS to recruit additional molecules and proteins [49], thereby contributing to amyloidogenesis.

GAGs are usually found linked to a protein backbone, and this GAG:protein complex yields a proteoglycan (PG). As major components of all mammalian tissues, PG play a vital role in mammalian physiology by acting as local regulators of tissue function and homeostasis as well as structural elements [51, 52]. They are expressed ubiquitously on all cell surfaces and are present within extracellular matrices. It has been widely accepted that GAG and PG are involved in the recruitment of a range of proteins which typically aid in tissue remodeling during inflammatory and disease-induced immune responses [51, 53]; additionally, GAGs bind to matrix metalloproteinases and their inhibitors which, together, can be sequestered by PG to promote enzymatic regulation following tissue injury [51, 53]. Given these properties of tissue PGs, the GAG associated with amyloid deposits may recruit proteins and other components to the amyloid which contributes to its complexity. Immunohistochemical techniques have established that HS, likely linked to perlecan core protein, is the PG most commonly associated with amyloid deposits in man and animals [5, 26, 54]. Heparan sulfate proteoglycans are found in all amyloid deposits and, as mentioned above, are structurally and electrochemically different from the HSPG contributing to healthy tissue physiology. It has been suggested that these non-protein components undoubtedly contribute to the pathological process of fibril formation; additionally, GAGs should be considered a part of amyloid—not just “associated” materials [5, 55].

1.2.3. Serum amyloid P component

Another biological constituent of amyloid is the pentameric glycoprotein, serum amyloid P component (SAP) which is a normally, soluble, circulating plasma protein that is known to bind to all types of amyloid fibrils in a calcium-dependent manner (Figure 1.6) [16, 56, 57]. The biological function of SAP is not fully understood, but it has been shown to avidly bind to the DNA in chromatin that has been released into extracellular spaces in apoptotic or necrotic conditions [58, 59]. Once bound, the SAP is able to stabilize and protect the DNA from degradation [59]. Additionally, reproducible experimental observations suggest that SAP may contribute to a host's innate immunity against certain bacterial and parasitic infections [57, 59]. When mice with targeted deletion of the SAP gene, SAP knockout (KO) mice, were injected with lethal doses of smooth *E. coli* strain O111:B4, they were significantly more susceptible to infection than mice with inherent SAP. The role of SAP in amyloidogenesis is not entirely understood, but studies suggest that it may be able to prevent proteolytic digestion of amyloid fibrils, thus, contributing to their stability *in vivo* [8, 16, 59, 60]. Tennet *et al.* studies showed that SAP can prevent fibril digestion in an *ex vivo* assay but only if SAP is bound to them; the addition of SAP in the presence of its specific ligands, a cyclic pyruvate acetal, inhibited its binding to fibrils and rendered it incapable of protecting fibrils from proteolysis [60]. Additionally, *in vivo* studies have been conducted which reveal that SAP KO mice have delayed and reduced AA amyloid deposition when compared to SAP "sufficient" mice that underwent amyloid induction [8, 61, 62]. These studies suggested that SAP may indeed play a role in amyloidogenesis; however, Botto *et al.* also concluded that SAP is not a requirement for amyloid formation because, when

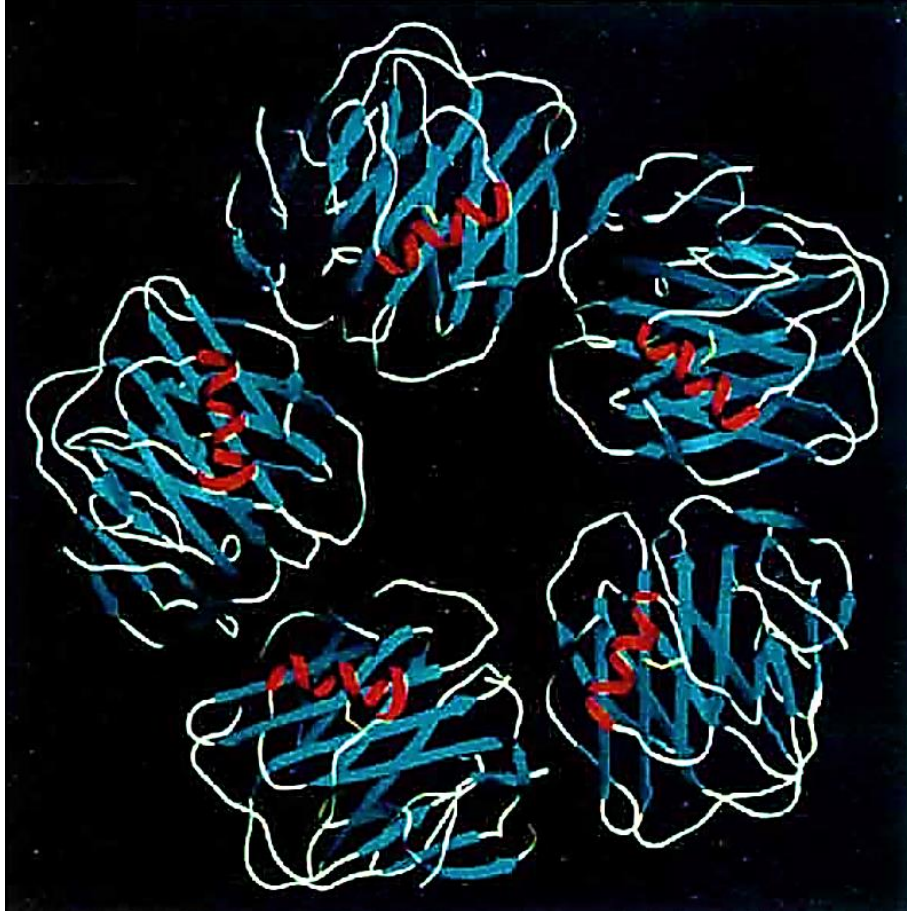


Figure 1.6: Crystal structure of serum amyloid P component. (Image taken from [16]).

given a sufficient stimulus, AA amyloid readily formed in SAP-deficient mice in the system as well [8, 62]. Therefore, SAP is associated with, and may provide stability to, all amyloid deposits.

Certain countries, mainly in Europe, utilize the amyloid fibril-binding property of SAP as a method of non-invasively detecting and monitoring visceral amyloid deposits in patients [56, 57, 63-66]. The use of SAP as a non-invasive amyloid imaging agent will be discussed further in a later section.

Recently, anti-SAP antibodies have been developed for use as a novel immunotherapy for systemic amyloidosis [67]. Bodin *et al.* reported the therapeutic efficacy of anti-SAP antibodies in a transgenic mouse model of AA amyloidosis which produces human SAP instead of mouse SAP (Notably, mouse SAP does not bind amyloid as well as human SAP) [67-69]. Prior to antibody administration, the mice were injected with (R)-1-[6-[(R)-2-carboxypyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid (CPHPC), which has a high affinity for human SAP and can, thus, clear the majority (~ 90%) of SAP from the circulation [67, 69, 70]. Some residual SAP remains within the amyloid deposits, and this was the material targeted by the opsonizing antibody [68, 70]. Since amyloid is typically an acellular deposit with no surrounding indication of inflammation, the observance of cell infiltration at 24 hours post injection (pi) of anti-SAP antibody was noteworthy [67]. These cells stained strongly with antibodies to F4/80 indicating they were likely macrophages; this was not observed in amyloid deposits from mice that did not receive the antibody [67]. The anti-SAP antibody treatment initiated a cellular response which resulted in nearly complete amyloid clearance by day 16 pi; at this point, there was evidence of restored tissue

architecture [67]. Given these findings, this therapeutic approach was deemed to be clinically relevant and has been implemented into an ongoing clinical trial (NCT01777243).

Based on the foregoing discussion, biological reagents that bind specifically the components of amyloid, such as the small chain variable fragment NS4F5 [71-73] and SAP [67] can be expected to yield novel diagnostic and potentially therapeutic agents for patients with amyloidosis. The research presented in this thesis will further explore and exploit this potential by studying a novel class of amyloid-reactive peptides.

2. Clinical Manifestations

2.1. Overview

The clinical impact of the amyloid diseases depends on the type of amyloid and the location of the pathological deposits.

2.2. Light chain amyloidosis – AL

Of the human amyloidoses, light chain amyloidosis (AL), presenting as either a systemic or localized disease, is the most common form with an incidence of approximately 1 case in 100,000 per year in Western countries [17, 74, 75]. AL amyloidosis is a form of plasma cell dyscrasia that results in an overproduction of monoclonal immunoglobulin light chains (IgLCs), the precursor proteins that initiate the amyloid cascade for this disease. Not all secreted monoclonal IgLCs form amyloid. In

patients with multiple myeloma, a related plasma-cell dyscrasia, only 12-15% of patients develop amyloidosis regardless of elevated levels of free IgLCs [17]. Additionally, it has been stated that the presence of monoclonal free IgLC in the serum or urine of individuals over the age of 50 is not uncommon; however, only a minor fraction of these individuals will progress to develop AL amyloidosis [1, 75, 76]. Of the 2 IgLC isotypes (λ and κ), the λ isotype, in particular, has been shown to have greater amyloidogenic propensity [1, 19]. For example, two germline genes that encode for the variable region of lambda light chains ($V\lambda$), the 6a and 3r germ lines, contribute to 42% of cases of lambda IgLC-related amyloidosis, approximately $\frac{1}{3}$ of all patients with AL [17, 75, 77, 78]. In fact, (with singular exception, protein Jto), products of the λ 6a germline gene have been found exclusively in AL patients and are rarely seen in the normal light chain repertoire [1, 19, 77, 79]. One $V\lambda$ 6a amyloid-related IgLC protein, isolated from patient Wil, has 11 somatic mutations and was shown, by using heat and chemical denaturation experiments, to be less thermodynamically stable and have a faster kinetic rate of fibrillogenesis *in vitro* relative to the non-AL associated Jto protein and other λ 6 proteins [1, 80, 81]. These studies were among the first to demonstrate an inverse correlation between the thermodynamic stability of a native state folding domain and the propensity for, and rate of, fibril formation *in vitro* [81]. A similar correlation using a series of $V\kappa$ 4 IgLC proteins has also been shown [82]. The fibrillogenic characteristics of $V\lambda$ 6 Wil have been exploited in numerous *in vitro* studies to further elucidate the biochemical and biophysical factors that render IgLCs prone to fibrillogenesis [1, 77, 80]. In cases of AL amyloidosis, secreted free IgLCs circulate in the blood, and under certain conditions, i.e. when their local concentration increases, following proteolytic digestion, or after

other modifications of unfolding of the variable domain, they self-associate into amyloid fibrils. A summary of the mechanism of AL amyloidosis, as illustrated by Blancas-Mejía and Ramirez-Alvarado, is shown in Figure 1.7.

The clinical implications of AL amyloidosis depend on the organ involvement. Merlini estimated that approximately 25% of AL patients present with only single organ involvement; this particular organ would determine the prognoses for these individuals [17]. However, the vast majority of AL patients (~ 75%) have multiple organ involvement or, albeit less common, deposition of amyloid localized at a single anatomic site, including the brain, as a tumor-like amyloidoma [1, 83, 84].

The site of AL deposition likely depends on numerous different environmental factors, e.g., localized high IgLC concentration and low pH, both of which favor the formation of fibrils [1, 17, 85-87]. The tissues most typically affected by AL amyloidosis are the kidneys, heart, spleen, gastrointestinal tract, peripheral nerves and liver [1, 17, 88]. Gross clinical manifestations in several organs are shown in Figure 1.8. The lardaceous, white material present within the kidneys is amyloid deposition (Figure 1.8A). The gastrointestinal tract may be affected, and if so, it can lead to internal bleeding (Figure 1.8B). The liver and heart, both of which are enlarged, have been stained with Lugol's iodine solution; the evidence of bluish discoloration after the application of dilute sulfuric acid indicates regions that are rich in starchy components—in these cases, amyloid (Figure 1.8C and 1.8D) [17, 89].

Cardiac deposition of AL is usually observed within the myocardium as evidenced by thickening of the interventricular septum, and the distribution in the liver is

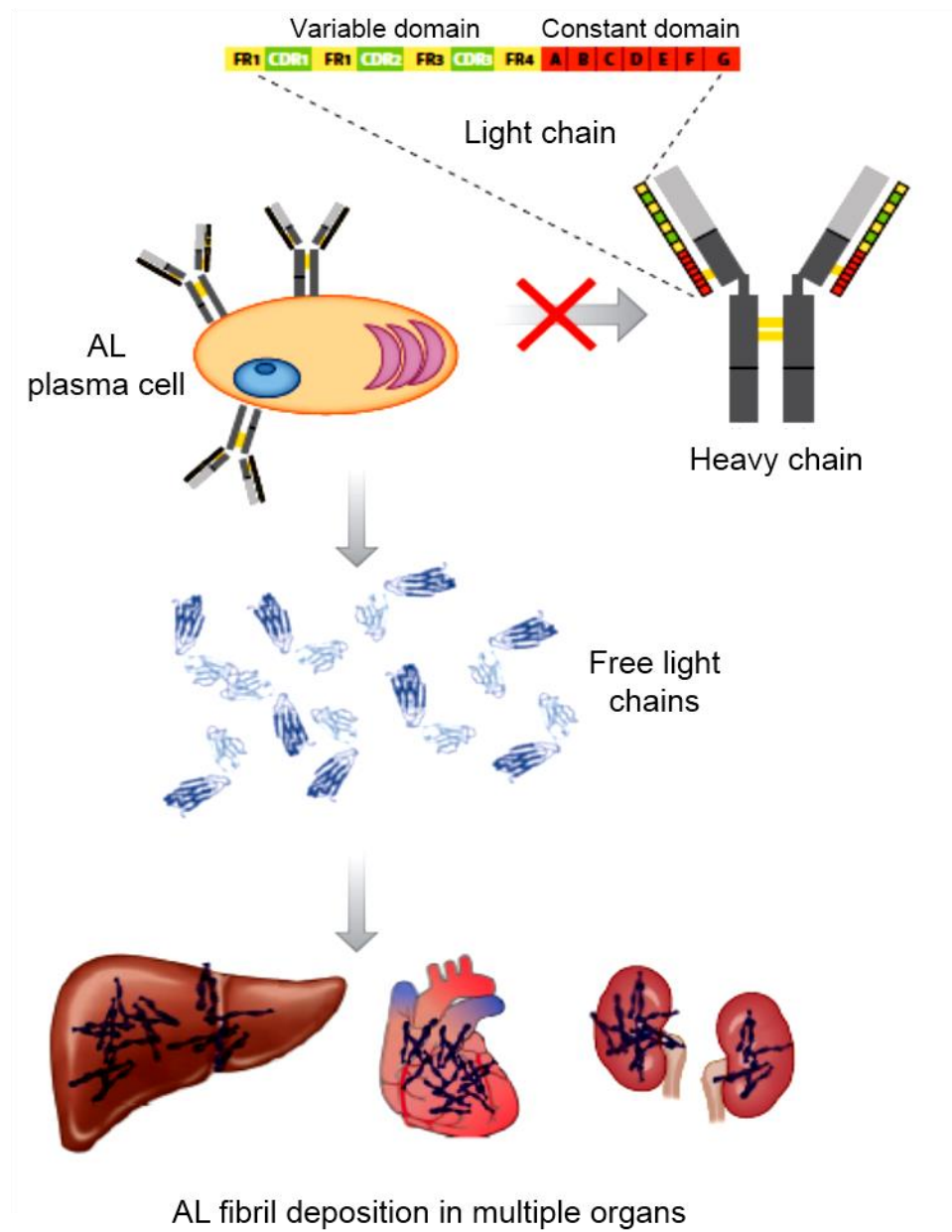


Figure 1.7: Basic mechanism of AL amyloidosis (Taken from [1]).

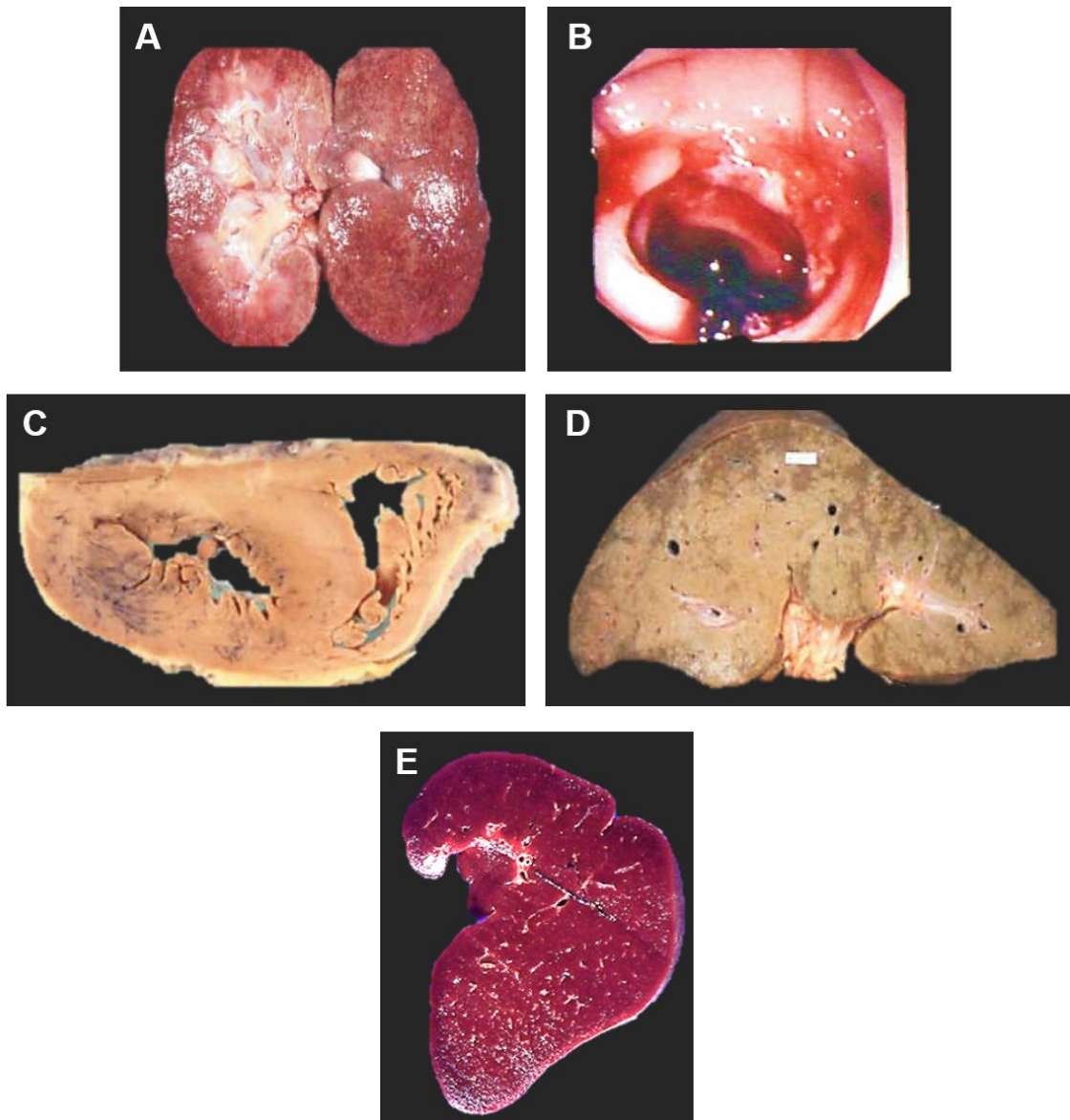


Figure 1.8: Clinical manifestations of AL amyloid in various tissues. (A) *Kidney*, (B) *Gastrointestinal tract*, (C) *Heart*, (D) *Liver* and (E) *Spleen* (All images adapted from [17] except the spleen which came from [89]).

generally localized to the space of Disse between the hepatocytes and sinusoids [17, 89]. Amyloid deposition in the spleen often appears similar to the kidney with the formation of dense, white material (Figure 1.8E); typically, the area around the follicles is the primary region of splenic deposition, but the amyloid can infiltrate further into the red pulp as well. Patients with renal or cardiac AL amyloidosis have the poorest prognoses with a median survival of one year, or less [90-93]. This is likely due to dysfunction caused by the disruption of sensitive tissue architecture in these organs in response to small amounts of amyloid, leading to progressive organ failure, co-morbidities and ultimately death. Therapeutic approaches for AL amyloidosis typically involve targeting the amyloidogenic plasma cell clones with chemotherapy which reduces the production of free IgLCs; this treatment, in rare cases, has been shown to improve cardiac and renal function even without the resolution of amyloid deposition, but the approach is not curative [17, 94-96].

2.3. Transthyretin amyloidosis – ATTR

Certain potentially amyloidogenic proteins contain heritable (familial) mutations which cause the protein to be either kinetically or thermodynamically less stable than the native, non-mutated variant. This is the case in patients with familial amyloid polyneuropathy (FAP) which is associated with the deposition of mutant transthyretin (TTR)—a homotetrameric protein that binds and transports thyroxine and retinol in the circulation [97-100]. The crystal structure of human transthyretin has been determined, and it includes 4 units comprising primarily beta sheets with one helix per unit (Figure

1.9). Unlike AL amyloidosis, the amyloid precursor protein, TTR, is not secreted by plasma cells; instead, TTR is produced exclusively by the liver. It is worth noting that, in addition to the familial form of ATTR, certain patients (normally > 60 years of age) present with a non-heritable form of the disease associated with the aggregation of “wild-type” protein [101]. This disease, formerly known as senile systemic amyloidosis (SSA), has recently been given the name wild-type TTR (wt-TTR) by the amyloidosis nomenclature committee which was announced at the XIVth International Symposium on Amyloidosis.

More than 100 point mutations have been characterized in TTR and most result in destabilization of the natively folded protein and/or cause dissociation of the TTR tetramer [102]—the critical event that leads to formation of the unstable structural conformation and precedes fibril formation [99, 103-106]. ATTR is a progressive, eventually fatal, disease that is typically characterized by sensory, motor, and autonomic neuropathy in addition to cardiomyopathy; thus, with its phenotypic variability, the disease may be difficult for physicians to recognize and properly manage [107-110]. In fact, under-diagnosis and misdiagnosis is quite common in these patients, which results in years of inappropriate treatment and increased likelihood of irreversible damage to the affected tissues [111]. The genetic inheritance of TTR-FAP is autosomal dominant; the variant TTR protein circulates from fetal life into adulthood, but no amyloid forms until later in life which suggests the importance of host factors, such as immune surveillance, in disease progression [111-113]. The clinical manifestations of ATTR-FAP paint a grim picture of diverse complications including peripheral neuropathy which initially affects the lower limbs. With time, numbness and discomfort make

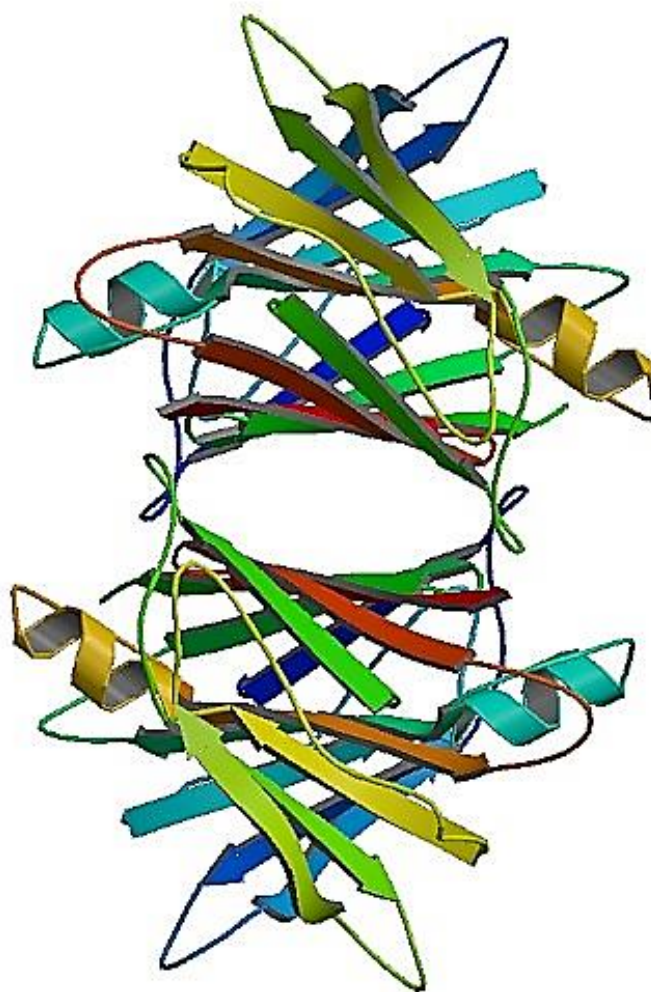


Figure 1.9: Crystal structure of homotetrameric transthyretin. The structure shown includes all 4 units of TTR which is almost entirely made of beta sheets (PDB accession number: 1DVQ).

walking more difficult. Some amyloid may accumulate locally which can cause carpal tunnel-like symptoms. The autonomic nervous system can also be affected which leads to gastrointestinal complications. Additionally, amyloid can infiltrate cardiovascular structures including atrial and ventricular myocardium; this causes thickening of the left and right ventricular walls. In some cases, heart failure progresses due to amyloid-associated restrictive cardiomyopathy [111, 114, 115].

TTR amyloidosis is the most common form of familial amyloidosis. Some germline mutations that are commonly observed clinically include Val122Ile, Ile68Leu, Thr60Ala and Leu111Met which generally result in cardiomyopathy as the predominant feature, and Val30Met, the most common TTR mutation, is primarily associated with peripheral neuropathy [111, 116]. Due to its heritability and late onset, there are populations around the world associated with higher prevalence of the disease [117-119]. For instance, in Portugal, Sweden, Japan and Brazil, the Val30Met mutation is predominant making this mutation the most frequently seen world-wide [120-122]. A region in northern Portugal is estimated to have an incidence of 1 in 538 individuals with the Val30Met mutation [123]. In the US African American population, the most commonly seen mutation is Val122Ile which has an incidence of approximately 4 percent in this population [124, 125].

Of the approximately 120 TTR mutations that have been characterized, only 13 have been deemed as non-amyloidogenic [111, 114, 126]; this, as one might expect, results in remarkable variability in the disease presentation in these patients. Given the high incidence of cases in various regions around the world, there are likely many cases of ATTR that are not recognized or misdiagnosed [111, 127, 128]. Knowledge of the

disease and detection strategies to diagnose TTR amyloidosis sooner would benefit patients worldwide.

2.4. Reactive amyloidosis – AA

Amyloid that results secondary to a chronic inflammatory stimulus or infection is known as secondary, reactive (AA) amyloidosis [9]. As described in a seminal review by Röcken and Shakespeare, diseases that can cause AA amyloidosis include: rheumatic diseases (arthritis); idiopathic disease (sarcoidosis, Crohn's disease and ulcerative colitis); inherited diseases (familial Mediterranean fever); infectious diseases (tuberculosis) and some malignant tumors (mesothelioma and Hodgkin's disease) [9, 129, 130].

AA amyloidosis is initiated by the production of serum amyloid protein A (SAA), which is the precursor of amyloidogenic protein, AA; SAA is produced principally by hepatocytes in the liver under the influence of certain pro-inflammatory cytokines such as IL-6 and TNF- α [131-133]. There are two types of SAA acute phase proteins, SAA1 and SAA2 in humans [9]. Both types are synthesized by the liver in response to an acute phase inflammatory response. During this response, the serum concentrations of these two proteins can reach 1-2 mg/mL which is 1000-fold higher than normal [9, 132, 133]. Once synthesized, 80% of SAA1 and SAA2 proteins bind to lipoproteins with approximately 90% complexing with high-density lipoprotein (HDL). The SAA bound to HDL is trafficked through the blood prior to uptake by certain tissues—predominantly the spleen, liver or kidneys—where there are macrophages known to

express receptors for SAA-HDL [9, 134-136]. The cellular compartment for the displacement of SAA from HDL following receptor-mediated endocytosis is unknown, but data suggests that it may occur in the late endosome as was demonstrated with SAA conjugated with a fluorophore and monitored with scanning confocal microscopy [135]. In response to the acidic environment, SAA leaves the HDL particle and undergoes proteolysis to form AA fragments which are typically N-terminal fragments spanning the first 66 – 76 amino acid residues of the 122 amino acid protein [137]. The pathogenetic pathway of AA amyloidosis was summarized by Röcken and Shakespeare (Figure 1.10) [9].

In the acute inflammatory state, SAA (and perhaps any AA amyloid that forms) is degraded, but in individuals with chronic inflammatory disorders, fragments of SAA persist, undergo fibrillogenesis and lead to amyloid deposition in a wide variety of tissues [138]. Interestingly, the proteolytic digestion of SAA *in vivo* resulted in products of varied amino acid length depending on which protease was used [139-141]. It has been postulated that certain cleavage sites are protective and, once cleaved, render the products incapable of forming amyloid fibrils [141, 142]. More specifically stated, cleavage at the N-terminal of SAA has been shown to prevent amyloid formation while C-terminal cleavage can yield potentially amyloidogenic protein fragments [9, 143]. Figure 1.11 illustrates various possible outcomes of SAA proteolysis.

The deposition of AA amyloid in man is thought to initially occur in the spleen (mediated by the perifollicular macrophages), followed by the liver and the kidneys before involving the vasculature and other visceral organs [9]. The most prominent clinical feature of the disease is renal involvement (> 90% of patients), which results in

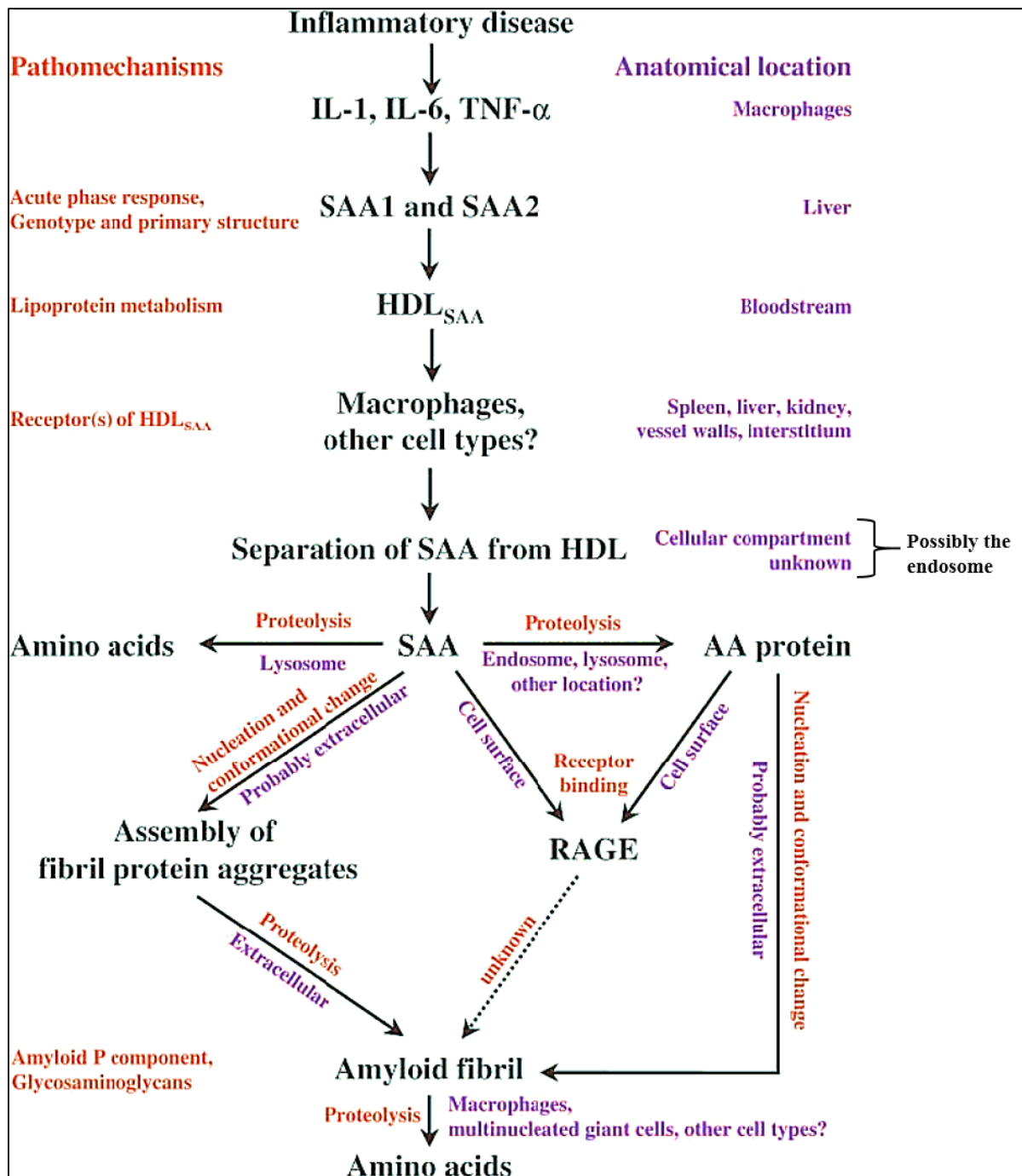


Figure 1.10: Mechanism of AA amyloidogenesis. This diagram illustrates the biological processes involved in the development of reactive amyloidosis beginning with the necessary inflammatory stimulus and ending with fibrillogenesis (Taken from [9]).

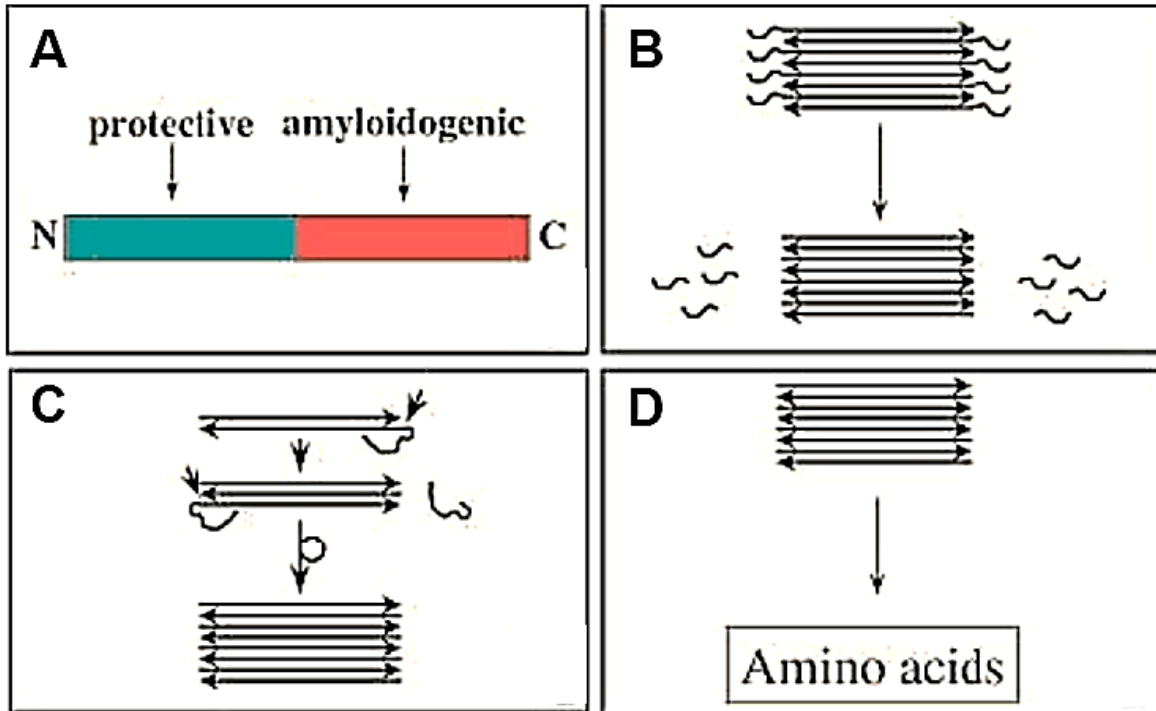


Figure 1.11: Possible outcomes of SAA proteolysis leading to fibril formation. (A) *Site of SAA cleavage dictates amyloidogenic potential.* The pathogenic SAA fragments that have been identified contain intact N-termini which suggests that cleavage in this region may be protective against fibril formation [143]. (B) *Cleavage of SAA post fibrillogenesis.* This outcome demonstrates that cleavage at this point in the pathogenic mechanism of fibrillogenesis may rid deposits of redundant peptides [144-146]. (C) *Cleavage of SAA during the formation of fibrils.* It is thought that cleavage during fibril assembly may assist in the process because it facilitates the removal of protein regions that might be hindering fibril formation [9]. (D) *Cleavage after fibril formation.* After fibrils have assembled, cleavage may have no effect, or it could result in complete degradation of the protein into amino acids [9] (Image taken from [9]).

proteinuria [147]. Cardiac AA amyloidosis, albeit rare in humans (~ 2% of patients), can disrupt tissue architecture and lead to restrictive cardiomyopathy and organ failure [148]. Gastrointestinal involvement, which presents as diarrhea and malabsorption, is also observed in ~ 20% of patients [149]. AA amyloidosis, unlike the other types of amyloidosis previously discussed, can be reversed if the serum concentration of SAA is substantially decreased, i.e. to < 10 µg/mL. Thus, if the underlying inflammatory cause of increased SAA levels is treated, the progression of AA amyloidosis can be delayed, halted, or even reversed [9, 150, 151]. While the incidence in man is typically low, experimental animal models of AA amyloidosis are one of the primary models used for researching amyloid diseases.

2.5. Other types of amyloidosis

In addition to the major forms of visceral amyloidosis mentioned above, there are other diseases associated with amyloid formation (see Table 1.1). For patients that must undergo long-term kidney dialysis, usually for 10 years or longer, an accumulation of β_2 -microglobulin (β_2m) leads to dialysis-related amyloidosis (DRA) [1, 152]. Under normal physiological conditions, β_2m is synthesized by all nucleated cells and associates with major histocompatibility complex class I (MHC I) which assists in the binding and presentation of short peptide sequences to appropriate CD8⁺ T-lymphocytes as part of an immune response [152-154]. The average life-time of MHC class I molecules varies from seconds to days [155, 156], but at the time of their degradation, the β_2m component is released into circulation [157]. Nearly all of the β_2m

is filtered through the kidney glomeruli and then reabsorbed by the proximal tubular cells where endocytosed β_2m is degraded [152, 158, 159]. Consequently, less than 0.1% of the filtered β_2m appears in the urine [160]. In patients that must undergo long term dialysis, the glomerular filtration rate is hindered due to renal dysfunction, and as a result, the serum concentration of β_2m remains elevated. The dialysis membrane molecular weight cut off is near or below the molecular weight of β_2m ; thus, the protein accumulates *in vivo* despite the renal filtration assistance of dialysis [152]. This sustained elevated concentration of β_2m (60-fold higher than healthy individuals) ultimately leads to DRA which tends to target cartilage associated with osteoarticular tissues leading to bone destruction (See Figure 1.12) [161-163].

Another amyloid-associated disease is type II diabetes (T2D) in which there is an accumulation of the amyloidotic islet amyloid polypeptide (AIAPP; also known as amylin) [164]. The deposition of AIAPP solely in the Islets of Langerhans in the pancreas is a pathological characteristic of T2D [31]. Human IAPP (hIAPP) is stored in the β -cell secretory granules, in association with insulin [165] and is released in response to events that lead to insulin secretion [166, 167]. While hIAPP it is a highly fibrillogenic protein, in non-diabetic individuals, it is normally soluble and unfolded in its native state [164]. Given its propensity to form fibrils, hIAPP must be protected in non-diabetic individuals in order to circumvent inevitable aggregation [164]. Association with insulin is thought to be the primary factor in the inhibition of hIAPP fibril formation as evidenced by several studies demonstrating the inhibitory effect of insulin on fibrillogenesis [164, 168-172]. Thus, the insulin dependency of individuals with T2D may predispose them to conditions in which IAPP is not fully protected from

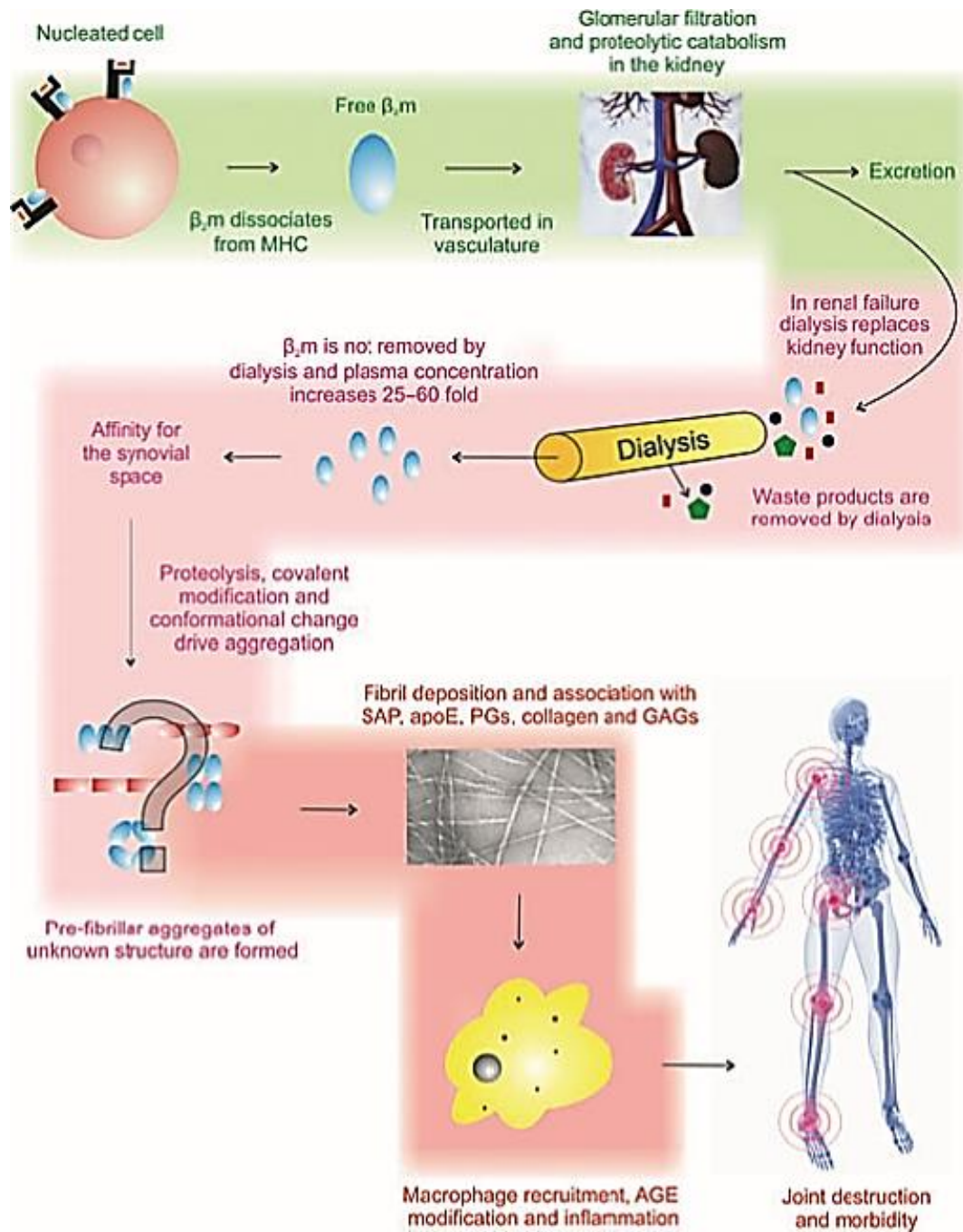


Figure 1.12: Mechanism of β_2m pathogenicity leading to dialysis-related amyloidosis. This illustration demonstrates how individuals on dialysis can develop an over-accumulation of β_2m which aggregates and forms amyloid deposits. The amyloid leads to the destruction of joints and osteoarticular tissues (Taken from [161]).

aggregation. It has been determined that AIAPP in the islets is not the cause of T2D, but it is widely accepted that amyloid formation leads to pancreatic β -cell dysfunction and cell death—a manifestation that leads to the loss of islet β -cell mass and insulin deficiency [31, 33, 173-175]. The normal physiological roles of IAPP have not been fully elucidated, but they may include controlling gastric emptying, maintaining glucose homeostasis, suppressing glucagon release and controlling satiety [31, 164, 176, 177]. Studies to determine whether or not the deposition of amyloid leads to islet β -cell death have been conducted. The data indicate that it is the oligomeric intermediate species that form prior to fibril elongation that are more cytotoxic *in vitro* [174, 178]. The addition of fully formed AIAPP fibrils to islet cells in culture did not induce apoptosis, but there was evidence of amyloid on the viable cells; in contrast, addition of an aqueous solution of hIAPP to islet cells resulted in apoptosis, and electron microscopy revealed the presence of nonfibrillar IAPP oligomeric species that had disrupted the cell membranes and permeated the cells [33, 174]. These data suggest that oligomers, formed prior to, but on the pathway to, fibril formation are more cytotoxic than the fibrils themselves; this is analogous to the pathology seen in Alzheimer's patients [10, 37, 179]. Several hypotheses have been offered to explain why islet amyloid forms in individuals with T2D, the simplest of which is that there is an increased production of IAPP as a result of increased secretory demand on the β -cell due to insulin resistance [177, 180]. This overproduction may lead to intracellular IAPP aggregation and extracellular amyloidogenesis [181, 182] as is the case with elevated SAA yielding AA amyloid deposition (see Figure 1.10). Additionally, Montane posits that there may be defects in the β -cell handling of IAPP due to impaired β -cell function associated with

T2D [177, 183]. More specifically, the granules within β -cells—where IAPP is produced and harbored until secretion—may not possess mature functionality under secretory stress. Indeed, the pH of β -cell granules under secretory stress is elevated which favors aggregation [177, 184]. While the mechanisms of AIAPP fibrillogenesis are not fully understood, two recent hypotheses have been outlined in the literature (Figure 1.13A and 1.13B); in addition to these, a compilation of “proposed mechanisms of IAPP-induced toxicity during amyloid formation” is also presented (Figure 1.13C) [31, 185-192]. Regardless of the pathway, AIAPP is associated with β -cell loss in individuals with T2D. Early detection of amyloid deposition could benefit T2D patients by implementing therapies sooner and sparing excessive loss of β -cells; thus, it may be of clinical import to develop non-invasive imaging strategies to detect and monitor AIAPP in patients.

Perhaps the most well recognized amyloid-associated disorder is Alzheimer’s disease (AD). The amyloid pathology with AD is localized to the brain and cerebral vasculature and arises from the misfolding of the $A\beta$ peptide—arising from the proteolytic cleavage of the amyloid precursor protein (APP) [32, 179]. Under normal conditions, $A\beta$ peptide cleaved by certain secretase enzymes remains soluble, is released into the cytosol and plays a role in nuclear signaling and induction of transcription and gene expression [193, 194]; however, in patients with AD, the peptide fragments [e.g. $A\beta(1-40)$ or $A\beta(1-42)$; [195, 196]] adopt conformations that are rich in β -sheets (Figure 1.14A) rendering them more prone to aggregate and form extracellular amyloid plaques in the brain and vasculature [32, 197-199]. An example of amyloid plaques in brain tissue can be seen in Figure 1.14B; these plaques have been stained

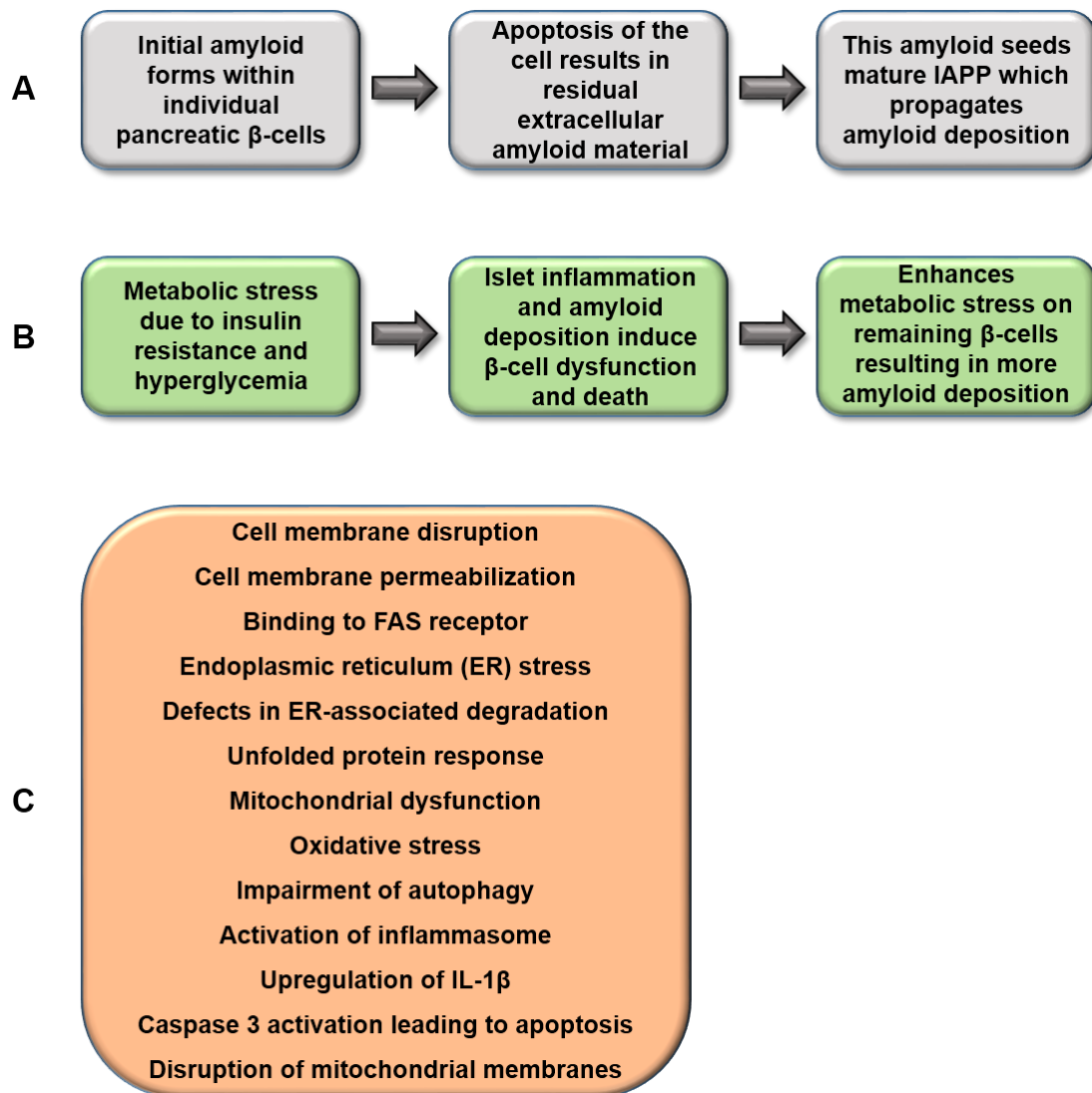


Figure 1.13: Proposed methods of A1APP toxicity leading to β -cell death and amyloid formation associated with type 2 diabetes. (A) Intracellular amyloid leads to apoptosis forming an amyloid seed propagating extracellular deposition [adapted from [164]]. (B) Metabolic stress leads to β -cell dysfunction causing amyloid deposition [adapted from [177]]. (C) List of other possible mechanisms leading to A1APP-induced cellular toxicity (compiled from [31]).

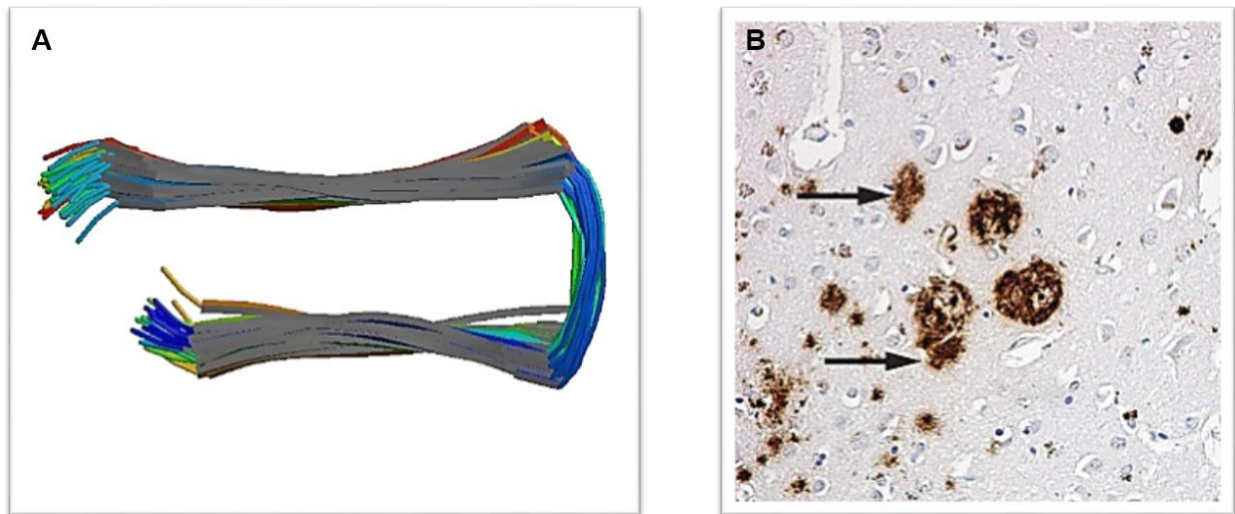


Figure 1.14: Crystal structure of $A\beta(1-42)$ peptide fragment and demonstration of the fragment within amyloid plaques of a sample of AD brain tissue. (A) *Crystal structure of the β -sheet rich, fibrillogenic $A\beta(1-42)$ peptide* (PDB accession number 2BEG). (B) *Immunohistochemistry of AD brain tissue with anti- $A\beta(1-42)$ antibody.* The tissue specimen demonstrates the presence of $A\beta(1-42)$ peptide within amyloid plaques (arrows) (Image adapted from [200]).

with an anti-A β (1-42) antibody indicating the presence of this fibrillogenic peptide within the AD amyloid deposits. There are two types of AD; one is a rare hereditary condition (< 3% of cases), in which APP is mutated leading to altered metabolism of the protein and an increase in A β peptide concentration. The other form of AD is more commonly seen; it likely involves the sporadic overproduction of aggregation-prone A β peptides and/or the reduced degradation and clearance of these species [32, 34]. According to the amyloid cascade hypothesis of AD, which suggests that AD is a result of A β amyloid accumulation in the brain, the formation of β sheet-rich A β peptide and fibrillogenesis are the first events that start the pathological changes which lead to dementia [201, 202]. Much focus has been directed toward understanding the pathogenesis of AD due to its devastating clinical and socio-economic impact. It was estimated that “in 2012, more than 15 million family members and other unpaid caregivers provided an estimated 17.5 billion hours of care to people with AD and other dementias—a contribution valued at more than \$216 billion” [203]. Investigation of the disease has now led many researchers to believe that it is the oligomeric species, which result from the association of multiple improperly folded A β peptides, that are more toxic to microglial cells than amyloid plaques [38] and are the underlying causative agent of the disease [36, 39, 40]. Given this, many therapeutic approaches involving the development of monoclonal antibodies targeted towards the A β peptide monomer in addition to a variety of A β dimers and oligomers produced prior to amyloid deposition have been investigated [38, 204]; however, treatment of the disease still remains a challenge [38].

Prion diseases, also known as transmissible spongiform encephalopathies (TSE), are fatal neurodegenerative disorders of the central nervous system (CNS) and are either inherited, arise sporadically or are acquired from an infection [205-207]. Several prion diseases are known to affect humans while others are typically seen in other mammals. The most common human TSE are Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome and fatal familial insomnia disease [208]. A less common TSE, one of the few acquired human diseases that make up 5 percent of all TSE cases, is called Kuru which was found associated with villages in Papua New Guinea [209]. The clinical features of TSE include widespread neuronal loss, astrogliosis, spongiform changes within the gray matter and amyloid plaque deposition in the blood vessels of the central nervous system which, in humans, presents with loss of motor coordination, neurophysiological deficits and dementia due to synaptic dysfunction [207, 210-213]. The pathogenicity of these diseases initiates with the misfolding of the inherently circular prion protein (PrP^c)—a glycosylphosphatidylinositol (GPI)-anchored cell surface glycoprotein with no certain function that is abundantly expressed by neurons in the CNS [214-216]. The pathogenic form of this protein (PrP^{Sc}) is rich in β -sheet which increases its likelihood to associate with other misfolded prion proteins resulting in the deposition of fibrillar protein aggregates - amyloid [217]. Numerous mutations in the PrP^c gene account for the inheritable forms of these diseases, and it is thought that the transformation of the protein into the PrP^{Sc} isoform occurs spontaneously under the influence of the mutation [217, 218]. As with other neurological forms of protein-misfolding diseases, the intermediate species that develop as the protein progresses to amyloid plaque deposition may be the cytotoxic agents that

result in synaptic dysfunction and neuronal loss [217, 219, 220]. Perhaps the most remarkable feature of TSE was the identification that it was a “transmissible” condition – indeed, it was originally thought that the pathologic aggregates that could induce pathology in another human carried some sort of biological information akin to DNA. However, this phenomenon has since been explained by the ability of amyloid fibrils to seed and induce amyloid formation due to the presence of a preformed template (the seed or nucleus). It is now widely accepted that seeding with preformed PrP^{Sc} fibrils is the underlying mechanism for transmissibility. This observation has led to the finding that other forms of amyloidosis are also transmissible both naturally and experimentally – AA in goats [28] and in mice [221, 222], ApoA2c in mice [30] and A β in mice [223].

2.6. Non-human amyloid-associated diseases

Protein-misfolding diseases are not restricted to humans; they can affect other mammals as well [206, 207]. As previously stated, certain TSE are pathogenic in animals; Scrapie which affects goats and sheep, bovine spongiform encephalopathy (BSE) which is also known as “mad cow” disease [224] and chronic wasting disease (CWD) which affects both deer and elk are the most common [225].

In addition to TSE, animals can also develop AA amyloidosis which likely progresses, as it is seen in many cases in humans, coincident with chronic inflammation. Tissue samples from several different animals including, but not limited to, dogs, cats, ducks, guinea pigs, cheetahs, tigers, gazelles, rabbits, bats, chickens and hamsters have demonstrated presence of the disease [9, 226-228]. The

mechanism by which the amyloid forms in these animals is thought to follow the same progression as that seen in Figure 1.10—the proposed mechanism for human AA amyloidosis.

The islet amyloid polypeptide that is associated with T2D is present in many animals, but it is not always an amyloidogenic protein [229]. In addition to humans, cats and monkeys contain versions of IAPP with mutations in the central region of the protein that make them more likely to misfold leading to amyloid deposition and T2D [164, 229]; the appearance of a proline residue in the sequence of rat and pig IAPP renders these proteins non-amyloidogenic [229] unless, in the case of rats, they are genetically altered to express human IAPP [230-232]. Studies have been conducted comparing the IAPP homology between cats and humans which reveal that cat IAPP is not as amyloidogenic as human IAPP [233], but both can misfold and lead to AIAPP amyloidosis.

Given that there are naturally occurring animal models of protein misfolding disorders including TSE's, AA amyloidosis and T2D, the use of these disease models provides a tractable *in vivo* system in which amyloid-seeking agents can be evaluated for efficacy. These disease model systems are invaluable to investigators who are developing diagnostic and therapeutic agents for the treatment of amyloidosis. They provide important test beds prior to seeking FDA approval of new drugs before translation into human clinical studies.

3. Diagnosis

3.1. Pathobiology

Amyloidosis is considered an orphan disease (not including AD or T2D), which accounts for approximately one per thousand deaths in developed countries [8]; however, since it is not a clinical condition that physicians commonly see and typically involves multiple tissues in the disease presentation, missed or delayed diagnoses often occur [8]. While there is a considerable challenge in making an accurate diagnosis of any type of amyloidosis, it is important to strive for the development of tools, such as imaging agents, to foster earlier and more complete diagnoses; appropriate treatment plans being implemented sooner could provide better prognoses for affected individuals.

The realm of diagnostic tools for amyloidosis has somewhat expanded over the last decade, but the most widely used method for recognition of the pathology relies heavily on the oldest technique, i.e. histological examination of biopsied tissues [1, 8, 17]. Unlike other protein aggregates, the fibril composition of amyloid enables its binding to Congo red (CR)—a histological dye that, according to recent work, utilizes both hydrophobic and electrostatic interactions to bind parallel to fibrils that contain β -strands along the edge or in the middle of a β -sheet [21, 234]. A proposed localization of Congo red within amyloid fibrils was shown in Figure 1.3B. When viewed microscopically with cross-polarized light, the fibrils show green birefringence which is indicative of amyloid deposition within the tissue (see Figure 1.3A). Examination of CR-stained tissues has been the gold standard for amyloid detection for more than 50 years, but this technique has its challenges and limitations; it requires proper application

of the stain along with other chemicals and skilled interpretation for accurate results [1]. Additionally, the test requires adequate amounts of amyloid and sufficiently thick tissue sections—without these, the sensitivity of the test falters [8]. According to Pepys, the British National Health Service National Amyloidosis Center sees more than 1100 patients with known or suspected amyloidosis annually; thus, they have reviewed thousands of CR biopsies. He states that they continue to see both false positive and false negative CR diagnoses from many hospitals around the world which further elucidates the challenges that physicians face when diagnosing the disease [8]. Experiments have shown that CR can bind many “native proteins including cellular prion protein [235], elastin [236], RNA polymerase [237] and human prostatic phosphatase [238]” [234]. In addition, CR lacks secondary structure specificity which may suggest an explanation for the false-positive results that are commonly obtained [234]. While observing positive CR-stained tissues may provide a diagnosis of amyloid, the type of amyloid cannot be determined without further testing.

Once it has been detected, classification of the amyloid is a necessity for each individual patient so that the origin and possible pathogenesis of the amyloid disease can be recognized and appropriate therapeutic regimens implemented [239-243]. There are different approaches to amyloid typing including the macro- and micro-extraction of the amyloid protein from tissues and immunohistochemical analysis of formalin-fixed paraffin-embedded tissue samples [241]. The former has been performed on autopsy-derived amyloid-laden tissues by multiple centrifugations as described by Pras et al. [244]. This macroextraction method of separation and purification yielded approximately 1100 mg of amyloid protein within the resultant supernatants from 20 g of

amyloid-laden tissue which differed from the normal tissue controls in which no detectable protein was recovered by adhering to the same protocol [244]. The microextraction of amyloid proteins has also been performed on biopsy-derived tissue specimens [245-247] and formalin-fixed tissues [248]; once extracted, the derived proteins can be identified by amino acid sequencing or mass spectrometry [249, 250]. In addition to protein extraction and sequencing, amyloid classification can be determined with immunohistochemical techniques in which amyloid-specific antibodies to some of the most common amyloid proteins (e.g. AA, AL κ , AL λ , ATTR) are applied to fixed tissues [251-256]. This approach is relatively inexpensive [241], but it has limitations including the lack of appropriate antisera to conduct the studies [254]. Murphy *et al.* explains that “commercially available reagents are prepared against the native form of the amyloidogenic precursor” and may, therefore, not be suitable for amyloid binding [254]. As was previously demonstrated in the proposed mechanism of nucleation-dependent fibrillogenesis (see Figure 1.4), the process introduces conformational changes to the precursor protein. Due to the lack of standardization of the reagents used for amyloid typing and the lack of regularization of the processing of the tissue sample, results from immunohistochemical analysis may be inconclusive; however, if the appropriate antibody is available and stains amyloid within the sample strongly and uniformly, it is likely diagnostic [241].

Given that these methods of amyloid characterization may render inconclusive results, Murphy *et al.* explored the use of mass spectrometry to identify amyloid type within biopsy samples and fat aspirates [254]. The application of tandem mass spectrometry (MS/MS) to various samples of amyloid-containing biopsies or fat

aspirates that had previously been characterized (e.g. AL λ , AL κ , AA, ATTR) yielded fragmentation indicative of each of the amyloid types studied; a limitation to this procedure is the amount of material needed for MS/MS analysis—additional biopsy samples were often necessary [254].

Whether it be due to: the untrained observer's inability to detect scant amyloid birefringence; the difficulty associated with discerning between amyloid-associated congophilic material and native tissue components with cross-polarized birefringence when attempting amyloid detection; the lack of appropriate sample preparation or amyloid stains for amyloid characterization; or the limited availability of material necessary for MS/MS amyloid typing, there is an extreme challenge associated with detecting and characterizing amyloid deposition by these methods. Therefore, other techniques need to be developed and validated.

3.2. Imaging

In most instances, patients with amyloidosis begin their clinical path by seeing physicians who do not specialize in the treatment of the disease (most often oncologists, cardiologists and nephrologists). Consequently, accurate diagnosis of amyloidosis can take more than 18 months and usually requires consultation at one of the amyloid treatment centers such as the Mayo Clinic, Rochester. Therefore, more effective approaches need to be implemented for routine detection and diagnosis of amyloid in the general clinical arena. To this end, dedicated efforts have been made in the realm of medical imaging for the facile detection of amyloid diseases. Advances in

molecular imaging can help provide these patients with a non-invasive alternative for disease detection—prior to advances in the field of imaging, biopsy-derived histological test results were the only means of detecting the disease. Some of the imaging modalities that have been utilized for the detection of amyloid include magnetic resonance imaging (MRI), ultrasound (US), optical imaging (OI), computed tomography (CT), positron emission tomography (PET) and single photon emission computed tomography (SPECT).

3.2.1. Modalities

CT and MRI—anatomical imaging modalities

While anatomical imaging such as CT, MRI and US should not be used alone as diagnostic tools for amyloidosis, they can provide valuable information about the anatomic consequences of amyloid deposition. For instance, abnormalities present in CT/MRI/US can offer guidance as to where biopsies should be taken if amyloid disease is suspected [257-259]. Additionally, the presence of abnormalities can help discern the type of amyloid disease. If, upon CT examination, pulmonary deposits are detected, there is greater likelihood that the amyloid disease in question is AL and not ATTR or AA because, unlike AL, these amyloidoses rarely affect the lungs [260].

CT is an imaging modality capable of visualizing the anatomical characteristics of an individual (spatial resolution of clinical scanners is 0.5 – 1.0 mm) without having to collapse a 3D structure into two dimensional images like conventional radiography [261, 262]. The principles behind this imaging system rely on the ability of X-ray beams to physically traverse the cross-section of an object, be attenuated by the object and then

be detected [263]. Multiple projections are necessary to reconstruct a representative 3D image. As the X-rays travel through diverse types of material, they become variably attenuated; for instance, water, bone, muscle and air attenuate X-rays differently. Each attenuation is assigned a linear attenuation coefficient (μ) which is a measurement reflecting the degree to which the X-ray intensity was reduced by a certain material. These values are used in the formula below to determine the CT number, known as the Hounsfield unit (HU), associated with the materials detected in the CT acquisition [264]:

$$CT\ number = (\mu - \mu_{water}) / \mu_{water}$$

The assigned CT numbers represent a range of gray values which are used to generate an image (i.e. the HU of water is 0 and air is -1000). The materials of lowest density appear darker; air will look black in the image. More dense materials will appear lighter with bone looking white in the image (Figure 1.15). In some clinical cases, only certain regions of patients are of interest, and to efficiently visualize those regions, the HU window can be set to only display a particular range of values—alteration of the window width. This is an advantage of CT imaging and image analysis, as compared to MRI or 2D X-ray, because it can improve lesion detection and diagnostic accuracy [265-267]. Additionally, CT imaging is often much faster than MRI imaging which is beneficial for the patients and their level of comfort. While CT provides much more information than X-ray radiography, this imaging modality exposes individuals to more radiation [268]. To address this concern, faster multidetector CT scanners with the automatic exposure control have been implemented into many systems [268, 269].



Figure 1.15: CT image demonstrating visualization of air and bone in a patient. Air within the lungs of this individual is black which is assigned the HU value of -1000 (red arrow); bone, a more dense material, appears white which has the HU value of 1000 (green arrow). This is an unpublished image generated in Dr. Wall's lab at the University of Tennessee Medical Center, Knoxville, TN.

MRI is a non-invasive method for examining the tissue architecture that does not rely on an internal or external source of radiation. This imaging modality takes advantage of the magnetic properties within the body's composition, especially those associated with the nuclei of hydrogen atoms. With the Earth's magnetic field, atoms align, but once the body is subjected to the presence of another magnetic field, like that within an MRI system, the atoms move to align with the new magnet—this can be manipulated and measured to form an image. MRI can provide exquisite soft tissue imaging and is advantageous in that it does not require much energy absorbance. MRI has been used to image patients with amyloidosis, but most often, the anatomical imaging reveals tissue abnormalities that may have resulted due to amyloid deposition, not the presence of amyloid itself. The observed abnormalities, however, can guide the biopsy which will confirm, after histological examination, if amyloid is present in the tissue abnormality. Typical amyloid-associated MRI findings include myocardial and interventricular thickening associated with cardiac amyloidosis in patients with AL or ATTR [270, 271]. Additionally, patients with ATTR occasionally undergo MRI analysis to visualize neuronal amyloid deposits around the leptomeninges and brain stem [272]. In rare cases, patients with localized amyloidosis develop focal deposits known as amyloidomas; this pathology can be well visualized with MRI [273, 274]. When investigating cardiac abnormalities that are possibly attributed to amyloid deposition, post-contrast MRI has been shown to display “global, subendocardial late gadolinium enhancement” which is characteristic of cardiac amyloid, but this abnormality can be challenging to detect when amyloid has infiltrated the interstitium or when arrhythmias degrade the quality of the image [275-278]. Ultimately, MRI can assist in the

visualization of pathological sequelae arising from amyloid deposition, can assist in the detection of rare, focal amyloidomas and can display contrast-enhanced abnormalities indicative of cardiac amyloidosis. However, it cannot provide information about the whole body distribution of amyloid itself.

PET Imaging

In recent years, much effort has been directed toward the implementation of molecular imaging technologies for the quantitative detection of amyloid in patients. The goal of these protocols is to create images that capture the *in vivo* whole body distribution of the amyloid, to assess the molecular-based characteristics of diseases and to monitor therapeutic responses in both humans and animals [261]. PET imaging involves the detection of positron-emitting radionuclides; positrons are particles that have the same mass as electrons but the opposite charge and are emitted by proton-rich nuclei such as Fluorine-18 (^{18}F), Carbon-11 (^{11}C) and Iodine-124 (^{124}I) [261, 279]. These radionuclides are conjugated to tracers that are injected into or inhaled by the patient, and it is the distribution of the radiotracer that is imaged with PET. The signal that is detected with PET imaging is generated by the annihilation of a positron/electron pair which usually emits a 511 keV coincident gamma photon pair in opposite directions at 180° [261]. The line that could be drawn to connect the two detected gamma photons is called the line of response (LOR). Numerous photon pairs are detected by the 511 keV-sensitive crystal scintillation detectors which are typically positioned cylindrically around the imaged subject. The scintillation material is able to emit visible light when it absorbs energy from charged gamma rays [280-282]. The positron and electron do not always annihilate and create a 180° coincident event, also known as a

true event; some annihilations result in skewed emissions called scatter events or random events (Figure 1.16). Scatter events arise when the emitted photons, either both or just one, undergo Compton scattering—a photon encounters an electron causing the electron's energy to be released and the photon's direction to be changed [283]—prior to their detection. Thus, the lack of 180° opposing deflections does not generate a LOR that accurately pinpoints the origin of emission (Figure 1.16A). Random events occur when photons from distinct nuclei are generated and detected simultaneously, but the LOR between the two detections is not indicative of the origin from which either photon was emitted (Figure 1.16B). For an image to be processed, the detected photon energy which has been transformed into light energy by scintillators must be converted into electronic signals; this is accomplished with photomultiplier tubes (PMT) [280]. The sensitivity of PET imaging, which is greater than that of SPECT, is reliant upon both geometric and intrinsic detection efficiencies; the former pertains to the placement of detectors as close to the subject as possible to lessen the likelihood that any photons are emitted and not captured. The latter pertains to the material used within the detectors—thicker, denser materials will be more likely to stop 511 keV gamma rays, allowing for their detection. The use of PET imaging provides a unique capability for *in vivo* monitoring of the biodistribution of radiolabeled materials in real time—an acquisition known as dynamic PET imaging. Analysis of dynamic data can generate time activity curves (TAC) which can elucidate the biokinetic properties of radiotracers in the subject. The high sensitivity of PET imaging makes it a desirable modality for disease detection in both preclinical and clinical studies, but it has limitations. The noncolinearity of annihilation photons and the range of positron

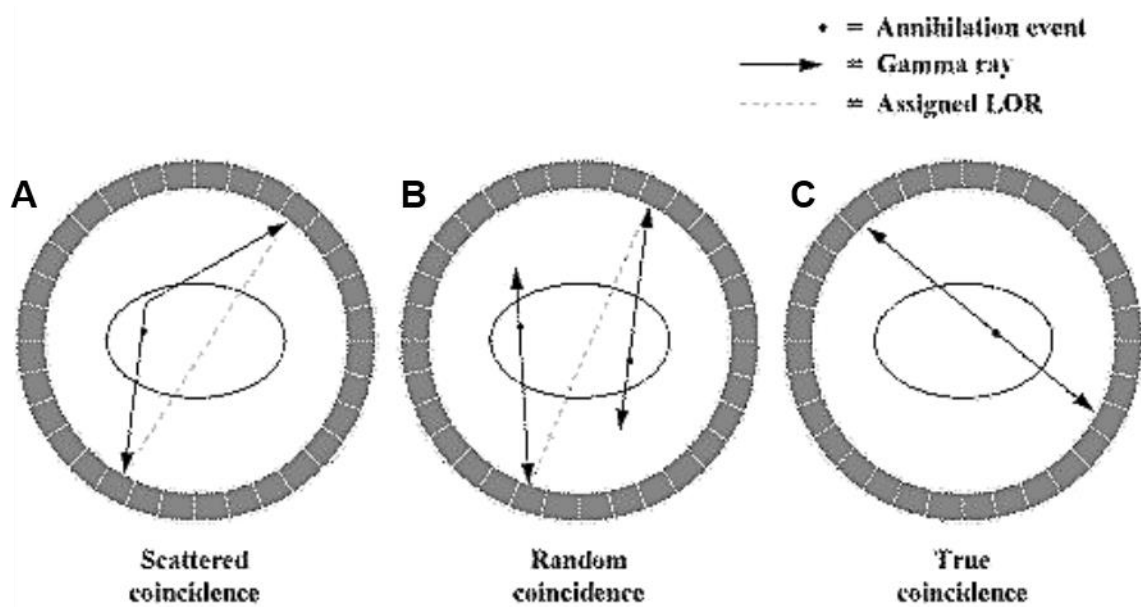


Figure 1.16: Types of coincidence events associated with PET photons (Image taken from [261]).

emissions hinder the system's ability to distinguish and detect subtle differences in molecular signals from miniscule structures (generally < 4 mm), known as the spatial resolution, of PET [261, 284]. Recent advances in Alzheimer's disease molecular imaging utilize PET to image the radiotracer, Amyvid™, in amyloid-laden regions of the brain that may be indicative of the disease (which will be discussed in a later section) [285].

SPECT Imaging

In addition to PET molecular imaging, SPECT imaging is also used to non-invasively detect diseases and clinical manifestations. SPECT is the most common molecular imaging modality in the clinic and requires the use of an injectable tracer which has been radiolabeled with isotopes that emit gamma ray photons. Some of the most commonly used gamma ray-emitting SPECT radionuclides are iodine-125 (^{125}I), iodine-123 (^{123}I) and technetium-99m ($^{99\text{m}}\text{Tc}$). Unlike PET imaging, where detection of the radionuclide is dependent upon the formation of a LOR generated from two high energy positrons through electric collimation, with SPECT, the one photon emission associated with each event must be detected through what is termed physical or geometric collimation [261, 284]. The collimators within a SPECT imaging system are usually made of a dense material such as tungsten or lead. They contain a configuration of pinholes through which photons that will contribute to the image enter; those that do not go through the pinholes will not be detected [261]. The pinholes, in combination with a position-sensitive detector, determine the LOR affiliated with photon emissions in SPECT systems. Much like that seen with PET systems, not all events that are recorded by the camera are true events that should contribute to the image;

events are considered true if the LOR that is drawn between the point of interaction with the detector and the photon emission location passes through a collimator pinhole [286]. Scatter events occur when photons enter the pinholes from skewed directions thus creating false events. With both PET and SPECT imaging modalities, scattered photons generate what is referred to as “background noise” within the image—this “statistical uncertainty” creates a haze and reduces the image’s contrast resolution [261, 287, 288]. The data from SPECT systems are acquired much like that seen in PET systems, but, instead of the detector elements being placed in a ring around the imaged subject, the detectors are on plates (one plate is required, but two plates increase the sensitivity of the system) which rotate around the individuals to generate 3D images. Pre-clinical and clinical SPECT imaging systems offer different advantageous properties as compared to PET. These include a range of radionuclides with longer physical half-lives than those routinely used in PET protocols, which may better estimate the biokinetics of radiotracers, and the ability to perform multiple energy studies simultaneously [280]. The latter refers to studies in which more than one radiotracer can be introduced into the imaged subject; the tracers are individually labeled with different radionuclides (i.e. ^{123}I with $^{99\text{m}}\text{Tc}$ or ^{125}I), and since the energies associated with these radionuclides do not overlap ($^{99\text{m}}\text{Tc}$ has an energy of 140 keV and ^{125}I has an energy of 20-35 keV), the two tracers can be discerned within the individual simultaneously [289]. This capability provides a means of comparing two imaging agents, perhaps the gold standard agent and a novel agent, under the same physiological conditions within the same individual; it also allows visualization of two different physical processes, such as the measurement of blood flow, metabolic rates or

protein binding, amidst the detection of other characteristics simultaneously [289-294]. The ability to detect and acquire multiple measurements within an individual subject with SPECT imaging circumvents the questionable reproducibility of *in vivo* conditions that arise with sequential imaging acquisitions when concurrent dual energy studies are not feasible. For example, an individual, amyloid-laden mouse which was simultaneously administered the gold standard amyloid imaging agent, SAP, radiolabeled with ^{125}I , and an amyloidophilic, synthetic peptide, p5, radiolabeled with $^{99\text{m}}\text{Tc}$, can be seen in Figure 1.17 [293]. This study illustrated a direct comparison of two radiotracers—insight into the relative efficacy of either (i.e. comparative effectiveness) can be ascertained with SPECT dual energy imaging in a single subject.

Dual Modality Imaging

In many instances, CT imaging is informative as a singular modality; however, recently, CT is used in conjunction with PET and SPECT to provide enhanced capabilities. This approach yields images that provide anatomical reference to the radioactivity introduced by the PET or SPECT imaging agent. A review by Schöder *et al.* on the subject refers to this system as “an imaging technology that combines high-quality PET and CT in a single device and provides simultaneous structural and biochemical information under nearly identical conditions” [262]. Without an anatomic frame of reference, the precise location of radiotracers *in vivo* is not evident, and thus, the image may be misinterpreted. Studies have been conducted to assess the value of the dual modality system in which individual modalities and combined PET/CT modalities were used to detect pathology; results in each study show that the use of PET/CT enhanced detection of disease [262, 295, 296]. In addition to the benefit of

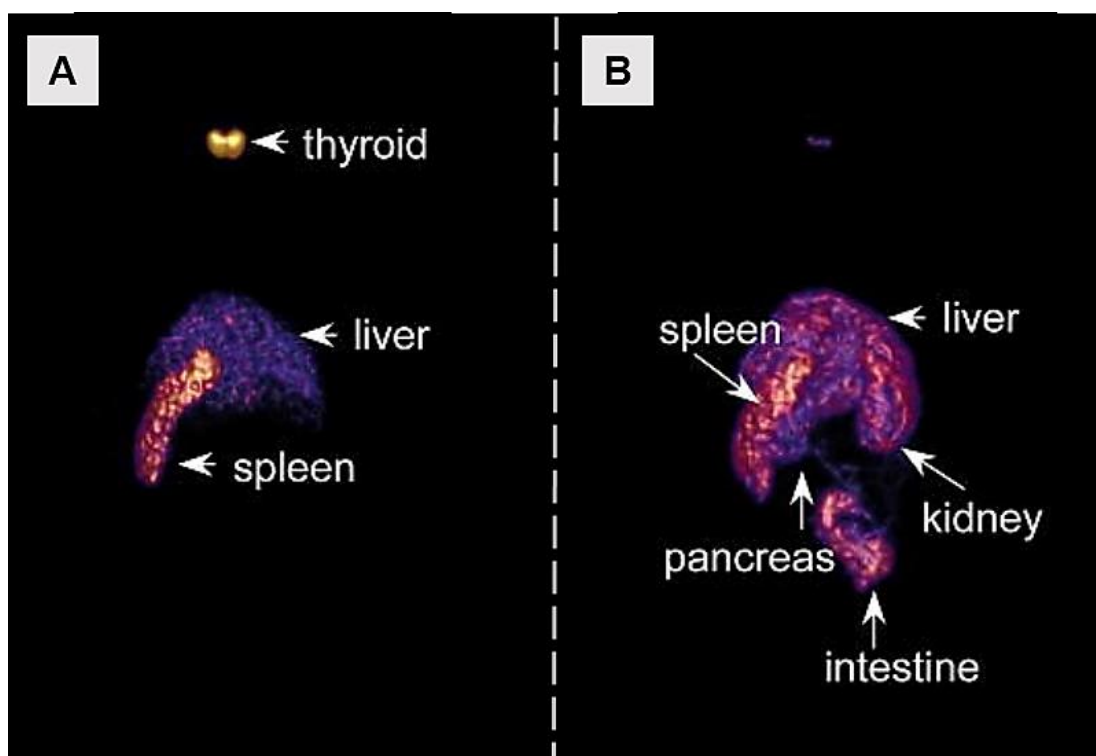


Figure 1.17: Dual energy SPECT imaging with ^{125}I -SAP and $^{99\text{m}}\text{Tc}$ -p5 in an amyloid-laden mouse. The use of two radiotracers allowed for simultaneous imaging of both SAP and peptide p5 in a single mouse. (A) *In vivo* distribution of ^{125}I -SAP. The radioactivity has localized to amyloid in the liver and spleen of this AA mouse with slight accumulation of free iodide in the thyroid. (B) *In vivo* distribution of $^{99\text{m}}\text{Tc}$ -p5 in the same mouse. The image demonstrates accumulation of radiotracer in the liver, spleen, kidneys, pancreas and intestine. Direct comparison of SAP and p5 suggests that p5 targets more of the amyloid load in this mouse. (Adapted from [293]).

anatomical reference, CT can be used to provide attenuation correction to the PET image data which results in better image quality and a reduction in whole body scan time by at least 40% [279] . Without attenuation, the uncorrected PET image can form “a rim of apparently increased activity along the periphery of the body and relatively lower tracer uptake in organs in the center of the body” [262]. Thus, attenuation correction is necessary in order to provide more accurate image display and interpretation.

Nowadays, all clinical and preclinical PET imaging is performed by using dual modality PET/CT systems. SPECT/CT systems have also been implemented to address many of the same concerns that single modality PET imaging faces. As stated by Mariani et al., “the main advantages of SPECT/CT are represented by better attenuation correction, increased specificity and accurate depiction of the localization of disease” [297]. Additionally, dual SPECT/CT imaging improves visualization of functional and metabolic information that is provided with nuclear medicine tests which monitor molecular changes that may not be observed with anatomical tests such as CT or MRI alone [297-299]. To demonstrate the advantage of anatomic localization using SPECT/CT, the same data presented in Figure 1.17 is displayed in Figure 1.18 as a SPECT/CT dual modality image.

3.2.2 Tracers

Molecular imaging in the form of PET or SPECT relies on the use of radiotracers which have been developed to target disease and metabolic functions. I will focus exclusively on those that relate to amyloid imaging. These agents can be categorized into one of the following types: small molecules, antibodies and proteins or amyloid-

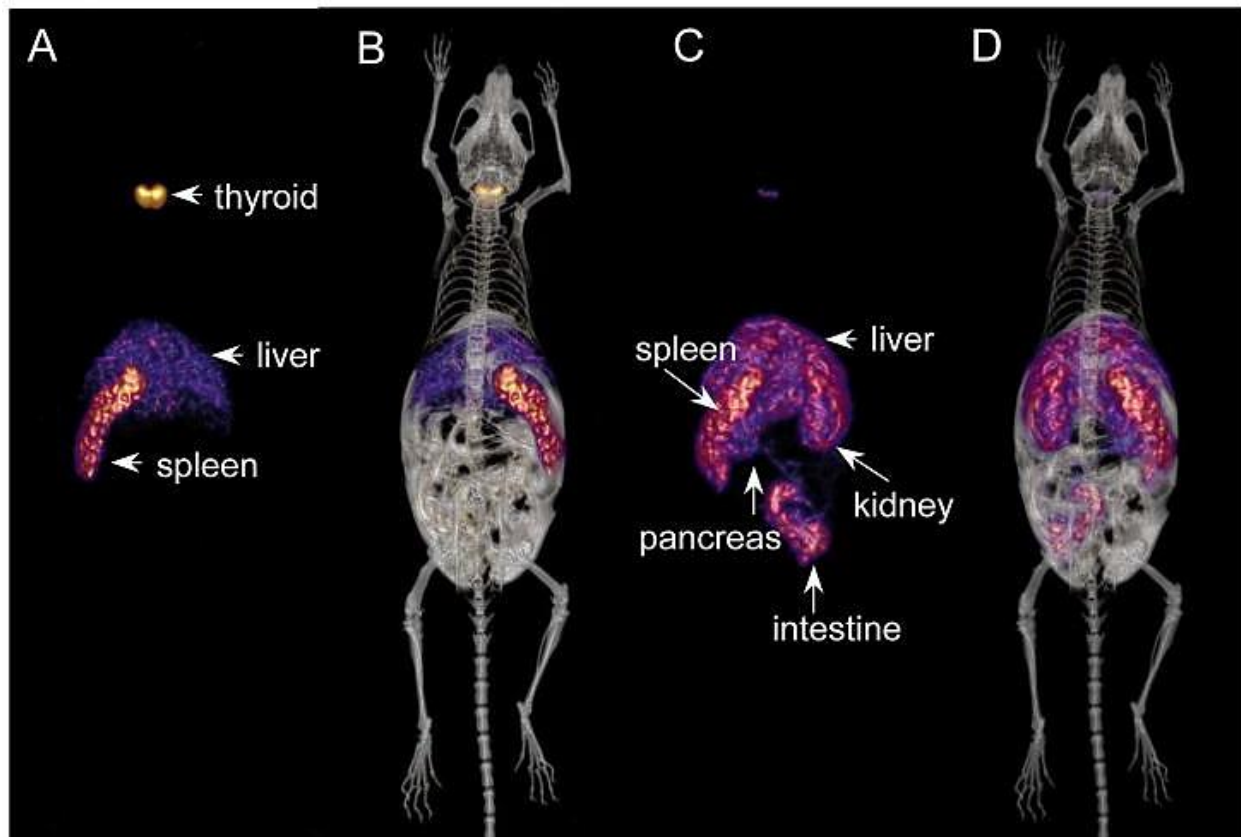


Figure 1.18: Dual modality SPECT/CT images. Co-registration of the CT data with the SPECT data (B and C) provides anatomical reference for the distribution of radiotracers. Thus, the labeled tissue accumulation shown (A and C) are more readily interpreted (Image taken from [293]).

associated materials. The tracers have been radiolabeled with positron emitters such as ^{18}F , ^{124}I , ^{11}C or gamma emitters such as ^{123}I or $^{99\text{m}}\text{Tc}$ for PET or SPECT detection, respectively.

3.2.2.1. Small molecules

Due to its immense socio-economic impact, much clinical focus has been devoted to the molecular imaging of amyloid associated with Alzheimer's disease (AD) which has been determined to be one of the "defining neuropathologic findings" of the disease [300]. The greatest risk factor for AD is advanced age, and with enhanced medical discoveries leading to longer life-spans, there is greater likelihood that more individuals will become afflicted with the disease [179, 301, 302]. According to the AD organization's annual review, it is estimated that 5.2 million Americans have AD; this estimate can be broken down into approximately 5 million that are over the age of 65 and 200,000 individuals under age 65 that have developed the disease with earlier onset [303, 304]. The report also indicates that "one in nine people age 65 and older (11 percent) has the disease"; this estimation is based on the U.S. population records projected by the 2013 U.S. Census Bureau [304].

The pathological, extracellular deposition of amyloid in the brain parenchyma and cerebral vasculature of AD patients and patients with mild cognitive impairment (MCI) results from the accumulation of misfolded beta-amyloid peptide fragments as amyloid [32]. The discovery of extensive and site-specific amyloid in AD patients warranted the development of an amyloid-seeking agent which could aid in detection of the disease.

The first amyloid imaging agent to be approved by the FDA for clinical assessment of the amyloid load in patients suspected of AD is Amyvid™ (formerly known as Florbetapir or AV-45; Avid Radiopharmaceuticals/Eli Lilly, Indianapolis, IN). Several multicenter trials have been conducted in which Amyvid™ was administered to patients with AD and patients with MCI along with a group of healthy controls (HC) [305-307]. PET/CT imaging was performed after ~ 50 – 70 minutes post injection of the radiotracer. The results from these studies indicated that the accumulation, determined visually and quantitatively using standard uptake values (SUV), in cortical regions was higher in AD and MCI patients than that seen in HC. While these findings were encouraging for both physicians and patients affected by AD, there are limitations to its clinical use. The quantitative information collected from each of these scans must be interpreted by a skilled and trained reader in order to get credible results; it has been reported that the discernment between AD/MCI patient data and HC data could be simplified if efforts are made to improve the spatial resolution of the images and if appropriate training is offered for nuclear medicine physicians [305]. News reports have indicated an additional limitation on the clinical use of Amyvid™ because of the decision, one that took many months to reach, by the Centers for Medicare and Medicaid Services (CMS) to not cover the drug's use for β -amyloid PET imaging for the diagnosis and treatment of neurodegenerative disorders [308, 309]. According to the detailed memo provided by the CMS, there was a lack of sufficient evidence to support the use of PET imaging with Amyvid™; however, there was a realization that the drug may be beneficial in specific cases in which the goal would be to exclude AD as a diagnosis (e.g., in patients with fronto-temporal dementia) [310]. They also believed

Amyvid™ could facilitate clinical studies seeking better treatment and prevention strategies (i.e., used as an endpoint in clinical trials). While there are positive results for Amyvid™'s ability to detect amyloid plaques within the brain, there is a lack of evidence that the radiopharmaceutical could be used for the whole body detection of systemic, visceral amyloid. A recent study using Amyvid™ to detect systemic amyloid deposition revealed that distribution of the radiotracer provided target-to-background ratios indicative of amyloid pathology in only the heart of amyloid patients [311]. Given that it was a pilot study, they were unable to calculate the specificity, sensitivity or diagnostic accuracy of the imaging agent, and they were unable to discern whether the amyloid was AL or ATTR in those patients with positive images. Additional, multicenter studies are being performed to further assess Amyvid's™ utility as a visceral amyloid imaging agent [311].

Another small molecule used for β -amyloid detection is known as Pittsburgh compound B (2-[4'-(methylamino)phenyl]-6-hydrobenzothiazole; PiB). The molecule also binds to A β amyloid in the brain parenchyma and blood vessel walls of AD patients [312-317]. Initial studies with the PiB used carbon-11 as the radionuclide. While ^{11}C -PiB has been shown to detect β -amyloid, the positron emitter hinders the effectiveness of ^{11}C -PiB due to its very short half-life (20.4 min)—essentially, in order to use the molecule, the *in vivo* image acquisition must be performed in an institution with a cyclotron for radioisotope preparation and immediate use [317, 318]. To circumvent the inconvenience of the short half-life of carbon-11, PiB has been radiolabeled with fluorine-18 ($T_{1/2} = 109.8$ min); this molecule, recently approved by the FDA, is known as ^{18}F -Flutemetamol (or clinically Vizamyl™: GE Healthcare, Arlington Heights, IL).

Studies in clinical trials have shown that the fluorinated molecule performs similarly to the parent ^{11}C -molecule and additionally provides wider accessibility for both clinical and research purposes [317].

However, it suffers from the same regulatory limitations imposed on AmyvidTM. Recent data indicate that ^{11}C -PiB, because of its retention by amyloid, can also be used to image cardiac amyloid deposits associated with both ATTR and AL amyloidosis [319]. This group concluded that the molecule could image cardiac amyloidosis within the myocardium of affected patients, but there was reversibility in the binding which would suggest early time point imaging in future studies [319]. Notably, the amyloid in other visceral organs was not imaged using this tracer. Studies using ^{18}F -Flutemetamol for imaging cardiac amyloidosis have yet to be published.

Many other small molecules have been utilized as amyloid-imaging agents also. These include $^{99\text{m}}\text{Tc}$ -labeled 3,3-diphosphono-1,2-propanodicarboxylic acid ($^{99\text{m}}\text{Tc}$ -DPD) and $^{99\text{m}}\text{Tc}$ -labeled hydroxymethylene diphosphonate ($^{99\text{m}}\text{Tc}$ -HDP). These $^{99\text{m}}\text{Tc}$ -labeled phosphate derivatives were developed decades ago as bone seeking agents to be used in nuclear medicine planar, gamma scintigraphy as a means of detecting bone abnormalities such as skeletal metastases and benign bone disease [320-322]. Calcifications that present in some amyloid deposits—especially those seen in the heart of patients with ATTR and those with SSA (wild type ATTR)—may explain the uptake of these tracers in cardiac amyloid [323]. Myocardial involvement associated with TTR amyloidosis, both mutant and wild type TTR amyloid, has been imaged with $^{99\text{m}}\text{Tc}$ -DPD; positive image data correlated with high amounts of amyloid fibrils [323-325]. The same tracer used in AL patients with cardiac amyloid deposition gave negative image data

[323, 324]. Thus, this imaging agent may be used to discern between ATTR and AL cardiac amyloid. A recent study using ^{99m}Tc -HDP concluded that scintigraphic analysis with this tracer may be able to detect cardiac amyloidosis in ATTR patients, and like DPD, the amount of uptake correlated with the severity of amyloid deposition [326].

These small molecules have been shown effective in certain types of amyloid diseases, but they are not suitable for whole-body detection of amyloid in the case of systemic disease. Thus, the small molecules available for amyloid detection do not address all clinical needs.

3.2.2.2. Antibodies and proteins

In addition to small molecules, certain antibodies and proteins have been developed as amyloid-targeting agents. Two antibodies that have been explored as therapeutic and/or diagnostic (“theranostic”) agents for amyloidosis are 11-1F4 [327-329] and 2A4 [330, 331]. Initially developed as therapies for the disease, these antibodies have since undergone PET and SPECT imaging studies to assess their efficacy for amyloid detection *in vivo*. In addition, the most commonly used protein that has been evaluated as an amyloid-imaging agent is aprotinin [332]. It was shown to be efficacious as an amyloid imaging agent, but, as with other tracers, there are limitations associated with its use.

The monoclonal antibody (mAb) 11-1F4 was generated using human light chain amyloid-related aggregates and recognizes an epitope common to fibrils associated with AL $_{\kappa}$ and AL $_{\lambda}$ [327, 328, 333]. Initial studies conducted with 11-1F4 revealed its ability to expedite resolution of subcutaneous (sc) human AL in “amyloidoma”-bearing

mice; these studies involved formation of a palpable sc AL amyloidoma by injection of ~100 mg of amyloid extract derived from autopsy tissue specimens [327]. After iv injection of a total of 600 µg mAb 11-1F4 (administered over a 12 day period), the amyloidomas in treated mice, by day 15, were reduced in size as compared to control mice which received equivalent amounts of an isotype matched control antibody or were left untreated altogether [150]. Furthermore, iv administration of radioiodinated 11-1F4 into mice with an AL amyloidoma and PET/CT imaging showed co-localization of ¹²⁴I-11-1F4 radiotracer in the amyloid xenograft [328, 329] as shown in Figure 1.19. Given the potential therapeutic and diagnostic potential of the mAb, an FDA-approved Phase 1 exploratory investigational new drug (eIND) trial to study the efficacy of ¹²⁴I-11-1F4 as a systemic amyloid targeting agent was initiated. The results of this study indicated that 9 of the initial 18 enrolled patients with known amyloid deposition demonstrated uptake of ¹²⁴I-11-1F4 in the liver, lymph nodes, intestines, bone marrow and spleen as shown in PET/CT images [329]. A representative patient from this study can be seen in Figure 1.20 where the image revealed uptake of the mAb in the amyloid-laden liver. For all imaged patients, the renal and cardiac deposits were not visualized, however. The lack of uptake in these affected organs was thought to be due to the absence or inaccessibility of the mAb's epitope. The conclusion from this study was that imaging with ¹²⁴I-11-1F4 could be a good predictor of patients that may benefit from a passive immunotherapeutic approach with this mAb (i.e., as a preliminary patient stratification tool before recruitment into an 11-1F4 therapy protocol).

The other mAb that has been generated to possibly assist in the detection and/or therapeutic removal of amyloid deposits is known as 2A4 [330, 331]. This mAb was

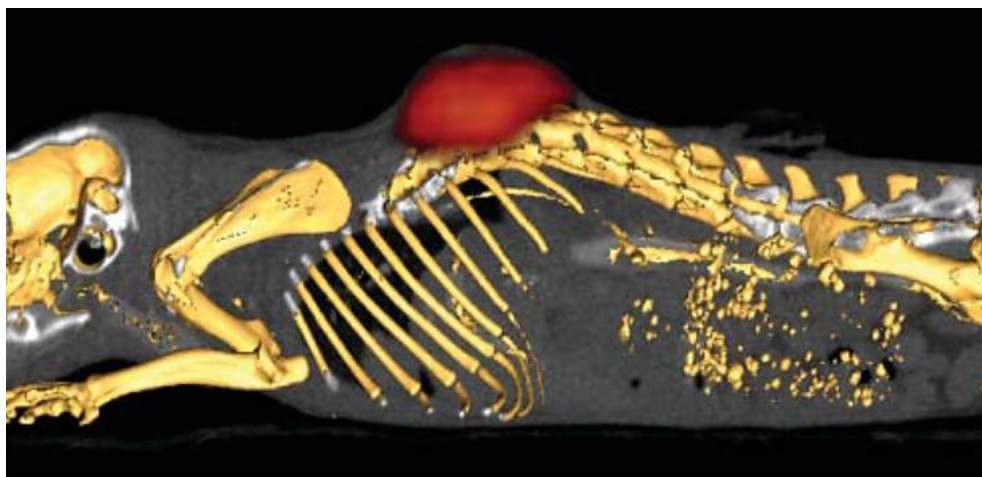


Figure 1.19: Co-localization of mAb ¹²⁴I-11-1F4 with a sc AL amyloid xenograft as evidenced by PET/CT. The image shows uptake of the radiotracer within the AL amyloidoma which is located between the scapulae of the mouse. This finding led to further development of the radiotracer as a potential imaging agent in patients with AL amyloidosis. (Adapted from [328]).



Figure 1.20: The mAb ^{124}I -11-1F4 can image amyloid-laden liver in an AL patient. The mAb was administered 5 days prior to imaging. As shown, ^{124}I -11-1F4 accumulated in the hepatic amyloid of this patient. (Taken from [329]).

developed to bind the carboxy-terminal epitope (amino acid sequence His-Glu-Asp-Thr; HEDT) which was created following proteolysis of serum amyloid A protein (SAA), the precursor protein for AA amyloidosis. The antibody does not recognize native SAA which contains 103 amino acids; it was shown to bind the -HEDT epitope of the cleaved SAA fragment (~ 75 residues) associated with AA amyloid fibrils [142, 330, 334, 335]. When radiolabeled with iodine-125 and injected into AA amyloid-laden mice, ¹²⁵I-2A4 co-localized with the amyloid deposits as evidenced by SPECT imaging (Figure 1.21A) and microautoradiography [330]. Surprisingly, further preclinical studies with this antibody indicated that it is also capable of *in vitro* binding to amyloid fibrils composed of immunoglobulin light chains associated with AL amyloidosis [331].

Furthermore, *in vivo* analysis revealed that administration of ¹²⁵I-2A4 to mice bearing sc human AL amyloidomas resulted in preferential SPECT imaging of the amyloid mass (Figure 1.21B), and treatment of these mice with the mAb expedited resolution of the amyloidomas when compared to control mice which received an isotype-matched antibody. Thus, preclinical studies provided evidence that 2A4, because of its ability to selectively bind amyloid *in vivo*, could be a valid agent for imaging and immunotherapy in patients with AL amyloidosis in addition to its diagnostic utility for AA amyloidosis [330, 331]. A humanized GMP version of the murine mAb 2A4 was generated, known as NEOD001 (Prothena; Dublin, Ireland), is currently in a Phase 1 clinical trial investigating the maximum tolerated iv dose in AL amyloidosis patients [336] (NCT01707264).

The protein that has been investigated most thoroughly as a potential amyloid imaging agent is ^{99m}Tc-labeled bovine aprotinin (Trasylol), a protease inhibitor. Since

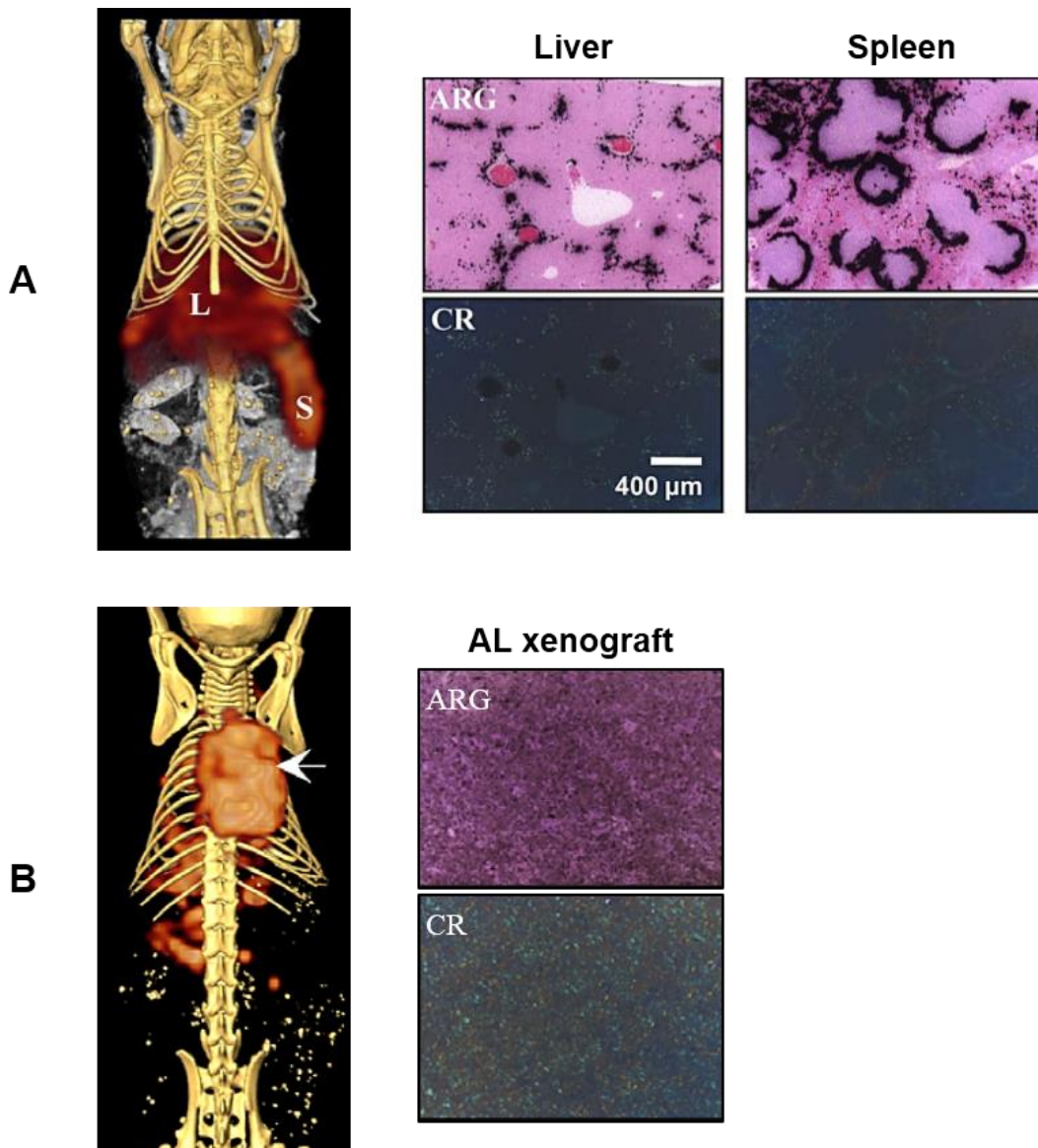


Figure 1.21: SPECT/CT imaging and ARG of both AA and AL amyloid with mAb ^{125}I -2A4. (A) Distribution of ^{125}I -2A4 in an AA amyloid-laden mouse. SPECT/CT imaging showed uptake of the mAb in the liver and spleen of an AA mouse. The black silver grains in the ARG of both liver and spleen tissues coincide with the birefringent regions in consecutive tissue slices stained with CR indicating co-localization of mAb with AA amyloid. (B) Uptake of ^{125}I -2A4 in a sc human AL amyloidoma. The SPECT/CT indicates radiotracer in the AL amyloidoma (arrow), and ARG/CR comparison confirmed co-localization of mAb with AL amyloid (Images adapted from [330, 331]).

amyloid deposits are known to contain antiproteinases such as α_1 -antichymotrypsin [332, 337, 338], it was hypothesized by Aprile *et al.* that aprotinin may react with this component of amyloid deposits [332]. There is evidence that this protein accumulated in the renal cortices as part of natural catabolism; thus, initial investigations with the imaging agent focused on the detection of extra-abdominal AL amyloid deposition by planar scintigraphy [332, 339-342]. Cardiac amyloid involvement in the 24 AL patients participating in this study was imaged in all patients with known myocardial deposition; additional amyloid deposits were also imaged in the lung, thyroid, salivary glands and tongue leading to the conclusion that ^{99m}Tc -aprotinin could be a cost-effective approach to imaging extra-abdominal amyloid involvement in AL patients [332]. A more recent study conducted in 2003 showed similar results in 23 patients with known or suspected AL amyloidosis; uptake of ^{99m}Tc -aprotinin was, again, visualized in the heart and other organs previously mentioned. Of note, this study included a patient which showed positive myocardial uptake of the radiotracer but had no clinical signs of cardiac involvement; thus, ^{99m}Tc -aprotinin was able to detect “clinically silent” amyloid pathology in an individual [343]. Conclusions from this study revealed that ^{99m}Tc -aprotinin may be useful for detection of extra-abdominal amyloid lesions, but, due to renal excretion, “it is not suited for detection of amyloid deposits in kidneys or bladder” [343]. Other limitations include the inability of the radiotracer to reveal amyloid within the central or peripheral nervous system and nonspecific binding in the liver. While aprotinin has been used by several institutions world-wide, it has been suspended from clinical use in the United States due to adverse effects seen in studies utilizing the drug to reduce bleeding during heart surgeries [344].

Thus, 11-1F4 and aprotinin have been developed for imaging amyloid deposition in patients, but there are limitations to each of their use—namely, the antibody has not shown efficacy in detecting cardiac or renal amyloid, and aprotinin, which has shown merit in cardiac amyloid detection, can no longer be used in the United States.

3.2.2.3. Visceral amyloid-related agents

Given the need for amyloid imaging agents, some investigators have explored the use of radiolabeled amyloid components as a strategy to detect the deposits. There are two amyloid-associated agents that have been utilized: serum amyloid P component (SAP), which is found ubiquitously in amyloid deposits and β -2-microglobulin (β 2M), which is found in amyloid fibrils related to dialysis-associated amyloidosis.

Of all the imaging agents developed for amyloidosis, SAP has been most widely investigated and utilized clinically, albeit exclusively in Europe (UK, Italy and the Netherlands). As previously stated in section 1.2.3, SAP is a 250 kDa glycoprotein that is associated with all types of amyloid deposits with the exception of A β plaques in the brain. In an individual with no amyloid, SAP circulates in the plasma at ~ 20 mg/L; the concentration of SAP is increased in the amyloid, and there can be 20,000 mg of SAP within the deposits as well as free SAP in circulation [8]. SAP binds to the fibrillar component of amyloid deposits in a calcium dependent manner [8]. Given this, the abundance of SAP associated with amyloidosis patients has been exploited as a means of amyloid detection. It was first introduced as a potential imaging agent by Hawkins *et al.* when they discovered that ^{123}I -SAP targeted amyloid after iv injection in a murine model of AA amyloidosis as evidenced by planar scintigraphy [65]. The imaging agent was introduced into patients with amyloidosis in a study conducted by Hawkins,

Lavender and Pepys in which 50 biopsy-proven patients were enrolled (25 AL and 25 AA patients) [345]. The protocol for this study included iv administration of ^{123}I -SAP into patients and control individuals which was then followed by whole body scintigraphic imaging 24 hours post injection. This landmark study revealed that ^{123}I -SAP imaged amyloidotic organs in all confirmed patients, and uptake was also seen in 6 individuals whom had negative biopsies prior to the study. These patients either underwent subsequent biopsies or tissue was analyzed at autopsy, and amyloid disease was later confirmed in all 6 patients [64]. The control individuals showed no uptake of the tracer. A representative image of a patient with AL amyloidosis alongside an image of a healthy control can be seen in Figure 1.22. The image shows uptake of the tracer in the liver, spleen and bone marrow of the AL patient while the healthy control reveals evidence of radiotracer in the blood pool and tissues involved with hepatobiliary clearance. The specificity of ^{123}I -SAP localization to amyloid deposits was confirmed at autopsy in 6 patients [64]. Additionally, the investigators found that the “density of amyloid deposition demonstrated histologically in the various tissues corresponded exactly to the quantity of tracer present in the *in vivo* images” [345]. This study indicated that, although the tracer cannot provide convincing images of cardiac amyloid, ^{123}I -SAP can be used as a diagnostic tool for amyloidosis and as a method to ascertain disease progression or regression for AL and AA patients [346]. With the success of this tracer, scintigraphic imaging with ^{123}I -SAP has been incorporated into the clinical management of many patients at multiple institutions in Europe [56, 64, 345, 347-350]; the tracer, however, is not FDA approved and cannot be used as a means of determining amyloid

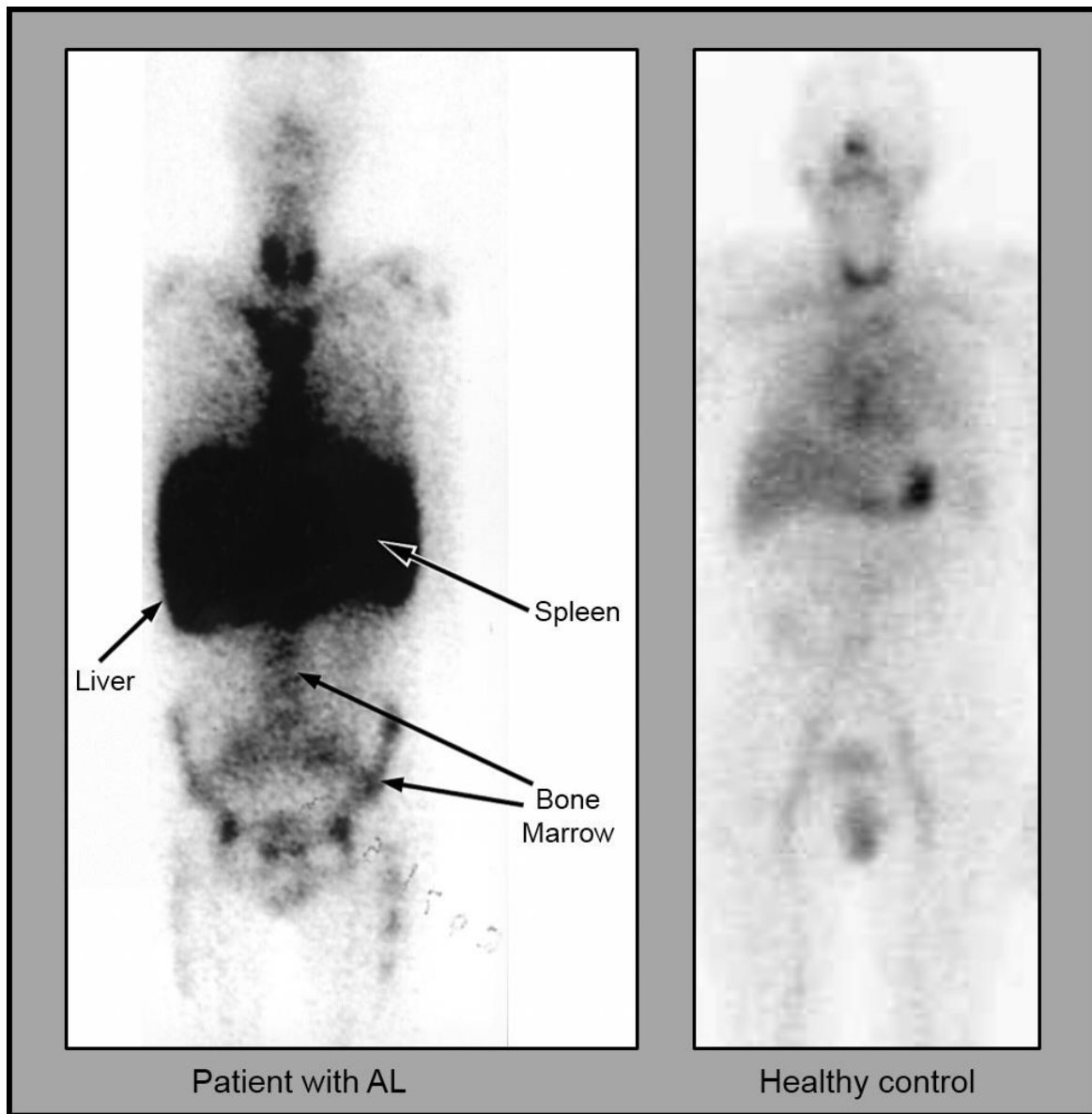


Figure 1.22: ^{123}I -SAP planar scintigraphy acquired 24 h pi in an AL patient and a healthy control. The image on the left shows uptake of the radiotracer in AL amyloid-laden tissues such as the liver, spleen and bone marrow of a patient. (Image taken from [351]). The distribution of ^{123}I -SAP (and free ^{123}I -iodide) in a healthy individual is shown on the right to account for non-specific accumulation of the radiotracer at 24 h pi. (Image taken from [352]).

distribution for patients in the US. An alternative whole-body imaging agent is needed for patients that cannot undergo an SAP imaging acquisition.

The final amyloid component that has been developed as an imaging agent for amyloidosis detection is ^{111}In - or ^{131}I - $\beta 2\text{M}$. Patients that have undergone chronic dialysis for longer than 10 years are at risk of developing dialysis related amyloidosis (DRA) which involves an over-abundance of circulating $\beta 2$ -microglobulin rendering it amyloidogenic ($\text{A}\beta 2\text{M}$) [161, 353]. The amyloid is typically deposited in soft tissues surrounding joints but can also affect the gastrointestinal tract, lungs and heart [1]. It has been reported that the relatively safe methods of amyloidosis diagnosis—Congo red histological analysis of a fat aspirate or rectal biopsy—fail in the cases of DRA [354-359]; additionally, the method that can, indeed, detect $\text{A}\beta 2\text{M}$ involves examination of amyloid within synovial fluid invasively extracted from joints such as the knee, ankle, wrist or shoulder [354, 358, 360]. As an alternative to these invasive strategies, imaging agents for $\text{A}\beta 2\text{M}$ detection have been investigated. While ^{123}I -SAP can detect some amyloid deposits in long-term hemodialysis patients, accumulation in other sites such as joints in the hips and shoulders were not accomplished with this tracer [361]. Studies conducted by Floege *et al.* demonstrate that scintigraphy with radiolabeled $\beta 2\text{M}$ could adequately visualize $\text{A}\beta 2\text{M}$ amyloid deposition in patients with confirmed DRA; patients that underwent hemodialysis but had no evidence of amyloid deposition were also administered the tracer, and no uptake was detected [361]. They used either ^{131}I - $\beta 2\text{M}$ or ^{111}In - $\beta 2\text{M}$ to assess amyloid deposition, and they determined that the ^{111}In - $\beta 2\text{M}$ provided improved image contrast and sensitivity along with reduced radiation exposure. This imaging agent could offer a non-invasive approach to diagnosing DRA

in patients with end-stage renal failure [360], but it is not applicable to other forms of systemic amyloidosis.

4. Preclinical evaluation of amyloidosis

4.1. Model systems

Based on the previous description of amyloid-specific radiotracers, there is a clear need to develop alternatives, particularly for whole body imaging in the US. To discover and optimize new amyloid-targeting imaging agents, preclinical models of the disease have been developed. Proper assessment of novel techniques and reagents must be performed prior to their introduction into a clinical setting; this section discusses many of the model amyloid systems, both *in vitro* and *in vivo*, that are utilized by investigators and employed in this thesis for studying new potential imaging agents for amyloidosis.

4.1.1. Synthetic fibrils

Given that all types of amyloid deposits contain proteinaceous fibrils, one of the earliest model systems that has been developed for *in vitro* studies of amyloidosis is synthetic fibrils. The use of synthetic fibrils gives insight into the structural, biochemical and biophysical properties of protein polymerization, or fibrillogenesis, and has been employed to study numerous types of protein fibrils [81, 182, 362, 363]. This model system can elucidate protein-protein interactions in a simple, non-biological environment—an advantage and limitation of this method. A certain dye, known as

thioflavin T (ThT), is a fluorescent marker that binds to all amyloid fibrils; upon binding, the emission intensity of ThT at 490 nm, as measured with a fluorimeter, increases by orders of magnitude which indicates the presence of fibrils or the process of fibril formation if measurements are taken over time [364, 365]. This type of assay can also be used to evaluate potential inhibitors of synthetic fibril formation [366]. Additionally, the *in vitro* formation of amyloid-like fibrils facilitates high resolution structural studies of the fibrils and the intermediates that exist on the pathway of fibrillogenesis. Techniques such as X-ray diffraction [367], solid state nuclear magnetic resonance [368, 369] and cryo-electron microscopy [370] have been performed on various amyloid-like synthetic fibrils—processes that would not be amenable using tissue amyloid extracts due to the heterogeneity and complexity associated with such samples. Synthetic fibrils composed of the variable domain of λ 6 light chains, e.g. V λ 6Wil, are generated [81] and used routinely in our laboratory.

4.1.2. Tissue extracts

Since there are more components to amyloid deposits than solely the fibrils, amyloid extracts derived from tissues provide a more complex model system of the disease. Tissue extracts derived from autopsy materials (livers and spleens), of patients with AL amyloidosis have been used in characterization studies of potential amyloid-binding agents [363, 371]. Patient amyloid extracts are often prepared using techniques which removed extraneous materials leaving amyloid fibrils, proteoglycans and minimal SAP components within the samples [244]. In contrast, tissue amyloid extracted from patient material or from mouse organs (e.g., with AA or ApoA2c amyloidosis) yields homogenate samples which contain all the essential amyloid

components mentioned above, but they might be complicated by the presence of other polymers such as intracellular materials. When compared to synthetic fibrils, the use of tissue extracts provides an *ex vivo* environment that more closely resembles the composition of amyloid material *in vivo*. This model system is amenable to characterization studies which assess the binding affinity of certain amyloid-targeting probes—namely those to be utilized *in vivo* as amyloid imaging agents (as is presented in this thesis). Specificity of such agents can be established using this amyloid substrate by various techniques such as sodium chloride (NaCl) titrations (to assess the strength of electrostatic interactions) and heparinase treatments (see later sections). Heparinase treatment has been applied to AL patient extracts to remove the HSPG component and determine if amyloid-binding agents can interact with amyloid in the absence of this prevalent component [363]. These studies were used to elucidate the heterogeneity of patient AL extracts as indicated by the vastly differing binding abilities of a single agent on diverse AL λ and AL κ preparations. These component-specific studies would not be possible without access to “complete” amyloid present in extracts; synthetic fibrils alone would not be as informative for analyzing amyloid-peptide interactions. The amyloid extract model system can help characterize amyloid-targeting agents, but the possible hindrances that exist *in vivo*, such as tissue vascularity and competing “sinks” for the agent, are absent in this system; thus, positive findings *ex vivo* may not fully translate to the *in vivo* system—something I explore in this thesis.

4.1.3. Mice

Experimental *in vivo* models of amyloidosis are the most informative system for evaluating amyloid-targeting agents and/or potential therapeutics. Given that there are

several amyloidoses which are known to affect various tissues, many mouse models have been developed to facilitate the investigation of these diseases; however, the repertoire is incomplete. Although there are several mouse models of Alzheimer's disease which deposit localized A β amyloid, they are beyond the scope of this thesis. The following section discusses mouse models of visceral amyloidosis (e.g., AA, ApoA2c, ATTR, A β 2M and AL).

The most historically used model is that of AA amyloidosis which relies on the induction of amyloid deposition by the injection of compounds including casein [372], silver nitrate [373] and lipopolysaccharide [374]—all of which stimulate the production of pro-inflammatory cytokines, such as IL-1 and IL-6, and the subsequent secretion of SAA [27]. The repeated induction of an inflammatory stimulus causes an overproduction of SAA ultimately leading to AA amyloidosis. This model has been used extensively to study the disease and is believed to mimic amyloid distribution seen in patients with AA amyloidosis [375], but in the absence of the stimulus, amyloid deposition can resolve spontaneously with time [27, 376]; thus, there are limitations to the types of studies which can be conducted.

An alternative model for AA amyloidosis is a transgenic mouse which constitutively expresses human IL-6 leading to the overproduction of SAA. The transgenic mouse strain, B6(C)-Tg(H2-Ld_IL-6)Kish(H2/huIL-6), was developed at the National Institutes of Health as a model for studying myelomas and plasmacytomas [377, 378]. The C57BL/6 mouse germ line was modified by the introduction of human IL-6 cDNA with transcriptional control mediated by the murine major histocompatibility complex class I (H-2Ld) promoter. These mice (H2/IL-6) were crossed with mice of

BALB/c genetic background [379], and after more than 20 generations had been performed, spontaneous AA amyloidosis was detected in the spleen, liver, tongue, heart, intestines and kidneys by 8 to 9 months of age [378].

The amyloid deposition associated with both mouse models of AA could be induced by injection of AEF [380, 381], but the transgenic model circumvents the need for repeated inductions of inflammatory stimuli, and it provides a system to study early-onset, spontaneous, chronic AA disease which has proven suitable for both amyloid-targeting and therapeutic studies.

As an experimental model for mouse senile amyloidosis,—a disease which demonstrates progression in an age-associated manner—transgenic mice were developed that overexpress apolipoprotein A-2c (ApoA2c) under the cytomegalovirus immediate early gene enhancer/chicken beta promoter on the apoA-II gene [382]. The ApoA2c protein self-associates to form amyloid fibrils (AApoA2c) in these mice as they age. Other allelic variants of this gene, namely ApoA2a and ApoA2b, are unable to form amyloid fibrils in transgenic mice [383]. An abundance of ApoA2c in combination with administration of pre-formed AApoA2c fibrils (8 to 10 µg) generated severe amyloidosis which can be observed in the testes, liver, kidneys, heart, lungs, spleen, tongue, stomach and intestine [382]. While the human form of AApoA2c amyloidosis is extremely rare (< 10 cases described in the literature), this model of the disease offers another *in vivo* system in which potential amyloid-targeting agents can be evaluated, particularly in the heart where significant amyloid is observed.

Mouse models of human ATTR amyloidosis have been produced, but “they have not been as successful as investigators would wish” [384]. These models involve the expression, as transgenes, of both mutant and wild-type human TTR genes, and while deposition does occur in some tissues, the model does not consistently generate amyloid deposits; additionally, the deposition does not recapitulate the neuropathology seen in patients [384]. Some studies involve the introduction of TTR genes into C57Bl/6 x DBA/2 F1 mice in which the inserted human genomic DNA contained all elements known to regulate tissue specific expression; this technique, however, led to deposition in organs that are not known to synthesize the protein [385]. Later studies found that approximately 80 % of transgenic mice modified with wild type human TTR older than 18 months of age developed non-fibrillar deposits; the remaining 20% of these mice developed lesions consistent with the human condition [386]. A model utilizing insertion of human genomic DNA from a patient with FAP confirmed these same findings in that mice most commonly generated what was considered to be “non-fibrillar, amorphous aggregates” as indicated by the presence of human TTR with immunohistochemical analysis and a lack of Congo red staining in histological examination [384]. In mice where amyloid is deposited, the hepatic and cardiac deposits are similar to those seen in patients; additionally, the late onset of the disease is analogous with the age-associated manner of disease progression that affects patients [387-390]. Overall, these studies suggest that since there is inconsistent amyloid deposition in mice, this system may be most useful for only evaluating TTR aggregates which may form prior to amyloid deposition.

As previously stated, patients that undergo long-term hemodialysis are at a risk of developing DRA by the deposition of amyloidotic β 2M [391, 392]. Studies indicate that murine β 2M is not amyloidogenic; thus, a transgenic model generated to overexpress human β 2M (h β 2M) in mice with β 2M knockout background has been developed [393, 394]. The concentration of h β 2M in mouse plasma measures 100 times greater than that seen in normal human plasma, and it is more than 4 times that measured in long-term dialysis patients [394]. In regard to these plasma levels, the mice are similar to patients seen clinically. The amyloid deposition that is associated with patients, however, was not observed in the transgenic mice; even by 2 years of age, there was no birefringent material detected in the mice when tissues were examined with Congo red stain [394]. The model may provide information on the diseased state of animals with elevated h β 2M levels, but the lack of amyloid deposition makes it unlike the human condition. These data also imply that certain pro-amyloid host factors may be present in man but lacking in mice.

Given that AL is the most common form of amyloidosis in humans, a mouse model of the disease for experimental studies of potential amyloid-targeting agents for detection or therapy would be of great value. Investigators have used different approaches to reproduce the disease in mice, but a model that closely resembles the pathology seen clinically is still lacking. One model involved the intraperitoneal injection of various amounts of λ 6 Bence Jones protein (BJP; extracted from AL patient urine) into C3H/HEJ mice [395]. These mice received multiple injections over several weeks. It was found that a single injection of 300 mg BJP was unsuccessful at producing amyloid deposits in mice euthanized 48 hours post injection; similarly, repeated

injections of 100 mg over 2 to 5 weeks, which was the equivalent of 900 to 2200 mg of protein, resulted in no amyloid deposition [395]. Slight renal deposition was detected only after mice had received ~ 5300 mg BJP over a 12 week period. Since this requires large amounts of patient material, they employed a strategy that had been previously used to accelerate the induction of renal casts in mice receiving BJP—dehydration of the mice (48 hour without water prior to BJP injection) increased the extent of deposition [395, 396]. Amyloid deposition occurred in mice that were dehydrated for 24 hours prior to each of ten 200 mg injections of BJP over an 8 week period (2 g BJP total)—renovascular deposits within vessel walls and the interstitium were Congo red positive. This model proved to be useful for the investigation of amyloidogenicity of certain BJP, but as previously stated, there is a need for large amounts of patient materials which is not ideal for an experimental animal model since a limited amount of patient-derived BJP may hinder experimental protocols. Additionally, the repeated injection of BJP in immunocompetent mice could result in a mouse-anti-human immunological response [397]. The administration of AL patient-derived BJP into mice was used in another model in which the light chains were injected (1 mg per day) through the mouse penile vein; renal tissues were examined for amyloid deposition at various time frames, up to 14 days, post-injection [398]. This model was generated as a means to investigate mesangial glomerular amyloid deposition that contributes to the renal failure seen in AL patients. By 12 h pi of patient light chains, mice had extracellular deposition of amyloid fibrils; at 7 d pi, amyloid deposition was clearly evident, but by 14 d pi, amyloid formation was abundant as evidenced by transmission electron microscopy and polarized Congo red stain. As stated by Teng et al., this model system “is potentially a

significant scientific advance toward future development of new therapeutic avenues to ameliorate the progressive renal failure which represents the typical clinical course of patients with AL amyloidosis with renal involvement” [398]. Since the aforementioned AL mouse models involve the introduction of potentially limited patient-derived materials, and there is potential for a mouse immune response to injected materials, the use of this model for long-term, *in vivo* AL studies may be challenging. To circumvent these obstacles, a transgenic mouse model was generated by insertion of cDNA from a $\lambda 6$ AL patient who had amyloid involvement in the heart, liver, kidney, spleen and adrenals [397]. While the transgenic mouse was able to produce human light chains, Congoophilic amyloid material was only found in the lumen of gastric glands of the stomach. The amyloid was of light chain origin, but the clinical manifestation of AL amyloid is not typically localized to the stomach nor presents with this type of distribution. This transgenic mouse model is useful for AL amyloid detection which is amenable to potential oral therapeutics [397], but it is not representative of the disease state which affects humans.

4.1.4. Naturally occurring disease models

For certain amyloid diseases, there are naturally occurring animal models. As previously mentioned, AL is the most common amyloid disease to affect humans. In other mammals, however, AA amyloidosis is the most common form of the disease. Two of the most widely recognized naturally occurring models are in Chinese Shar pei dogs and Abyssinian cats. AA amyloidosis in the former, which presents much like an inherited disorder known as familial Mediterranean fever (FMF) in humans, causes renal disease; however, the gene responsible for FMF differs from that seen in dogs [399,

400]. Studies suggest that Chinese Shar pei dogs with renal amyloidosis in combination with recurrent fever of unknown origin may be useful as a naturally occurring model of human disease due to the common organ distribution [400]. In Abyssinian cats, renal medullary and glomerular amyloidosis along with papillary necrosis has been observed as a result of familial predisposition; studies suggest that this amyloid is AA and that these animals may be a naturally occurring model of familial secondary amyloidosis or FMF leading to papillary necrosis [399-402].

In addition to naturally occurring cases of AA amyloidosis, feline diabetes mellitus is another common naturally occurring model of amyloidosis in that there is deposition of IAPP detected in the pancreatic tissue, most commonly within the islets of Langerhans, of cats [403-405]. It has been postulated for years that AIAPP could play a significant role in the impairment of islet cells in individuals with Type 2 diabetes mellitus; islet amyloid deposition occurs in more than 90% of humans with the disease and more than 65% of adult diabetic cats [166]. Given that diabetic cats generally exhibit the same amyloid distribution detected in human pancreatic tissue, this naturally occurring animal model may prove valuable for investigating IAPP amyloid-targeting agents and can be used to evaluate *in vivo* efficacy and toxicology.

4.2. Preclinical amyloid-targeting agent: Peptide p5

Since the clinically available amyloid-targeting agents discussed above (Section 3.2.2) have limitations to their use, much focus has been directed toward the preclinical development and translation of novel agents. For example, the camelid antibody, B10,

has been shown to target a particular epitope on A β and AL fibril fragments through an electrostatic interaction by using *in vitro* binding studies [362, 406, 407]. In addition, we have shown that the small chain variable fragment (scFv), NS4F5, which interacts with a unique hypersulfated form of HS, also preferentially binds amyloid deposits as evidenced by SPECT/CT imaging [49, 71-73, 408]. These preclinical agents have demonstrated the utility of electrostatic interactions for targeting amyloid deposits. They also have limitations to their use. For example, B10 has not been shown effective as a pan-amyloid binding agent [362], and the amyloid-NS4F5 interaction was transient, and this probe was incapable of detecting amyloid at 4 h pi [73]. These limitations led to our further investigation of synthetic peptides as an alternative amyloid-targeting agent.

Since the HSPG in amyloid appears to have sulfation patterns more like heparin [7] and is distinct from the HS in all other tissues and organs, recent studies have focused on the idea of amyloid-targeting through GAG involvement and have explored the use of heparin-binding peptides for amyloid detection [409]. The attention shifted from scFv to peptide development because “peptides can be chemically synthesized, are rapidly cleared from the circulation when not bound to target, are relatively small, can penetrate blood vessel walls and are generally nonimmunogenic” [409-413]. Seven heparin-binding peptides were radiolabeled with ¹²⁵I and investigated by small animal SPECT imaging, biodistribution measurements and micro-autoradiography in H2/IL-6 AA mice (previously described in section 4.1.3). These peptides, referred to as p1 through p7 in the comparative study, all exhibited a polybasic amino acid motif but revealed variable affinities for AA amyloid *in vivo*, as evidenced by SPECT/CT, biodistribution measurements and microautoradiography studies [409]. The lead

candidate peptide after image analysis at both 1 hour post injection and 4 hours post injection was peptide p5—a 31 amino acid peptide whose parent structure, referred to in the literature as K8, was initially synthesized for heparin neutralization [414]. The polybasic sequence of p5 includes a heptad repeat Lys-X-X-Lys-X-X-X (where X is Ala or Gln) which provides a +8 net positive charge and was predicted to adopt an α -helical conformation. Interestingly, the secondary structure prediction illustrated that the basic lysine residues aligned to one face of the helix as seen in Figure 1.23. Previous circular dichroism studies with K8 indicated that the peptide is, indeed, α -helical in the presence of heparin [414]. These experimental data, together with the predicted structure of the peptide, led to the hypothesis that the binding of p5 with HSPG in amyloid deposits may be the result of an electrostatic interaction between the polybasic surface of the α -helical peptide and the enhanced sulfation pattern uniquely seen in amyloid-associated HSPG. Microautoradiography of tissue samples obtained after the comparative peptide SPECT imaging study revealed that p5 preferentially bound amyloid as evidenced by the co-localization with amyloid in multiple organs (Figure 1.24). This specificity has been further demonstrated by staining amyloid with biotinylated p5 in formalin-fixed tissue sections from patients with e.g., AL κ , AL λ , ATTR and A β [409].

Given the amyloid-targeting specificity of peptide p5 in the aforementioned SPECT imaging study of AA mice, a dual energy SPECT/CT comparative effectiveness imaging analysis was performed by comparing p5 and the clinically available agent, SAP. This study allowed for the direct comparison of p5 distribution with SAP distribution in the same H2/IL-6 AA mouse [293]. SAP was radiolabeled with ^{125}I and injected into the lateral tail vein of mice; at 22 h pi, $^{99\text{m}}\text{Tc}$ -p5 was injected into the same

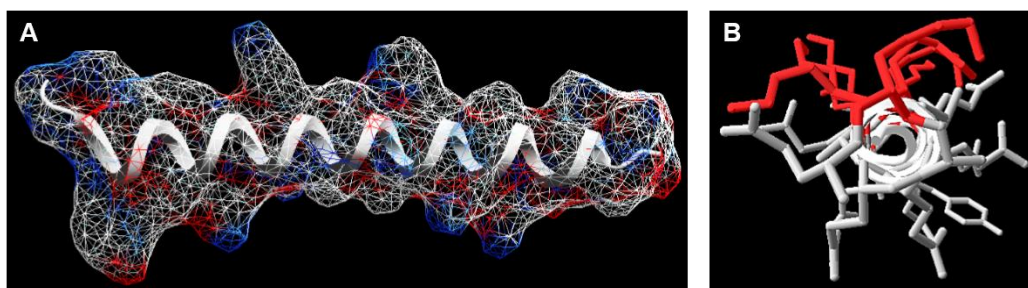


Figure 1.23: Predicted structure of peptide p5. Structure was generated by I-Tasser [415], and images were adapted from [416].

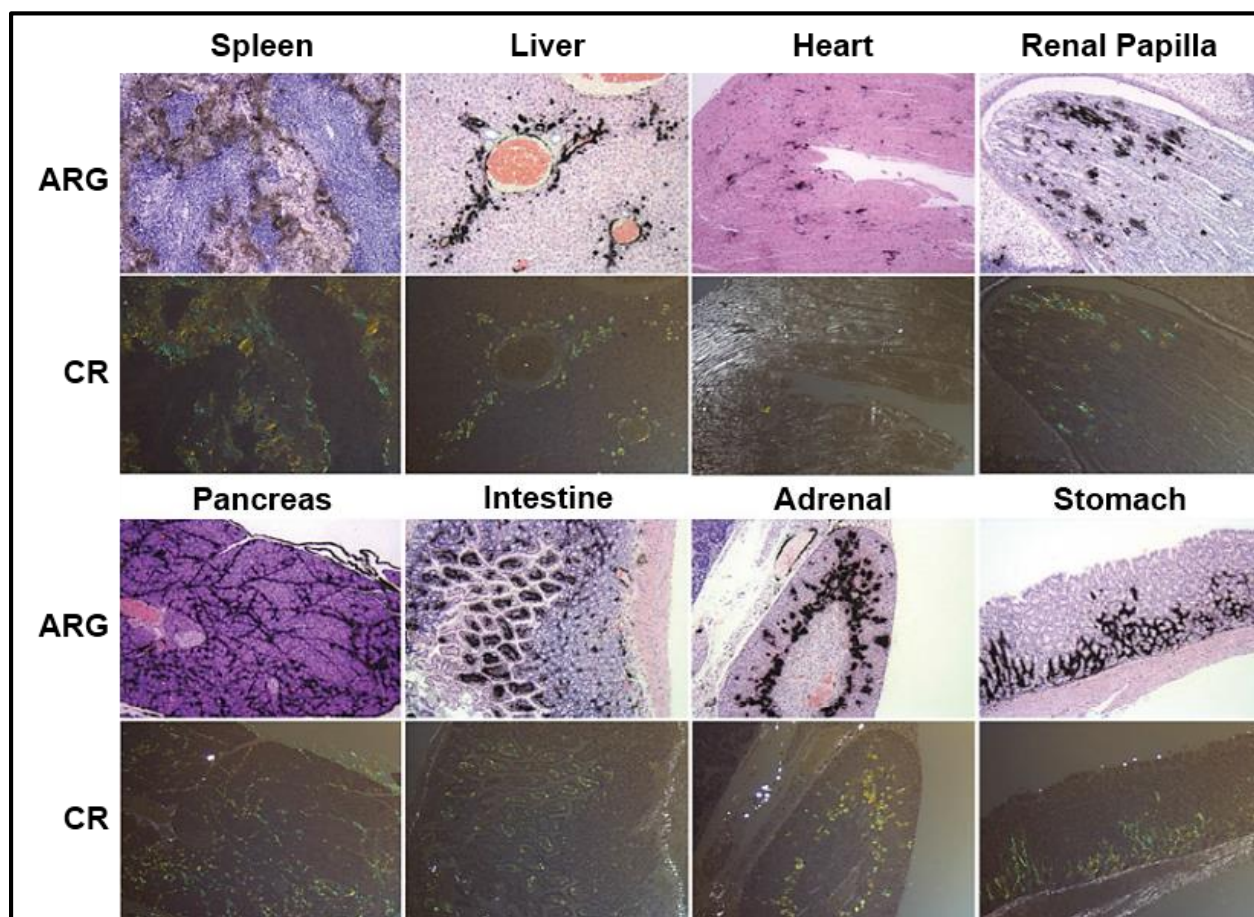


Figure 1.24: Microautoradiography (ARG) of ^{125}I -p5 distribution in various AA mouse tissues and Congo red (CR) histological confirmation of peptide co-localization with amyloid. (Image taken from [409]).

mice. They were euthanized and imaged at 24 h pi of ^{125}I -SAP (adhering to clinical protocol) and 2 h pi of $^{99\text{m}}\text{Tc}$ -p5 [293]. The resulting images reveal that both SAP and p5 were able to localize to hepatic and splenic amyloid, but p5 bound to pancreatic and intestinal amyloid more effectively than SAP (Figure 1.25). Conclusions from this comparison suggest that p5 was a valid alternative to SAP and could fill a clinical need for patients in the United States.

Additional studies with p5 have been conducted to show that the amount of ^{125}I -p5 that localizes to amyloid-laden mouse tissues after injection correlated with the amount of amyloid within the tissue [417]. This study compared amyloid load assessed by quantification of Congoophilic, birefringent material in tissue sections with ^{125}I -p5 uptake, i.e. biodistribution measurement (percent injected dose of peptide/gram of tissue), of the same tissue. The amount of peptide p5 uptake and birefringent material correlated linearly with a correlation coefficient of > 0.7 and a p value less than 0.00001 [417], which indicated that p5 could not only detect amyloid deposition but that it could do so in a quantifiable manner.

While the initial hypothesis implied that peptide p5 binds to amyloid-associated HSPG, recent work has demonstrated that heparinase-treated, human AL amyloid extracts could still exhibit an interaction with p5. This study compared p5 binding to various AL patient amyloid extracts before and after heparinase treatment utilizing an *ex vivo* binding assay [363]. The data indicated that binding of p5 to many of the extracts was decreased, but not completely abolished, after the removal of the HSPG sulfate groups, suggesting that p5 may have another target in amyloid deposits. This was confirmed when the peptide was shown to bind synthetic AL amyloid fibrils composed

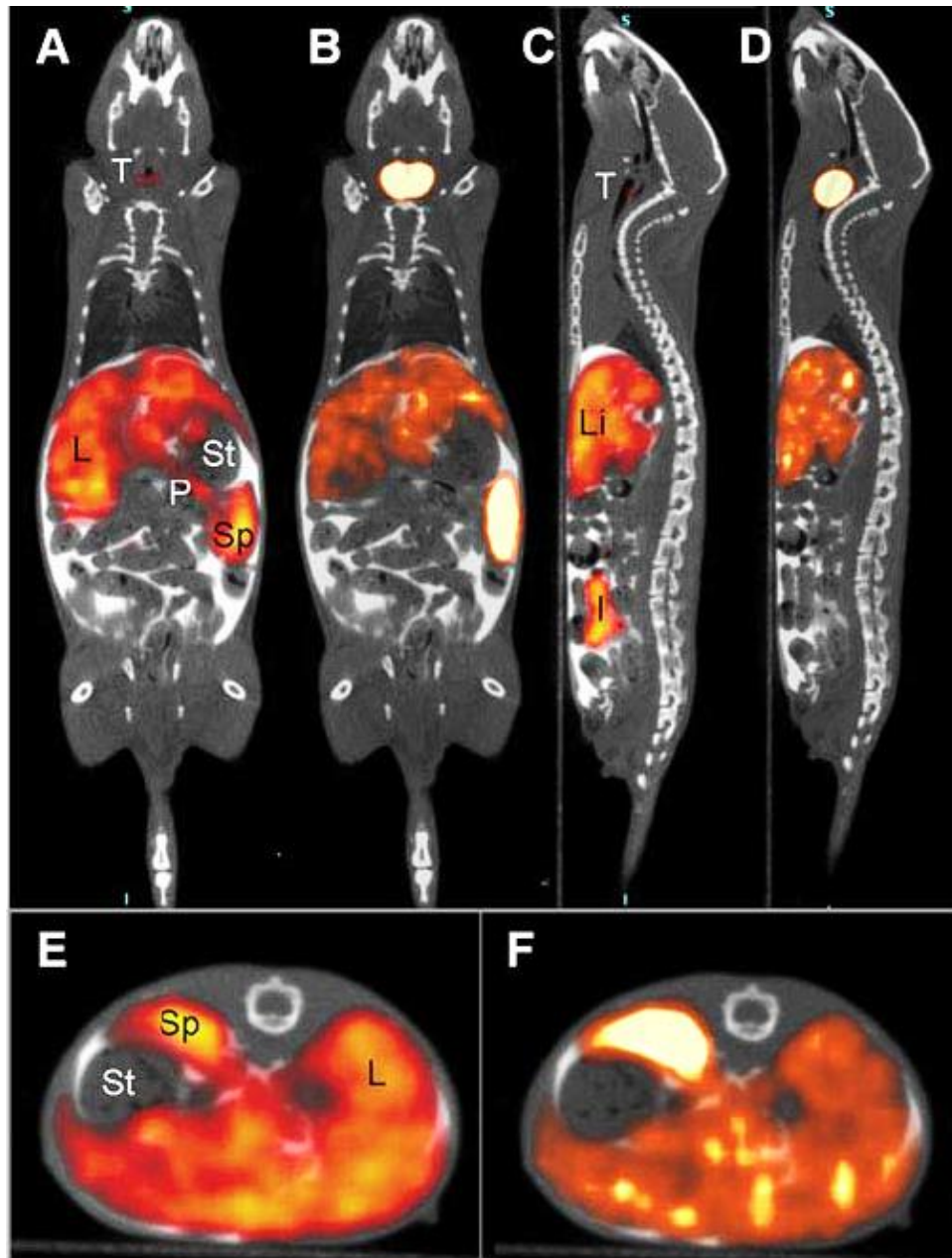


Figure 1.25: Dual energy SPECT/CT images illustrating $^{99m}\text{Tc-p5}$ and $^{125}\text{I-SAP}$ distribution in an individual AA mouse. (A, B and E) Distribution of $^{99m}\text{Tc-p5}$. There was uptake in the liver (L), spleen (Sp) pancreas (P) and intestines (I). The stomach (St) and thyroid (T) were devoid of radiotracer. (C, D and F) Distribution of $^{125}\text{I-SAP}$. The image revealed uptake in the liver and spleen. The stomach showed no uptake of radiotracer, but the thyroid sequestered free ^{125}I -iodide as a result of radiotracer dehalogenation. These images depict that radiolabeled p5 provided more information to the whole body amyloid burden than radiolabeled SAP. (Image taken from [293]).

exclusively of rV λ 6Wil [363]. Furthermore, Scatchard analyses supported the “multiple target” hypothesis of p5 revealing that the peptide, in the presence of tissue amyloid homogenate, has 2 binding sites which are, hypothesized to be HSPG and fibrils [371].

Although the peptide has proven efficacious at targeting amyloid in multiple systems and in various studies, certain biological parameters of p5, specifically the kinetics of the radiotracer *in vivo* and the stability of peptide-amyloid interaction, were undeterminable using the methods mentioned in previous studies. To gain insight into the biodistribution and pharmacokinetics of p5 in diseased and healthy animals, dynamic PET imaging acquisitions were performed, and the time activity curves generated from the data illustrated that the peptide is rapidly cleared from the blood pool in both WT and AA mice. The accumulation of ^{124}I -p5 in amyloid-laden liver and spleen began abruptly after blood pool clearance and remained stable throughout the 2 h duration of the acquisition; in healthy mice, at ~ 7 min pi, the radiotracer uptake peaked in the kidneys then washed out leaving no accumulation of intact ^{124}I -p5 in any tissues—only free isotope was detected in the stomach and thyroid due to dehalogenation in the kidneys. The kinetic profiles of diseased and healthy mice were drastically different which could facilitate diagnosis of amyloidosis (Figure 1.26). Additionally, this study revealed that the dehalogenation of p5 in the absence of amyloid target was important and could be beneficial for imaging renal amyloid. The stability of p5 binding was also assessed, and there was evidence of ^{125}I -p5 bound to amyloid in SPECT/CT images acquired at 72 h pi (Figure 1.27). With such stability comes the advantage of waiting “extended periods of time post injection without the loss of positive signal” [416]. This allows for clearance of background noise and enhances the

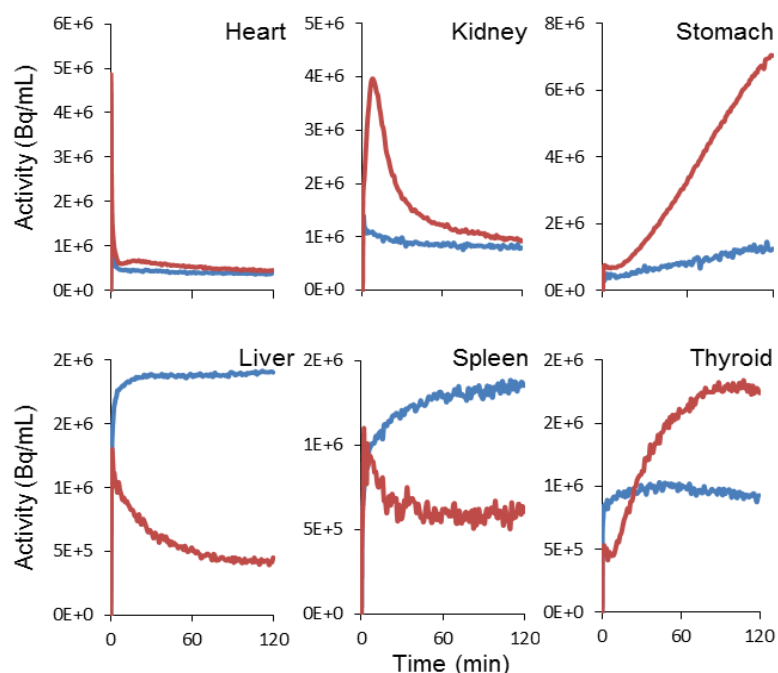


Figure 1.26: ^{124}I -p5 PET dynamic time activity curves. The radiotracer distribution throughout a 2 h acquisition in AA mice (blue) and WT mice (red) is shown. Notice the rapid blood pool clearance (heart) in both diseased and healthy mice. Additionally, there was evidence of radiotracer clearance through the kidney in WT mice which led to stomach and thyroid accumulation. The liver and spleen of diseased mice showed rapid ^{124}I -p5 accumulation. These distinct kinetic profiles could identify diseased mice. (Image adapted from [416]).

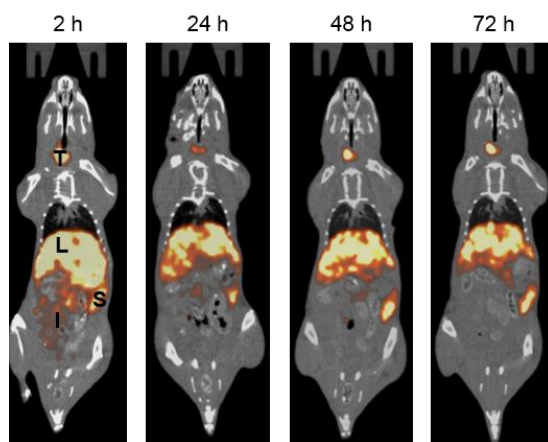


Figure 1.27: SPECT/CT images revealing the stability of ^{125}I -p5 binding to amyloid. AA mice were administered ^{125}I -p5 and imaged multiple times pi. The images indicate radiotracer stability in amyloid-laden tissues for at least 72 h. (Image taken from [416]).

likelihood of the critical detection of small deposits that form in early stages of the disease—a valuable property of an imaging agent for any disease but especially valuable for the under-diagnosed, yet deadly, amyloidosis.

Chapter 2

Project Rationale and Summary

The systemic amyloidoses are particularly devastating diseases which are thought to be clinically underdiagnosed due to their heterogenous nature and the, often times, inconclusive presentation of clinical symptoms. With the most common forms of systemic amyloidosis, namely light chain amyloidosis and hereditary or wild type transthyretin amyloidosis, renal and cardiac pathological involvement contribute to the most ominous prognoses for patients. Since these organs are typically affected in the later stages of the disease, it is of the opinion of many researchers and clinicians that early diagnosis could lead to the implementation of appropriate treatments and, thus, better prognoses for patients. Currently, the accepted method of amyloidosis detection and diagnosis involves histological (Congo red) evaluation of biopsy-derived tissue samples – an invasive procedure prone to clinical complications. The use of Congo red for amyloid detection in tissue samples has been employed for decades, but for untrained observers, the samples can be difficult to interpret. Additionally, this method is subject to sampling error and cannot offer insight into the whole body distribution or the extent of amyloid deposition in an individual. Therefore, a more facile, non-invasive method of amyloid detection would be valuable to both physicians and patients.

While histological examination of tissue specimens remains the gold standard for disease diagnosis, efforts directed toward the development of amyloid-targeted imaging agents have been made. The following imaging agents are clinically available or are in

human clinical trials for systemic amyloidosis: ^{123}I -SAP, $^{99\text{m}}\text{Tc}$ -aprotinin, $^{99\text{m}}\text{Tc}$ -DPD/HDP, ^{11}C -PiB, ^{18}F -PiB, ^{18}F -florbetapir, and ^{124}I -11-1F4. These agents, however, all have limitations which include the lack of availability in the United States, the inability to detect cardiac amyloid deposits, isotope half-life constraints and inconsistent findings, i.e. the inability to detect all known amyloid-laden organs in a patient. Given these restrictions, the development of novel amyloid-imaging agents is of clinical import.

Since the composition of amyloid deposits contains a unique, hyper-sulfated form of heparan sulfate proteoglycan which “looks” biochemically and electrochemically similar to heparin, we have investigated a series of heparin-binding peptides as potential amyloid-targeting and imaging agents. These studies elucidated positive binding characteristics of one peptide in particular, known as p5. This peptide has been shown *in vivo* to preferentially co-localize to amyloid deposits found in the murine H2-IL6 model of AA amyloidosis, and comparative studies of p5 and SAP in this mouse model indicate that the peptide may be a valid imaging agent for AA amyloidosis. Histological examination of the peptide on tissue samples of various other visceral amyloid diseases—AL κ , AL λ and ATTR—demonstrated co-localization of the peptide with these forms of amyloid also. Since there are no well-defined experimental or naturally-occurring animal models of AL or ATTR in which to further validate the peptide, another murine animal model of amyloidosis, an ApoA2c transgenic model, was used for additional *ex vivo* and *in vivo* testing of p5.

Although the peptide p5 has been shown to interact with multiple types of amyloid including human AL, AA and ATTR as well as murine AA, p5 was not detected in ApoA2c tissue homogenates in an *ex vivo* peptide-binding assay. This lack of

interaction was also seen *in vivo* where radiolabeled p5 peptide was incapable of binding and imaging amyloid in ApoA2c mice. This conundrum led us to develop other synthetic peptides as prospective imaging agents, based on the structure of the parent p5 peptide.

In order to optimize peptides as agents for enhanced ApoA2c amyloid-targeting, multiple p5 variants were synthesized that focused on biochemical characteristics that govern amyloid detection. Initially, the modification consisted of substituting all lysine residues with arginine residues to potentially increase the α -helicity of the peptide and, in turn, the strength of amyloid binding. Next, extended peptides with four additional basic amino acid residues were created with the hypothesis that an increased net positive charge would enhance the peptide-amyloid interaction, which is principally electrostatic in nature. A peptide with an N-terminal polyethyleneglycol (PEG) modification was synthesized to investigate the supposition that extended half-life via an increase in size would improve amyloid binding and coverage. Finally, peptides with the incorporation of non-natural D-amino acids at the N-terminal were also developed and tested to hypothetically extend the half-life without altering the size of these amyloid-targeting agents and, thereby, permit greater amyloid uptake.

The work described in this thesis investigates these peptide variants for their ability to bind amyloid in both the AA and ApoA2c mouse models. Surprising findings seen using the p5 peptide and peptides evaluated herein indicate that amyloid is a complex pathology and that it may be unreasonable to speculate that one peptide could detect all amyloid types. Nonetheless, peptides optimized for the detection of certain amyloid diseases could be of clinical import for diagnosing the diseases and monitoring

responses to appropriate therapies. An optimized peptide would be invaluable and permit earlier detection of amyloidosis.

Chapter 3

Materials and Methods

3.1 Peptides and proteins

The peptides used in the following studies were synthesized by Keck Laboratories (New Haven, CT) and RS Synthesis (Lexington, KY). A table of the peptides along with their sequences, molecular weights and net charges is included (Table 3.1). Synthetic recombinant V λ 6W λ l fibrils were produced as described [81] by expression in *E. coli*. The pelB leader resulted in accumulation of the protein in the periplasmic space from where it was isolated by osmotic shock and purified by reverse phase HPLC using a C8 solid phase matrix with a gradient of acidified acetonitrile as the mobile phase.

3.2 Reagents

Ascorbic acid.....Sigma Aldrich (St. Louis, MO)

Blue dextran Sigma Aldrich (St. Louis, MO)

Chloramine T.....Sigma Aldrich (St. Louis, MO)

Heparinase I.....Sigma Aldrich (St. Louis, MO)

Iodogen Pierce (Rockford, IL)

Table 3.1: Physical properties of synthetic peptides

Peptide	Amino Acid Sequence	Molecular Weight (Da)	Net Charge (+)
p5	GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q	3257.7	8
p5R	GGGYS RAQRA QARQA RQAQR AQRAQ ARQAR Q	3481.8	8
p5R+14	GGGYS RAQRA QARQA RQAQR AQRAQ ARQAR QAQRA QRAQA RQARQ	5102.6	12
p5+14	GGGYS KAQKA QAKQA KQAQK AQKAQ AKQAK QAQKA QKAQA KQAKQ	4766.4	12
PEG-p5R+14*	PEG-GGGYS RAQRA QARQA RQAQR AQRAQ ARQAR QAQRA QRAQA RQARQ	~ 25,000.0	12
p43**	AQAYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q	3356.8	8
p50	AQAYS KAQKA QAKQA KQAQK AQKAQ AKQAK Q	3356.8	8

*The polyethyleneglycol modification is 20 kDa.

**Those in red are D-type amino acids.

The molecular weights and net charges were calculated using ProtParam [418].

Isoflurane	Abbott Laboratories (Chicago, IL)
Leupeptin	Thermo Scientific (Rockford, IL)
Lyophilized gelatin.....	Bio-Rad Labs (Hercules, CA)
NaCl	Fisher Scientific (Fair Lawn, NJ)
NaN ₃	Sigma Aldrich (St. Louis, MO)
NuPAGE™ LDS sample buffer 4X	Novex (Carlsbad, CA)
NuPAGE™ sample reducing buffer 10X	Invitrogen (Carlsbad, CA)
Phenylmethylsulfonyl fluoride	Sigma Aldrich (St. Louis, MO)
Phosphate buffered saline.....	Sigma Aldrich (St. Louis, MO)
Sodium metabisulfite	JT Baker (Phillipsburg, NJ)
Tween-20	Fisher Scientific (Fair Lawn, NJ)

3.3 Mice

Transgenic mouse models of AA and ApoA2c amyloidosis were used in the following studies.

The H2-IL6 mouse model of AA amyloidosis was generated by the incorporation of human interleukin 6 (IL-6) cDNA into the C57BL/6 germ line with transcriptional control mediated by the murine major histocompatibility complex class one promoter (H-

2L^d). These transgenic mice were then mated with BALB/c mice for >20 generations. The overabundance of constitutively expressed IL-6 in these mice led to spontaneous AA amyloidosis by 8 to 9 months of age [379]. A breeding pair of H2-IL6 mice on the BALB/c background were kindly provided by Dr. Michael Potter from the Laboratory of Genetics at the National Cancer Institute of the National Institute of Health, Bethesda, MD.

An experimental model of senile ApoA2c amyloidosis was generated by the overexpression of apolipoprotein A-2c, controlled by the cytomegalovirus immediate early gene enhancer/chicken beta constitutive promoter [382]. Production of high concentrations of the amyloidogenic ApoA2c protein, in combination with an iv injection of preformed AApoA2c fibrils (amyloid enhancing factor (AEF): 10 – 100 µg), yielded severe systemic amyloidosis. The initial breeding pair was kindly provided by Dr. Keiichi Higuchi from the Department of Aging Angiology at the Shinshu University School of Medicine, Matsumoto, Japan.

Wild type BALB/c mice from Charles river or non-transgenic H2/IL-6 littermates were used as controls in these studies.

All animals were housed in microisolator cages and provided food and water *ad libitum*. The University of Tennessee Medical Center vivarium is an IAAALAC-accredited facility, and all procedures described in this thesis were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) approved protocols and in accordance with the Guide for the Care and Use of Laboratory Animals (8th Edition, National Academic Press, Washington, D.C. 2011).

3.4 AL amyloid extract preparation

Human AL amyloid was extracted from autopsy-derived tissues, both liver and spleen were used for this purpose. The water floatation method used to separate amyloid proteins from unwanted tissue was previously described [244]. Briefly, approximately 20 g of tissue and 400 mL of 0.15 M NaCl were homogenized using a Polytron homogenizer (Binkmann Instruments, Westbury, NY) and then centrifuged at 10,000 rpm ($\sim 15,000 \times g$) for 30 minutes. The pellet was then mixed with 400 mL of 0.15 M NaCl, and then homogenized and centrifuged, again, as described above. This was repeated six or seven times; the supernatant from each centrifugation was discarded because it contained unwanted soluble proteins and non-amyloid tissue components. After serial homogenization in 0.15 M NaCl the final pellet was resuspended in 300 mL of distilled H₂O followed by further homogenization and one hour centrifugation (25,000 rpm). This step was repeated using 200 mL of distilled H₂O for two more consecutive runs; the final run was performed with 70 mL of distilled H₂O for 3 hours at 10,000 rpm. The supernatants from the final four centrifugation steps were pooled and contained the amyloid material. The pooled amyloid suspension was lyophilized using a VirTis system (Gardiner, NY) and stored at room temperature in glass vials until used. This material was supplied to me by the Human Immunology and Cancer Program at the University of Tennessee Graduate School of Medicine.

3.5 Radiolabeling peptides with Iodine-125 or Iodine-123

Several methods of radioiodination have been reported [419, 420], but the method used for our *in vivo* and *ex vivo* studies with iodine-125 (^{125}I) or iodine-123 (^{123}I) utilized the oxidative property of chloramine T [328, 421] to incorporate the radioiodide into the phenol ring of tyrosine residues within the peptide sequence. The radiolabeling procedure was described previously [328, 363, 409, 416]. Briefly, and with slight modification, a 5-mL Sephadex G-25 resin-packed column (GE Healthcare, Buckinghamshire, UK), with glass wool as a stopper was equilibrated with at least two bed volumes of carrier protein solution (0.2 μm pore size-filtered 0.1% gelatin in phosphate buffered saline [PBS], pH 7.2). Peptides were dissolved in H_2O to obtain a stock solution of $\sim 1\text{--}2\text{ mg/mL}$, and for radiolabeling, $\sim 50\text{ }\mu\text{g}$ of peptide (25 – 50 μL) was used in each reaction. The peptide was added to 5 μL of NaPO_4 buffer (pH 7.6) in a 1.5 mL-volume microfuge tube. The ^{125}I (Perkin Elmer, Waltham, MA) or the ^{123}I (Nordion, Ottawa, ON, Canada) was then added to the reaction mixture—amounts of radioiodide varied with the planned experiment. For example, *in vivo* imaging experiments required more radiolabeled peptide and, therefore, more radioactivity than *ex vivo* tissue binding assays (described in Section 3.9). The purchased isotope contained $\sim 1\text{ mCi}$ of radioactivity per 10 μL of ^{125}I or ^{123}I . Therefore, between 5 (500 μCi) and 25 μL (2.5 mCi) was used for radiolabeling peptides for *ex vivo* and *in vivo* experiments, respectively. Next, 10 μL of 2 mg/mL chloramine T dissolved in distilled H_2O was added to initiate the labeling reaction. As an oxidative agent, chloramine T was able to assist in the iodination of peptides by oxidizing iodide (I^-) into iodine monochloride which was highly reactive and bound covalently to the aromatic rings

present in tyrosine side chains [422]. The iodination reaction was complete within one minute of chloramine T addition [328]; however, any residual reaction was quenched by the addition of 10 μ L of 2 mg/mL $\text{Na}_2\text{S}_2\text{O}_5$ (sodium metabisulfite) dissolved in distilled water. Thirty microliters of blue dextran exclusion marker ($\text{MW} \sim 2 \times 10^6$) and 50 μ L of carrier protein solution were added to the reaction mixture prior to chromatographic separation of free radioiodide. The reaction mixture was introduced onto the Sephadex G-25 column and elution buffer (0.1% gelatin in PBS) added drop-wise to the column. Once the blue dextran reached the column tip, 10 “30 second fractions” ($\sim 200 \mu\text{L}$) were collected into plastic tubes to ensure that the radiolabeled peptide was collected. A constant flow rate was maintained by continuous manual addition of carrier protein solution to the top of the column. A small amount ($\sim 1 \mu\text{L}$) of each fraction was transferred into a glass tube and the radioactivity measured using a gamma scintillation counter (Packard Cobra II, Perkin Elmer, Waltham, MA) set to detect either ^{125}I or, using a larger energy detection window, ^{123}I . The fractions containing the majority of radioisotope (generally 3 fractions) were pooled and used for experiments. The radiolabeling efficiency (i.e. the yield of radioiodinated peptide relative to the starting material) was typically $> 50\%$.

3.6 Radiolabeling peptides with Iodine-124

The method used for radiolabeling with iodine-124 (^{124}I : IBA, Richmond, VA) used the same principles as those mentioned with $^{125}\text{I}/^{123}\text{I}$ radiolabeling; however, it was possible that stock solutions of ^{124}I from the manufacturer contained oxidized iodine (I_2),

instead of iodide (I^-). Therefore, ^{124}I reactions were reduced prior to the oxidation step and reaction with the peptide [416]. The protocol began with the addition of 10 μ L of 0.5 M $NaPO_4$ buffer at pH 7.6 to a 1.5 mL microfuge tube. The radioisotope was then added in an amount appropriate for the planned experiment—these were usually *in vivo* studies so the number of mice to be used was an important consideration. The stock solution was decay-corrected for 1 mCi per 10 μ L; therefore, volumes between 10 - 50 μ L were generally used. To reduce the $^{124}I_2$ to ^{124}I -iodide, 1 μ L of 2 mg/mL fresh ascorbic acid in PBS, pH 7.2, was added. The peptide, 50 μ g dissolved in H_2O (10-25 μ L), was brought up to 100 μ L by the addition of PBS and was then added to the reaction mixture. At this point, oxidation of the radioisotope was achieved by addition of 7 μ L of 1 mg/mL Iodogen dissolved in 100 % acetonitrile. Two minutes were allotted for the reaction. To fully quench the reaction, 50 μ L of 2 mg/mL ascorbic acid was then added. Approximately 20 μ L of blue dextran exclusion marker and 50 μ L of filtered 0.1% gelatin in PBS were added to the reaction mixture before being introduced to a G-25 size exclusion column (Section 3.5).

Fractions were manually collected after the blue dextran marker reached the column tip. The first fraction was collected for 30 sec and the following three fractions collected for 1 min each. Another 30 sec fraction was collected and the remaining volume discarded once it eluted. The radioactivity of the fractions were measured with a dose calibrator (Capintec, Inc., Ramsey, NJ) set to detect ^{124}I . The majority of the radiolabeled product was within the 2nd and 3rd “one-minute” fractions, but the 4th fraction also often contained a salvageable amount of radiolabeled peptide product. The radiolabeling efficiency of this protocol was also typically 50%.

3.7. Radiolabeling peptides with Technetium-99m

To conduct SPECT imaging studies which utilize multiple radiotracers (i.e. dual energy SPECT imaging), ^{99m}Tc -radiolabeling [293] of one of the tracers was necessary. The technique used to radiolabel with ^{99m}Tc yielded incorporation into the peptides.

To prepare for this technique, ~ 1 mg of SnCl_2 and ~ 1 mg of cysteine were weighed in separate 1.5 mL microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). A 5 mL Sephadex G-25 column was poured as described in Section 3.5. In the assigned reaction mixture tube, 20 μL of 0.15 N NaOH was added prior to the addition of ~ 20 μg of CGGY-p5 peptide. To the tube containing SnCl_2 , 1 N HCl was added to yield a 100 mg/mL suspension which was then diluted 100-fold by the addition of ultra-pure water. A 1:10 dilution of this solution in water yielded a final concentration of 0.1 mg/mL in Sn in 0.01 N HCl. The cysteine was made up to 1 mg/mL by addition of PBS. The ^{99m}Tc solution was supplied by the Nuclear Medicine department at the University of Tennessee Medical Center in Knoxville, Tennessee. An appropriate amount of ^{99m}Tc was added to PBS in a separate tube. At this point, there were 4 separate tubes: 1) SnCl_2 solution; 2) Cysteine solution; 3) ^{99m}Tc solution; 4) the reaction mixture containing the peptide.

Approximately 2 μL of the SnCl_2 solution was applied to side of the reaction tube just above the peptide mixture. The entire ^{99m}Tc solution was then added to the reaction tube, and the 2 μL of SnCl_2 washed into the reaction mixture. The reaction was allowed to take place for ~ 1 min at room temperature prior to the addition of 1 μL of the cysteine solution which quenched the reaction. To the mixture, 2 drops of carrier

protein solution (0.1 % filtered gelatin in PBS) and ~ 20 μ L of blue dextran solution were added. The reaction mixture was then passaged through the Sephadex G-25 size exclusion column, and 4 fractions were collected once the blue dextran reached the glass wool at the tip of the column: fraction 1 was collected for 30 seconds and fractions 2 – 4 were collected for 1 minute. The radioactivity in these was measured using the Capintec (^{99m}Tc setting), and those with the majority of radiolabeled peptide were pooled for further use.

3.8. Radiochemical purity testing

For all radiolabeled peptides, it was necessary to ensure that the product was radiochemically pure, i.e., that there was little free radioisotope present. To do this, one μ L of a 1:100 dilution of the radiolabeled peptide in PBS or 0.1% gelatin solution was analyzed using 10 % acrylamide SDS-PAGE gel electrophoresis (Novex, Carlsbad, CA). To the peptide was added 7.5 μ L of NuPAGE™ LDS sample buffer (4X) and 10 μ L of distilled H₂O, and the solution was then boiled for 10 min. A second sample of peptide, prepared by addition of 7.5 μ L of SDS buffer, 10 μ L of distilled H₂O and 2.5 μ L of NuPAGE™ sample reducing buffer (10X), was also boiled for 10 min. Approximately 10 μ L of both reduced and non-reduced peptide samples were loaded onto the gel. If the sample was pure and did not contain aggregates or contaminants, both the reduced and non-reduced preparations migrated similarly through the gel (if the peptides contained no cysteine residues). Electrophoretic separation was achieved with an FB200 system (Fisher Scientific, Dubuque, IA) set at a constant voltage of 200 V and a

current of $\sim 1.0 \text{ } \mu\text{A}$ for 30 min. To visualize the peptides, the gel was removed, covered with plastic wrap, and placed on a phosphor imager screen (Cyclone Storage Phosphor System, Perkin Elmer, Shelton, CT) for up to 1 h depending on how much radioactivity was in the loaded sample (samples with greater radioactivity required less exposure time). In certain cases when very little radioactivity was loaded onto the gel, the exposure time was extended beyond 1 h with only slight increases in non-specific background occurring. Image analysis, i.e. measurement of radiochemical purity, was performed using the OptiQuant software package (Packard, Downers Grove, IL). If more than one band was observed, there may be aggregates or contaminants in the preparation. Free radioisotope, when present, was seen at the gel “running” front. In general, the radiochemical purity of the peptides used in these studies was $> 95 \%$ [328]. This procedure can be used for quality assurance of peptides labeled with many radioisotopes, i.e. ^{125}I , ^{123}I , ^{124}I and technetium-99m ($^{99\text{m}}\text{Tc}$), but the half-life of the radioisotope should be taken into consideration so that this assay is performed in a timely manner.

3.9. Tissue homogenate preparation

Preparation of the tissue homogenates used in all *ex vivo* peptide binding assays followed a procedure that has been reported [371]. The procedure remains unmodified for most experiments. To begin, mouse tissues, namely liver, spleen, heart and lung, from two or three amyloid-laden mice (AA amyloid or ApoA2c amyloid) as well as control (healthy mice) were harvested at necropsy. A portion of tissue from each mouse

was combined to yield a “pooled sample” and placed within a pre-weighed 50 mL plastic conical tube (Fisher Scientific, Pittsburgh, PA). The total combined amount of each tissue was ~ 200 mg – 1500 mg net weight (depending on the tissue) and a 10-fold volume of PBS added to the sample. To reduce the risk of tissue proteolysis, serine protease inhibitors, leupeptin and phenylmethylsulfonyl fluoride (PMSF), were included at 100 µg/mL each. The solution was then mixed vigorously (speed 6) with 3 x 10 s bursts using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) before being centrifuged at 4,000 x g for 10 min. The supernatant was discarded, and the pellet was resuspended in another 10-fold volume of PBS with 0.05 % Tween-20 (PBST) to prevent the homogenized material from sticking to the sides of the tube and 0.05 % NaN₃ to inhibit bacterial growth. This solution was then homogenized a second time (as described above), before being stored at 4 °C for use in *ex vivo* peptide binding assays. This same procedure was used for healthy, amyloid-free (WT) tissues.

3.10. *Ex vivo* peptide binding assays

For these binding assays, tissue homogenates prepared from: AA, ApoA2c, or WT mice; human AL amyloid extracts, or; synthetic recombinant λ6 Wil variable domain (rVλ6Wil) fibrils were used. The general procedure for all materials was the same, but some parameters were unique. The method for tissue homogenate (specifically AA liver) binding assays and AL extract/rVλ6Wil fibril binding assays have been described in the literature [363, 371].

3.10.1. Tissue homogenates

For an *ex vivo* peptide binding assay using tissue homogenate as the substrate, 50 µL of the tissue homogenate was centrifuged at 21,000 x g for 3 min in a 1.5 mL-volume microfuge tube. This was done in duplicate (i.e., 2 samples per peptide). The resultant supernatants were discarded and the pellets resuspended in 400 µL PBST. A 1:100 dilution of radiolabeled peptide in either 0.1 % gelatin/PBS or PBST was added to the tissue samples. Twenty microliters of peptide (~ 200,000 counts per minute [cpm] of radioactivity) were added and the samples rotated for 1 h (Tube Rotator, Scientific Equipment Products, Baltimore, MD) at room temperature. Samples were then centrifuged at 10,000 x g for 3 min; the supernatants, which contained unbound peptide, were carefully aspirated and placed in glass tubes, and the pellets were resuspended gently by hand in 400 µL PBST and washed by centrifugation, as described above.

Supernatants from the second wash were added to those previously collected, and pellets were resuspended in 800 µL PBST and transferred into glass tubes. The radioactivity in both the pooled supernatant samples (*free peptide*) and the pellet samples (*bound peptide*) were measured using a gamma scintillation counter with a 1 min acquisition time and an energy window optimized for detection of ¹²⁵I. The percentage of radiolabeled peptide bound to the pellet was determined using the following formula:

$$\% \text{ peptide bound} = \left[\text{Pellet} \frac{\text{CPM}}{\text{Pellet CPM} + \text{Supernatant CPM}} \right] * 100$$

3.10.2. AL extracts and rV λ 6Wil fibrils

For *ex vivo* peptide binding assays using human AL amyloid extracts and rV λ 6Wil fibrils, the protocol was essentially the same as described in Section 3.10.1 with the following modifications. The amount of amyloid extract or fibril used was 25 μ L (~ 25 μ g of protein). Since this procedure used half the reaction volume of the tissue homogenate protocol, only 10 μ L of a 1:100 dilution of radiolabeled peptide (~100,000 cpm) was added to all samples prior to their 1 h rotation at room temperature. The samples were washed by centrifugation at 15,000 x g for 10 min because the pellets formed in this assay were not as “tightly packed” as tissue homogenates when using the standard protocol of 10,000 x g for 3 min. Increasing the speed and time of the centrifugation for the human AL extracts and rV λ 6Wil fibrils yielded a more reliable (“stable”) pellet for the assay. The reactivity in the supernatant and pellet fractions was measured as previously described, and the percent of radiolabeled peptide bound to the extract or fibril was determined (Section 3.10.1).

3.11. Assessment of relative peptide affinity to amyloid using NaCl titration

Since the binding of poly-basic peptides to amyloid is thought to be due to principally electrostatic interactions of the peptide with the negatively-charged sulfate moieties presented by the hyper-sulfated heparan sulfate proteoglycans associated with amyloid, the peptide binding assay was modified slightly to determine a measure of the relative affinity of peptide for the substrate (e.g., tissue amyloid, AL extract or rV λ 6Wil

fibrils). This was achieved by performing the binding reaction in increasing concentrations of NaCl which will perturb the electrostatic binding interactions.

The protocol was altered, from that described above (Section 3.10.2) after the initial centrifugation; the pellets were resuspended in either 0.25, 0.5, 0.75, 1.0 or 2.0 M NaCl instead of PBST. The volumes of each NaCl concentration were, as before, 400 μ L for tissue homogenates and 200 μ L for AL extracts or rV λ 6Wil fibrils. The addition of NaCl at this step was designed to perturb the binding of radiolabeled peptide to the substrate during the 1 h incubation. All wash and resuspension steps after the rotation used PBST, and the protocol was performed as described (Sections 3.10.1. and 3.10.2).

3.12. Imaging acquisitions

To assess the *in vivo* amyloid-targeting peptides, SPECT, dynamic PET and CT small animal imaging were performed. All imaging data were acquired using the Inveon trimodality SPECT/PET/CT platform with the Inveon Acquisition Workplace software version X (Siemens Preclinical Solutions, Knoxville, TN).

3.12.1. SPECT imaging

SPECT imaging was used for visualization of ^{125}I - or $^{99\text{m}}\text{Tc}$ -radiolabeled tracer distribution in mice. The imaging protocol for the studies described herein has been previously reported [371, 416] with only slight modifications tailored to each study. The basic protocol included injection of the radiotracer (i.e., ^{125}I -peptide, ^{125}I -SAP, $^{99\text{m}}\text{Tc}$ -

peptide) iv in the lateral tail vein of amyloid-laden or WT mice. At the appropriate time post injection (dependent upon the radiotracer used), mice were euthanized, by isoflurane overdose, for post mortem imaging or anesthetized in an induction chamber with 3 % isoflurane in air at 2 L/min for live imaging. For live imaging, paralube ophthalmic veterinary ointment (Dechra, Overland Park, KS) was applied to the eyes of the mice prior to positioning in a heated imaging chamber (M2M, Cleveland, OH) with a nose cone delivering 2 % isoflurane for the duration of the acquisition. The Inveon imaging system acquired gamma photons (^{125}I : 25 – 45 keV; $^{99\text{m}}\text{Tc}$: 140 keV) at 60 different projections, each acquired for 16 seconds, over 90 mm of scanner bed travel. To enhance the image resolution, the multi-pinhole (Mouse Whole Body) collimator [261] was used with a 30 mm radius of rotation. SPECT data were reconstructed using a 3D ordered subset expectation maximization (OSEM) algorithm with 8 iteration and 6 subsets. The resulting image was displayed onto an 88 x 88 x 312 matrix with isotropic 0.5 mm voxels.

For studies utilizing two radiotracers in a single animal, a dual energy SPECT acquisition was performed. This protocol has been described previously [293], but there were minor modifications for these studies. Both radiotracers (e.g., 2 different peptides labeled with ^{125}I and $^{99\text{m}}\text{Tc}$) were injected simultaneously with similar amounts of radioactivity (~100 μCi / isotope). After an appropriate uptake time (e.g., 2 h pi), mice were euthanized as described above. The comparison of 2 radiolabeled peptides was achieved by performing 2 consecutive SPECT acquisitions (as described above).

3.12.2. Dynamic PET imaging

Dynamic PET imaging was used to visualize the biodistribution of radiolabeled peptides and to obtain their kinetic biodistribution *in vivo*. The protocol has been outlined previously [416]. Mice were injected with ^{124}I -labeled peptides following anesthesia and placement in the imaging chamber on the PET scanner bed—anesthetic delivery and mouse positioning were performed as described in section 3.12.1 for live SPECT imaging. The data acquisition was initiated ~ 5 sec prior to injection of the radiotracer—this time delay was used to obtain the full kinetic biodistribution of the radiolabeled peptide after injection.

AA amyloid-laden or WT mice received ~ 20 μg of ^{124}I -radiolabeled peptide (200 – 400 μCi) in ~ 200 μL of sterile gelatin/PBS solution with 1 mM ethylenediaminetetraacetic acid (EDTA), which prevented blood coagulation upon injection. PET data were acquired for either 60 or 120 min, depending on the study. The data were histogrammed for ^{124}I with appropriate scatter and attenuation correction using a low resolution CT dataset (see section 3.12.3). Reconstruction of the images was completed with the following binning parameters: 10 frames $\times$ 1 sec, 10 frames $\times$ 30 sec, 30 frames $\times$ 114 sec and 60 frames covered the remainder of the data. Reconstruction was performed using a 3D OSEM with the Maximum A Posteriori (MAP) algorithm, and the image matrix was $256 \times 256 \times 159$ with a pixel size of $0.0215 \times 0.0215 \times 0.796$ mm.

3.12.3. CT imaging

Low-resolution CT data were acquired in conjunction with both SPECT and dynamic PET image acquisitions, which provide anatomical reference correct for attenuation and scatter, respectively. Images were acquired with an x-ray voltage biased to 80 kVp with an anode current of 500 μA . Two bed positions were acquired to obtain a CT image of the entire mouse body. Each acquisition comprised 121 projections with a 225 ms x-ray exposure collected over 220° of continuous rotation around the imaged subject. Low resolution (i.e., binning of 4) were used. Reconstruction of the data was performed with an implementation of the Feldkamp-filtered back-projection algorithm onto a $224 \times 224 \times 635$ matrix with isotropic 212.1 μm voxels.

3.13. Image analyses

Both SPECT and dynamic PET data were analyzed with the 2D Inveon Research Workplace visualization software (IRW; Siemens Preclinical Solutions). The methods of analysis for SPECT/CT data and dynamic PET/CT data were described by Martin et al.[416].

3.13.1. SPECT/CT analysis

The SPECT data were automatically co-registered with CT data using IRW. To obtain measurements of radioactivity (displayed as voxel intensities in arbitrary units), regions of interest (ROI) were manually drawn over multiple organs including the liver,

spleen, heart, lungs, leg muscle, stomach, pancreas, kidneys, intestines and bladder. A 3D ROI drawing tool (spherical or cubical) was used to encompass multiple CT slices of the organ. This method provided a better representation of the radioactivity within the ROI. The mean voxel intensity in the SPECT data associated with each ROI was used as a measure to compare radiotracer accumulation in each tissue and to compare the accumulation between amyloid-laden (AA or ApoA2c) and WT mice.

3.13.2. Dynamic PET/CT analysis

The 2D IRW software automatically co-registered PET data with CT data. Prior to drawing ROI in the same manner as described in section 3.13.1, all image frames for the entire acquisition were summed. The ROI for kinetic biodistribution analyses included the vena cava (a measure of blood pool), heart, liver, kidneys, stomach, thyroid and bladder of each mouse. The software's kinetic analysis tool generated time activity curves (TAC) for each ROI drawn. The TAC data were fit to exponential equations using Prism version 6.0 (GraphPad Software Inc., La Jolla, CA).

3.14. Necropsy

Mice were euthanized by isoflurane overdose in accordance with IACUC-approved animal protocols and amyloid-laden and healthy tissues were harvested for tissue homogenate preparation, biodistribution measurements, histological examination and microautoradiography. The tissues that were harvested for homogenate preparation (section 3.9) to be used in the *ex vivo* assay (section 3.10) were the liver,

spleen, kidneys, heart, lungs, intestines and pancreas. These tissues were taken in full and stored in -20°C until used.

When performing experiments with radiolabeled peptides, tissues were harvested for biodistribution measurements and histological examination or microautoradiography (when ^{125}I or ^{124}I was used). The tissues taken for these procedures were leg muscle, liver, pancreas, spleen, kidneys, stomach, ~ 2 inches of upper intestine (taken from the base of the stomach), ~ 2 inches of lower intestine (taken near the anus), heart, lung and, in some cases, tongue. The contents of the stomach and intestinal samples were emptied prior to analyses. A small portion of each tissue was used for biodistribution measurements, and the remainder of the tissue was placed in 10 % buffered formalin to be fixed for histology and microautoradiography. After use, the radioactive carcasses were held in a biohazard/radioactive freezer for radioisotope decay and disposal.

3.15. Biodistribution measurements

The amount of radioactivity within tissues was measured as described previously in several articles [378, 381, 409]. Samples of tissue, harvested at necropsy, were placed into tared plastic vials (Wheaton, Millville, NJ) and weighed. The radioactivity (^{125}I , ^{123}I , ^{124}I or $^{99\text{m}}\text{Tc}$) in each sample was measured for 1 minute using a Wizard 3 gamma counter (1480 Wallac Gamma Counter; Perkin Elmer, Boston, MA). Additionally, 10 μL of a diluted sample of the injected dose of radiotracer was added to 2 separate vials and counted. These were considered “decay-corrected” standards by

which to determine the number of counts equivalent to the injected dose. The biodistribution data were determined with the following formula and the data expressed as a percent injected dose per gram (%ID/g):

$$\%ID/g = (\text{counts per min}[cpm]/\text{total cpm}) \times (100/\text{tissue sample weight}[g])$$

3.16. Autoradiography and Histology

Confirmation of radiotracer localization to amyloid deposits in tissue sections was obtained by comparing microautoradiography and Congo red (CR)-stained histological tissue sections [378, 381, 409]. Tissues harvested at necropsy were fixed in 10% buffered formalin for at least 24 h prior to being embedded in paraffin blocks. For autoradiography, sections ~ 4 – 6 µm thick were cut from the blocks and placed onto Plus™ microscope slides (Fisher Scientific, Pittsburgh, PA). The slides were then dipped in NTB2 emulsion (Eastman Kodak, Rochester, NY) and stored in the dark for a predetermined amount of exposure time depending on the study [423]—tissue specimens taken from imaging experiments were typically stored for 4 days prior to development; studies of biodistribution only, that did not require as much radioactivity, were exposed to the photographic emulsion for 10 days prior to development. The sections were then counter-stained with hematoxylin and eosin and examined with light microscopy. Radiotracer accumulation was evidenced by the presence of black silver grains within the tissue sections.

Consecutive 4 – 6 µm-thick slices of the same paraffin-embedded specimens were then stained with freshly prepared alkaline CR solution. For the detection of CR

birefringence, all sections were examined by light microscopy (DM500, Leica, Bannockburn, IL) using cross-polarized illumination. Microscopic images were taken using a cooled, charge-coupled device camera (SPOT, Diagnostic Instruments, Sterling Heights, MI). Some studies involved imaging CR fluorescence using epifluorescent illumination with a rhodamine (red) filter [416].

3.17. Statistical analyses

Statistical analyses (e.g., unpaired/two-tailed/student t-tests and correlation analyses) were performed using the Prism software package (GraphPad, LaJolla, CA). Estimation of statistical significance was based on $p < 0.05$.

Chapter 4

Peptide characterization studies

4.1. Overview

The peptides to be studied in this thesis for amyloid binding were characterized using several *in vitro* and *ex vivo* studies. Previously, circular dichroism of the peptide p5 revealed that it has a propensity to form an α -helix in a solution of more than 30% trifluoroethanol (TFE)—a chemical known to promote the formation of helical secondary structure [371, 424]. Additionally, the peptide from which p5 was derived, K8, was shown to adopt an α -helix in the presence of heparin [414]. These studies suggested that p5 adopts a secondary structure in the presence of its ligand (or “when bound to target”); the notion of helical peptide structure was reiterated in the predicted structure of the peptide [363, 371, 415]. A linear array of positive charges aligned to one face of the peptide was predicted by I-TASSER modeling; this structural property was thought to facilitate in p5-GAG interactions [371]. Given these structural properties which likely make the peptides more amenable to bind all amyloid, we characterized the interaction of peptides to a battery of amyloid-related substrates. The *ex vivo* binding assays described in section 3.10 were used. This chapter surveys the interactions of peptides p5, p5R and p5R+14 with various amyloid-laden materials including AL patient-derived amyloid extracts, recombinant V λ 6W μ l fibrils, and AA or ApoA2c amyloid-laden mouse tissue homogenates. Although AL and ATTR are the most common forms of human visceral amyloidosis, there are no good experimental models of these diseases;

therefore, we used murine models of AA and ApoA2c amyloidosis which represent the most widely studied models of visceral amyloidosis.

4.2. AL extract binding studies with ^{125}I -p5

Patient-derived AL amyloid extracts that contain fibrils, HSPG and numerous accessory molecules were used to study peptide binding. The study outlined below was recently published in Biochemical and Biophysical Research Communications [363]. Briefly, 9 patient samples derived from either the liver or spleen of confirmed AL amyloid patients was evaluated; additionally, the collection included both AL_κ and AL_λ amyloid types so as to not bias the study to one isotype of AL fibril. Radioiodinated p5 was incubated with approximately 25 µg of amyloid extract (which contains HSPG and fibrils but only negligible amounts of SAP and no DNA components) and a peptide binding assay performed (Section 3.10.2).

Results

As seen in Figure 4.1, results of this study indicated that the reactivity of p5 with the extracts was quite variable (% peptide bound to the amyloid pellet ranged from 2% to 74%). Since amyloidosis in patients is such a heterogenous disease in terms of amyloid load per organ [1, 19], the inconsistent amyloid-binding was not entirely unexpected; however, as evidenced by Alcian blue measurements, these extracts contained sulfated GAGs, a major ligand of p5, making the near lack of p5 binding, in some cases, perplexing.

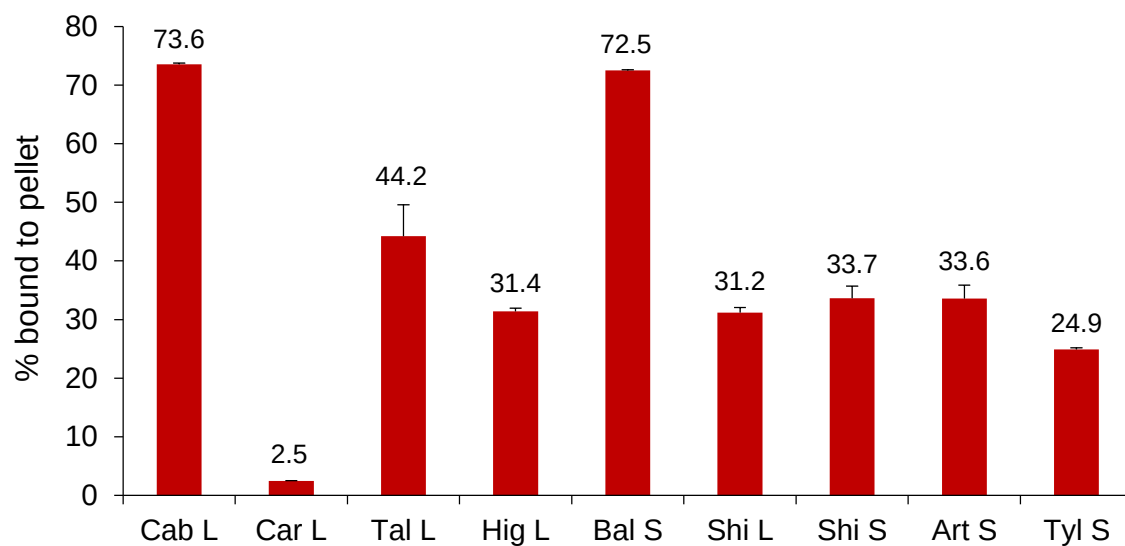


Figure 4.1: ^{125}I -p5 binding to AL extracts. The heterogeneity of AL patient-derived extracts was demonstrated by the variable reactivity of ^{125}I -p5 with the extracts in the peptide binding assay. There was a wide range of peptide reactivity in the nine samples tested. (L) liver, (S) spleen.

Therefore we used the GAG-clearing enzyme, heparinase I, to remove the heparan sulfate GAGs [363]. We posited that without the hypersulfated GAGs, a known target of p5, there would be diminished binding in all of the samples. Heparinase I was added to each AL extract prior to performing the *ex vivo* peptide binding assay. We observed a decrease in p5 binding to all samples; however, there was still significant binding. This finding suggested that there must be another component in the amyloid extracts to which p5 can bind. In the heparinase-treated samples, a positive linear correlation was found between p5 binding and ThT fluorescence (a measure of the fibrils) in the 5 samples which exhibited the highest ThT measurements (Figure 4.2). These data suggested that, in the absence of HSPG, there was a positive correlation with p5 binding relative to the fibril content. Thus, we hypothesized that p5 may also be able to interact directly with AL amyloid fibrils.

It is known that synthetic fibrils display an electronegative surface to which the primarily positive antigen binding site of camelid antibody, B10, can interact [362, 407]. Furthermore, the polybasic nature of p5 with a predicted linear array of positively-charged amino acids positioned on one face of the peptide may render the peptide capable of also binding fibrils. This was tested using synthetic rV λ 6Wil fibrils, which lack GAGs and other amyloid associated proteins, in the peptide binding assay. Our data showed that p5 interacted with synthetic fibrils with ~ 80% peptide bound (Figure 4.3). Therefore, p5 was shown to interact with both the HSPG and the fibril components of AL amyloid, and our studies demonstrated that: 1) amyloid is an extremely heterogenous material; and 2) p5 can recognize two of the primary components in amyloid deposits.

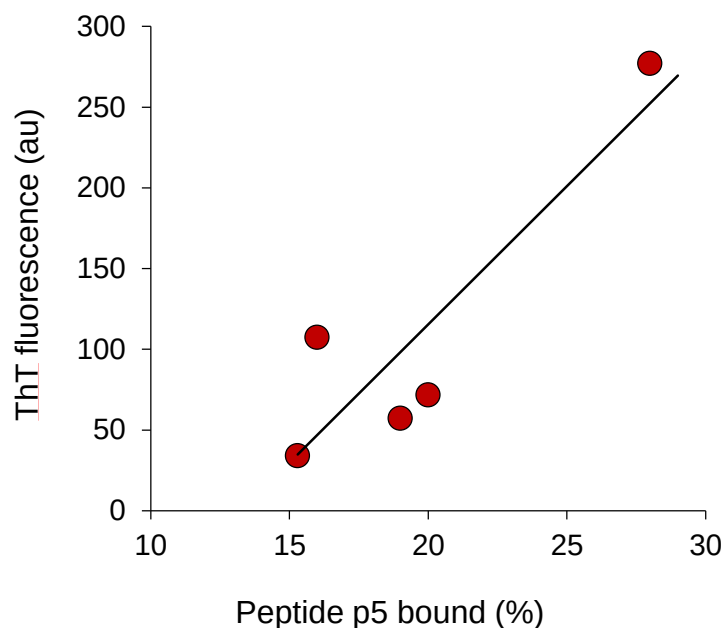


Figure 4.2: Positive correlation of ^{125}I -p5 binding and ThT fluorescence in heparinase-treated samples. The five AL extracts with the highest ThT measurement showed a positive correlation with ^{125}I -p5 binding after heparinase treatment. This proposed that peptide p5 may bind to amyloid fibrils directly. (Image adapted from [363]).

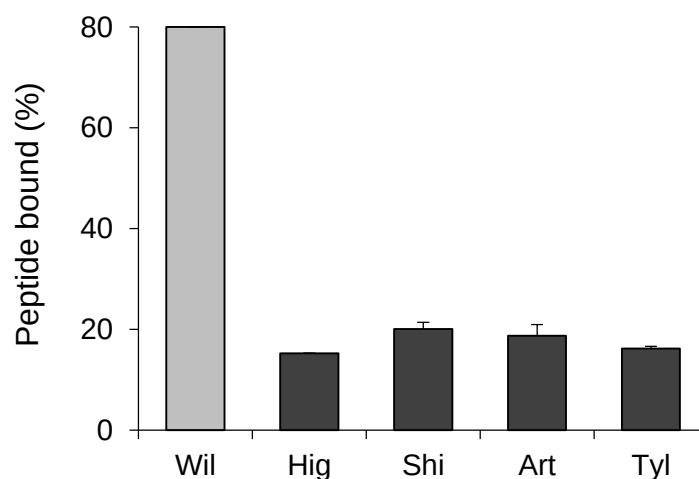


Figure 4.3: ^{125}I -p5 binds to synthetic recombinant V Λ 6Wil fibrils. The binding of ^{125}I -p5 to heparinase-treated AL samples and V Λ 6Wil fibrils supported the interaction of this peptide with fibrils directly. (Image taken from [363]).

4.3. Peptide binding to amyloid-laden tissue homogenates

Mouse tissue homogenates were used to assess peptide binding with a more “complex” amyloid system which includes HSPG, fibrils, SAP, as well as DNA and other cellular debris that was not fully separated from the amyloid in the tissue homogenate preparation. One of the experiments routinely performed when screening new peptides for amyloid binding was an *ex vivo* binding assay utilizing liver homogenate from H2-IL6 transgenic mice with confirmed AA amyloidosis. The assay was performed as described in section 3.10.1. The peptides tested were: the parent peptide, p5; an arginine derivative of the peptide, termed p5R; and an extended version of p5R, known as p5R+14. The arginine variant of p5 was analyzed because literature suggested that arginine has a higher propensity to form an α -helix which may enhance binding with amyloid [425]. Thus, it was hypothesized that p5R would exhibit greater binding to AA amyloid-laden tissue when compared to p5. The extended version of these peptides, p5R+14, combined both the higher propensity to form an α -helix with an additional 14 amino acids that included four more arginine residues making the resultant net charge +12 instead of +8 as seen with p5 and p5R. It was postulated that, due to these two properties (enhanced helicity and increased charge), p5R+14 would bind more effectively than p5R. The reasoning behind this stemmed from a recent study which investigated the *in vivo* binding efficacy of a 24 amino acid p5R variant with a +6 charge. Data suggested that this shortened p5R peptide did not bind to amyloid as well as the intact peptide [371]. Therefore, we hypothesized that an increase in peptide charge may enhance amyloid binding. Based on this hypothesis, we expected to see the following peptide pattern relative to their binding to amyloid-laden tissue

homogenates: $p5 < p5R < p5R+14$. For these studies, we used organs from the H2/IL-6 and ApoA2c transgenic mice.

Results

Our studies indicated that all 3 peptides were able to bind to AA amyloid-laden homogenates from liver, spleen, kidney, pancreas and intestines in a peptide binding assay (Figure 4.4A). The lung had much less binding with all peptides, but this was expected because very little, if any, AA amyloid forms in the lungs. The heart revealed increasing binding from p5, to p5R, to p5R+14, but even with the p5R+14, there was less interaction than with all other tissues—again, due to the scant deposits in this tissue. The differential binding of the peptides ($p5 < p5R < p5R+14$) was also seen in the kidney and the intestines (Figure 4.4A). Thus, with some tissues, the expected pattern of peptide binding, based on our conceptual model, was observed, but this was not the case for all tissues studied. The liver and spleen generally have more amyloid relative to the other tissues (Congo red scoring of these 2 tissues was typically 3+ to 4+ on a scale of 0 – 4+) which could explain the higher percentages with all peptides in these tissues (nearly all were > 50%).

The binding of these peptides to WT tissues revealed that, in all tissues, the p5R+14 peptide had the most non-specific binding (Figure 4.4B). In some tissues, namely the pancreas and the intestine, p5R also displayed more off-target binding than that seen with p5. The greater net positive charge associated with p5R+14 may have enhanced its affinity for other components within the homogenates, possibly the negatively charged DNA. Regardless, all peptides exhibited much greater binding to

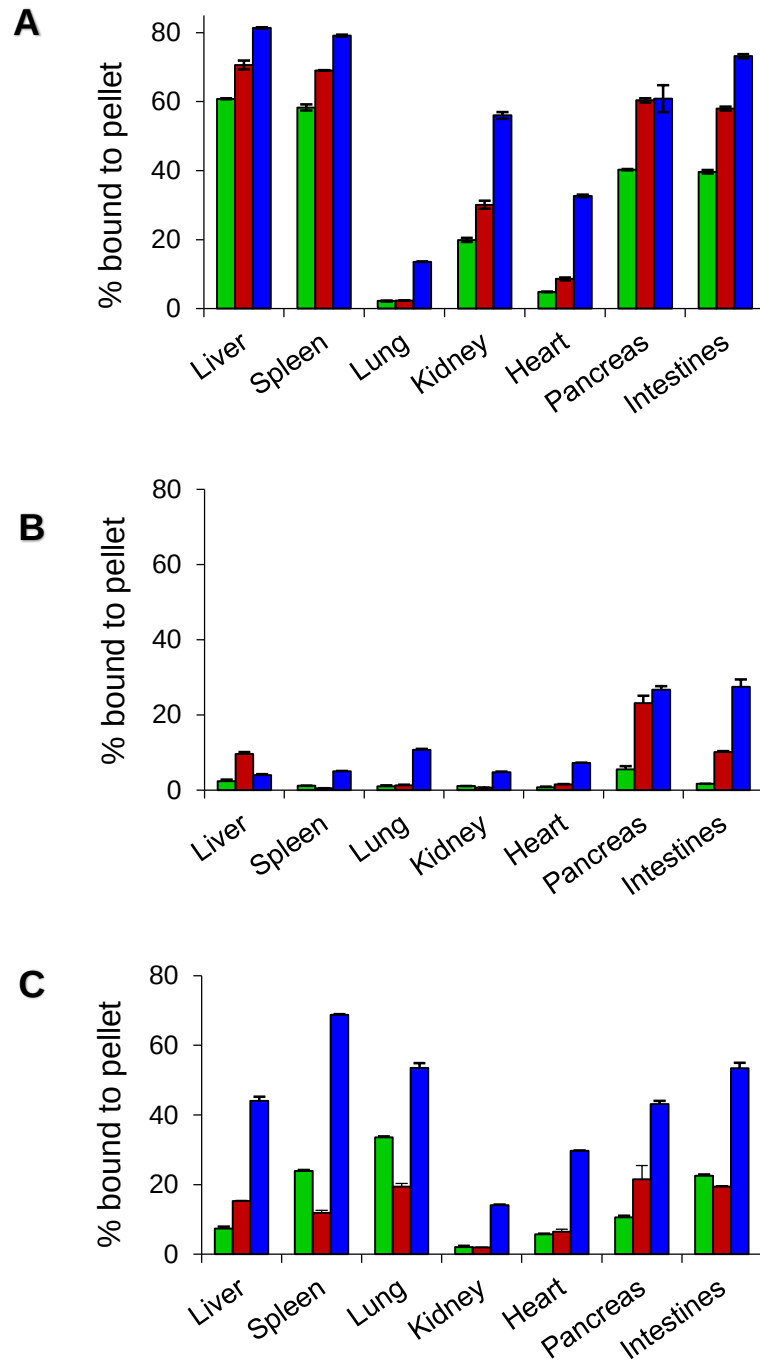


Figure 4.4: Peptide binding to tissue homogenates. (A) Peptide reactivity with AA tissues. (B) Non-specific binding of peptides to WT tissues. (C) Peptide reactivity with ApoA2c tissues. In all graphs: ^{125}I -p5 ■, ^{125}I -p5R ■ and ^{125}I -p5R+14 ■.

the highly AA amyloid-laden homogenates, suggesting that each of these are capable of specifically interacting with *ex vivo* AA amyloid deposits.

Given these findings for the 3 peptides, they were further tested in the same assay to determine their efficacy in binding to ApoA2c amyloid-laden mouse tissues (Figure 4.4C). The distribution of amyloid in these tissues varies slightly from that seen in AA mice; the main difference is that there is much more amyloid deposition in the lung and heart relative to AA tissues. The data indicated that p5 and p5R interacted much more weakly with ApoA2c amyloid in this system relative to the binding with AA amyloid. The spleen, a site known to be largely affected with ApoA2c amyloid, exhibited minimal binding with the p5 and p5R peptides. In contrast, positive binding was seen in all ApoA2c tissues with p5R+14 (Figure 4.4C).

These findings suggest that AA and ApoA2c amyloids, although they do contain HSPG and fibrils, are remarkably physico-chemically different. Given that the homogenized mixtures should, theoretically, expose the amyloid for binding with no hindrances to the interaction (e.g. access to the vasculature), the structural composition of the deposits themselves must be different—e.g. p5 and p5R do not recognize their targets within ApoA2c amyloid as compared to AA amyloid. The ApoA2c tissues appear to require a peptide with higher net positive charge to achieve the best peptide-amyloid interaction. There are numerous reasons why this may be the case: ApoA2c amyloid may contain a ratio of the 2 known peptide targets, hypersulfated HSPG and fibrils, which makes certain peptides less effective at binding to this type of amyloid; the ApoA2c amyloid deposits may consist of more apolipoproteins than AA amyloid

deposits, hindering peptide reactivity; or ApoA2c fibrils may have a different charge motif than AA fibrils, thus, decreasing peptide binding.

This study led us to investigate the relative strength of binding between the peptides and certain AA and ApoA2c tissues.

4.4. Binding studies in the presence of increasing concentrations of sodium chloride to assess the relative strength of electrostatic binding of peptides with amyloid

Since there were unexpected inconsistencies in binding between the peptides and the two amyloid types, sodium chloride (NaCl) titrations were performed to assess the strength of the electrostatic interaction between p5, p5R and p5R+14 with AA and ApoA2c amyloid. The tissues used included AA liver and spleen and ApoA2c liver, spleen, heart and lung—all were known to contain considerable amyloid for their respective disease. The pancreas and intestine were not used in these studies because of the inappropriate nature of the material after incubation with NaCl (i.e. the pellets were unable to be separated from the supernatants).

The same peptide binding assay was used in these studies with only slight modifications. For the NaCl titration studies, concentrations of NaCl (0.15 M (PBST), 0.25 M, 0.5 M, 0.75 M, 1.0 M and 2.0 M) were used in the peptide-homogenate incubation step to attempt interference with the peptide-amyloid electrostatic interaction. Even though we observed that all peptides bound impressively to AA liver and spleen tissues, we hypothesized that it would take a higher concentration of NaCl to inhibit

binding by 50% in samples containing p5R+14 due to the +12 net charge on the peptide. For the ApoA2c tissues, we hypothesized that the concentration of NaCl needed to interfere with binding would follow a similar pattern but would be less for all peptides when compared to the concentrations used in AA tissues—a hypothesis derived from the observation that the peptides interacted much less avidly with ApoA2c tissue in PBST as compared to AA.

Results

The data indicated that, with p5, binding to AA liver required ~ 0.4 M NaCl to inhibit 50% (IC_{50}) of the maximum binding observed with PBST. The IC_{50} for p5R with AA liver was ~ 0.9 M NaCl, and was > 2.0 M NaCl for p5R+14 (Figure 4.5). The same pattern was observed with AA spleen with IC_{50} values for each peptide: IC_{50} ~ 0.5 M NaCl for p5, IC_{50} ~ 1.7 M for p5R and IC_{50} > 2.0 M NaCl for p5R+14 (Figure 4.5). The NaCl titration on AA tissues indicated that there was an ionic interaction between the peptides and amyloid since binding decreased with increasing salt concentration. Additionally, the strength of the ionic binding for the tested peptides followed the expected pattern for AA tissues: $p5 < p5R < p5R+14$. Interestingly, these data suggest that, for peptide p5R+14, binding other than electrostatic interactions may be occurring.

Titration of peptides on ApoA2c tissues demonstrated lower IC_{50} values for most samples, which was consistent with the decreased binding seen earlier. For both p5 and p5R, the IC_{50} for each of the 4 tissues tested was < 0.35 M NaCl. However, for p5R+14, there was a much wider range of NaCl concentrations needed to interfere with tissue amyloid binding. For the heart and liver, the IC_{50} of p5R+14 was < 0.4 M NaCl, whereas the spleen IC_{50} was 0.47 M, and the lung required a concentration >0.8 M

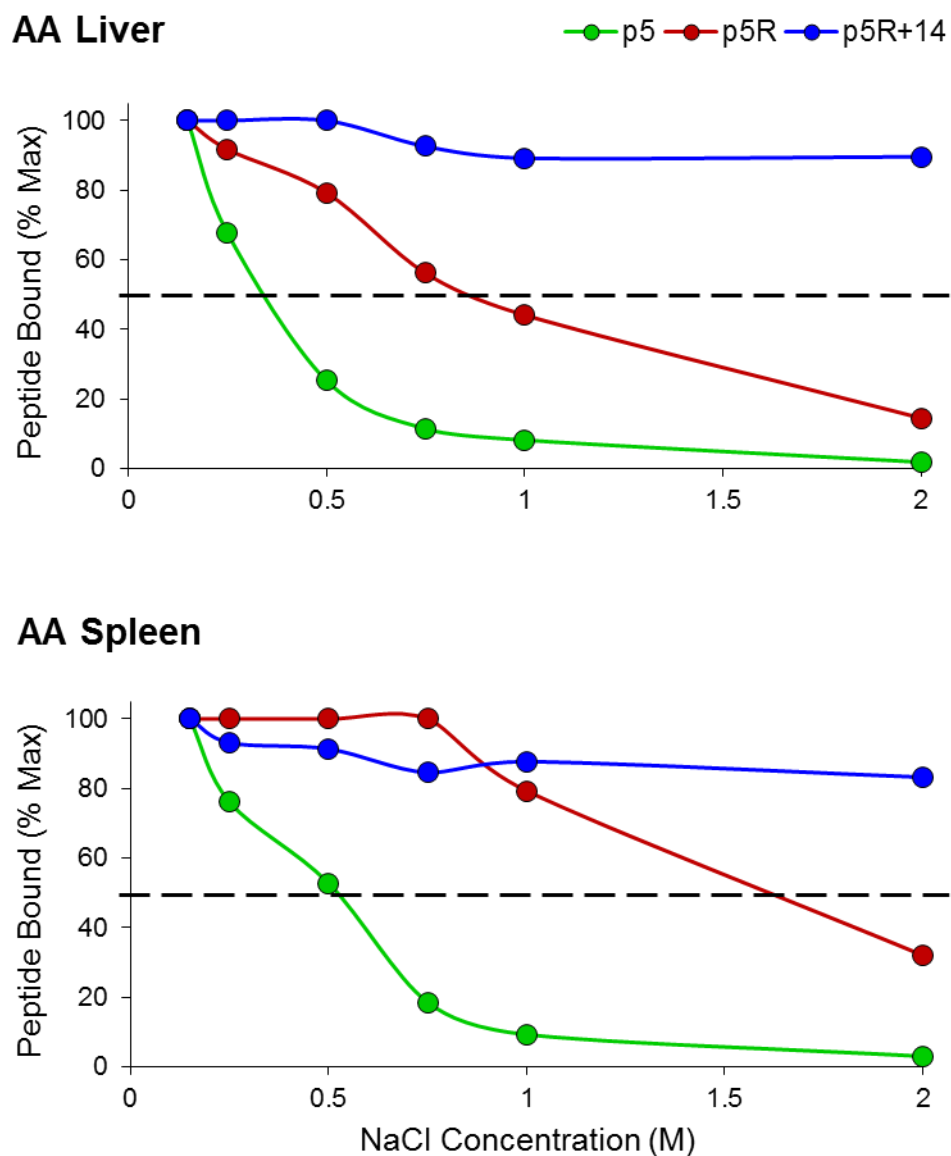


Figure 4.5: Peptide binding to AA tissues in the presence of NaCl. The NaCl titration indicated that the peptides bind to AA liver and spleen with differing affinities. For this type of amyloid, peptide binding followed the hypothesized pattern: $p5 < p5R < p5R+14$.

NaCl. These data, summarized in Figure 4.6, suggested that the peptides had a lower binding affinity for ApoA2c amyloid than AA amyloid, but of the 3 peptides, p5R+14 exhibited the strongest binding to most tissues, consistent with our hypothesis.

The differences observed in the NaCl titration reiterate that AA and ApoA2c amyloids, while comprised of similar components, are obviously “different”. The varying degrees of peptide binding may be the result of differing amounts of HSPG or fibrils in the tissues and the different affinity that each peptide has for these components. If peptides exhibit a greater affinity for HSPG but the composition of the deposit contains more fibrils than HSPG, the peptides would not be expected to bind as well to these samples. Since p5 and p5R bind with much lower affinity than p5R+14, perhaps the former 2 peptides have a greater propensity for a target that is underrepresented in ApoA2c amyloid. A report in 2013 by Wall *et al.* indicates that p5 and p5R both bind immobilized heparin, but p5R (~ 1.3 M NaCl) has a greater affinity than p5 (~ 0.9 M NaCl) [371].

Affinity for immobilized heparin may suggest that p5 and p5R preferentially bind to HSPG in amyloid deposits. All 3 peptides were subjected to a NaCl titration binding study with rV λ 6Wil fibrils to directly compare their affinity for fibril-binding in the absence of HSPG. The titration revealed that p5R+14 expressed a much higher affinity for synthetic fibrils than the other two peptides (Figure 4.7); an IC₅₀ of between 0.75 M and 1.5 M NaCl was seen previously with p5 and p5R. On the contrary, p5R+14 appeared to plateau at 2.0 M NaCl as evidenced by > 50% bound even in the presence of 4.0 M NaCl. Thus, there was a very high affinity of p5R+14 for this synthetic fibril.

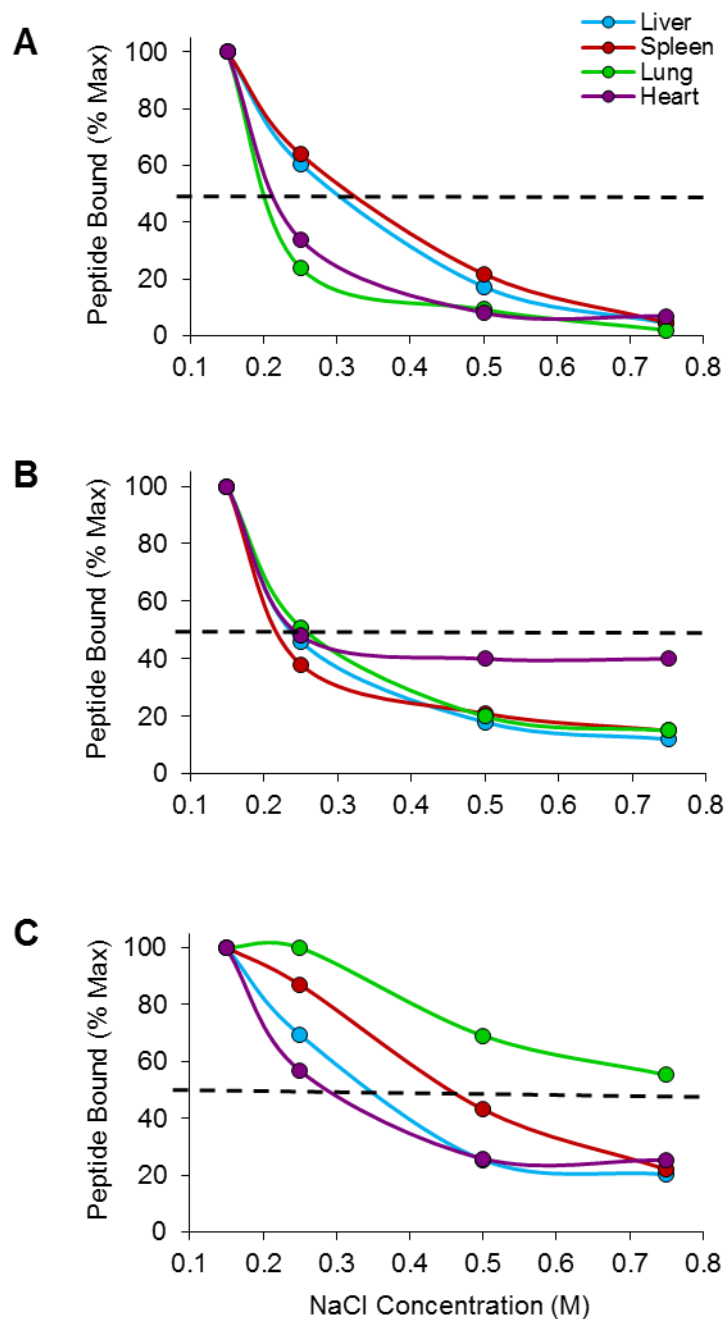


Figure 4.6: Peptide binding to ApoA2c tissues in the presence of NaCl. (A) ^{125}I -p5 reactivity. (B) ^{125}I -p5R reactivity. (C) ^{125}I -p5R+14 reactivity. The peptide reactivity with ApoA2c amyloid required a much lower concentration of NaCl than was seen with the AA tissues, but the same pattern was observed: p5 < p5R < p5R+14. (Many thanks to Brianna Rader and Angela Lee for their assistance in obtaining the NaCl titrations).

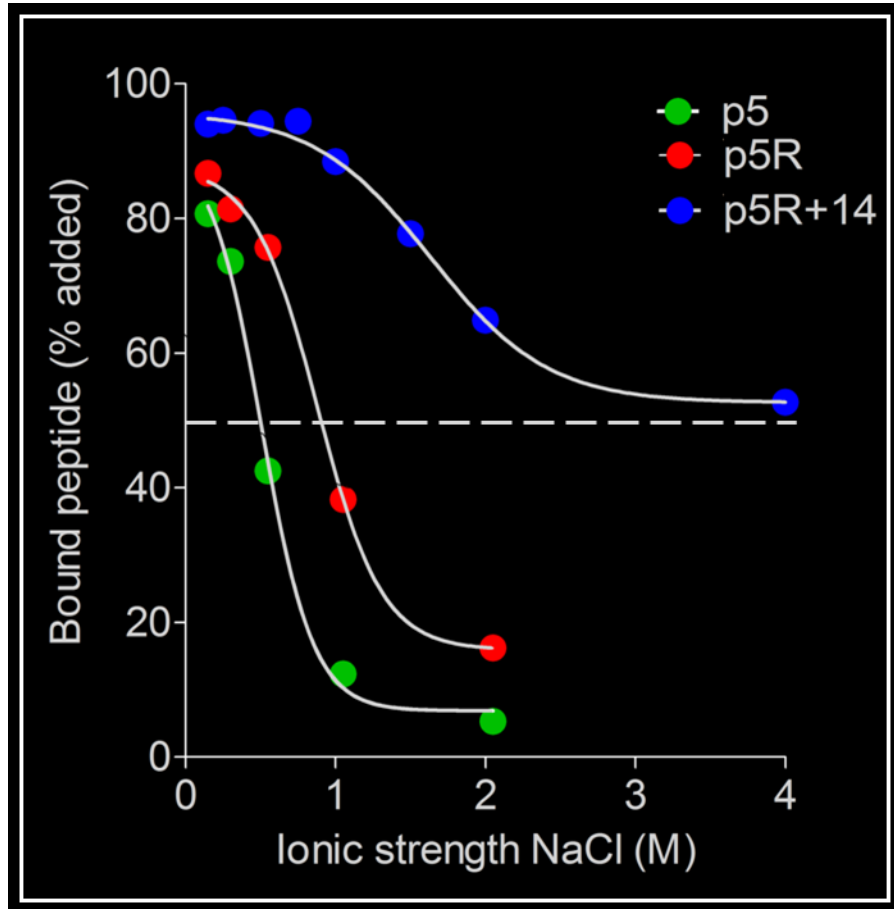


Figure 4.7: Peptide affinity for recombinant synthetic V λ 6W μ l fibrils measured by NaCl titration. The peptides showed variable affinity for fibrils. Notice that p5R+14 would require > 4.0 M NaCl for 50% binding inhibition. (Thank you to Mrs. Angela Williams for the historic p5 and p5R NaCl titration data).

Since the binding inhibition of p5R+14 plateaued, it was worth speculating that the binding may be more than simply ionic in nature. To test this, we examined the binding of p5R+14 to rV λ 6Wil in the presence of 4.0 M urea which should disrupt peptide-fibril hydrophobic interactions. As shown in Figure 4.8, urea treatment resulted in a decrease in p5R+14 binding to rV λ 6Wil fibrils by ~ 20% compared to fibril binding in PBST. To ensure that the 4.0 M urea treatment reduced peptide-fibril interactions without diminishing fibril structure, four different concentrations of urea were added to plated fibrils alone; after 1 hour at room temperature, ThT fluorescence was measured at 490 nm. The data indicated that there was no significant difference between the fluorescence emissions in any of the fibril samples, treated or untreated with urea (Figure 4.9); therefore, the fibrils were intact. The data suggested that p5R+14 binding to fibrils may consist of both ionic and hydrophobic interactions; the two tested treatments were unable to completely inhibit binding, however. These experiments could assist in explaining the various peptide-ApoA2c amyloid interactions. In short, the molecular composition of ApoA2c amyloid may contain more fibrils with respect to HSPG, resulting in greater p5R+14 binding and decreased affinity of peptides p5 and p5R.

4.5. Conclusions

The studies presented in this chapter were designed to characterize the interactions of peptides p5, p5R and p5R+14 with synthetic and “native” amyloid and to assess the nature of those interactions. The data support the thesis that peptides can be used to specifically target amyloid in various forms—patient extracts, tissue

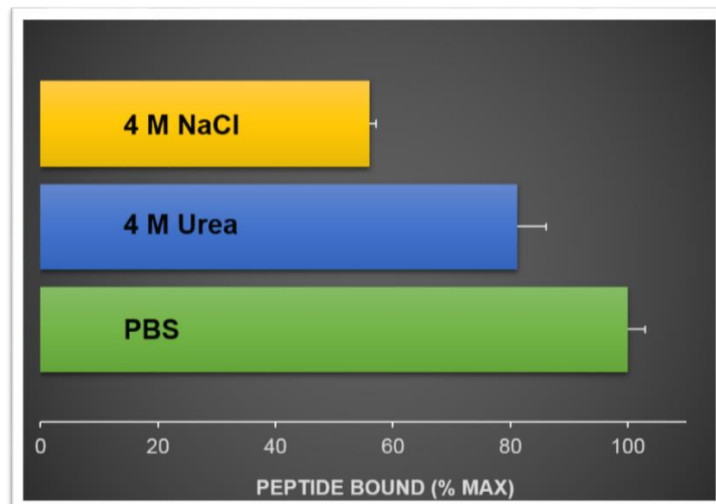


Figure 4.8: ^{125}I -p5R+14 binding to rVλ6Wil fibrils in the presence of both NaCl and urea treatments. Peptide binding in PBS alone represented maximum binding. After 4.0 M urea treatment, ~ 20% of the maximum peptide reactivity with fibrils was lost. As was previously determined, 4.0 M NaCl interfered with ~ 50% of the maximum binding. These treatments and the resultant peptide reactivity indicated that p5R+14 may use both hydrophobic and ionic interactions to bind to synthetic fibrils. Neither treatment resulted in full interference of peptide binding, however.

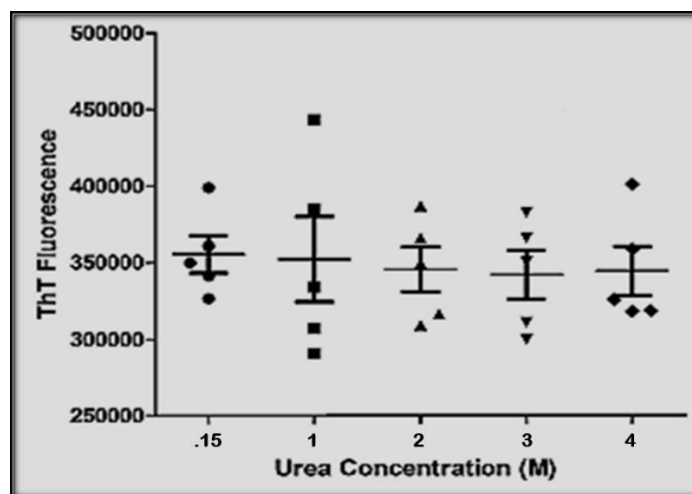


Figure 4.9: Urea treatment of rVλ6Wil fibrils. The one-way ANOVA test revealed that up to 4.0 M urea treatment resulted in no significant difference in fibril structure as measured by ThT fluorescence ($p = 0.9649$).

homogenates and synthetic fibrils. The model systems used proved that the modifications made to p5 resulted in stronger amyloid binding (in most cases). The data also indicated that AA and ApoA2c amyloids are structurally different regardless of the ubiquitous presence of fibrils and HSPG in both types of deposits. The peptides did not behave identically in each of the model systems, which suggested that they may each prefer binding to HSPG or fibrils differentially. These *ex vivo* studies were used to characterize how p5 and its variants interact with multiple amyloid types; this information was the foundation on which the following experiments were designed and conducted to examine amyloid-targeting with various peptides.

Chapter 5

Dual isotope SPECT/CT imaging of peptides in ApoA2c mice

5.1. Overview

In our laboratory, we have found that many peptides interact with amyloid materials in *ex vivo* assays. The previous chapter indicated that peptides p5, p5R and p5R+14 bound with varied affinities to AA and ApoA2c amyloid extracts likely due to preferential interactions with GAGs and fibrils, but these data could not guarantee that similar peptide behavior would be observed *in vivo* since possible hindrances to peptide-amyloid reactivity (i.e. weak vascularization of amyloid-laden tissues and serum half-life of the peptide tracer) are factors that must be considered. The literature provides data pertaining to the *in vivo* reactivity of the parent peptide, p5, in the transgenic H2/IL-6 model of AA amyloidosis [293, 409]. With this peptide, SPECT/CT imaging studies have been conducted and revealed positive and specific uptake in amyloid-laden organs; the murine model deposited ample AA amyloid in several tissues, and autoradiographs (ARG) showed preferential binding and co-localization of ¹²⁵I-p5 with amyloid deposits (see Figure 1.24; [409]). To ascertain amyloid-reactivity in a different model of the disease, this chapter focuses on the *in vivo* analyses of peptides p5 and p5R+14 in transgenic ApoA2c mice. To induce amyloidosis, the mice were injected with 50 µg of ApoA2c intestine-derived AEF at 14 weeks of age, and they were used at 12 weeks post AEF induction when sufficient amyloid was present. Prior to this study, no *in vivo* small animal imaging of ApoA2c mice had been conducted or reported.

With that, we were uncertain which, if any, peptides would target amyloid in these mice such that the pathology could be visualized. Given that p5 is the parent peptide to these studies and that it provided positive amyloid imaging in AA mice, we opted to investigate it first in this model of amyloidosis; additionally, we investigated p5R+14 since it bound most effectively of all the tested peptides to ApoA2c tissue homogenates in the *ex vivo* binding assay (Figure 4.4C). The method by which these 2 peptides were comparatively evaluated was dual-isotope SPECT/CT imaging; by radiolabeling the two peptides with different radioisotopes, in this case ^{99m}Tc and ^{125}I , a direct comparison within a single mouse was possible. This type of study was previously conducted in which peptide p5 and SAP were compared [293]. In addition to imaging, tissues were harvested in order to obtain biodistribution measurements for both radiotracers, and for the ^{125}I -labeled p5R+14, ARG was performed to confirm peptide-amyloid co-localization.

5.2. Dual-isotope SPECT/CT imaging of peptides in ApoA2c mice

As previously stated, radioiodinated p5 successfully imaged AA amyloidosis with significantly higher tissue biodistribution measurements compared to WT controls [423]. Experimental conditions from those studies involved injection of the radiotracer and imaging using mice at approximately 3 – 6 weeks post induction of amyloidosis with AA spleen-derived AEF. The transgenic model of AA amyloidosis yielded abundant amyloid deposition within this time frame. For ApoA2c transgenic mice, however, 9 – 11 weeks post AEF induction were required to yield a comparable amount of amyloid. For the peptide comparison study, p5R+14 was radiolabeled with ^{125}I (Section 3.5), and peptide p5 was radiolabeled with ^{99m}Tc (Section 3.7). For each radiotracer,

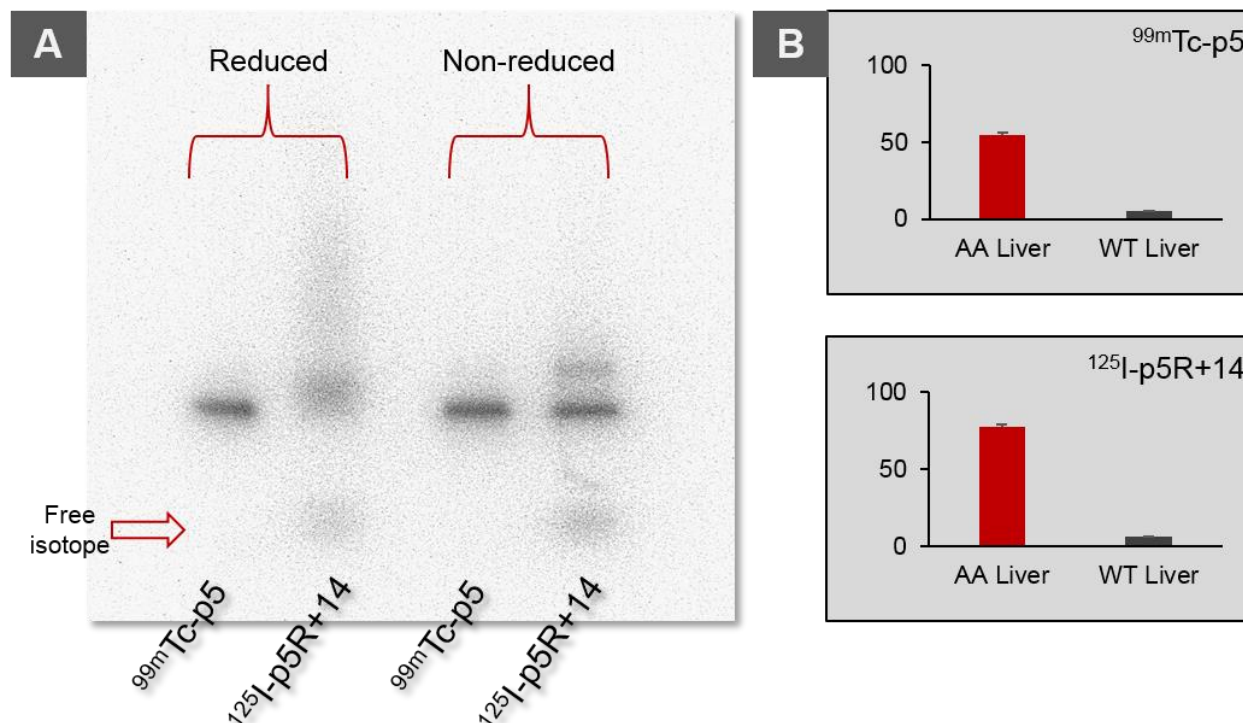


Figure 5.1: Quality assurance of radiolabeled p5 and p5R+14. (A) Results of the SDS-PAGE radiopurity analysis for $^{99m}\text{Tc-p5}$ and $^{125}\text{I-p5R+14}$. Both the non-reduced and reduced samples of peptides are included. The gel indicates that there was a small quantity of free ^{125}I in the p5R+14 product and no detectable ^{99}Tc in the radiolabeled p5 product. (B) Peptide binding assays demonstrating amyloid reactivity. Both radiolabeled peptides show positive reactivity with AA liver homogenate and minimal reactivity with WT liver.

1 μL of 1:100 diluted product in 0.1% purified gelatin was tested for radiopurity with SDS-PAGE (Section 3.8). Little free isotope was detected for either peptide (Figure 5.1A). To ensure that both radiolabeled peptides were amyloid-reactive, they were tested by binding to AA liver homogenate in the *ex vivo* (pull down) assay (Section 3.10.1; Figure 5.1B), and both peptides were shown to be amyloid-reactive. An approximately equal amount of radioactivity of each tracer ($\sim 200 \mu\text{Ci}$) was injected into the lateral tail vein of WT and ApoA2c mice (total volume $\sim 200 \mu\text{L}$ per mouse). Mice were euthanized at 2 h pi, and biodistribution of each radiotracer was established with SPECT/CT imaging. The imaging protocol consisted of 2 consecutive SPECT acquisitions—the first to acquire only high energy ($\sim 140 \text{ keV}$) $^{99\text{m}}\text{Tc}$ photons and the second for low energy ($\sim 30 \text{ keV}$) ^{125}I photons. A single CT acquisition provided anatomical reference for both SPECT datasets.

Since the peptide tracers were radiolabeled with different isotopes, both their catabolism and their distribution in mice were not expected to be equivalent (e.g. dehalogenation of only the radioiodinated peptide). To determine the “background” *in vivo* distribution of radioactivity associated with these tracers, approximately the same activity equivalent was injected into healthy mice.

Results

SPECT/CT imaging of WT mice at 2 h pi revealed that ^{125}I -p5R+14 underwent dehalogenation when it encountered deiodinase enzymes in both the liver and kidneys [416, 426]; this resulted in the release of radioiodide and its uptake in the stomach and thyroid. In contrast, the $^{99\text{m}}\text{Tc}$ -p5 radiotracer accumulated in the kidneys and intestines as a result of peptide clearance (N.B. $^{99\text{m}}\text{Tc}$ is not prone to dehalogenation activity). A

representative WT mouse demonstrating these two different radiotracer distributions is shown (Figure 5.2). The bladder was emptied naturally when mice were euthanized prior to image acquisition and was not visible with either radiotracer in the image.

Although peptide p5 has previously been shown to interact with AA amyloid in all affected organs such that whole-body amyloid burden could be visualized with SPECT/CT imaging [293], the visual biodistribution of p5 in ApoA2c mice provided much less information about the amyloid burden due to decreased reactivity with ApoA2c amyloid. ^{99m}Tc -p5 in the ApoA2c mouse revealed minimal uptake in amyloid-laden tissues; the image was dominated by radiotracer accumulation in the kidneys in addition to hepatobiliary clearance evidenced by radiotracer in the intestines (Figure 5.3). For ApoA2c mice, renal amyloid pathology is typically less extensive than that seen in AA mice, and the kidneys are not considered a major organ for amyloid deposition [382]; thus, the enhanced renal uptake in ApoA2c mice is presumably associated with radiotracer clearance rather than amyloid-reactivity. Since amyloid deposition within the intestines of ApoA2c is a common manifestation, the radioactivity displayed in the intestines of the ApoA2c mice could be considered amyloid-specific, but the biodistribution measurements suggested otherwise (Figure 5.4). After the removal of feces from the upper and lower intestines, there was little ^{99m}Tc -p5 associated with these organs in ApoA2c or WT mice. SPECT images of ^{99m}Tc -p5 distribution demonstrated similar patterns between diseased and healthy mice—this further suggested that presence of ^{99m}Tc -p5 in these tissues was associated with radiotracer clearance and not amyloid uptake. Biodistribution measurements (reported as % ID/g) were made for the liver, spleen, upper/lower intestines, heart, kidneys, lungs and

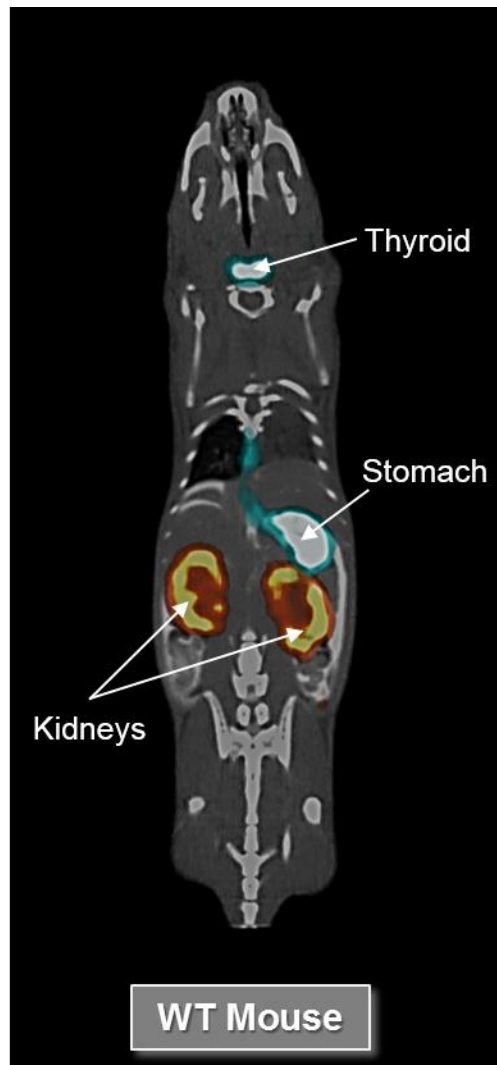


Figure 5.2: Dual energy SPECT/CT image representing the distribution of ^{125}I -p5R+14 and $^{99\text{m}}\text{Tc}$ -p5 at 2 h pi. The standard distribution of ^{125}I -p5R+14 is shown in blue and is present in the thyroid and stomach due to peptide dehalogenation. The distribution of $^{99\text{m}}\text{Tc}$ -p5 is shown in orange and is seen in the renal cortices due to peptide clearance and, potentially, retention of the $^{99\text{m}}\text{Tc}$ nuclide in the proximal tubules.

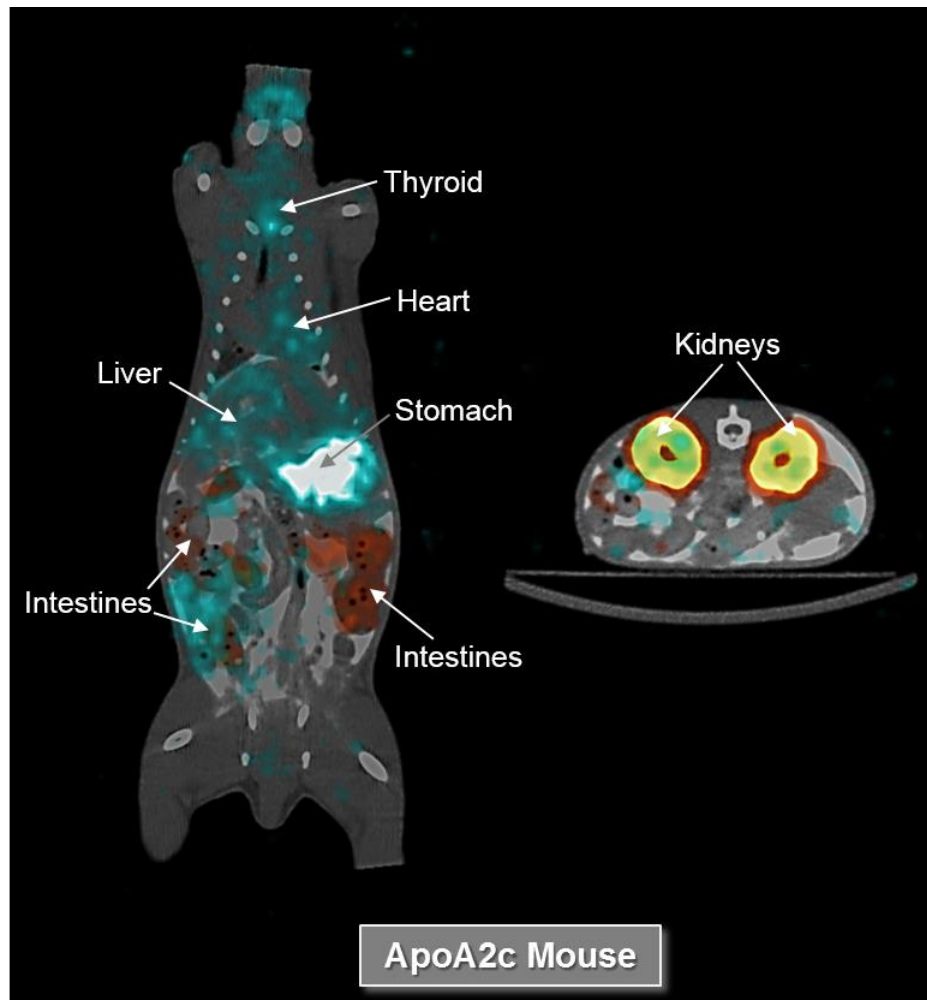


Figure 5.3: Dual energy SPECT/CT image demonstrating the distribution of both $^{99m}\text{Tc-p5}$ and $^{125}\text{I-p5R+14}$ in a representative ApoA2c mouse. The image shows uptake of $^{99m}\text{Tc-p5}$ (yellow-orange) in the kidneys and intestines which are both likely associated with peptide clearance. Distribution with $^{125}\text{I-p5R+14}$ (blue) was more extensive and can be seen in the liver and heart. Intestinal uptake may be associated with clearance or amyloid reactivity. Accumulations in both the thyroid and stomach, however, are a clear result of radiotracer dehalogenation and free iodide sequestration. The image demonstrates that $^{125}\text{I-p5R+14}$ provided a more informative picture of amyloid deposition.

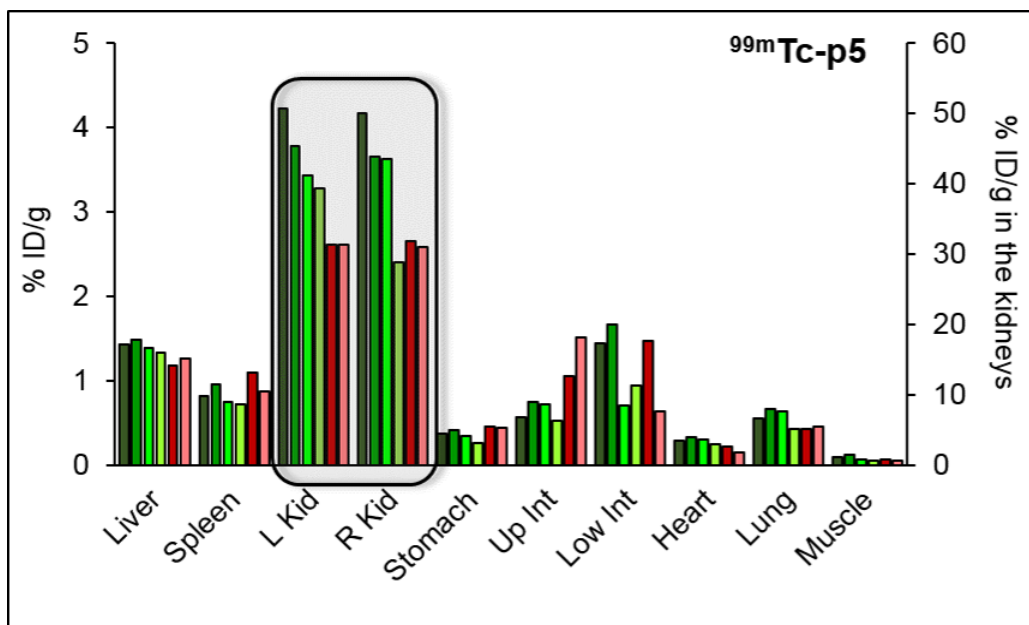


Figure 5.4: Biodistribution measurements of ^{99m}Tc-p5 in ApoA2c and WT mice at 2 h pi. These measurements revealed that the uptake of radiotracer in each tissue did not differ between diseased and healthy mice. Since the tracer was not dehalogenated, the route of clearance, the kidneys, displayed a much higher % ID/g than other tissues. The kidneys were plotted against the right axis due to their high level of uptake.

stomach of both ApoA2c and WT mice (Figure 5.4). These quantitative measurements of tracer tissue distribution indicated that the difference in uptake of ^{99m}Tc -p5 between diseased and healthy mice was not significant in all tissues, with the exception of the observed renal activity. Although not shown, SPECT/CT imaging of WT mice with free ^{99m}Tc shows uptake in the renal cortices; therefore, it is likely that the radionuclide itself gets retained in the proximal tubules. This suggests that the uptake observed with ^{99m}Tc -p5 in ApoA2c mice was not amyloid-specific but was associated with peptide clearance and radionuclide retention.

Images and biodistribution measurements of ^{125}I -p5R+14 in ApoA2c mice were quite disparate to those seen with ^{99m}Tc -p5. The SPECT/CT imaging data revealed uptake of ^{125}I -p5R+14 in amyloid-laden organs including the liver and heart (see Figure 5.3). There was also evidence of radiotracer dehalogenation with stomach and thyroid uptake of radioiodide. As was stated before, the intestinal accumulation seen with ^{125}I -p5R+14 could have been associated with amyloid, or it might be associated with hepatobiliary clearance of the peptide. It was more likely in this case, however, that the intestinal uptake was amyloid-specific because: 1) the biodistribution measurements obtained after imaging showed enhanced lower intestinal ^{125}I -p5R+14 uptake compared to that seen in WT mice, and 2) the peptide would be dehalogenated in the liver and, therefore, not visible in the intestines. A greater accumulation of ^{125}I -p5R+14 was observed in most ApoA2c tissues (% ID/g) when compared to the WT tissues, but this finding was particularly noticeable in the spleen, lower intestines and lung, sites of significant ApoA2c amyloid accumulation (Figure 5.5). To directly compare the uptake of both tracers in each of the tissues and accounting for any possible off-target binding,

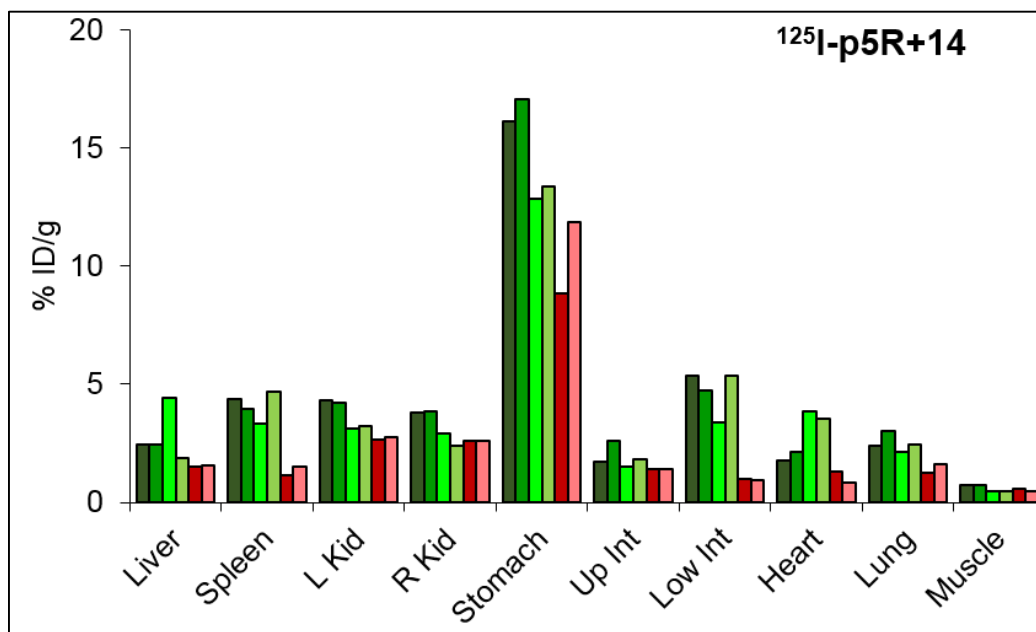


Figure 5.5: Biodistribution measurements of ^{125}I -p5R+14 in ApoA2c and WT mice at 2 h pi. A higher % ID/g of ^{125}I -p5R+14 accumulated in most ApoA2c tissues (green) when compared to WT tissue (red) accumulation. This was especially noticeable in the spleen, lower intestines and the heart.

the % ID/g of the tracers in each tissue was scaled by the average uptake obtained in the respective tissue within WT mice (i.e. ApoA2c mouse/mean WT). These comparisons for each mouse independently revealed that ^{125}I -p5R+14 dominated the accumulation profiles in most organs as compared to p5, due to its increased reactivity with ApoA2c amyloid (Figure 5.6).

To further evaluate the accumulation of these two peptide radiotracers in ApoA2c mice, image analysis of the dual isotope acquisition was performed by drawing regions of interest over known ApoA2c amyloid-laden tissues such as the liver, heart and lungs. Although the uptake in these tissues was not clearly visible in each mouse, image analysis revealed a higher mean voxel intensity (reported as arbitrary units [au]) of ^{125}I -p5R+14 radiotracer in each of these organs when compared to $^{99\text{m}}\text{Tc}$ -p5 distribution (Figure 5.7). Since the intestinal uptake visible in the images could not discern whether the radioactivity was associated with clearance or with specific amyloid-binding, the intestines were not included in the image analysis. Image analyses confirmed the findings in the tissue biodistribution data—p5R+14 accumulated in ApoA2c amyloid-laden tissues more effectively than p5.

To ensure that the ^{125}I -p5R+14 tissue uptake was amyloid-specific, portions of the harvested tissues were analyzed with microautoradiography (ARG). Tissues were fixed in 10% buffered formalin for 24 h and developed after an emulsion exposure of 4 days (Section 3.16). Examination of the ARG revealed that ^{125}I -p5R+14 co-localized with amyloid deposits (seen as CR birefringent material in consecutive slides; data not shown). Although peptide p5R+14 clearly bound amyloid, there was evidence in the ARG that not all of the amyloid within certain tissues—namely the spleen and lung—

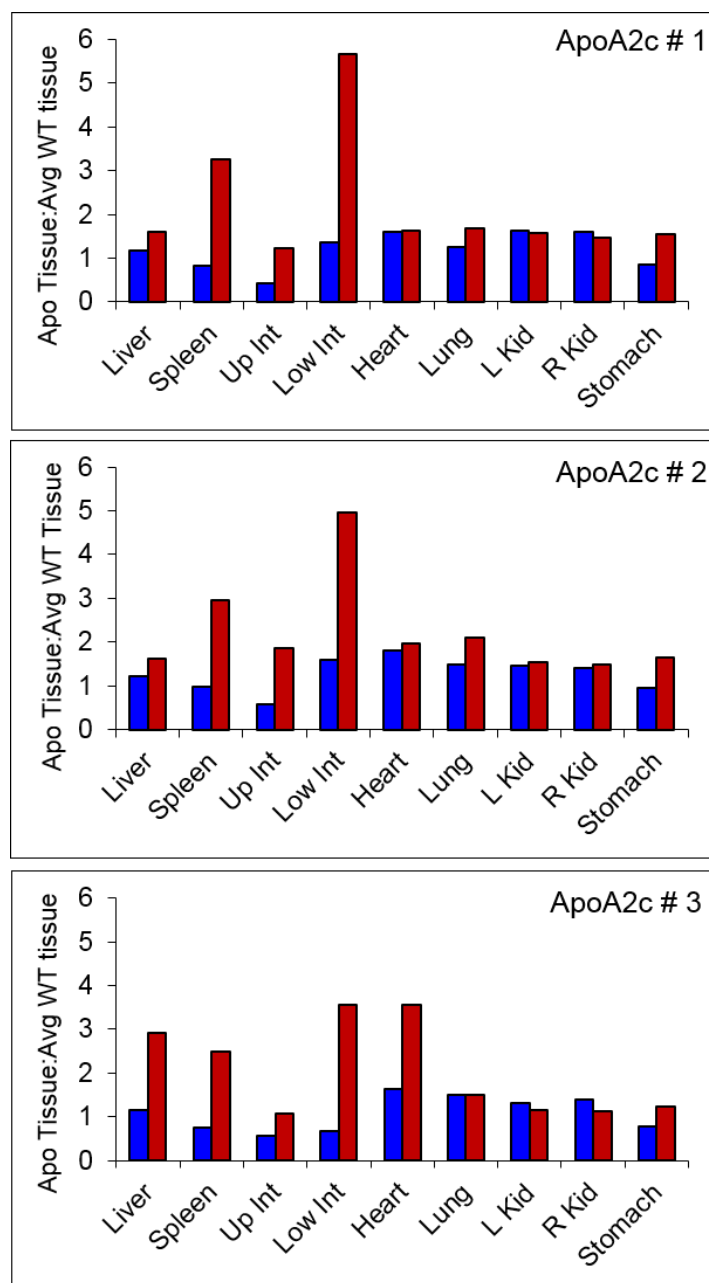


Figure 5.6: Scaled comparisons of $^{99m}\text{Tc-p5}$ and $^{125}\text{I-p5R+14}$ uptake in ApoA2c mice. These graphs display the ratio of radiotracer uptake in ApoA2c tissues to the average uptake in respective WT tissues. This accounts for any non-specific binding that biased radiotracer accumulation. There was greater $^{125}\text{I-p5R+14}$ (red) than $^{99m}\text{Tc-p5}$ (blue) accumulation in most tissues but most notably in the lower intestines and spleen. In ApoA2c # 3, both the liver and heart also showed enhanced accumulation of $^{125}\text{I-p5R+14}$. Error bars are not shown because these data are ratios.

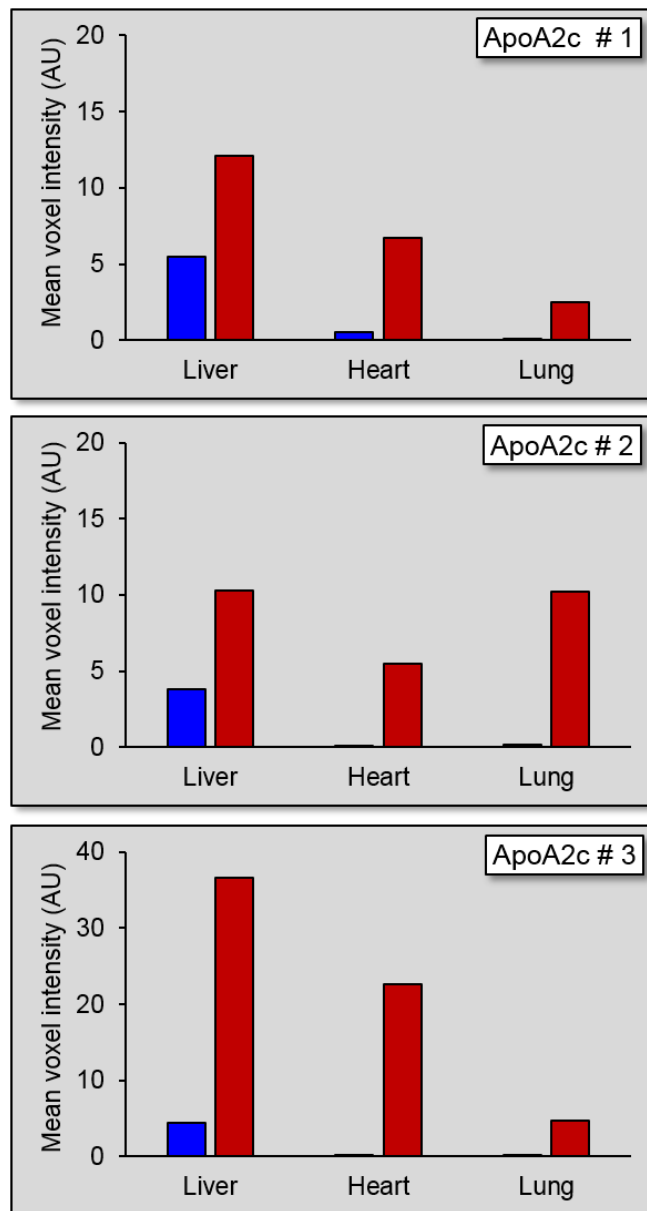


Figure 5.7: Image analyses revealed greater ^{125}I -p5R+14 uptake in amyloid-laden organs. These data were acquired by drawing ROI over the liver, heart and lung. In each mouse, it was evident that a more ^{125}I -p5R+14 (red) bound as compared to ^{99m}Tc -p5 (blue). Since intestinal activity could have been amyloid-specific or a result of clearance, this organ was removed from the analysis. Error bars are not shown because these data represent individual animals.

was covered by silver black grains. This meant that the amyloid deposits were not entirely targeted with the peptide. Additionally, there was no reactivity with healthy tissue in the evaluated organ specimens (Figure 5.8).

5.3. Conclusions

Perhaps the most remarkable finding in these studies was positive cardiac imaging which was made possible with ^{125}I -p5R+14 in ApoA2c mice. We have observed that, unlike the AA model of amyloidosis which inconsistently develops small amounts of cardiac amyloid, the ApoA2c transgenic mice unfailingly generate a large amount of cardiac amyloid. The biodistribution measurements associated with ^{125}I -p5R+14 in ApoA2c tissues yielded a diseased heart tissue:healthy tissue ratio of ~ 3 in one mouse. This value was high enough to clearly visualize the radiotracer within the heart (see Figure 5.3). Since cardiac amyloid is a particularly ominous manifestation in patients with amyloidosis, the ability of this radiotracer to visualize amyloid burden in this critical organ may be clinically relevant.

In summary, dual isotope imaging provided a facile method for assessing two different peptide radiotracers in individual mouse, thus subjecting them to the same *in vivo* conditions. This direct comparison generated data suggesting that peptide p5 was not suitable for ApoA2c amyloid detection; peptide p5R+14, however, was able to detect ApoA2c amyloid effectively. Both tissue biodistribution measurements and image analyses revealed that p5R+14 was superior to p5 in ApoA2c amyloid reactivity and that it could positively detect cardiac amyloidosis. The previous chapter discussed *ex vivo*

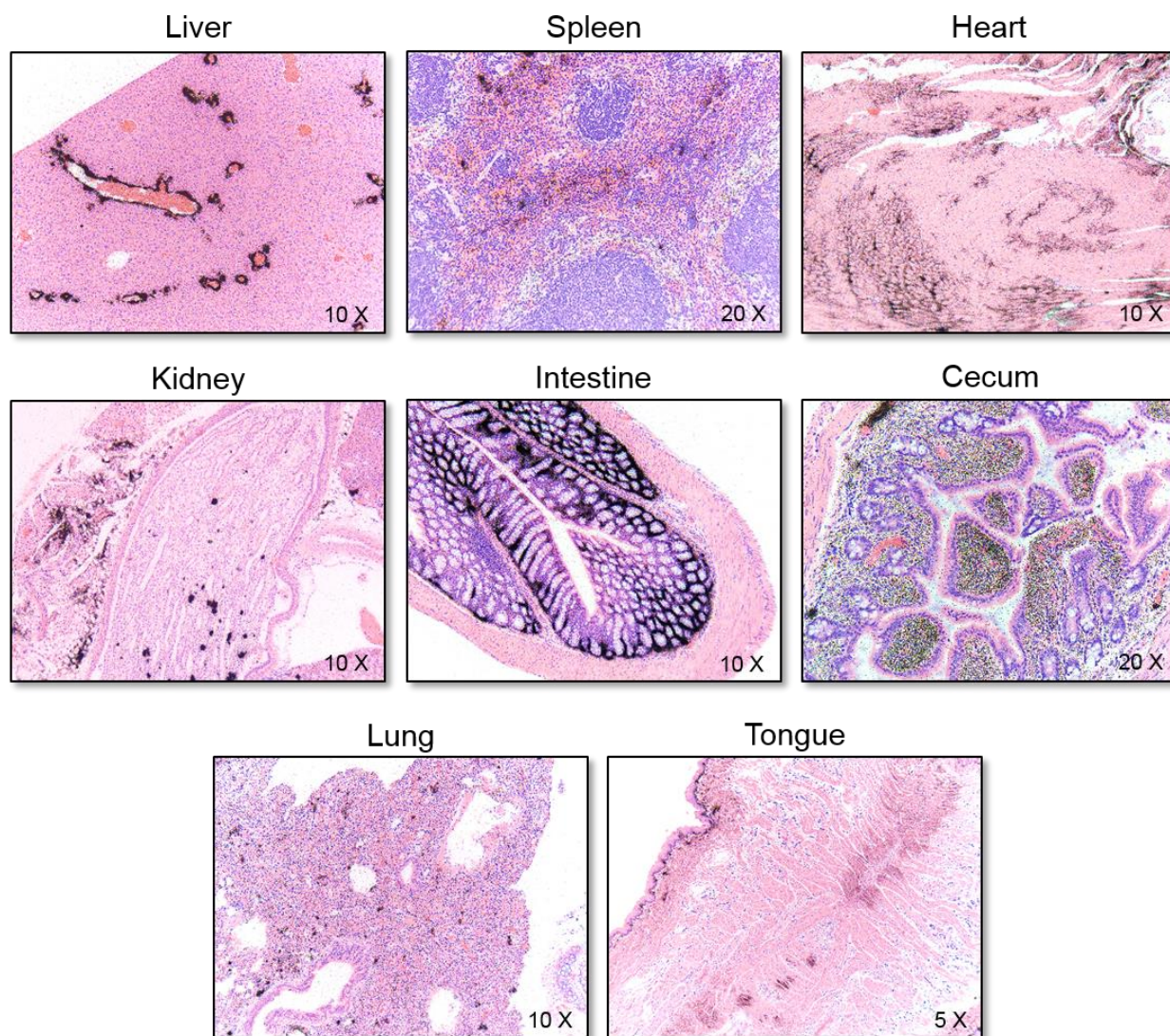


Figure 5.8: Autoradiographs of ^{125}I -p5R+14 in ApoA2c tissues at 2 h pi. These ARG indicate that peptide, seen as black silver grains, avoids reactivity with healthy tissue in these samples and preferentially binds to amyloid deposits.

findings which indicated that p5R+14 bound to ApoA2c tissue homogenates better than both p5 and p5R peptides; the translation of these findings *in vivo* was made credible through the dual isotope study. Additionally, p5R+14 ARG and CR tissue examination confirmed that the peptide co-localized with amyloid in all tissues, but it also revealed that amyloid coverage did not reach entirety with the radiotracer which is the focus of the next few chapters.

Chapter 6

Comparison of in vivo and ex vivo ApoA2c amyloid binding with p5R+14

6.1. Overview

The *in vivo* reactivity of p5R+14 with ApoA2c amyloid was clearly established by using dual isotope imaging, tissue biodistribution and microautoradiography (Chapter 5). Autoradiographs of various tissues revealed that the peptide co-localized specifically with the amyloid deposits, leaving healthy tissue unaffected. The amyloid reactivity in tissues with extensive, “waxy deposition”, however, was incomplete with p5R+14 (i.e. not all of the amyloid was decorated with peptide). This effect was observed most commonly in the spleen. There are a number of possible explanations for the lack of full amyloid coverage by ^{125}I -p5R+14 *in vivo*: 1) There was a lower concentration of peptide binding sites in splenic amyloid compared to other tissues; 2) dense amyloid deposition rendered the peptide incapable of fully penetrating the pathological material; or 3) the binding site became buried in the thick amyloid depots which commonly form in the spleen. Peptide reactivity with ApoA2c tissues in the *ex vivo* binding assays (Chapter 4) demonstrated that p5R+14 bound to amyloid-laden spleen with a greater percentage relative to its reactivity with most other tissues and the reactivity of other peptides with ApoA2c spleen. Thus, the peptide binding site must be present in splenic tissue amyloid and is likely no less pronounced than in other tissues. Since the much larger SAP radiotracer (MW 250,000 Da) could fully penetrate ApoA2c splenic amyloid *in vivo* (Figure 6.1), it seemed unlikely that the peptide would be incapable of doing the same.

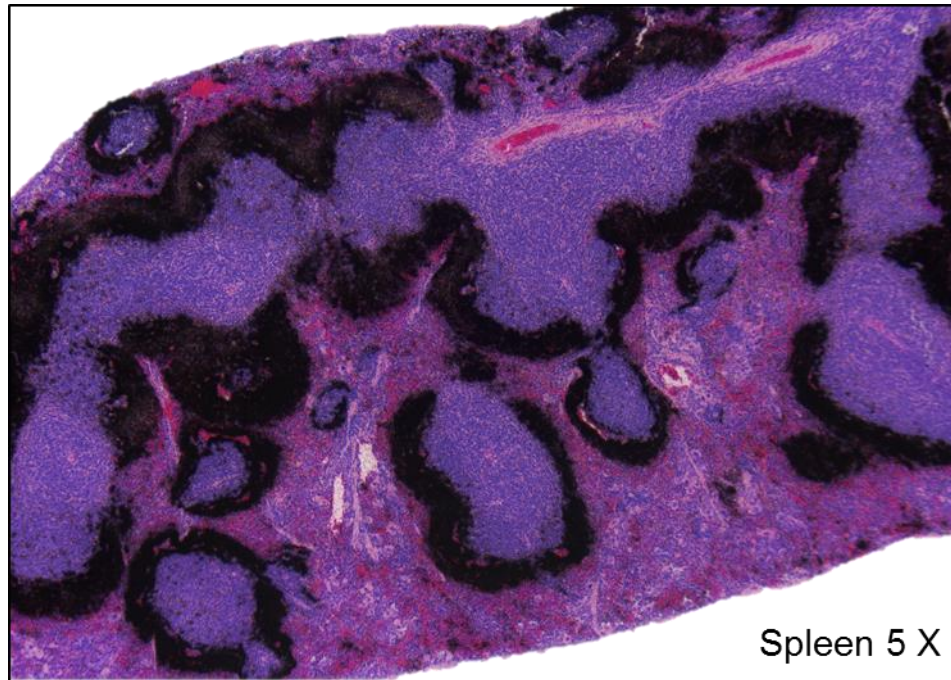


Figure 6.1: Autoradiograph of ^{125}I -SAP in ApoA2c amyloid-laden spleen. The black silver grains deposited perfollicularly in the splenic tissue sample demonstrated full ApoA2c amyloid coverage with ^{125}I -SAP. The healthy red pulp and white pulp were unaffected by the radiotracer which suggested that SAP preferentially bound amyloid in this tissue. (Image provided by Dr. Jonathan Wall).

Therefore, it appeared most likely that the binding site for the peptide became buried or more inaccessible within *in vivo* conditions as the deposits grew denser over time. If this were the case, p5R+14 reactivity with tissue amyloid *in vivo* may underrepresent the full burden of amyloid, especially if these tissues had developed extensive deposition. This hypothesis was tested by performing a study that compared the *in vivo* reactivity of p5R+14 in ApoA2c mice using tissue biodistribution measurements with the *ex vivo* reactivity of the peptide in the pull down binding assay utilizing tissues harvested from the same mice. To achieve this, the peptide was radiolabeled with I-123 for the *in vivo* biodistribution study because it has a half-life of only 13 hours which would allow for a full radioactive decay of the harvested tissues (10 half-lives as recommended by UT Radiation Safety) within 5 days. These tissues could then be prepared as homogenates for the *ex vivo* assay. In order to have enough material to homogenize, a sample of each tissue was not collected for ARG in this study. Thus, the p5R+14 peptide was tested under both conditions using the same tissues to determine if the under-representation of amyloid burden, when analyzed *in vivo*, was due to inaccessibility of the peptide binding site.

6.2. Biodistribution measurements of ¹²³I-p5R+14 in ApoA2c and WT mice

To obtain *in vivo* measurements of peptide binding, p5R+14 was radiolabeled with I-123 as described in the methods chapter (Section 3.5) with the help of Dr. Stephen Kennel. Radiopurity was assessed, and the peptide was evaluated in the *ex vivo* binding assay with AA liver (Figure 6.2). Mice were injected with approximately 20 µCi of radiolabeled peptide with an uptake time of 4 hours. Mice were then euthanized

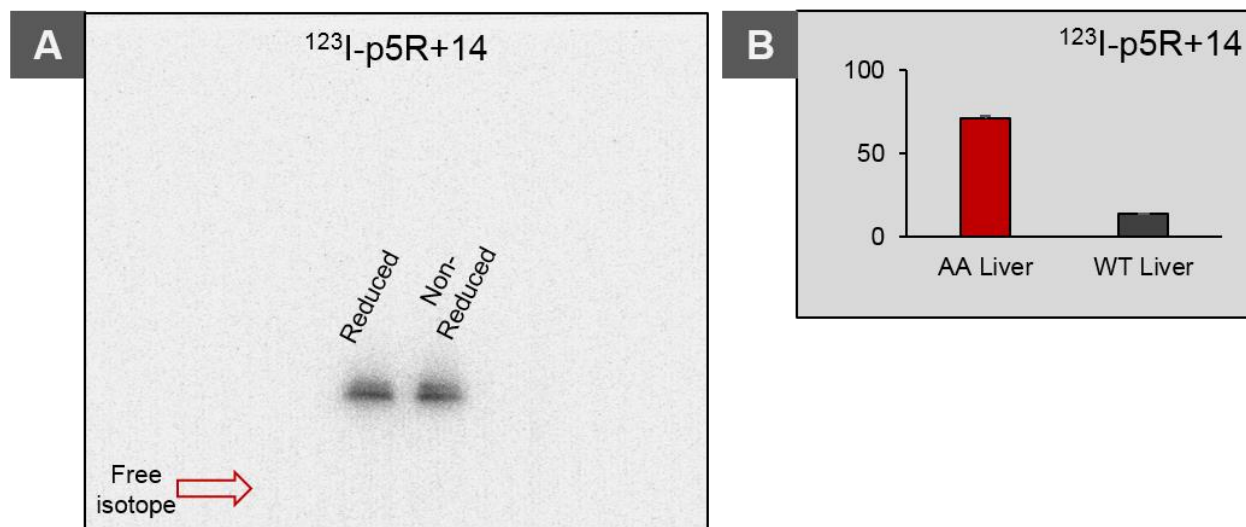


Figure 6.2: Assessment of radiopurity and amyloid-reactivity of ^{123}I -p5R+14. (A) *SDS-PAGE analysis of radiolabeled peptide.* The reduced and non-reduced samples migrated identically on the gel and revealed no oligomeric species within the product. Additionally, no free isotope was detected. (B) *Results of the ex vivo peptide binding assay.* The radiolabeled p5R+14 binds amyloid-laden tissue and yields low reactivity with healthy tissue.

by inhalation overdose of isoflurane, and tissues of interest (liver, spleen, kidneys, intestines, lungs and heart) were harvested for biodistribution measurements. As indicated in Figure 6.3, there was a higher percent injected dose per gram in most ApoA2c tissues relative to the amount detected in an average of 2 WT mice for each tissue; however, the uptake (% ID/g) was, with the exception of the heart, not more than 2 or 3-fold higher than the non-specific reactivity of p5R+14 with healthy tissue, and no measurement was higher than 7 %ID/g for any tested tissue. With biodistribution values less than 7% in all examined tissues, it was unclear whether or not these ApoA2c mice had actually developed significant amyloid or if the pathological deposits were incompletely targeted. Since samples were not harvested for Congo red histological examination, an additional study was performed to test for the presence of amyloid.

6.3. ¹²⁵I-p5R+14 binding assay on ApoA2c tissue homogenates

As a means of confirming amyloid deposition and comparing *in vivo* peptide reactivity with *ex vivo* binding data, the harvested tissues were frozen for 5 days for radioactive decay (10 half-lives) and homogenates prepared for the *ex vivo* peptide binding assay (Section 3.10.1). Each of 6 individual tissues from 3 ApoA2c mice and 2 WT mice were homogenized as previously described (Section 3.9) and a pull down assay performed. Peptide p5R+14 was radiolabeled with ¹²⁵I for the *ex vivo* binding assays due to cost efficiency and radioisotope half-life ($T_{1/2} = 59$ days; the assays took 3 weeks to complete). Our data indicated that p5R+14 bound to all ApoA2c tissues (> 20%) to a much greater degree than the non-specific binding associated with WT tissues (< 10%; Figure 6.4). Given that the peptide can bind the amyloid-laden tissue

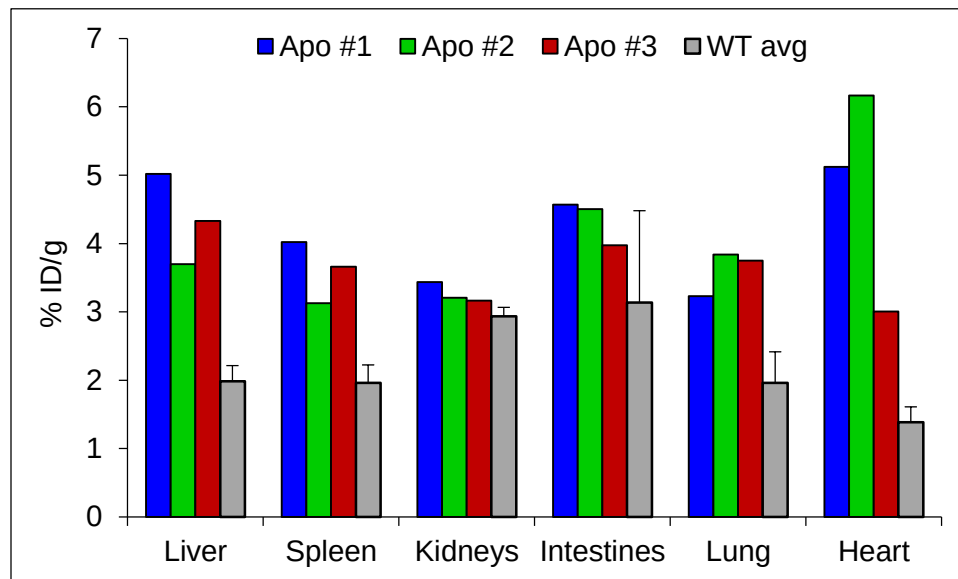


Figure 6.3: Biodistribution measurements of ^{123}I -p5R+14 in ApoA2c and WT mice at 4 h pi. The radiotracer accumulation in various tissues harvested from 3 individual ApoA2c amyloid-laden mice is shown. In addition, the mean plus SD of peptide accumulation obtained from 2 WT mice is included to represent any non-specific binding within the tissues. Notice that the % ID/g for the liver, spleen, lung and heart of each ApoA2c mouse exceeded the average WT value. The kidneys, a tissue less affected by ApoA2c amyloid, revealed similar values for diseased and healthy mice. After removal of the feces, the ApoA2c intestines did not appear to accumulate much more peptide than that seen with WT mice.

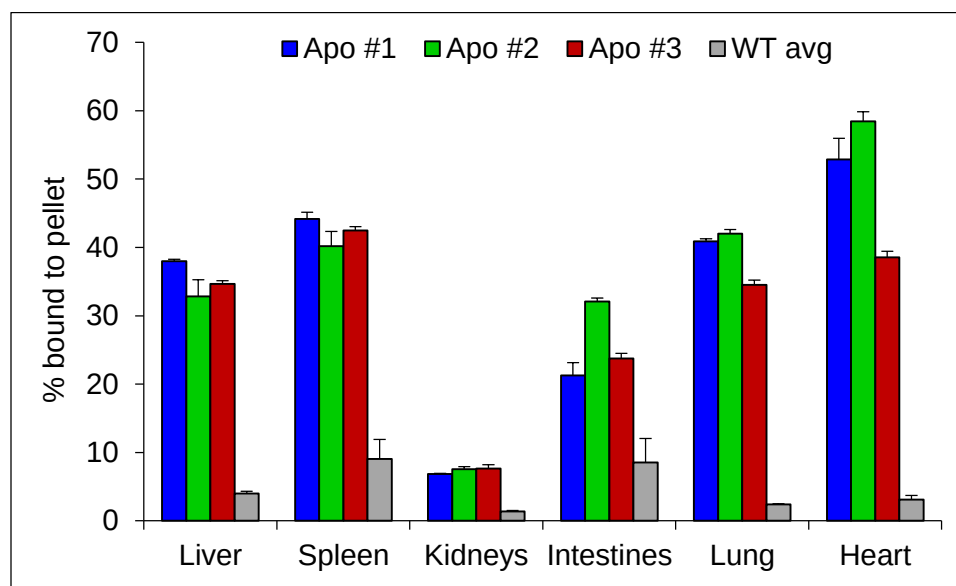


Figure 6.4: *Ex vivo* assessment of ^{125}I -p5R+14 binding to ApoA2c and WT homogenized tissues. The tissues analyzed in this assay were harvested from the 3 ApoA2c mice and 2 WT mice used in section 6.2. The data indicate that there was greater peptide reactivity with all diseased tissues as compared to that seen in an average measurement of non-specific peptide binding to amyloid-free tissue homogenates.

from the healthy tissue for each organ as suggested by the percentage of radiolabeled peptide bound to the pellet in this assay, it seemed logical to assume that p5R+14, when exposed to the multitude of binding sites presented after tissue homogenization, can effectively target ApoA2c amyloid.

6.4. Comparison of *in vivo* and *ex vivo* peptide binding data

In order to directly compare the *in vivo* and *ex vivo* data, the amount of radioactivity (counts per minute; cpm) in each tissue was reported relative to the weight of the tissue. In the *ex vivo* binding assay, approximately 5 mg of tissue and 100,000 cpm were used in each sample. Thus, if complete binding was achieved, 1 mg of tissue would bind 20,000 cpm. For the biodistribution study, each tissue was weighed, and a percentage of the injected dose (measured in cpm) in the tissue was recorded. With these data, the *in vivo* analyses could be directly compared to the data generated from the binding assay. An example of the analysis can be seen in Figure 6.5, and the results from each individual mouse are presented in Figure 6.6.

The direct comparison of p5R+14 binding to amyloid *in vivo* and *ex vivo* showed that the peptide underrepresented the amount of amyloid in half of the tested tissues—notably the spleen, lungs and heart. Since the tissues harvested at necropsy were to be homogenized and used as substrate for the *ex vivo* pull down assay, there was not enough of each sample to develop ARG. Thus, we were unable to visually locate regions of amyloid in these specific tissues that were not completely targeted by the peptide radiotracer. Previous studies utilizing p5R+14 in ApoA2c mice produced ARG

Tissue homogenate preparation

- 50 μL of a 1 g/10 mL PBS solution was used in each Eppendorf tube for the assay.
- 1 g/ 10 mL = 0.0001 g/ μL (0.0001 g/ μL x 50 μL) = 0.005 g = **5 mg tissue**

Approximately 100,000 counts (cts) of radiolabeled p5R+14 was added to 5 mg tissue; thus **20,000 cts per 1 mg**.



Scaling biodistribution data for comparative analysis with ex vivo data

For a liver weighing 937 mg and an injected dose (ID) of 12,167,870 cts:

- (937 x 20,000) = 18,740,000 cts = theoretical maximum (TM; 100% binding)
- Prior to making further calculations, the average amount of counts in 2 WT livers was subtracted
 - measured counts in liver = 572362.4
 - average counts in WT liver = 238280.5

} 334081.9 cts

$$\left\{ \left[\left(\frac{\text{Remaining cts in liver}}{TM} \right) \right] \times \left(\frac{TM}{ID} \right) \right\} \times 100 = \% \text{ used in comparative analysis}$$

$$\left\{ \left[\left(\frac{334,081.9}{18,740,000} \right) \right] \times \left(\frac{18,740,000}{12,167,870} \right) \right\} \times 100 = \mathbf{2.7\%}$$

Figure 6.5: Method for analyzing ex vivo and in vivo data with comparable peptide binding values. Since there was a different amount of tissue used in the ex vivo binding assay (5 mg as compared to the whole organ used in the in vivo biodistribution measurements) and a different amount of radioactivity, the two methods were scaled by a theoretical maximum of counts bound to a certain weight of tissue. The example shown revealed a theoretical maximum of 2.7% binding in vivo for the amount of peptide that could bind to an equivalent piece of tissue ex vivo. Additionally, non-specific binding to each tissue was accounted for by the subtraction of peptide reactivity observed with respective WT tissues. All data were scaled according to the provided example in order to compare the datasets.

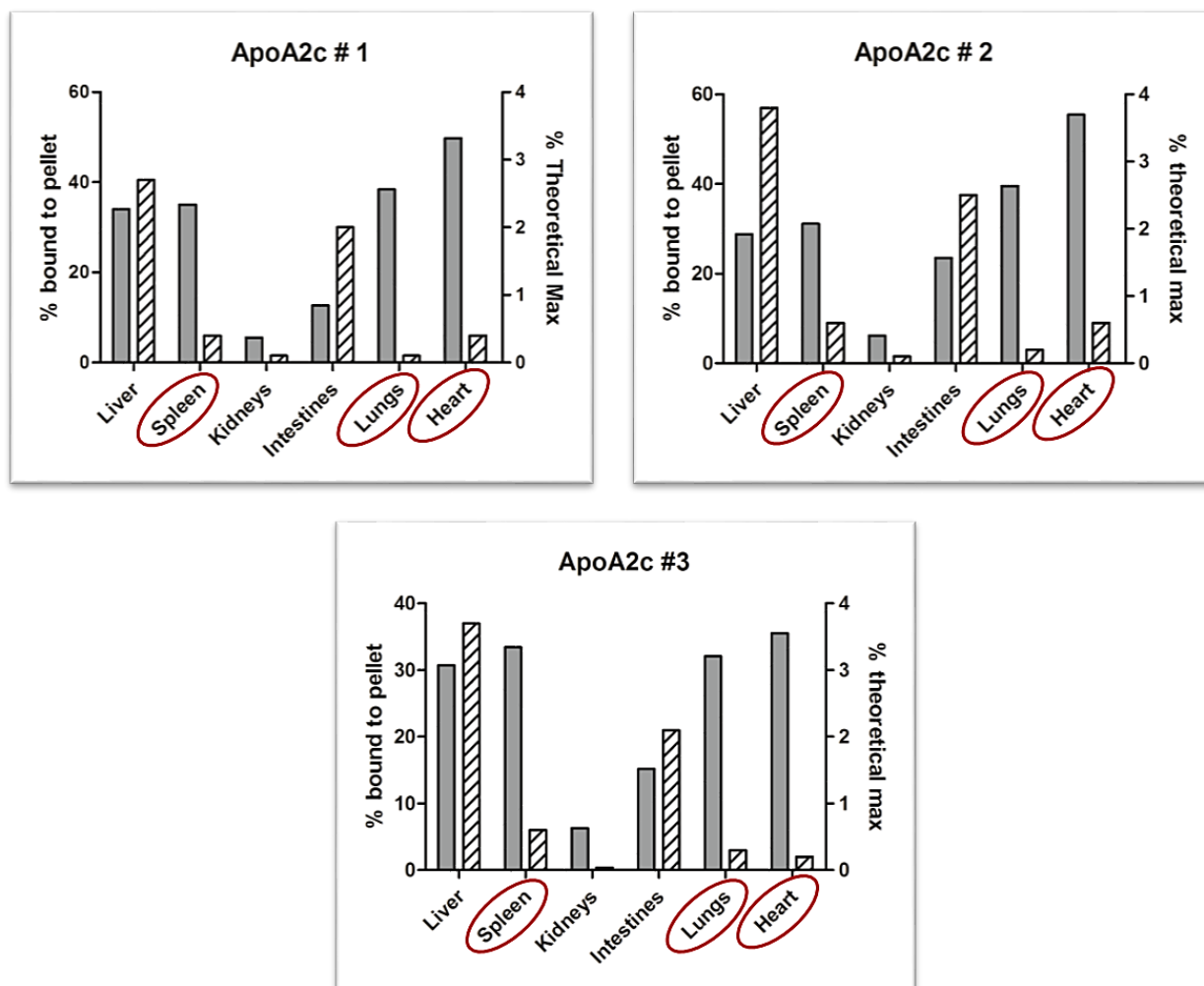


Figure 6.6: Comparative analysis of *ex vivo* peptide-binding data and *in vivo* biodistribution data in 3 ApoA2c mice. The *ex vivo* data (solid bars) are plotted against the left axis and *in vivo* data (striped bars) are plotted against the theoretical maximum axis on the right. In all 3 mice, there was a good representation of the liver and intestines with both the *ex vivo* and *in vivo* data. Since there is not typically a large amount of renal ApoA2c amyloid, the low kidney binding with both techniques was also expected. In the spleen, lungs and heart, however, there was a consistent under-representation of amyloid in the *in vivo* biodistribution analysis. This suggested that, in these tissues, radiotracer accessibility to the amyloid may be hindered *in vivo*.

which demonstrated the lack of complete amyloid coverage in the spleen, lung and heart (Figure 6.7). These observations further confirmed that peptide p5R+14 quantitatively underestimated the ApoA2c amyloid burden *in vivo*.

6.5. Conclusions

The data presented here indicate that even though p5R+14 was the optimal agent for imaging ApoA2c amyloid, it bound to ApoA2c tissues more effectively in the *ex vivo* assay for many of the tested tissues—particularly the spleen, lungs and heart. These findings indicate that the peptide binding site within amyloid that is associated with these organs was more readily available in the homogenized tissue samples than in the pathological tissue deposits *in situ*. Therefore, to enhance the reactivity of peptide with ApoA2c amyloid *in vivo*, a different peptide might be necessary. According to the literature [371], comparison of peptides p5 and p5R (the variant of p5 with arginine residues substituted for all lysine residues) in a murine model of AA amyloidosis revealed that the arginine variant had a higher affinity for amyloid resulting in a “binding site barrier effect” which limited amyloid coverage by the peptide in tissues with extensive amyloid deposition [371]. Given that p5R+14 has twelve arginine residues, it may also suffer from a similar effect in the extensive ApoA2c amyloid deposits. Additionally, it is worth considering that the biological half-life of a peptide is generally relatively short [410-412], due to its small size, which may have hindered the peptide’s ability to access the entirety of amyloid deposition due to its rapid catabolism and removal from the blood pool. To address this specific issue, we tested modified peptides which are discussed in the next chapter.

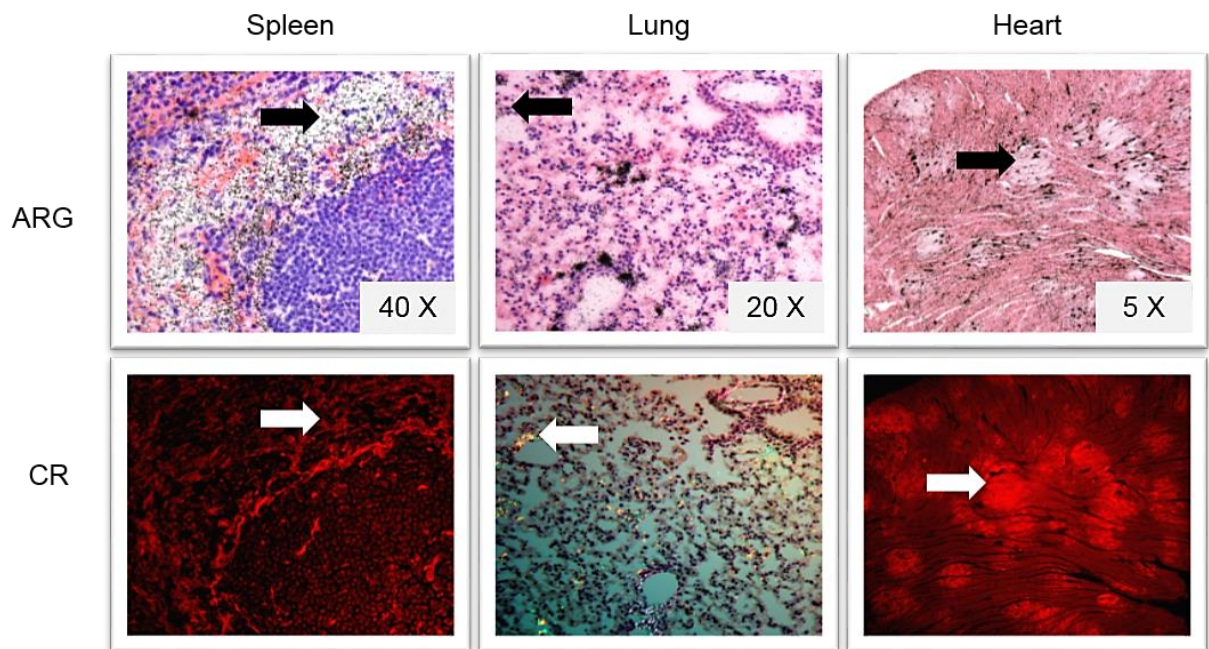


Figure 6.7: ApoA2c amyloid deposits which were unidentified by the ^{125}I -p5R+14 radiotracer in various tissues. Autoradiographs (ARG) of ApoA2c spleen, lung and heart demonstrated that the peptide, visualized as black silver grains, was incapable of fully targeting the entirety of amyloid deposition in these tissues. The regions of amyloid are shown as bright red in the Congo red (CR) fluorescent images of splenic and cardiac tissue, and the amyloid within the lung tissue appears as birefringent greenish-gold material in the center CR image. The black arrows indicate regions of amyloid that are not fully covered with ^{125}I -p5R+14 in these tissues. The white arrows indicate regions of amyloid which lack radiotracer co-localization. (Heart image was supplied by Dr. Jonathan Wall).

Chapter 7

Assessment of modified peptides designed to enhance quantitative ApoA2c amyloid detection

7.1. Overview

It has been shown that peptide p5R+14 effectively binds to ApoA2c amyloid such that many amyloid-laden organs can be non-invasively visualized with SPECT/CT imaging. Observations obtained from the *in vivo* vs. *ex vivo* amyloid binding experiment suggested that the peptide could not bind ApoA2c amyloid deposition in its entirety, however. In mice with advanced deposition, tissues accumulated dense pathological deposits of amyloid that the peptide was unable to bind to or fully penetrate. Thus, the representation of ApoA2c amyloid *in vivo* was underestimated with ¹²⁵I-p5R+14, notably in the spleen, lung and heart. While the peptide imaging agent may be effective at detecting amyloidosis, the lack of full amyloid coverage could be detrimental to the clinical use of this peptide in monitoring quantitative response to therapy aimed at clearing dense tissue amyloid deposits. We hypothesized that this phenomenon may be likely due to: 1) the high affinity of p5R+14 for the amyloid resulting in a binding site barrier effect, or 2) the short half-life of the peptide in the circulation did not allow for complete uptake of the peptide by poorly “vascularized”, dense amyloid lesions. To address these potential concerns, modified peptides were designed and tested in ApoA2c mice. These peptides addressed issues pertaining to amyloid binding affinity and serum half-life in an attempt to enhance uptake by dense amyloid.

7.2. Studies using ¹²⁵I-p5+14: A peptide addressing amyloid affinity and binding site barrier effects

A recent study using amyloid-reactive peptides suggested that enhanced affinity to the target resulted in a binding site barrier effect which hindered full amyloid coverage in extensive deposits [371]. This study utilized heparin-binding peptide, p5, and a variant of this peptide, p5R. The latter peptide was modified to contain arginine residues in place of all lysine residues, resulting in a greater propensity of the peptide to adopt an α -helical secondary structure [371, 425]. Since the helical structure of peptide p5 has been postulated as an important property required for amyloid-binding [371, 409], the potential for a more helical peptide, which could result in higher-affinity amyloid-targeting, was appealing. The arginine variant, indeed, had a higher affinity for heparin and AA amyloid as evidenced by HPLC and *ex vivo* NaCl titration, respectively [371]. Additionally, p5R was shown to co-localize with congophilic material in a murine model of AA amyloidosis indicating preferential *in vivo* binding to amyloid. Autoradiographs with ¹²⁵I-p5 or ¹²⁵I-p5R in tissues harvested from AA mice, with “1+” and “4+” splenic amyloid burden, were compared (Figure 7.1). Results of ARG studies indicated that, in mice with less amyloid, the peptides were equally efficient at quantitatively targeting all deposits, i.e. full coverage of the amyloid by peptide was achieved. In mice with greater amyloid burden, however, the high affinity of peptide p5R hindered its ability to penetrate dense deposits, resulting in a “binding site barrier effect”; thus, the lower affinity peptide p5 was more effective at quantitative amyloid coverage in mice with extensive deposition [371]. These findings introduced the notion that enhanced affinity of a peptide may lead to a binding site barrier effect causing less

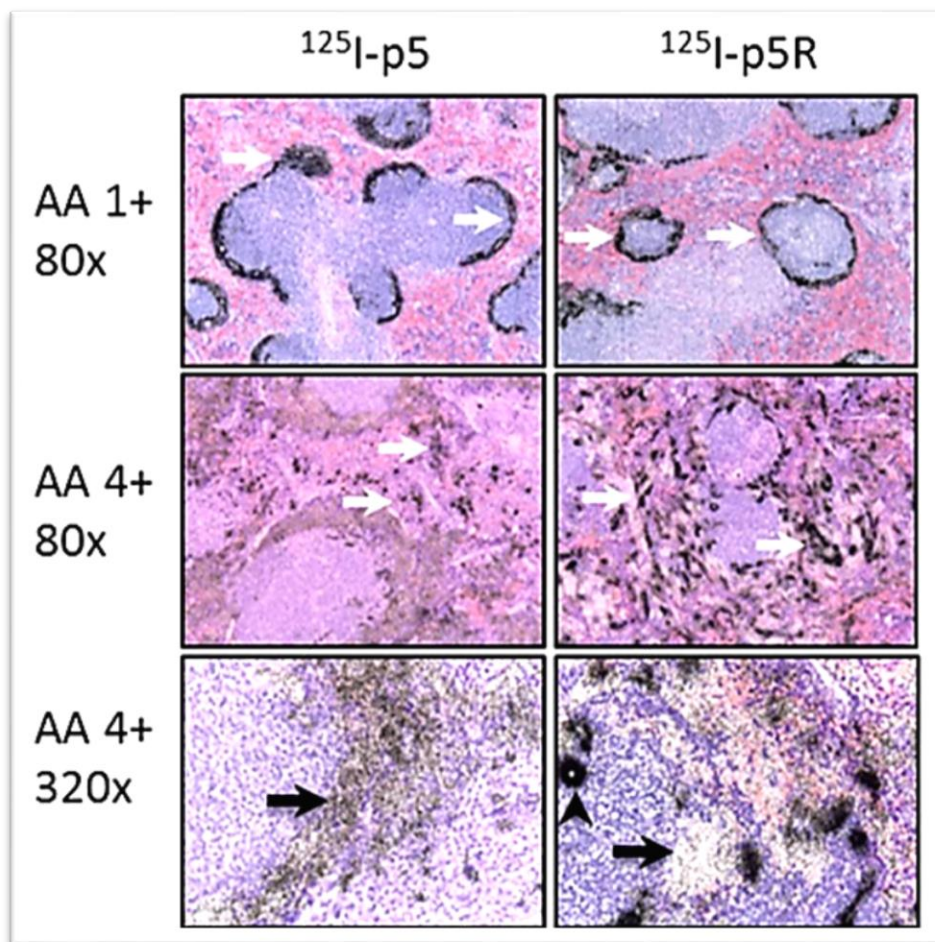


Figure 7.1: Autoradiographs (ARG) indicating disparate AA amyloid targeting with two radiotracers. These ARG demonstrate that amyloid coverage with peptides p5 and p5R decrease as the amount of amyloid within tissues increases. The coverage in mouse tissue with little amyloid (AA +1) looks similar with both peptides. Coverage of amyloid in tissue with more pathological deposition (AA 4+) revealed a pattern of p5 distribution which appeared less dense than that seen with p5R, but the coverage seemed to be more complete. The last row in the figure shows full amyloid coverage on the left (p5; black arrow) and incomplete coverage on the right (p5R; black arrow). The black arrowhead points to a region near a blood vessel that contains dense peptide deposition—thought to be the presence of a “binding site barrier effect”. (Image taken from [371]).

than complete target coverage. Given that incomplete ApoA2c amyloid coverage was observed with p5R+14, a peptide with 12 arginine residues, we posited that substitution of these 12 residues with lysines, thus slightly reducing the amyloid affinity, may visibly increase *in vivo* amyloid coverage observed with ARG and increase radiotracer accumulation seen in biodistribution measurements and SPECT/CT image analyses.

Results

For this study, peptides p5+14 and p5R+14 were both radiolabeled with ¹²⁵I (Section 3.5) and tested for radiochemical purity and amyloid-reactivity (Sections 3.8 and 3.10.1) prior to *in vivo* analyses. Additionally, affinity of both peptides for rVλ6Wil fibrils was assessed by performing a pull down assay with NaCl titration. It was determined that: there was no free isotope in the radiolabeled products; both peptides showed positive reactivity with AA amyloid liver; and a higher concentration of NaCl was required to interfere with rVλ6Wil fibril reactivity for peptide p5R+14 as compared to the lysine variant, which was expected (Figure 7.2). The radiolabeled peptides were intravenously injected into the lateral tail vein of ApoA2c mice that had been induced with ApoA2c AEF ~ 12 weeks prior to the imaging study (n = 5 with p5+14 and n = 4 with p5R+14). Necropsies were performed on all mice at 2 h pi, and harvested tissues were analyzed by using tissue biodistribution measurements and microautoradiography (Sections 3.14 – 3.16).

The accumulation of the radiotracers in harvested tissues was reported as a percent of the injected dose per gram of tissue (Figure 7.3). Results indicated that, on average, mice that received p5R+14 had greater accumulation of radioactivity in all tissues as compared to mice that received p5+14. The hypothesis that a peptide with

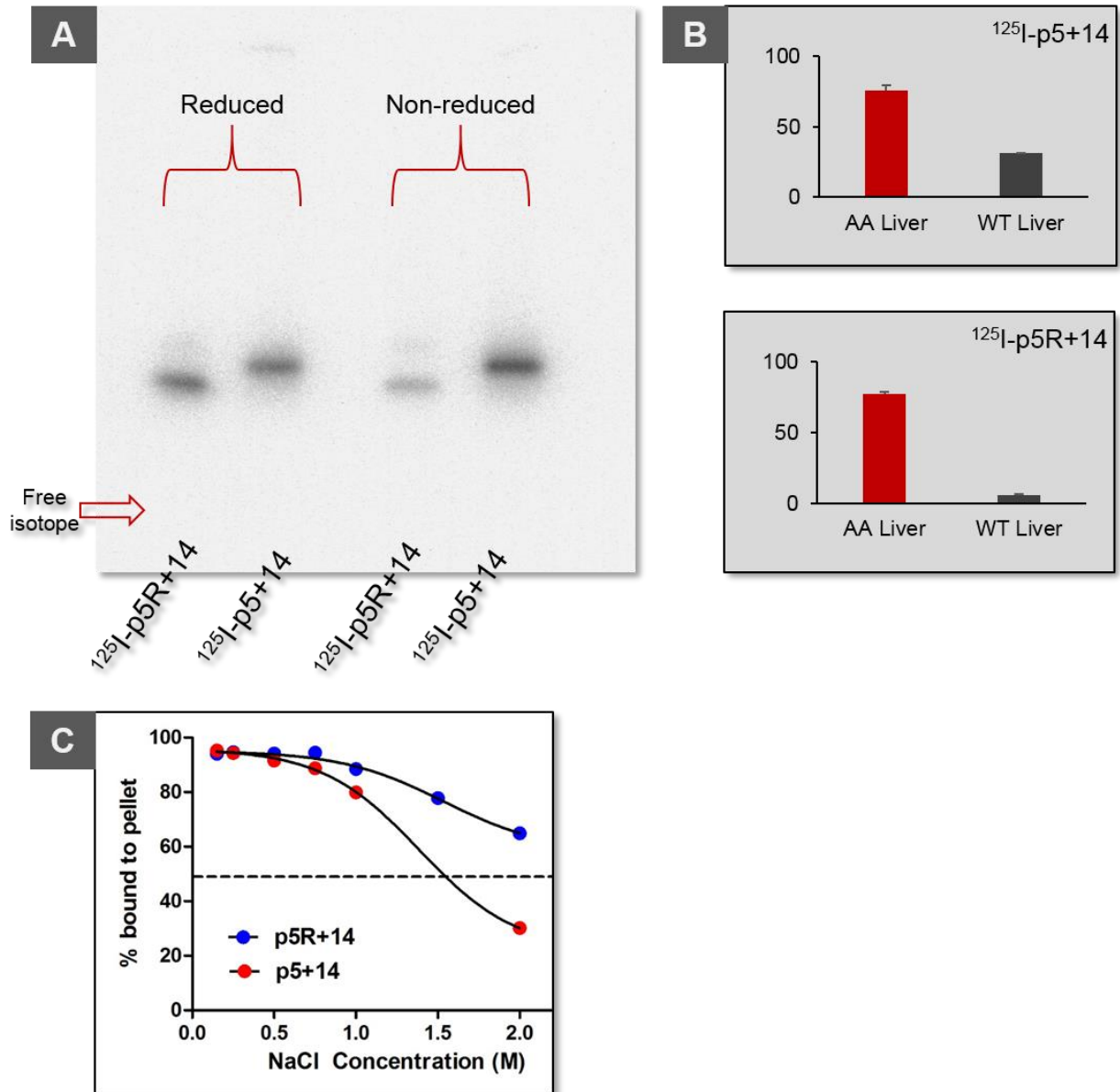


Figure 7.2: Assessment of radiopurity and amyloid-laden tissue and synthetic rVλ6Wil fibril reactivity. (A) *Electrophoresis of radiolabeled peptides.* The SDS-PAGE revealed that reduced and non-reduced samples migrated similarly. Additionally, no free isotope was detected in the products. (B) *Ex vivo peptide binding assay.* Amyloid-reactivity as evidenced by enhanced binding to amyloid-laden liver compared to binding to WT liver. (C) *NaCl titration with peptides on rVλ6Wil fibrils.* The titration indicated fibril reactivity with both peptides but higher affinity with p5R+14 compared to the lysine variant, p5+14.

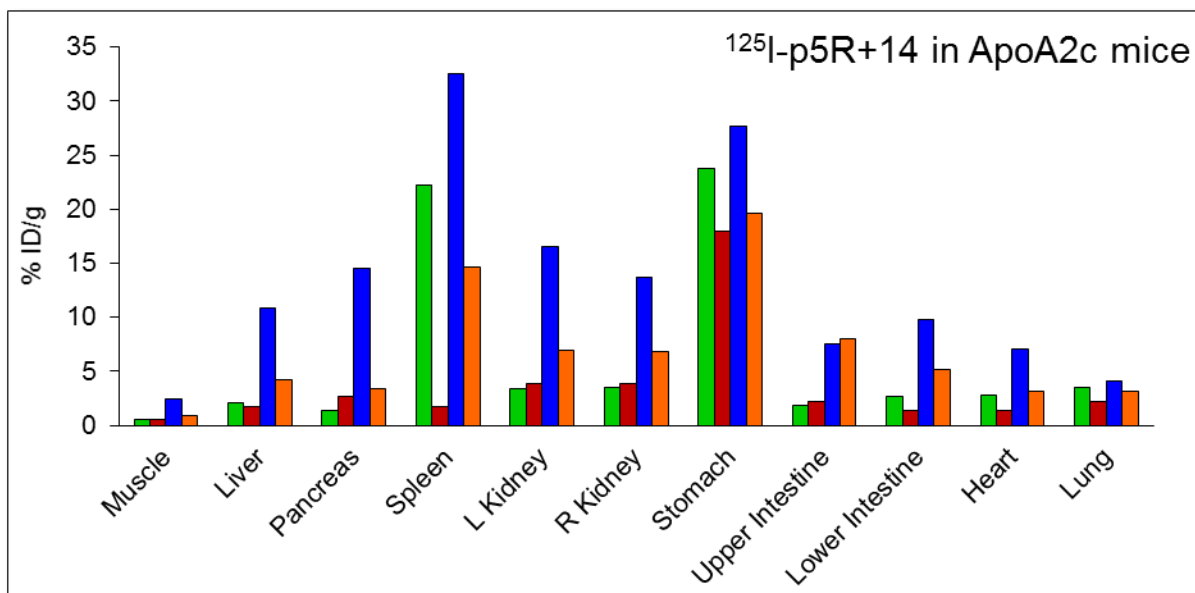
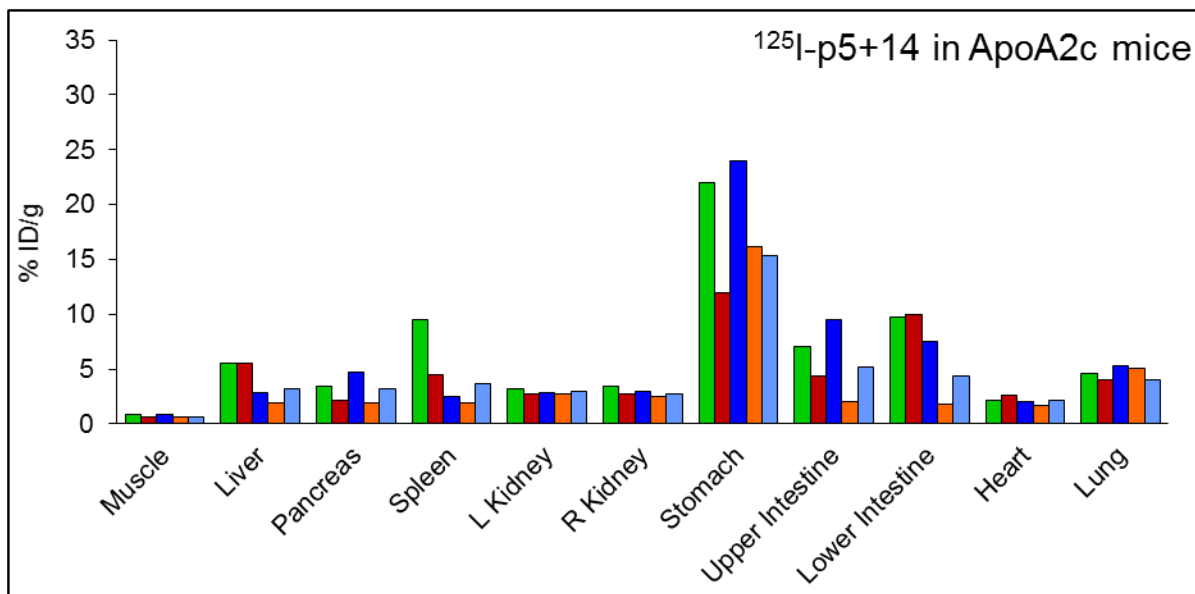


Figure 7.3: Biodistribution measurements 2 h pi of either ^{125}I -p5+14 or ^{125}I -p5R+14 in ApoA2c mice. The *in vivo* amyloid reactivity of both peptides showed that, in most tissues, there was greater ^{125}I -p5R+14 accumulation than ^{125}I -p5+14. One mouse, however, appeared to have very little ^{125}I -p5R+14 accumulation in any tissue. This mouse (red bar in bottom graph) likely had less amyloid than the other mice since only the stomach had comparable uptake which was due to free iodide sequestration after peptide dehalogenation. Each bar on the graphs represents individual mice; thus, no error bars are included.

less affinity may provide enhanced amyloid coverage did not hold true based on the biodistribution measurements alone—peptide p5+14 would likely have had greater accumulation if its lower affinity for amyloid improved coverage. To confirm that the peptides were specifically associated with amyloid deposits in the tissues, ARG was performed. The appearance of black silver grains seen in representative tissues confirmed that both peptides co-localized specifically with amyloid material, but p5R+14 achieved enhanced amyloid coverage as compared to peptide p5+14 (Figure 7.4). The binding site barrier effect was again present with p5R+14, but this peptide remained the most effective peptide for ApoA2c detection due to its high affinity for this type of amyloid.

These data suggested that the low affinity, lysine-rich peptide was less efficient than p5R+14 at detecting ApoA2c amyloid *in vivo*. In these experiments, contrary to the hypothesis, the peptide with a lower amyloid affinity did not enhance target (amyloid) coverage. Instead, the p5R+14 peptide that exhibited the binding site barrier effect in the lung and spleen was surprisingly the most effective at ApoA2c amyloid detection *in vivo* as evidenced by tissue biodistribution measurements and ARG. Given these data, we turned our attention to the second hypothesis and attempted increasing the serum half-life of the peptide without altering the arginine amino acids responsible for the strong interaction with amyloid. We posited that this peptide may achieve improved target coverage if it remained in circulation longer and was, therefore, “exposed” to the amyloid for longer periods.

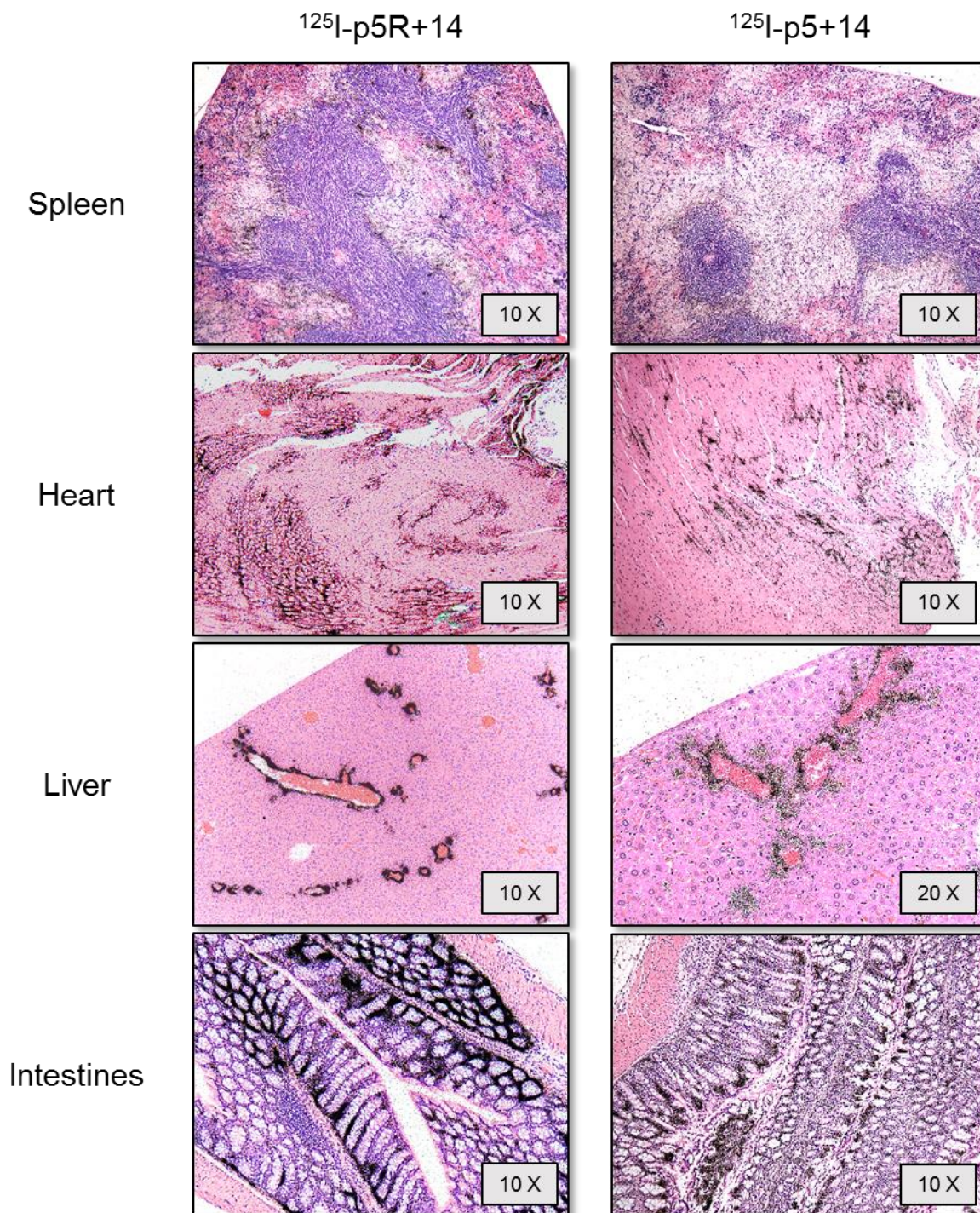


Figure 7.4: Autoradiographs demonstrating radiotracer accumulation in amyloid-laden tissues. ARG revealed that both peptides bound to amyloid within the tissues, but ^{125}I -p5R+14 exhibited greater accumulation when compared to ^{125}I -p5+14 as evidenced by the density of black silver grains within the amyloid deposits.

7.3. Studies using PEGylated ¹²⁵I-p5R+14: A peptide to study the effects of longer serum half-life

As stated in Chapter 1 (Section 4.4), there are several advantages to using peptides as imaging agents: they can be chemically synthesized; they are generally non-immunogenic; they clear rapidly when not bound to target; they can penetrate vessels due to small size [409-413]. Given that peptides are relatively small biological agents, in comparison to other potential radiotracers (i.e. antibodies, scFv's and proteins), their biological half-life is shorter compared to larger agents. For example, when the ~ 3100 Da peptide, p5, was radioiodinated and injected into WT mice, it was determined that the effective half-life of the peptide was ~ 2 – 3 min [416]. In contrast, when healthy mice were injected with the 250 kDa SAP, the half-life was determined to be ~ 7.5 h [427]. While rapid clearance of unbound radiotracer can be considered advantageous in molecular imaging studies because it can improve the signal-to-noise ratio of images, the short half-life of peptides could potentially limit the extent of ApoA2c amyloid-binding. The affinity of p5R+14 for ApoA2c amyloid *in vivo* and *ex vivo* surpasses all other tested peptides, but although it successfully detects deposits, it has not been shown to fully penetrate dense amyloid material. Therefore, a true, quantitative representation of the amyloid load in a mouse (or a patient) injected with p5R+14 might not be achievable.

In an attempt to extend the serum half-life of p5R+14—which may enhance the peptide's ability to penetrate ApoA2c amyloid—we incorporated a 20 kDa polyethyleneglycol (PEG) moiety to the N-terminus of the peptide (synthesized by RS Synthesis, Lexington, KY). This approach, also termed PEGylation, has been well-

documented in the literature for its utility, safety and success in extending the half-life of various molecules including those already approved by the FDA and in clinical use [428-431]. Since PEG is a “neutral, hydrophilic polyether”, it usually “exhibits little [immuno] reactivity” [432]. The incorporation of a PEG structure to an imaging agent, thus, yields a non-immunogenic, non-antigenic conjugate with an increased molecular weight which may alter its physicochemical properties and rate of catabolism within the organism; this may result in extended serum half-life [428, 432]. Without modification, peptide p5R+14 had a molecular weight of ~ 4,500 Da; after N-terminal addition of PEG, the conjugate had a molecular weight of ~ 24,500 Da. We tested both p5R+14 and the PEGylated p5R+14 (PEG-p5R+14) *in vivo* with expectation of detecting both enhanced serum half-life and amyloid coverage with the latter peptide.

Results

For *in vivo* studies, predetermined concentrations of the peptides to be studied (equal to 50 µg per peptide) were radiolabeled for comparable results. To determine the appropriate concentration of PEG-p5R+14, a BCA peptide concentration analysis was conducted with the assistance of Mrs. Angela Williams. Since the PEG portion of the conjugate contains no amino acids, it is not detected with this assay. Thus, unless the PEG interfered with the analysis, the resulting concentration was likely a direct indication of the p5R+14 content. The peptide portion of the conjugate is ~ 1/5 of the molecular weight; therefore, the calculated peptide concentration obtained from the analysis was multiplied by 5 which yielded a concentration of ~ 1 mg / mL. For the following *in vivo* studies, ~ 50 µL (~ 50 µg) of PEG-p5R+14 was radiolabeled with I-125. The non-PEGylated p5R+14 (~ 50 µg) was also radioiodinated, and both peptides were

tested for radiochemical purity (Section 3.8) and amyloid fibril reactivity (Section 3.10.2) prior to their utilization in the following studies. The SDS-PAGE analysis indicated that both peptides were similar in size—a finding that was initially puzzling since the PEGylated peptide should be 20,000 Da larger than the other. It was concluded that the SDS buffer used in the gel electrophoresis did not interact with the neutral, PEG portion of the conjugated peptide; thus, the analysis only represented the polybasic, p5R+14. No free iodide was detected, and the binding assay confirmed positive reactivity with synthetic fibrils (Figure 7.5). There was evidence of non-specific binding to WT liver tissue homogenate with the PEGylated peptide—a finding not entirely unexpected due to the size of the peptide but one that must be taken into consideration when evaluating *in vivo* data. Autoradiography was used to determine if there was non-specific binding to tissue *in vivo*.

Prior to the injection of PEG-p5R+14 in ApoA2c amyloid-laden mice, the PEGylated peptide and p5R+14 were administered to cohorts of WT mice in a pilot study to assess potential off-target binding and to investigate the extended serum half-life of PEG-p5R+14. Mice were injected with ~ 100 µCi of either ¹²⁵I-PEG-p5R+14 or ¹²⁵I-p5R+14, and uptake times were 15 min (n = 2 for PEG-p5R+14 and n = 1 for p5R+14) or 1 h (n = 2 per peptide). SPECT/CT imaging was performed on mice that were allotted an uptake time of 1 h (n = 1 mouse per peptide). All seven mice underwent necropsies to obtain tissues for biodistribution measurements and ARG. Due to the size of PEGylated conjugates, clearance through tissues such as the kidneys, lungs, spleen and lymph nodes is typically lower than that observed with non-PEGylated peptides [428]. Thus, renal clearance was expected to be lower with

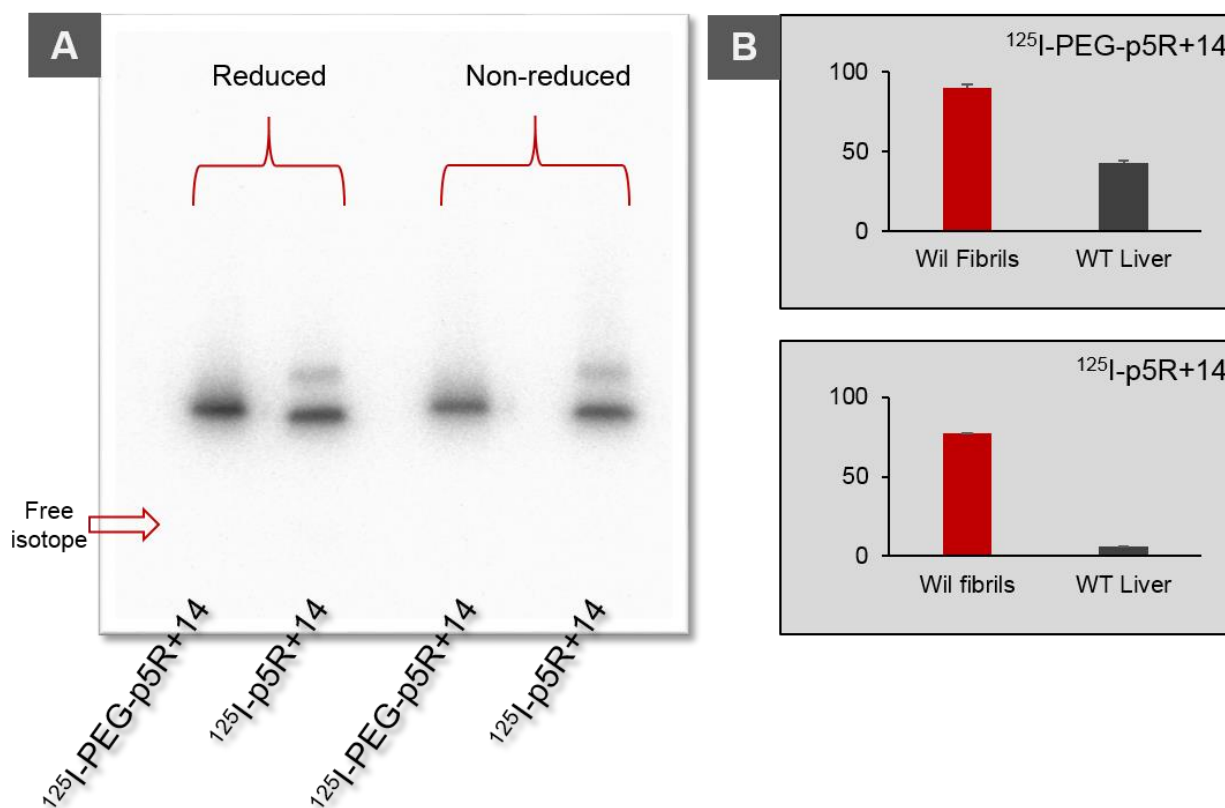


Figure 7.5: Radiopurity and fibril reactivity assays. (A) *SDS-PAGE analysis of radioiodinated PEG-p5R+14 and p5R+14 after 40 min exposure.* No free isotope was detected in the samples. Since PEG was neutral, the SDS buffer did not interact with it making the peptides migrate as the same size in both reduced and non-reduced samples. (B) *Peptide binding assays.* Both peptides exhibited binding to rV λ 6Wil fibrils. The PEGylated peptide had higher background binding than ^{125}I -p5R+14 which was made evident with WT liver reactivity.

^{125}I -PEG-p5R+14 in comparison to ^{125}I -p5R+14 in healthy mice. Results from the biodistribution measurements indicated that, at 15 min pi, the presence of radiotracer in the kidneys was greater than 4-fold higher in the mouse that received ^{125}I -p5R+14; although less pronounced, the same trend was observed at 1 h pi with mice that received the ^{125}I -p5R+14 having a 2-fold higher % ID/g than mice injected with the PEGylated peptide. Additionally, the radioiodide accumulation in the stomach—a direct indication of the dehalogenation of injected radiotracers and uptake by halide symporters present in the stomach [416]—was 2-fold higher at both 15 min and 1 h pi for the non-PEGylated peptide (Figure 7.6). These data suggest that the peptides were processed differently within WT mice and that the dehalogenation, and likely the clearance, of ^{125}I -p5R+14 was accelerated versus the larger ^{125}I -PEG-p5R+14. Images comparing the distribution of these radiotracers at 1 h pi are shown (Figure 7.7).

The *in vivo* detection of ApoA2c amyloid with these peptides was next analyzed. Given that we had observed a distinct difference in biological processing of the two peptides in WT mice, the analysis in diseased mice was extended to 1 h and 4 h pi with the hypothesis that, after 4 h, the PEGylated peptide would have accumulated to a greater extent in the amyloid (determined with biodistribution measurements after confirmation of peptide:amyloid co-localization by CR and ARG observation) as compared to observations made at 1 h. For the *in vivo* study, ApoA2c mice (11 weeks post 100 μg AEF injection) were administered ~ 100 μCi of either radiotracer and were euthanized at 1 h or 4 h pi ($n = 3/\text{peptide}/\text{time point}$). The necropsies of all mice included harvesting portions of muscle, liver, pancreas, spleen, both kidneys, stomach, upper intestine, lower intestine, heart and lung for biodistribution measurements and

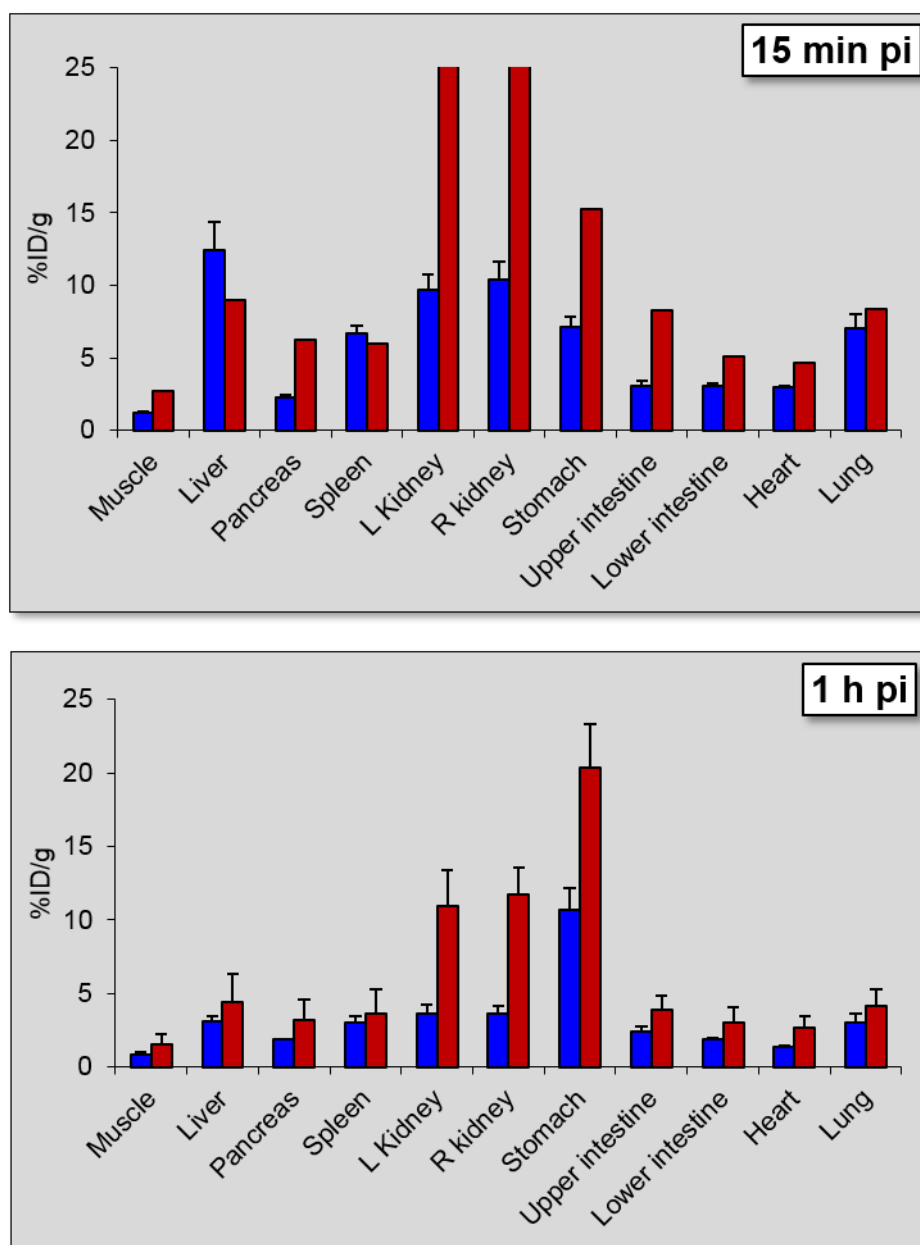


Figure 7.6: Biodistribution of ^{125}I -PEG-p5R+14 and ^{125}I -p5R+14 in WT mice at 15 min and 1 h pi. These biodistribution measurements revealed that the two peptides were processed differently in healthy mice. At 15 min pi, there was a higher % ID/g of ^{125}I -p5R+14 (red) in the kidneys and stomach indicating a higher level of clearance and dehalogenation in comparison to the larger peptide (blue). At 1 h pi, the same trend was present. These data support the notion that PEGylated p5R+14 is processed more slowly than p5R+14 in healthy mice.

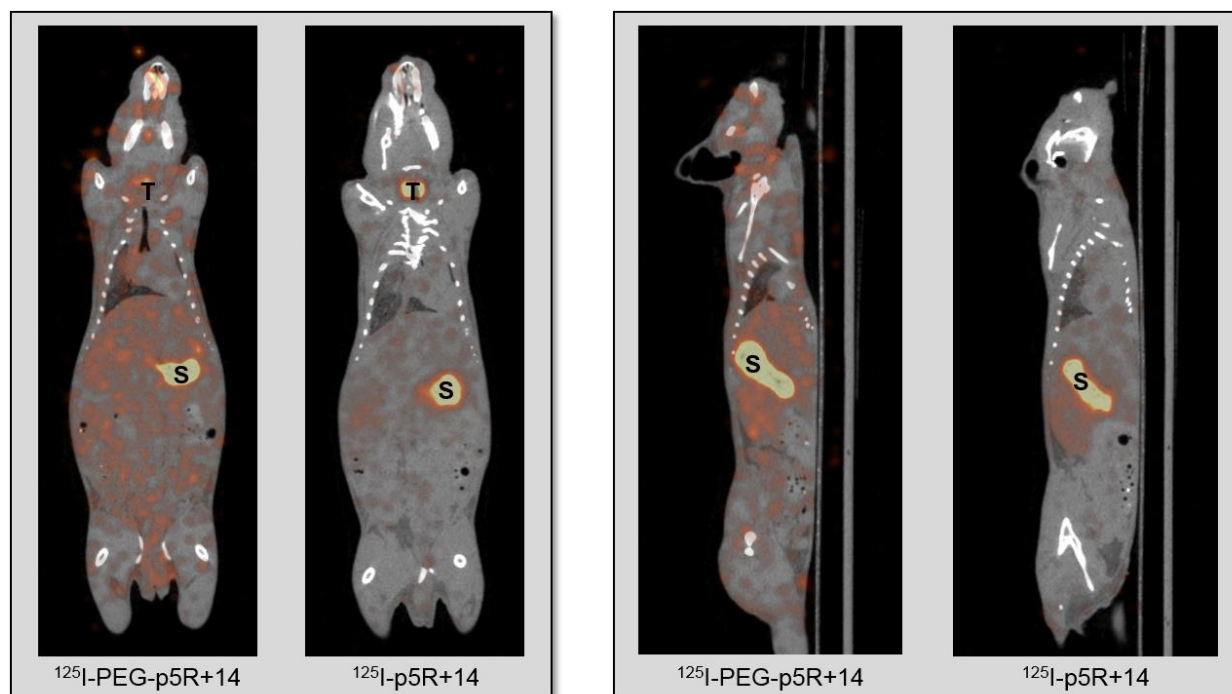


Figure 7.7: SPECT/CT images displaying the distribution of ^{125}I -PEG-p5R+14 and ^{125}I -p5R+14 in WT mice at 1 h pi. Both sets of images were scaled to the same threshold for comparable visualization. The images on the left show a coronal view of the distribution of these two radiotracers. At 1 h pi, there was uptake in the thyroid (T) and stomach (S) with both peptides. Sagittal views on the right also show uptake in the stomach of these mice. These regions of uptake are likely a result of dehalogenation of the peptide and accumulation of free iodide. There is evidence, however, that the PEGylated peptide may have remained in circulation for longer than the non-PEGylated peptide due to a visibly greater amount of disperse radioactivity seen with this peptide in both sets of images.

histological examination. Additionally, the tongue and the cecum—sites possibly affected by ApoA2c amyloid—were taken to be analyzed by ARG to better determine the severity of amyloid burden.

Results comparing the biodistribution of both peptides at 1 h and 4 h pi are displayed (Figure 7.8). These graphs display the average of 3 mice. There was no significant difference between the peptides at 1 h pi. However, there was slightly higher PEG-p5R+14 in the liver and lower intestines, this finding was also observed in ApoA2c tissues at 4 h pi. The increased accumulation of PEGylated peptide in the liver and intestines may have been due to the preferential route of clearance of the larger peptide (hepatobiliary) versus the renal clearance associated with the non-PEGylated p5R+14. These results suggest that the two peptides behave more similarly in the presence of amyloid *in vivo* than they do in healthy mice with no amyloid. Upon examination of tissue autoradiographs, amyloid coverage with ^{125}I -p5R+14 (see Figure 5.8) and ^{125}I -PEG-p5R+14 (Figure 7.9) confirmed similar amyloid-binding behavior between the two peptides.

7.4. Conclusions

A lack of full ApoA2c amyloid coverage had been observed in autoradiographs of tissue sections with ^{125}I -p5R+14; this led to the investigation of strategically modified peptides to potentially enhance ApoA2c amyloid-targeting based on two reasonable hypotheses. Data suggests that the arginine-rich, high affinity peptide may suffer from a

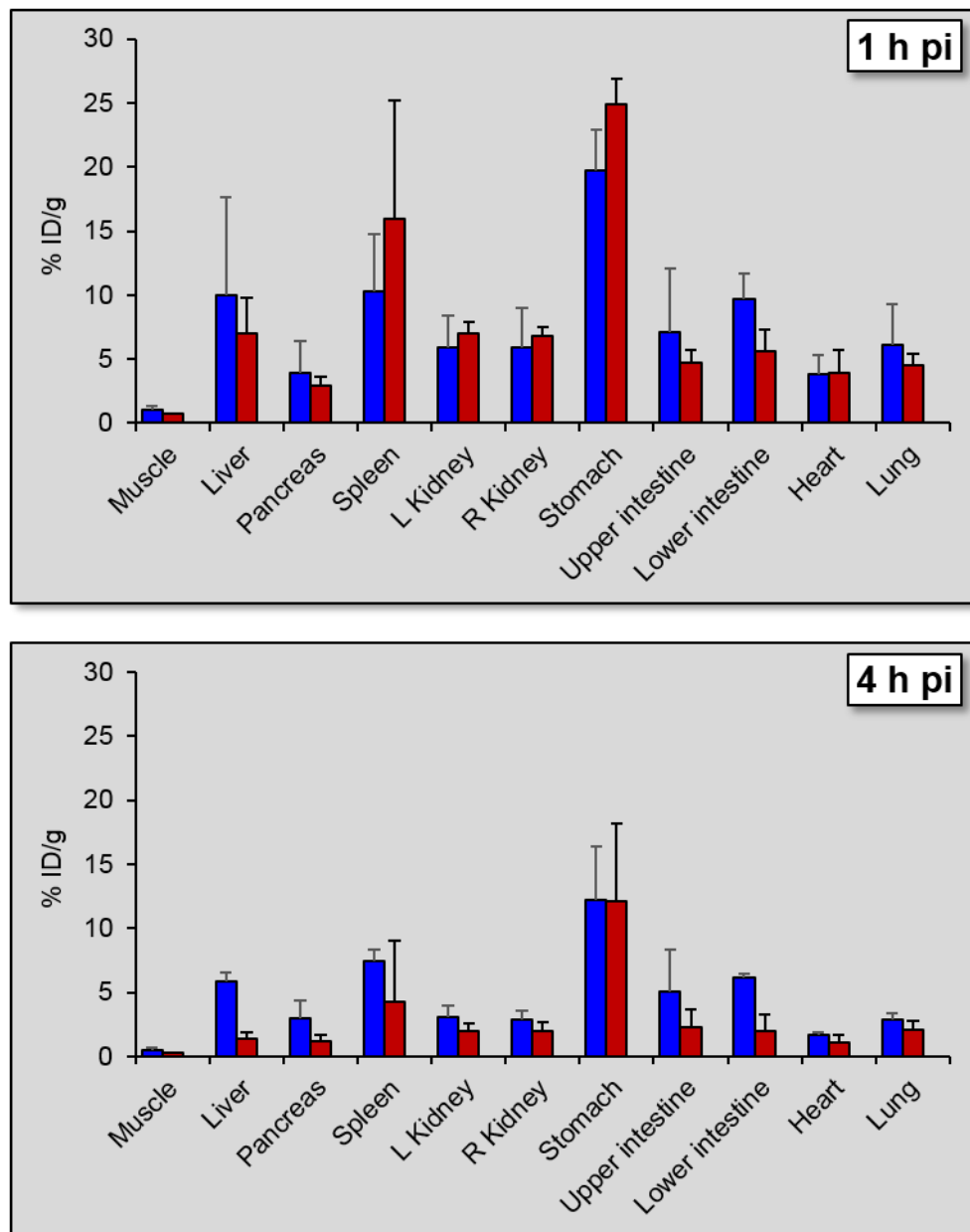


Figure 7.8: Biodistribution of peptides ^{125}I -PEG-p5R+14 and ^{125}I -p5R+14 in ApoA2c mice. At 1 h and 4 h pi, the peptides behaved similarly *in vivo*. These data suggest that PEGylation of p5R+14 may alter the metabolism of the peptide, but it did not improve amyloid targeting.

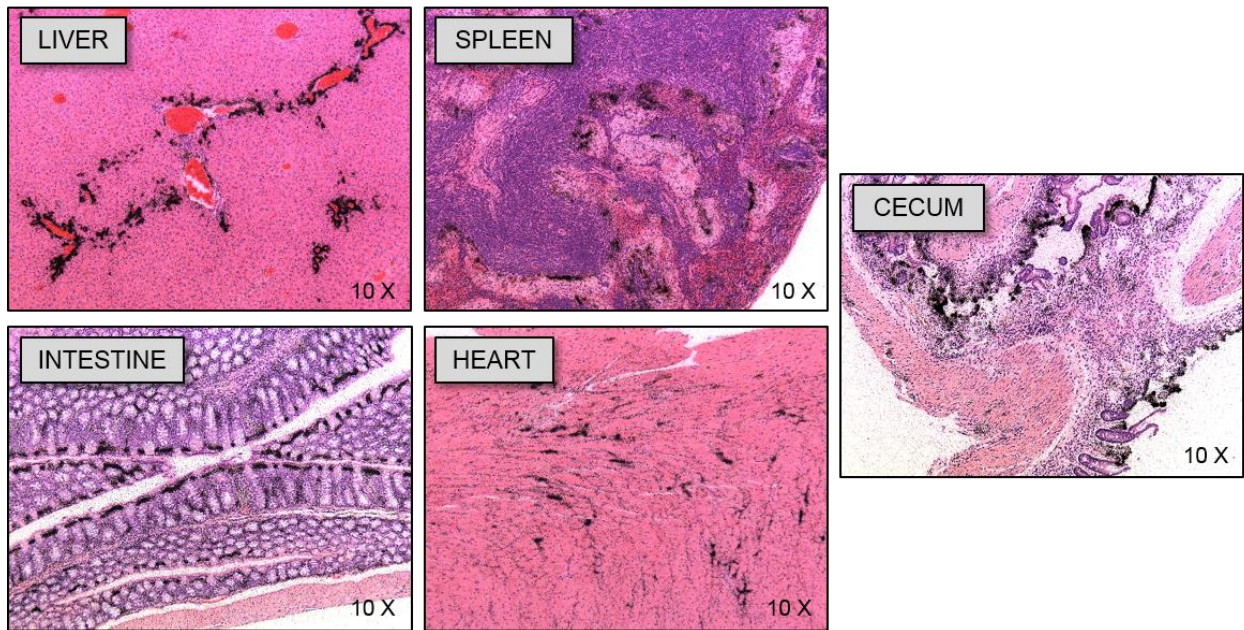


Figure 7.9: Autoradiographs of ^{125}I -PEG-p5R+14 in ApoA2c amyloid-laden tissues. The distribution of black silver grains confirmed peptide co-localization with amyloid in various tissues. Comparison of these images with those seen in Figure 5.8 suggest that ^{125}I -p5R+14 and ^{125}I -PEG-p5R+14 bind similarly to amyloid *in vivo*.

“binding-site barrier” effect which can hinder the extent of amyloid coverage *in vivo* [371]. This prompted the use of a lysine-rich variant of p5R+14, designated p5+14, with the hypothesis that a peptide with less affinity for amyloid would penetrate deeper into the mass and provide a better representation of the pathology. This study yielded results that were incongruent with the hypothesis—¹²⁵I-p5+14 did not improve amyloid coverage. Experiments were then conducted with a larger, N-terminal-PEGylated version of p5R+14 which was thought to enhance the serum half-life of the peptide without sacrificing the arginine residues believed to facilitate the observed peptide-amyloid interaction. A larger targeting agent was hypothesized to undergo altered catabolism (i.e. slower rate), thus increasing its serum half-life and allowing for enhanced amyloid contact and improved accessibility and coverage. Evaluation of PEG-p5R+14 in WT mice suggested that it was, indeed, catabolized differently as compared to the non-PEGylated peptide, but in ApoA2c amyloid-laden mice, the peptides were nearly indistinguishable which also did not support the hypothesis. Although full coverage was never reached, p5R+14 remained the most effective peptide for ApoA2c amyloid targeting *in vivo*.

Chapter 8

A non-dehalogenable, novel peptide for amyloid targeting

8.1. Overview

In the previous chapter, modified peptide variants of p5R+14 were examined as amyloid-targeting agents in the murine model of ApoA2c amyloidosis. Experiments with the lysine-rich and PEGylated variants revealed that altering the affinity or size of the peptide, respectively, was incapable of improving ApoA2c amyloid coverage as evidenced in ARGs. Given that peptides are typically cleared rapidly from circulation, an alternative technique addressing half-life extension as a way to counteract this and improve amyloid uptake—one which did not alter the size of the peptide—was employed.

There are two enantiomers of amino acids: the L-type conformation and, its “mirror image”, the D-type conformation (Figure 8.1). In nature, most amino acids are L-type; except for those found within some bacteria, the D-types of amino acids are commonly considered to be “non-natural” structures and are resistant to degradation—a characteristic of D-amino acids which has been exploited in the development of certain peptide-based drugs [433]. Data suggests that substitution of L-amino acids with the D-form can lead to stabilization through the increased resistance to protease degradation and prolonged half-life of peptides [433, 434]. Some clinically available peptides, which are analogues of luteinizing hormone releasing hormone (LHRH), have been developed as drugs (e.g., Triphorelin, leuprolide, buserelin, goserelin and nafarelin). These

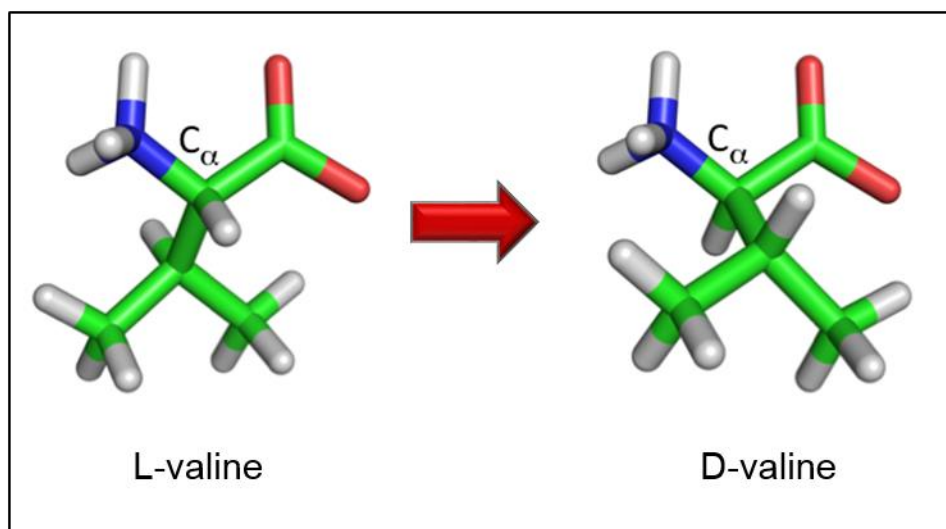


Figure 8.1: Illustration of both the L-type and D-type conformations of valine. With the exception of glycine, all amino acids can exist in one of two forms. This figure demonstrates both the L and D forms of the amino acid, valine. The two enantiomers are mirror images of each other. In nature, most amino acids exist in the L-type conformation. (Image taken from [433]).

reagents contain D-amino acids in certain positions, and as a result, they have prolonged reactivity *in vivo* [434, 435]. One clinically available, peptide-based antagonist of LHRH, Cetrorelix, incorporates D-amino acids at 5 different positions rendering the peptide resistant to degradation for up to 50 hours [436]. Other LHRH antagonists without non-natural amino acids are reported to circulate in the serum for only a maximum of a few hours before complete degradation [434, 437]. A more recent study evaluating the oostatic potential of a peptide containing D-amino acids indicates that the modified peptide had decreased catabolism and degraded at a significantly lower rate when compared to L-form of the peptide; this observation was likely due to decreased enzymatic degradation of the peptide bonds neighboring the non-natural D-amino acids [438].

Given that the incorporation of D-amino acids can enhance the serum half-life of peptides *in vivo* without increasing the size of the targeting agent, we hypothesized that this approach might result in improved ApoA2c amyloid uptake and detection. Therefore, we evaluated the amyloid-binding potential of a modified peptide p5 variant. This peptide, designated p43, was altered at the N-terminus by substitution of GGG at the N-terminal with D-amino acid forms of AQA (the GGG had to be substituted because there is no enantiomer for glycine). The p43 binding and catabolism characteristics were compared to historic p5 data to determine if D-amino acid incorporation could enhance half-life and amyloid uptake. Another peptide, designated p50, was synthesized as an additional control peptide for p43 to better determine whether any differences in reactivity were simply due to the amino acid substitution or the incorporation of D-enantiomers—p50 contained L-type AQA at the N-terminus.

These peptides were radiolabeled with I-125 and initially tested in the *ex vivo* binding assay to ensure that the modifications did not hinder reactivity of the peptide with amyloid-laden tissue. Observations in this assay prompted *in vivo* evaluations of the peptides in both AA amyloid and WT mice. The *in vivo* studies revealed that incorporation of D-amino acids altered more than was expected.

8.2. *Ex vivo* tissue binding assays with ¹²⁵I-p43 and ¹²⁵I-p50

To determine if D-amino acid incorporation these modifications of peptide p5 hindered the peptide-amyloid interaction, binding of radioiodinated p43 and p50 AA to amyloid-laden mouse tissue homogenates (Section 3.10.1) was tested. The reactivity of p43 with AA amyloid-laden liver and spleen confirmed that the modifications made to this p5 variant did not interfere with its binding to AA amyloid. In fact, the data showed that p43 had enhanced binding to these tissue homogenates as compared to the parent peptide (> 70% and > 60%, respectively) while maintaining the low, non-specific reactivity that p5 established with healthy tissues (~ 5%, Figure 8.2A). Interestingly, the reactivity of ¹²⁵I-p43 with AA tissues was not reproduced when ApoA2c tissues (liver, spleen, heart and lung) were tested. With all ApoA2c tissues, decreased peptide binding (even less than p5 binding), with the exception of the liver, was observed (Figure 8.2B). This was a counterintuitive finding since p43 bound well to AA amyloid, and a similar outcome was expected with ApoA2c amyloid. This finding was seen in two independent preparations of ApoA2c amyloid-containing tissue homogenates. Of all the peptides that have been evaluated, the most effective *in vivo* ApoA2c amyloid-targeting was p5R+14 (Chapter 4). The increased helicity achieved by incorporation of

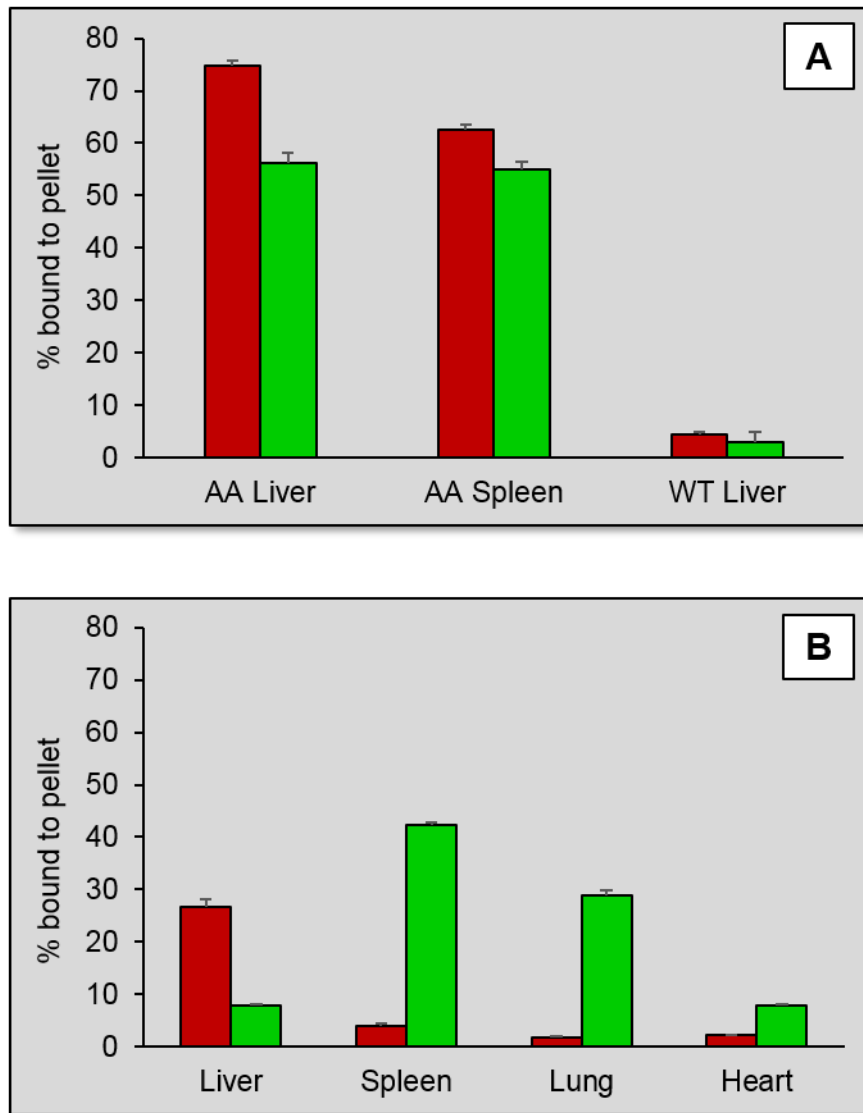


Figure 8.2: Ex vivo peptide-binding assays with ^{125}I -p43 and ^{125}I -p5. (A) *Peptide binding assays on AA and WT tissues.* The data showed that ^{125}I -p43 (red) exhibited greater binding to both AA liver and spleen compared to the parent peptide, ^{125}I -p5 (green). The enhanced binding with ^{125}I -p43 did not cause higher non-specific binding compared to ^{125}I -p5 as evidenced by similar percentages of the peptides bound to WT liver tissue. (B) *Peptide binding assays on ApoA2c tissues.* Binding with peptide ^{125}I -p43 to ApoA2c tissues was much less impressive than the binding established with AA tissues. There was minimal binding in each tissue with both peptides, but ^{125}I -p43 was, unexpectedly, even less effective than ^{125}I -p5 with this type of amyloid.

arginine residues as well as an additional 4 positively charged residues (total of 12 arginine residues) likely resulted in the optimal ApoA2c binding *in vivo*. Peptides p43 and p5, both containing 8 positively charged Lys residues, clearly did not possess the necessary characteristics for efficient ApoA2c amyloid-binding.

Peptide p50, the all-L-amino acid peptide control for p43, was not tested in the ApoA2c amyloid system due to p43's unimpressive binding. Instead, p50 was evaluated for positive AA binding with both liver and spleen homogenates. Reactivity with these tissues was similar to that seen with p5 and lower than the binding observed with p43 (Figure 8.3). Given that peptide p50 exhibited reduced AA amyloid interaction as compared to p43, the data indicated that an N-terminal composed of non-natural, D-type AQA residues enhanced binding compared to that observed with a peptide containing N-terminal L-type AQA residues or N-terminal GGG residues.

To assess the “affinity” of the binding with amyloid, the peptides were subjected to sodium chloride (NaCl) titrations on AA liver and AA spleen. It was hypothesized that p43 would bind with greater affinity to AA amyloid than p50 based on its increased retention in PBS. This hypothesis was tested using the following NaCl concentrations: 0.15 M (equivalent to PBS), 0.25 M, 0.5 M, 0.75 M and 1.0 M. The data revealed that p43, in the presence of both the liver and spleen homogenates, required a higher concentration of NaCl (~ 0.1 – 0.25 M greater) to interrupt the electrostatic peptide-amyloid interaction and reduce binding by 50% (IC₅₀) as compared to p50 (Figure 8.4). These data supported the hypothesis that p43 binds AA amyloid with slightly higher affinity as compared to p50. Also, because we observed greater binding to the amyloid of p43 at each salt concentration, it suggests that there are more “binding sites” for the

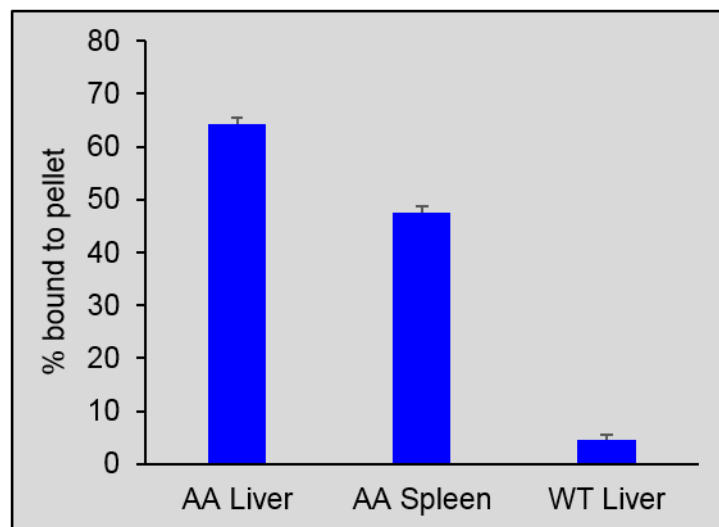


Figure 8.3: *Ex vivo* peptide-binding assay demonstrating ^{125}I -p50 reactivity with AA tissues and WT liver. Amyloid reactivity with the L-type N-terminal AQA peptide p5 variant was established, and data revealed that ^{125}I -p50 positively binds to AA amyloid in both the liver and spleen. The reactivity was less than that previously established with p43 in this assay, however. Non-specific binding to WT liver was low, and similar to that seen previously with both p43 and p5.

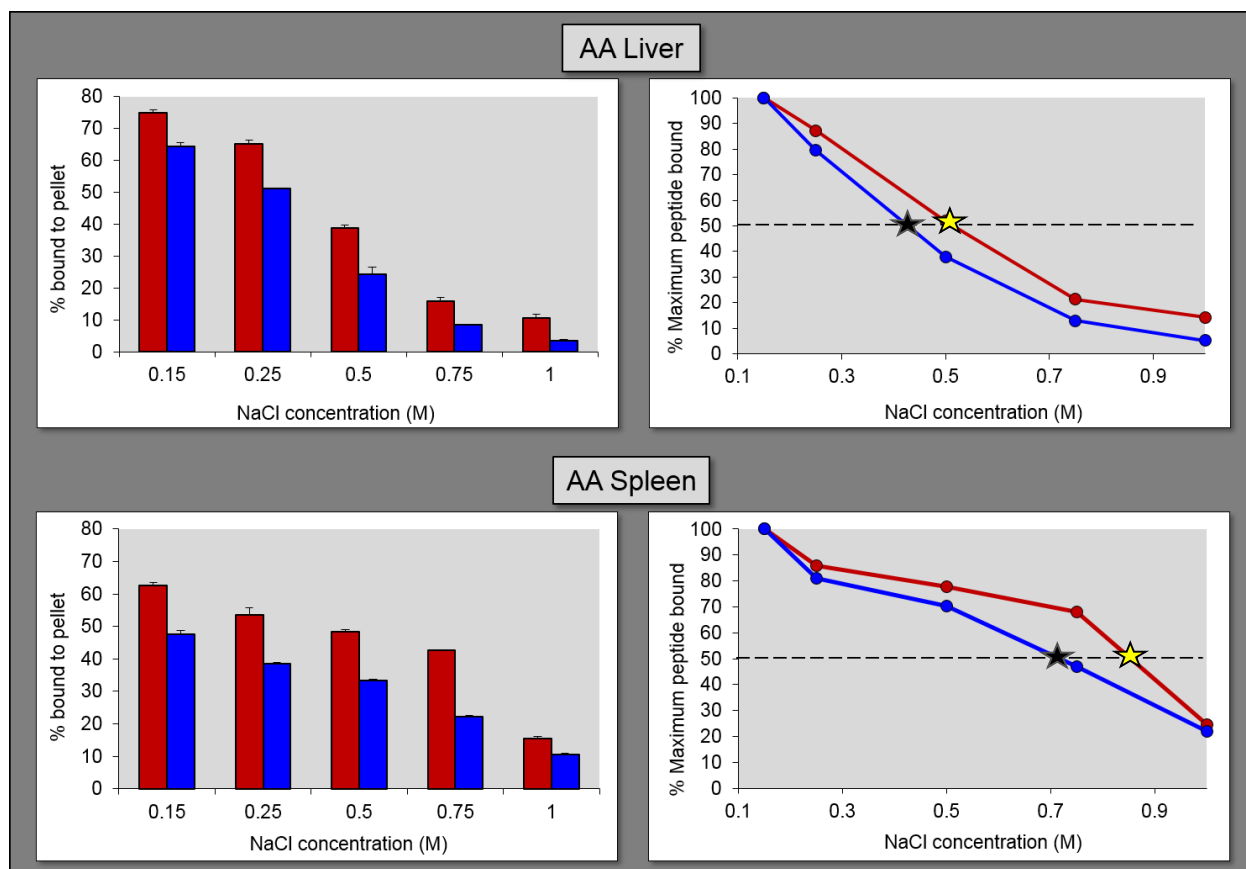


Figure 8.4: NaCl titrations assessing ¹²⁵I-p43 and ¹²⁵I-p50 affinity for AA liver and spleen homogenates. Concentrations up to 1.0 M of NaCl were used in the peptide binding assay to demonstrate the affinity of both ¹²⁵I-p43 (red) and ¹²⁵I-p50 (blue) to AA liver and spleen. As shown in the graphs on the left, there was a higher percentage of ¹²⁵I-p43 bound to both tissues in all concentrations of NaCl than was observed with ¹²⁵I-p50. When the data were plotted against the percentage of maximum peptide bound to pellet (value obtained in PBS alone), the IC₅₀ for ¹²⁵I-p43 (yellow stars) was determined to be slightly higher than the IC₅₀ for ¹²⁵I-p50 (black stars) with both tissues.

p43 peptide. It may be that p43 binds HS and fibrils in the amyloid, whereas p50 (and p5) may only bind HS. Since positive reactivity with AA amyloid had been established, we then assessed peptides p43 and p50 in the H2/IL-6 murine model of amyloidosis to elucidate whether they could effectively detect amyloid *in vivo*.

8.3. Time course biodistribution and SPECT/CT studies with ¹²⁵I-p43 and ¹²⁵I-p50

Peptide ¹²⁵I-p43

It was previously shown that both p43 and p50 bind to AA amyloid in the pull down peptide binding assay. Since p43 did not effectively bind to ApoA2c amyloid, further comparisons of p43 and p50 focused on the AA mouse. Given that p43 contained D-amino acids, we were uncertain of how the peptide would “behave” *in vivo*. The AA homogenate binding assay suggested that binding to AA amyloid was enhanced, which would facilitate uptake by amyloid in diseased mice. To determine if preferential AA amyloid binding of p43 could be reproduced *in vivo*, cohorts of AA mice (n = 3 per time point), at ~ 6 – 8 weeks post injection of AEF, were administered radioiodinated p43 or p50 iv in the lateral tail vein and euthanized at either 1 h, 4 h or 24 h pi for SPECT/CT imaging, tissue biodistribution measurements and microautoradiography. The radiopurity of the peptide samples was established by SDS-PAGE and amyloid reactivity confirmed by AA liver homogenate binding (Figure 8.5). To characterize potential non-specific, off-target binding associated with these peptides, WT mice were also administered the radiotracers and examined at the same time points post injection.

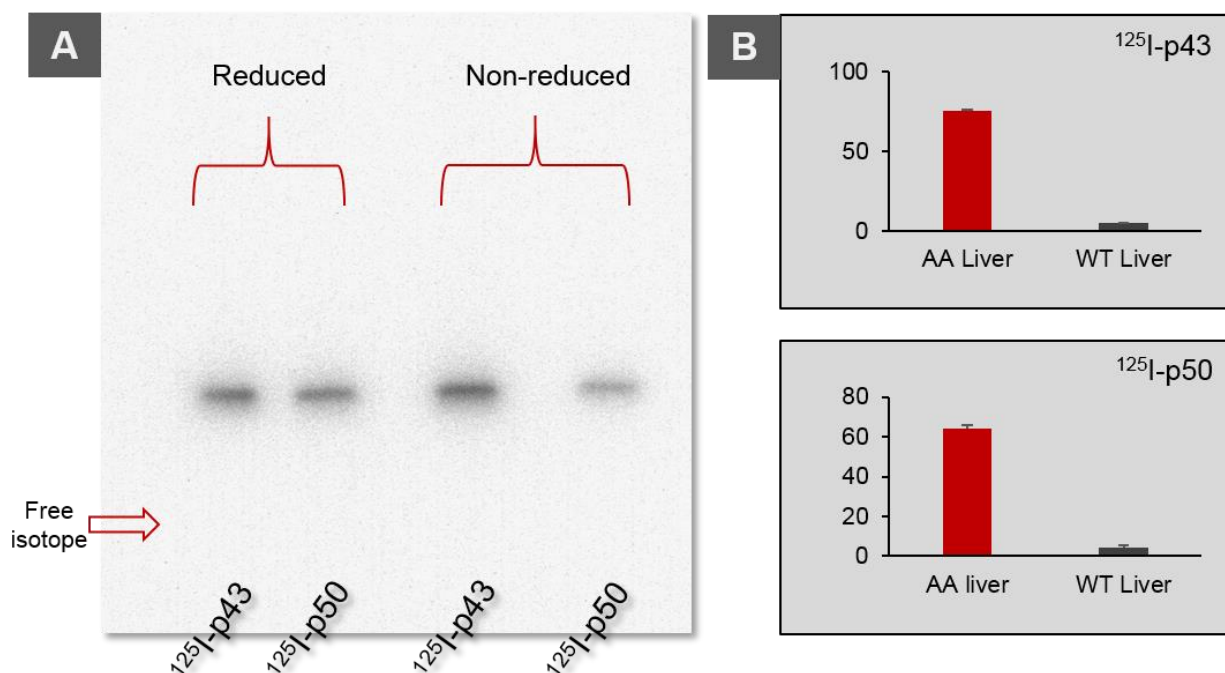


Figure 8.5: Radiopurity and amyloid-reactivity of the radiolabeled peptides. (A) SDS-PAGE analysis of radioiodinated p43 and p50. The gel shows there was no detectable free iodide in the peptide samples. (B) Ex vivo assessment of peptide reactivity with AA liver and WT liver. The data indicate that both peptides behaved as expected in the peptide-binding assay with positive reactivity to AA liver (~ 60 – 70%) and < 5% reactivity with WT liver.

The tissue biodistribution data generated by using gamma counting of ^{125}I -p43 in AA mice revealed uptake of the radiotracer ($\sim > 10\% \text{ID/g}$) in the liver, pancreas, spleen and intestines—all common sites of AA amyloid deposition (Figure 8.6). This uptake was observed at each time point, which indicated that the peptide-amyloid interaction occurred within 1 hour of radiotracer injection, and the interaction was stable for at least 24 h (Figure 8.6). The post mortem SPECT/CT imaging confirmed the biodistribution data, revealing uptake in tissues that exhibited the highest $\% \text{ID/g}$ and showing stability of ^{125}I -p43 binding to amyloid-laden tissues up to at least 24 h pi, i.e. liver, spleen and pancreas (Figure 8.7). This prolonged amyloid reactivity was previously observed with ^{125}I -p5 in the tissues of AA mice (see Figure 1.27) [416].

In WT mice, however, there was an interesting discrepancy in the biodistribution of ^{125}I -p43 when compared to the historical ^{125}I -p5 data. Accumulation of radioiodide—liberated when radioiodinated peptide tracers encounter deiodinase enzymes in the liver and kidneys [416]—in both the stomach and thyroid of mice injected with ^{125}I -p5 was observed due to the presence of sodium-iodide symporters in the stomach and thyroid. SPECT/CT imaging of ^{125}I -p5 in WT mice revealed expected uptake in both the stomach and thyroid within 1 h pi (Figure 8.8). In WT mice injected with ^{125}I -p43, however, dehalogenation was not present. This was evidenced by a lack of free iodide accumulation in the stomach and thyroid in images and tissue biodistribution (Figure 8.9) values that were lower than that seen for ^{125}I -p5 ($\sim 20\% \text{ID/g}$ with ^{125}I -p5 and $\sim 1\% \text{ID/g}$ with ^{125}I -p43) in the stomach. Instead, only the kidneys in WT mice with ^{125}I -p43 achieved a $\% \text{ID/g}$ high enough ($\sim 40\%$) to be visualized with SPECT/CT imaging (Figure 8.10A). The whole body ^{125}I -p43 activity was most prominent in the SPECT/CT

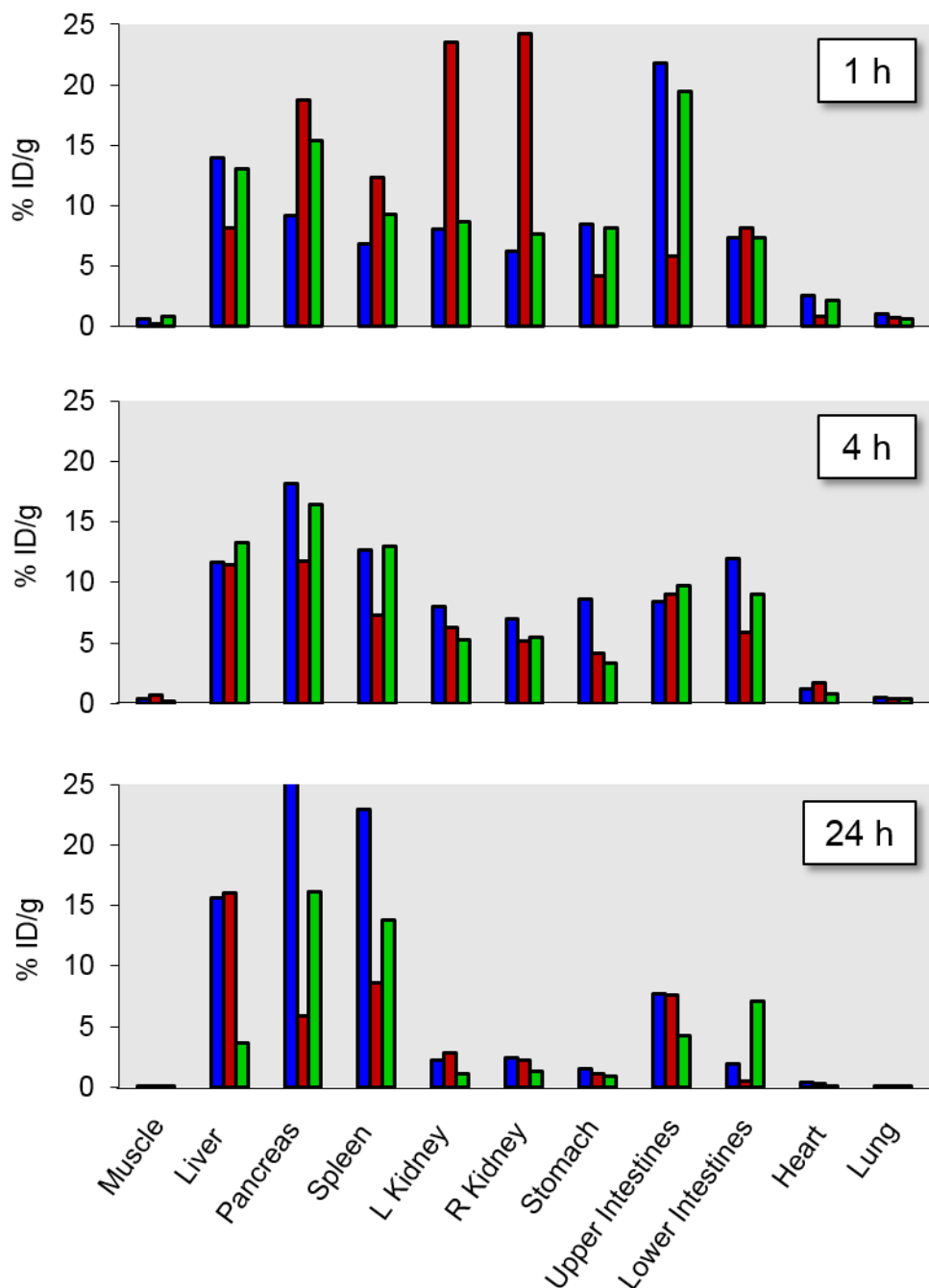


Figure 8.6: Biodistribution measurements of ^{125}I -p43 in a time course study with AA mice. These graphs demonstrated the uptake of ^{125}I -p43 in ApoA2c mice at various time points pi. There was evidence of accumulation of radiotracer in the liver, pancreas, spleen and intestines at each time point. The kidney binding gradually decreased over time as unbound radiotracer was catabolized. Each dataset on the graph represents an individual mouse.

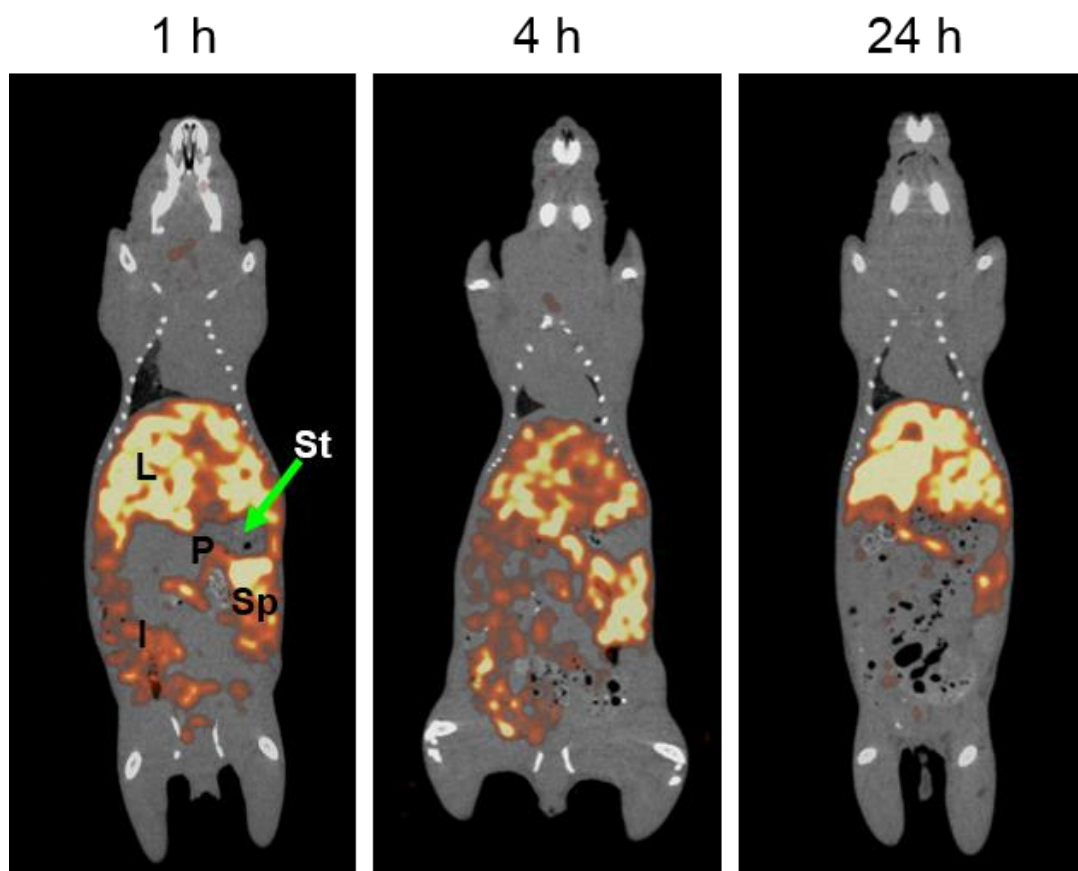


Figure 8.7: Representative SPECT/CT images of ^{125}I -p43 in AA mice at 1 h, 4 h and 24 h pi. The distribution of radiotracer ^{125}I -p43 at 1 h pi demonstrated that there was uptake in the liver (L), spleen (Sp), pancreas (P) and intestines (I). The stomach (St; green arrow) remained empty for the time course duration. There was evidence of ^{125}I -p43 accumulation in amyloid-laden tissues up to 24 h pi.

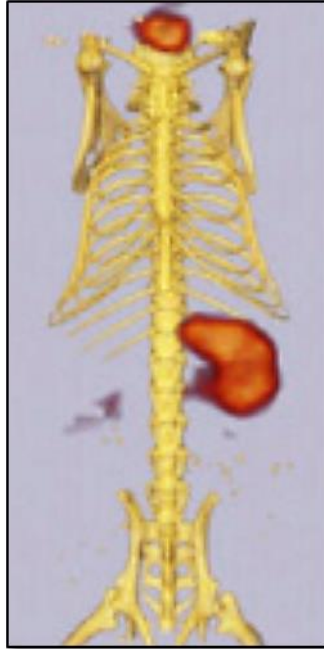


Figure 8.8: 3D SPECT/CT representation of ^{125}I -p5 in a WT mouse. This image displays uptake of liberated free iodide in the stomach and thyroid of a WT mouse at 1 h pi. (Image adapted from [409].

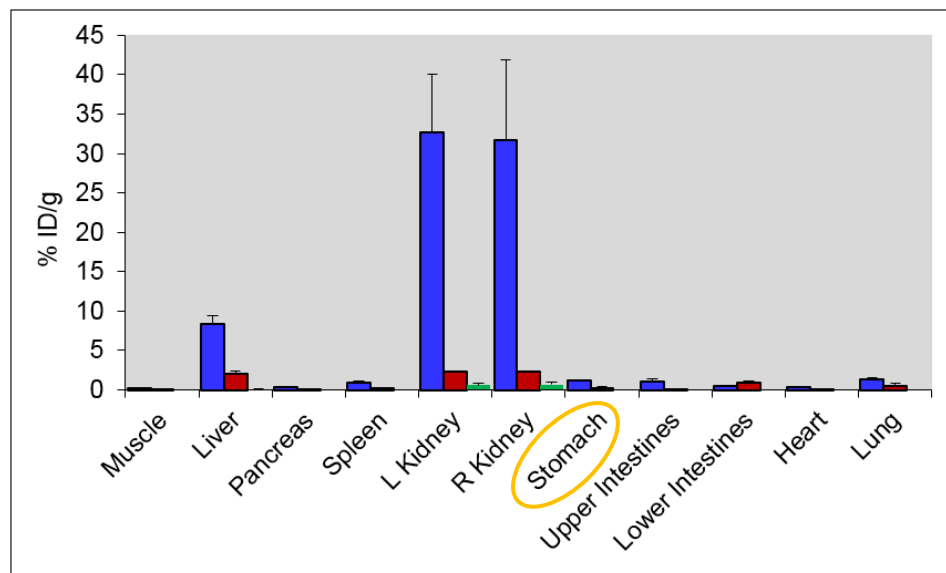


Figure 8.9: Biodistribution of ^{125}I -p43 in WT mice. The graph shows mean distribution ($n = 3$) in WT mice at each time point pi. At 1 h pi (blue), the radiotracer was present in the liver and kidneys. All tissues were “clear” by 4 h pi (red), and by 24 h pi (green), the biodistribution of ^{125}I -p43 was nearly completely diminished. Notice the lack of accumulation of free iodide in the stomach at any time point. This suggested that the peptide radiotracer was, unexpectedly, not dehalogenated *in vivo*.

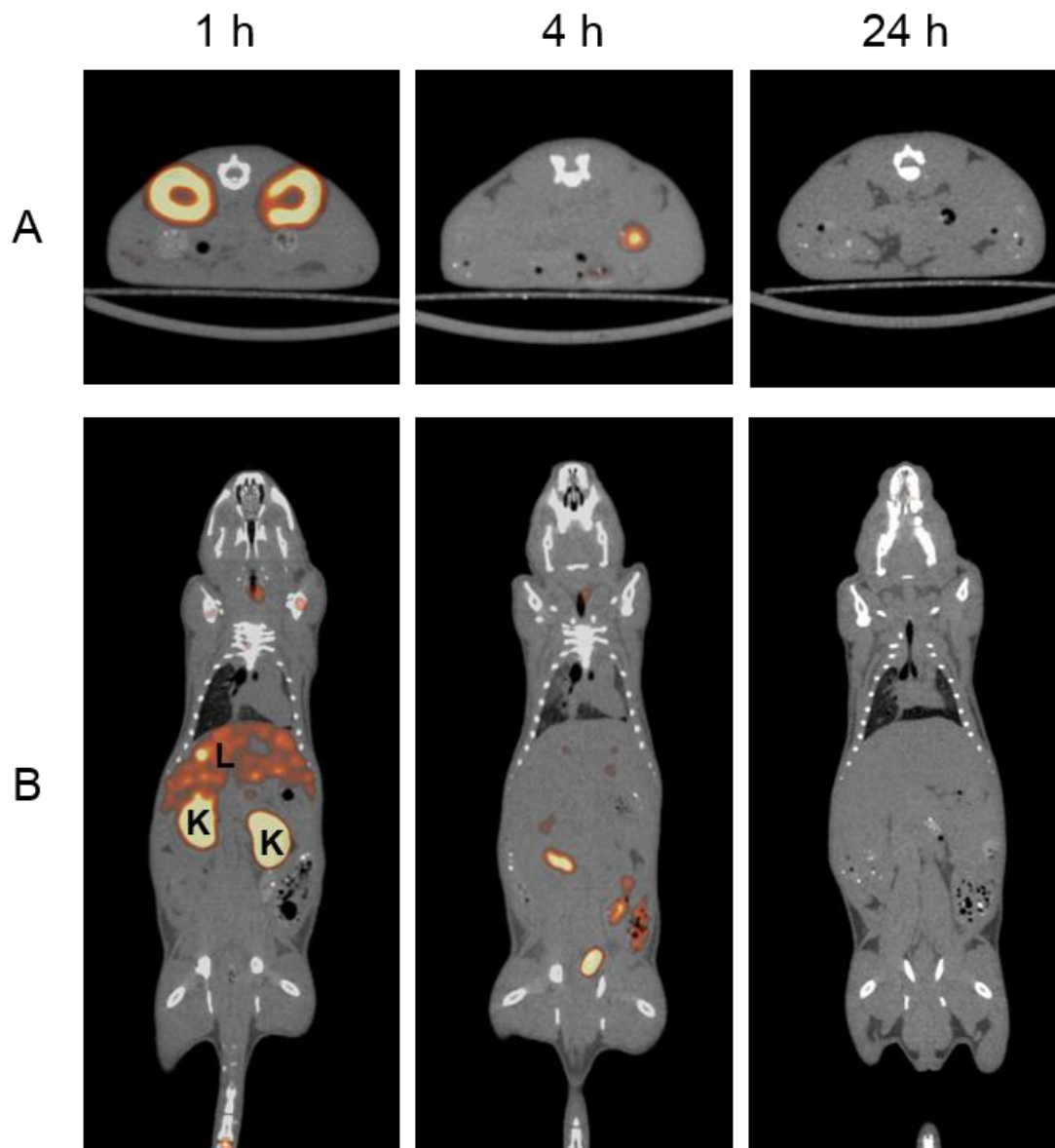


Figure 8.10: Representative SPECT/CT images of ^{125}I -p43 in WT mice at 1 h, 4 h and 24 h pi. (A) Axial images demonstrating renal accumulation at various times pi. The renal uptake of ^{125}I -p43 is most prominent at 1 h pi and is no longer evident by 4 or 24 h pi. (B) Whole body images demonstrating the distribution of ^{125}I -p43. These images display uptake of the radiotracer in the kidneys (K) and liver (L) at 1 h pi. The kidneys are cleared by the 4 h pi time point leaving only trace amounts of peptide clearing through the intestines. By 24 h pi, there was no evidence of any radiotracer in the image.

images at 1 h pi and progressively diminished in the 4 and 24 h pi images (Figure 8.10B). The lack of ^{125}I sequestration by the stomach and thyroid suggested that p43 had been catabolized without undergoing dehalogenation—an unexpected outcome. When compared to the parent peptide, p5, the incorporation of AQA D-amino acids at the N-terminal in p43 resulted in alternative catabolic processing of the radiotracer. However, instead of exhibiting a longer serum half-life prior to its dehalogenation and clearance (which was the expected biological behavior), the peptide avoided renal and hepatic deiodinases altogether. Since radiolabeling of peptides with ^{125}I results in incorporation into the L-tyrosine residue, we anticipated that the dehalogenation enzymes would “recognize” and act upon the iodinated amino acid; instead, the N-terminal D-amino acids appeared to have a “protective” effect over the radiolabeled tyrosine residue, resulting in no dehalogenation. Although this characteristic of p43 was unforeseen, we speculated that it could offer an advantageous property for AA amyloid imaging. With other peptides, dehalogenation in the kidneys makes renal amyloid imaging improbable at early time points pi because the tracer is slowly catabolized and ^{125}I is liberated; with p43, however, a lack of dehalogenation and rapid renal clearance could theoretically be exploited for imaging amyloid-laden regions in the kidneys by SPECT/CT imaging at very early time points.

The microautoradiographs (ARG) of ^{125}I -p43 in harvested AA tissues revealed that the radiotracer specifically co-localized with amyloid at all time points examined as demonstrated by the accumulation of black silver grains in regions of tissues that were also Congophilic (i.e. amyloid) in consecutive tissue sections. Representative ARG of various tissues at different time points are shown (Figure 8.11). In healthy tissues, ARG

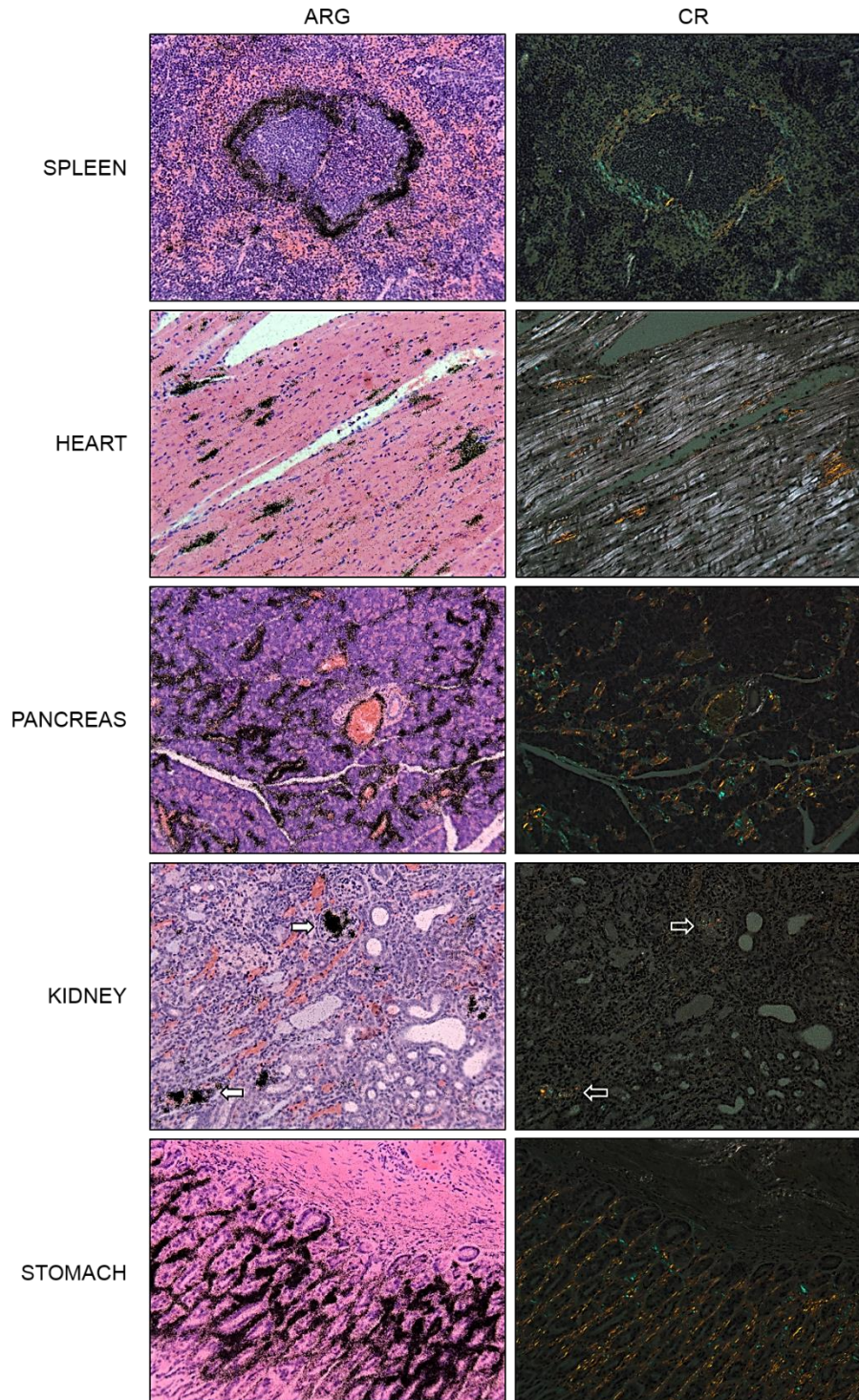


Figure 8.11: Co-localization of ^{125}I -p43 to AA amyloid at various time points pi. Microautoradiography (ARG; 20 X) shows distribution of the peptide (black silver grains), and Congo red (CR; 20 X) birefringence displays amyloid distribution in the tissues. Peptide is shown to co-localize with amyloid in all tissues. The distributions seen in the spleen and heart were at 1 h pi, the pancreas was at 4 h pi and the kidney and stomach were at 24 h pi.

indicated that there was dense accumulation of ^{125}I -p43 in the glomeruli, Bowman's capsules and proximal tubules (likely luminal) of the kidney, in addition to binding to, presumably, hyaline cartilage associated with tracheal tissue at 1 h pi. No other tissues revealed ^{125}I -p43 binding. Both the renal radioactivity and cartilage binding were much diminished at 4 h pi. The radiotracer distribution in the kidneys at this time point was detected in the distal tubules—indicative of natural radiotracer clearance. “Off-target” binding to cartilage was likely the result of peptide interaction with the glycosaminoglycan, chondroitin sulfate, in the tracheal cartilage. With a sulfation pattern similar to heparin (see Figure 1.5), the interaction of a heparin-binding peptide derivative, such as p43, with chondroitin sulfate would not be implausible. Indeed, we have seen this with most of our amyloid-reactive peptides. By 24 h pi, the black silver grains that were previously present in the kidneys and associated with cartilage tissue had diminished, indicating that the radiotracer had cleared and that the non-specific binding that ^{125}I -p43 demonstrated was seemingly weak and temporary (Figure 8.12).

Although the incorporation of D-amino acids in peptide p43 had not dramatically altered the blood half-life as was initially hypothesized, discovery of its new characteristic (i.e. non-dehalogenation) adds value to its potential clinical use. If, by 24 h pi, the peptide was fully cleared through the kidneys in a manner which did not result in dehalogenation of the radiotracer, then injection of radiolabeled p43 into a patient with a heavy renal amyloid burden could be used to specifically image amyloid rather than be attributed to radiotracer clearance. Since renal involvement is common in all AL, AA and ALect2 patients and yields a poor prognosis, the use of a radiotracer which

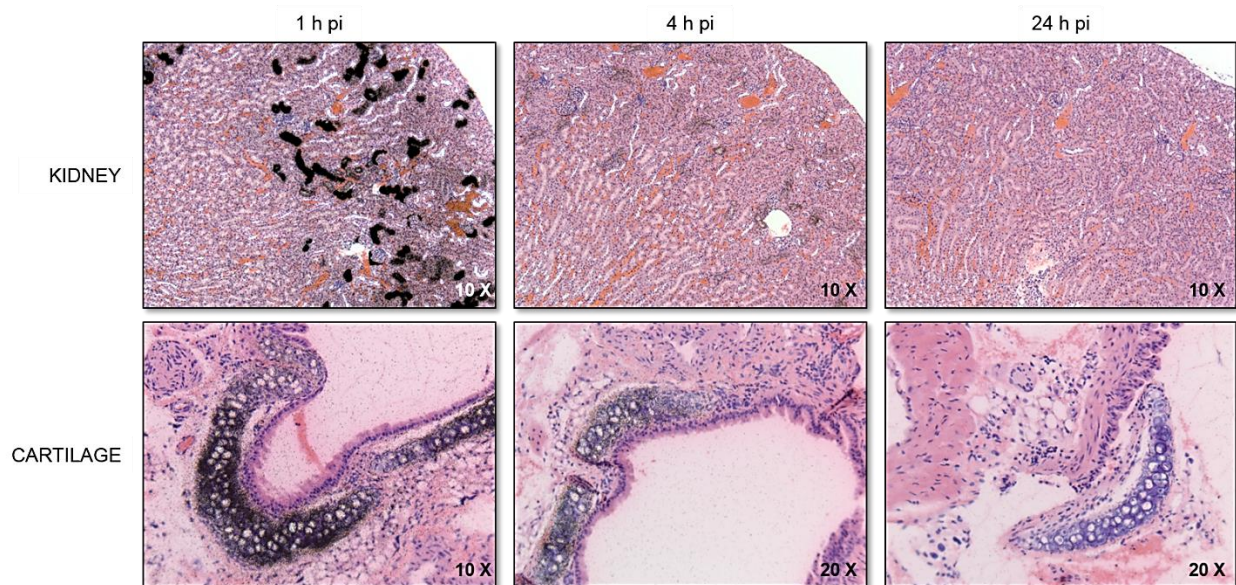


Figure 8.12: Autoradiograph visualization of ^{125}I -p43 binding to healthy kidney and cartilage tissue at various time points pi. The accumulation in the kidney seen at 1 h pi is primarily within the Bowman's capsules and renal tubules. At 4 h and 24 h pi, the density of radiotracer uptake in the kidney diminished which indicated clearance. The off-target binding observed in the tracheal cartilage was likely due to peptide reactivity with chondroitin sulfate. This interaction was transient, however, which was evidenced by decreased peptide binding observed at 4 h pi and a lack of peptide binding to cartilage at 24 h pi. No other tissues within WT mice demonstrated ^{125}I -p43 accumulation at any time point.

could determine the amyloid load in this particular organ may have clinical merit as a diagnostic agent or as a tool to monitor the response to therapies.

Studies with ^{125}I -p50

The previous studies determined that p43 exhibited different biological behavior *in vivo* when compared to historical p5 data. The incorporation of N-terminal D-amino acids was likely the factor which resulted in a non-dehalogenable product, but this hypothesis could not be confirmed without similarly testing p50 in AA and WT mice. Peptide p50 has the same amino acid sequence as p43, but there are no D-amino acids. Examining p50 in AA and WT mice would determine if the different biological behavior observed with p43 was a result of substituting N-terminal GGG residues with AQA or a result of incorporating D-amino acids. Therefore, p50 was analyzed in AA and WT mice to examine amyloid reactivity and dehalogenation. The same experimental conditions were used to examine the ^{125}I -p50 radiotracer in AA (~ 6 – 8 weeks post AEF induction) and WT mice. SPECT/CT imaging was performed after mice were euthanized at 1 h, 4 h or 24 h pi.

The images and mean tissue biodistribution measurements (at 1 h pi) of ^{125}I -p50 in WT mice indicated that this peptide behaved similarly to ^{125}I -p5 rather than ^{125}I -p43 *in vivo* as was suggested by the uptake of liberated ^{125}I in the stomach (31 %ID/g) and thyroid and a lack of radiotracer accumulation in the kidneys (9 %ID/g) (Figure 8.13A). Additionally, the images and mean tissue biodistribution measurements (at 1 h pi) of ^{125}I -p50 in AA mice revealed positive uptake in amyloid-laden tissues such as the liver

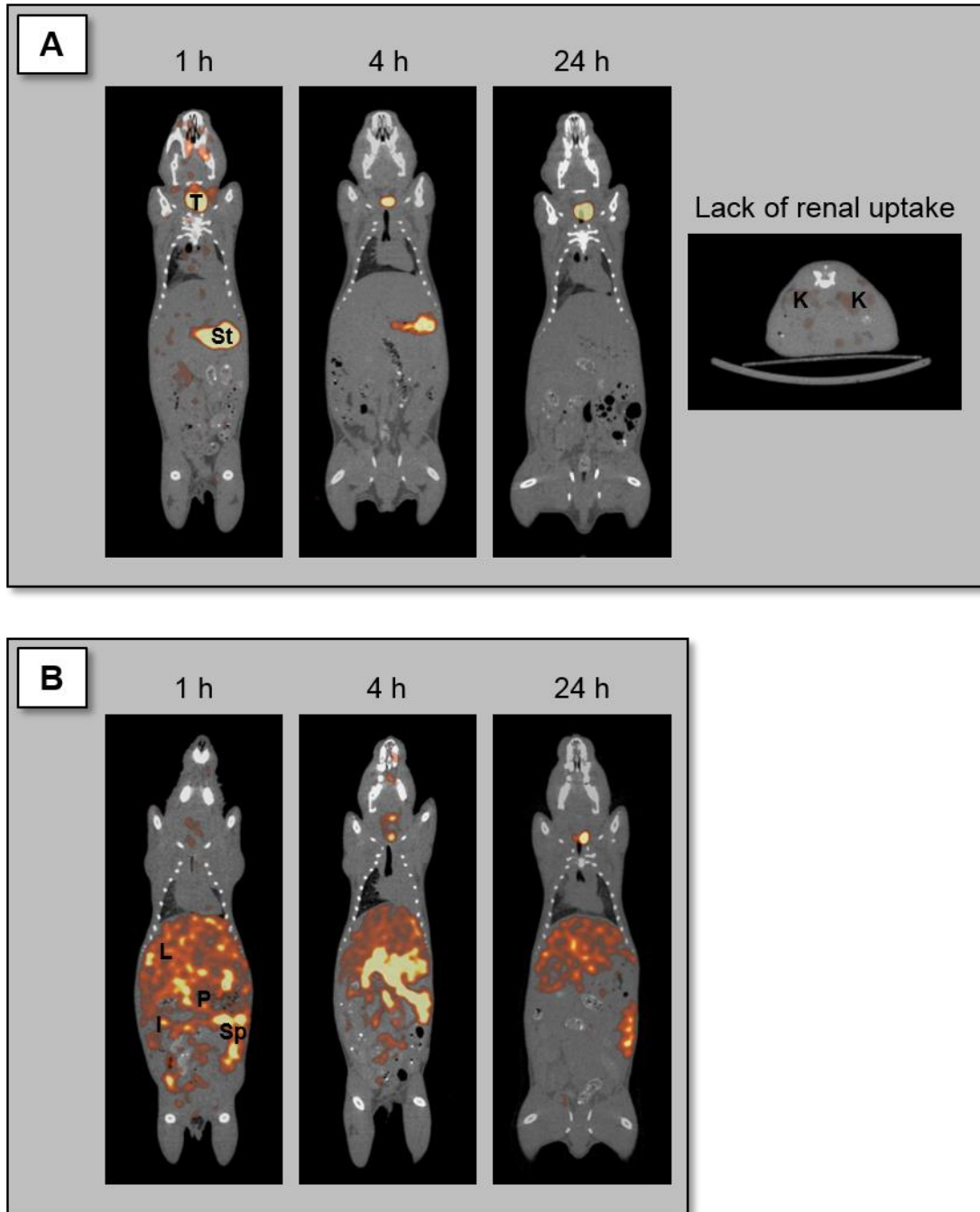


Figure 8.13: SPECT/CT images of ^{125}I -p50 in WT and AA mice at 1 h, 4 h and 24 h pi. (A) *Distribution of radiotracer in WT mice.* There was evidence of ^{125}I -p50 dehalogenation at all each time point due to free iodide uptake in the stomach (St) and thyroid (T). By 24 h pi, the stomach had cleared. Notice that at 1 h pi, there was no accumulation of radiotracer in the kidneys (K) which was in stark contrast to that seen with ^{125}I -p43. (B) *Distribution of radiotracer in AA mice.* ^{125}I -p50 accumulated in AA amyloid-laden tissues such as the liver (L), spleen (Sp), pancreas (P) and intestines (I). The radiotracer uptake was stable and was still visible at 24 h pi.

(17 %ID/g), spleen (19 %ID/g), pancreas (35 %ID/g) and upper/lower intestines (28 and 10 %ID/g, respectively) (Figure 8.13B). The uptake of ^{125}I -p50 was stable enough to provide positive amyloid imaging up to at least 24 h pi. The whole-body distribution in AA mice was similar to that observed with both peptides p5 and p43 indicating that all of the radiotracers, regardless of biological processing, were capable of targeting AA amyloid in multiple tissues. Confirmation of the preferential binding of ^{125}I -p50 to amyloid deposition over healthy tissue was similarly evidenced by ARG analysis of representative AA and WT tissue sections (Figures 8.14 and 8.15). Based on these findings, we speculate that, since ^{125}I -p50 behaved more similarly to ^{125}I -p5 than ^{125}I -p43 in AA and WT mice, it confirmed that it was the incorporation of AQA D-amino acids, not simply the N-terminal sequence, which resulted in a non-dehalogenable peptide capable of detecting AA amyloid.

8.4. Dynamic PET/CT analysis of ^{125}I -p43 in WT mice

Recently, we published a study in which dynamic PET/CT imaging was performed to better characterize the *in vivo* biokinetic profile of radioiodinated p5 in live WT and AA mice [416]. Analysis of dynamic PET/CT data, i.e. generation of time activity curves (TAC) for various organs of interest, was performed. These curves of the dynamic distribution of ^{124}I -p5 from the time it was injected, made possible the analysis of the uptake and clearance of the radiotracer in each organ. To gain insight into the non-dehalogenable nature of p43, dynamic PET/CT scans were conducted on WT mice, and data were compared to the published TAC for p5. Since it was thought that the

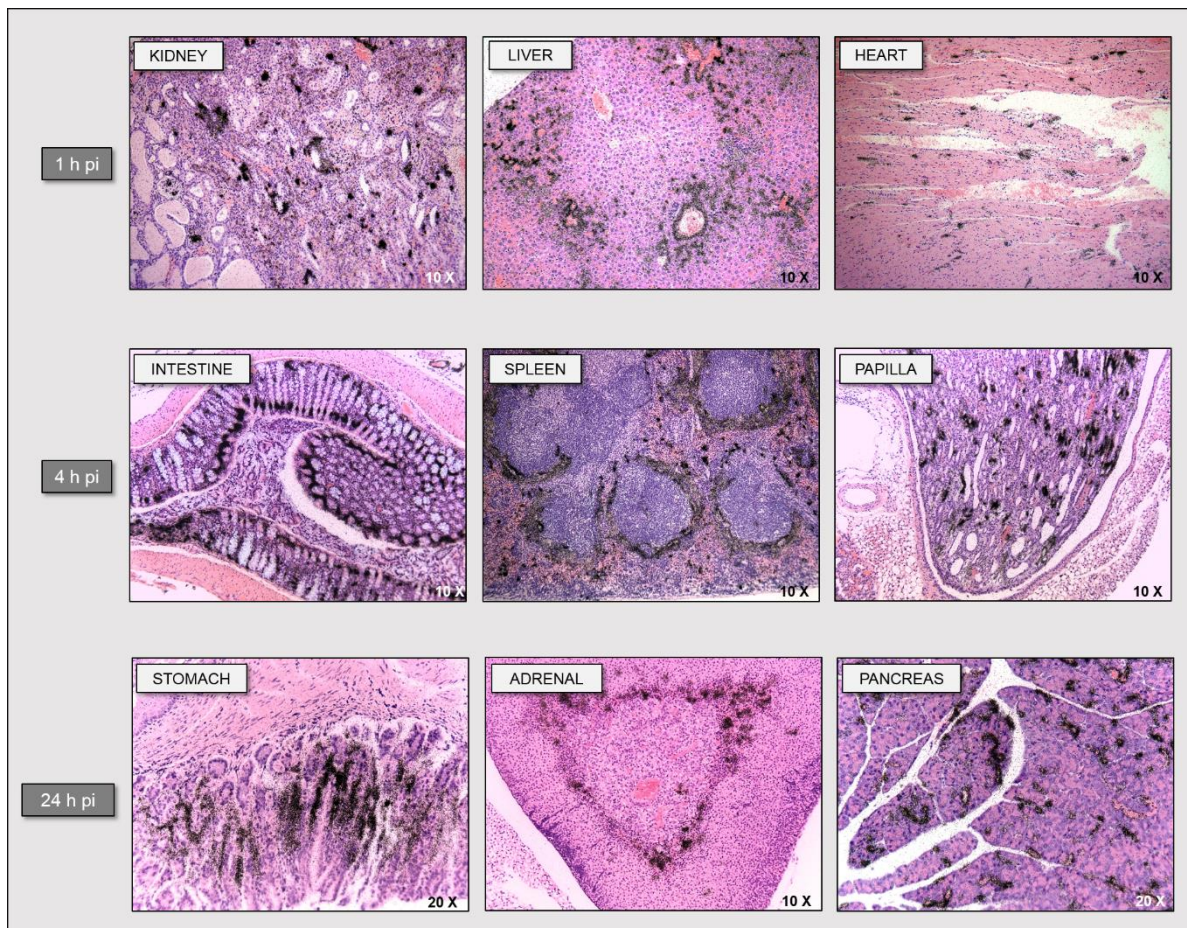


Figure 8.14: Microautoradiographs displaying ^{125}I -p50 in amyloid-laden tissues representative of various time points pi. These tissues demonstrate the ability of peptide p50 (black silver grains) to preferentially target amyloid within several tissues at 1 h, 4 h and 24 h pi. The radiotracer does not bind healthy tissue within these sections.

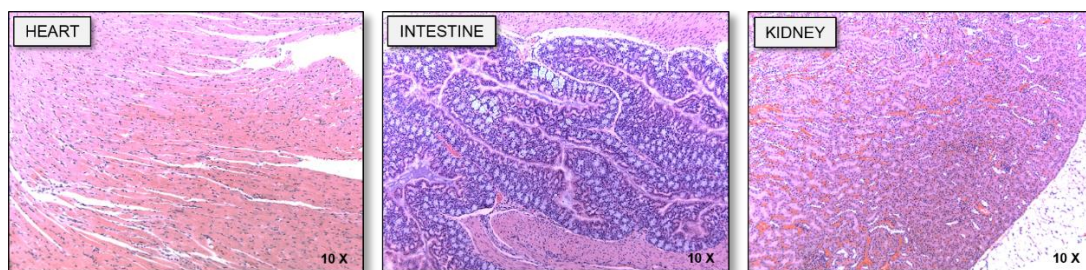


Figure 8.15: Microautoradiographs demonstrating a lack of ^{125}I -p50 accumulation in various WT tissues. These images confirm that ^{125}I -p50 does not bind healthy tissue *in vivo*.

dehalogenation of these peptides primarily takes place in the kidneys, the TAC for this organ was expected to differ between ^{124}I -p43 and ^{124}I -p5.

Dynamic PET/CT imaging requires the use of a positron emitting radioisotope. With a half-life of 4.2 days, radioiodination with iodine-124 (^{124}I) was performed for this study. Radiolabeling of peptide p43 with ^{124}I was achieved as described in Section 3.6. The product was diluted with sterile PBS such that each administered dose was ~ 200 μCi . Since this type of imaging was conducted with live animals, the mice were administered isoflurane anesthesia (2 % in air) prior to, and throughout the duration of the procedure (see Section 3.12.2). The PET data were analyzed using the Inveon Research Workplace software (Siemens). Time activity curves were created by drawing regions of interest over the heart, kidneys, thyroid and bladder of a co-registered PET/CT image since these organs represent blood pool and sites of catabolism and iodide uptake. Given that we expected dissimilarities between the dynamic renal distribution of ^{124}I -p43 and ^{124}I -p5, the generated TAC of the kidneys was compared to the historical p5 kidney TAC data [416].

The data indicated that the profiles of the radiotracers in WT mice did, indeed, differ in the kidneys (Figure 8.16). With ^{124}I -p5, the peak renal accumulation value occurred between 7 and 8 min pi followed by rapid clearance of the radiotracer from the organ; with ^{124}I -p43, however, the peak renal value occurred approximately 10 min later (~ 17 min pi), and the radioactivity was cleared at a much slower rate from the kidney than was observed with ^{124}I -p5. The delay in peak uptake that was observed with ^{124}I -p43 may suggest that this radiotracer had a slightly extended serum half-life prior to renal catabolism. Since p43 was designed as a p5 variant containing D-amino acids to

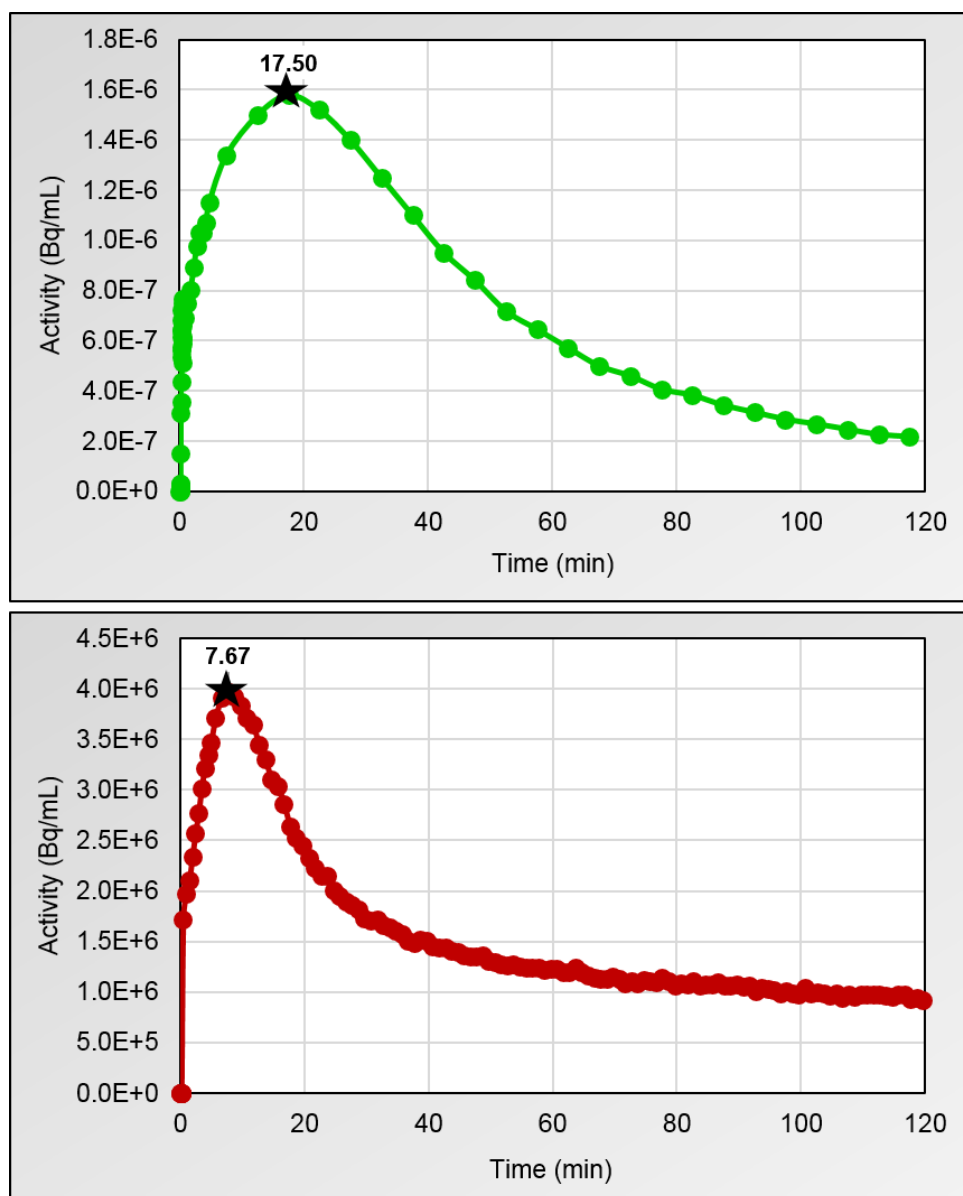


Figure 8.16: Time activity curves depicting renal accumulation and clearance of ^{124}I -p43 and ^{124}I -p5. The peak renal uptake values were measured. The curves demonstrate that ^{124}I -p43 (green) took ~ 10 min longer to reach maximum accumulation in the kidneys as compared to ^{124}I -p5 (red). The black stars pinpoint the respective peak renal activity time points. The two peptides differed in clearance of the tracers as well which was shown by the different shapes of the curves; ^{124}I -p43 had a broad peak in the kidneys, and ^{124}I -p5 had a sharp peak indicating much more rapid clearance of radioactivity of the latter. The y-axes are not scaled equally due to the differing amounts of radioactivity injected in the two studies. (Historical ^{124}I -p5 data was taken from [416] to create the image.)

possibly extend the half-life of the peptide, this was a gratifying finding that was not obvious in the previous SPECT/CT study.

In regard to the observed difference in renal clearance, ^{124}I -p5 was dehalogenated within the kidneys which resulted in a TAC that partially depicted the loss of free iodide to the blood instead of radiolabeled peptide to the bladder. This would further explain why the curve for ^{124}I -p5 revealed a sharper peak as compared to ^{124}I -p43. With the latter peptide, the TAC was broad, indicating that the radiotracer was retained within the kidney for a longer time period. Instead of monitoring the loss of liberated iodide from the kidney, the p43 TAC was indicative of the movement of radiolabeled p43, presumably intact, into the bladder. The previously described SPECT/CT time course also indicated the presence of ^{124}I -p43 in the renal tubules of WT mice at 1 h pi (see Figure 8.12), which further confirmed the extended presence of the radiotracer in the kidneys.

Since the ^{125}I -p43 radiotracer was resistant to dehalogenation, the stomach was devoid of radioactivity, and the thyroid only contained minimal amounts of radioiodide (Figure 8.10). Analysis of the dynamic PET/CT TAC for the heart, kidney, liver, thyroid and bladder was then performed. These curves indicated uptake and rapid clearance from the heart (blood pool), a delayed accumulation and prolonged clearance from the kidneys, slight uptake and steady level of radiotracer in the liver and minimal amounts of radiotracer in the thyroid as evidenced by very little accumulation toward the end of the 120 min acquisition (Figure 8:17A). The TAC for the bladder revealed steady accumulation that eventually plateaued; due to the high amount of radioactivity in this organ, it dominated the plot of TAC and was, thus, only shown in one of the graphs

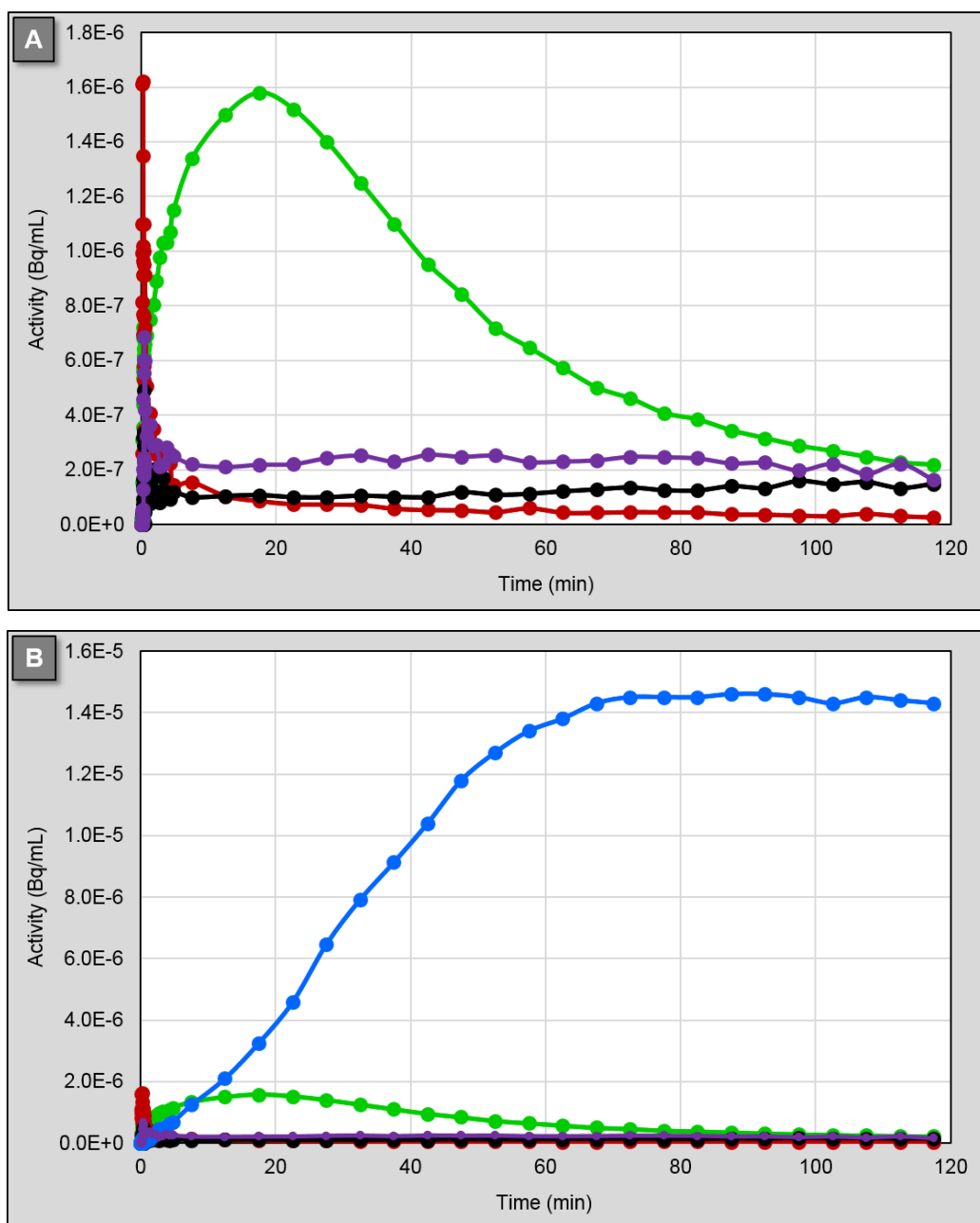


Figure 8.17: Time activity curves for ^{124}I -p43 in WT mice. (A) Curves depicting the accumulation and clearance of the heart, thyroid, liver and kidneys. The TAC showed rapid clearance from the heart (red), slight uptake in the thyroid (black), little accumulation in the liver (purple) that remained steady for the 120 min acquisition and delayed uptake in the kidney with slow clearance that was achieved by ~ 80 min pi. (B) TAC including bladder accumulation. The bladder (blue) began a steady accumulation of radiotracer at the beginning of the acquisition and eventually plateaued at ~ 60 min.

(Figure 8.17B). These curves support our hypothesis that ^{124}I -p43, in a WT mouse, exhibited slow renal clearance (relative to p5) and bladder accumulation which suggested that the peptide could be followed throughout the duration of the acquisition. It is noteworthy that little or no bladder radioactivity was observed when ^{124}I -p5 was injected into WT mice [416]. This is an especially interesting finding that could be clinically beneficial in certain circumstances. If, for instance, a patient were to receive a dose of ^{124}I -p43 peptide and be imaged at an appropriate time pi, the lack of dehalogenation of the radiotracer would, theoretically, allow for accumulation and visualization of peptide bound to scant amyloid deposits in the kidneys, stomach, pancreas and spleen of patients with amyloidosis. If the radiotracer is prone to dehalogenation, accumulation of free iodide in the stomach could make visualization of amyloid deposits in nearby regions nearly impossible.

8.5. Conclusions

Development of a peptide with non-natural, D-amino acid incorporation was an attempt to address issues with the relatively short half-life associated with peptide radiotracers. Incorporation of amino acids that are not naturally recognized by biological enzymes was the strategy to extend peptide half-life in order to enhance the time-frame in which the radiotracer could be taken up by amyloid *in vivo*. It was hypothesized that this type of modification would improve peptide bioavailability without altering the size, which may hinder its targeting capabilities.

Studies conducted *ex vivo* revealed that the D-amino acid peptide variant, p43, (like the p5 reagent) was incapable of binding to ApoA2c amyloid. In contrast, peptide p43 effectively targeted AA pathology by using both *ex vivo* and *in vivo* measures. The peptide was shown to bind more efficiently to amyloid-laden tissue homogenates *ex vivo* as compared to the parent peptide, p5. The alternative control peptide for these studies, p50 (no D-amino acids), was shown to bind in a similar fashion to p5. Thus, incorporation of D-amino acids modestly enhanced uptake and affinity for AA in the *ex vivo* binding assays.

The *in vivo* analyses with p43 revealed its effectiveness at targeting whole body amyloid burden in H2/IL-6 AA amyloid-laden mice. It was in WT mice, however, where the most interesting findings were made with p43. Instead of undergoing dehalogenation of the Tyr residue, which has been shown in multiple studies with ¹²⁵I-p5 [293, 416], the peptide avoided dehalogenase enzymes within the kidneys, and the radioiodination remained intact. This was shown by using SPECT/CT, biodistribution studies and analyses of TAC from dynamic PET/CT imaging. The control peptide made with only L-type amino acids, p50, did not display these properties. Additionally, dynamic PET/CT revealed that the peak renal uptake time of ¹²⁴I-p43 was approximately 10 min longer than the uptake time published for ¹²⁴I-p5 in healthy mice. Serum half-life extension may account for this delay. Thus, the incorporation of N-terminal D-type AQA immediately in front of the L-Tyr in p5 resulted in a non-dehalogenable peptide capable of effectively and specifically targeting AA amyloid *in vivo* with slightly extended serum bioavailability. This peptide could meet clinical needs that other amyloid-reactive peptides have not been able to achieve.

Chapter 9

Summary and future directions

Summary of data presented:

In the US, there are approximately 3500 new diagnoses of amyloidosis every year, and this number is expected to rise with greater clinical awareness and improved detection systems. The prognoses for these patients varies, but those with cardiac involvement have a considerably less favorable prognosis. In an attempt to facilitate a more complete and accurate diagnosis, we have begun a program developing molecular imaging techniques using amyloid-reactive tracers such that a non-invasive “picture” of amyloid burden in patients may be established. There is an unmet clinical need for a facile method of quantitatively detecting whole body amyloid burden in patients.

To this end, we have developed synthetic, amyloid-reactive peptides for use as radiotracers with SPECT/CT or PET/CT molecular imaging to detect amyloid deposition, using as a preclinical test-bed transgenic murine models of amyloidosis. In this thesis we tested a series of hypotheses and characterized a panel of peptides that could, one day, provide clinical benefit for the early detection of multiple types of amyloidosis.

Here I summarize the findings of this thesis describing our ongoing developmental and translational research:

- Polybasic, synthetic, heparin-binding peptides have been shown to bind both AA amyloid-laden tissue homogenates and synthetic AL fibrils in *ex vivo* pull down assays.
- Peptide affinity for ApoA2c amyloid-laden tissue homogenates *ex vivo*, however, increased according to the series p5, p5R and p5R+14, which exhibited the highest affinity for this substrate.
- Dual-isotope SPECT/CT imaging in association with tissue radioactivity biodistribution measurements, and SPECT image analyses revealed that peptide ^{125}I -p5R+14 provided more complete information on the whole body amyloid burden in ApoA2c mice as compared to $^{99\text{m}}\text{Tc}$ -p5 in the same animal.
- Autoradiographic analyses of ^{125}I -p5R+14 in ApoA2c amyloid-laden mouse tissues confirmed specific peptide co-localization with amyloid; however, amyloid deposits in certain tissues, notably the spleen, lung and heart, exhibited variable and incomplete uptake of the peptide radiotracer. Instead, a “binding site barrier” effect was observed in these tissues, likely due to the high affinity binding of the peptide to the dense ApoA2c amyloid deposits in these organs. This observation suggested that ^{125}I -p5R+14 imaging of amyloid may underestimate the extent of true amount of amyloid deposition *in vivo*.

- A comparative study of peptide p5R+14 binding to ApoA2c amyloid-laden tissues *in vivo* and *ex vivo* was performed to study the incomplete uptake of peptide by amyloid *in vivo*. These analyses showed that the peptide “underestimated” the amount of amyloid *in vivo* in the spleen, lungs and heart, as compared to *ex vivo* uptake of the peptide. These experiments showed that the peptide binding sites in these tissues may be “inaccessible” *in vivo* since *ex vivo* homogenization increased peptide reactivity.

- A modified peptide was developed to potentially improve *in vivo* ApoA2c amyloid-targeting and uptake. Since Wall *et al.* demonstrated that an arginine-rich peptide resulted in higher affinity for amyloid and a binding site barrier effect in AA amyloid-laden tissues [371], we hypothesized that a lysine variant of p5R+14, designated p5+14, would penetrate regions of dense ApoA2c amyloid deposits that were untouched by p5R+14 due to its lower affinity for the deposits. SPECT/CT imaging, tissue radioactivity biodistribution measurements and autoradiographic analyses of ¹²⁵I-p5+14 in ApoA2c amyloid mice yielded data that did not support this hypothesis, however, and revealed that ¹²⁵I-p5R+14 was a more effective at ApoA2c amyloid-targeting.

- While ¹²⁵I-p5R+14 specifically and effectively bound ApoA2c amyloid *in vivo*, the radiotracer was unable to completely decorate (cover) dense amyloid deposits. Lowering peptide affinity by substituting lysine for arginine residues in the peptide did not enhance amyloid coverage; thus, we developed another peptide

radiotracer which addressed a different physical property— the serum half-life. Peptides have short bioavailability *in vivo* due to their small size [410-412], which led to our hypothesis that extending the serum half-life of the peptide radiotracer may enhance amyloid-targeting due to its longer exposure to amyloid. Therefore, a PEGylated p5R+14 peptide (20 kDa PEG) was synthesized and evaluated in both WT and ApoA2c mice. Data from these studies indicated that catabolism of ¹²⁵I-PEG-p5R+14 differed from that seen with unmodified ¹²⁵I-p5R+14 as evidenced by hepatobiliary clearance of the larger peptide compared to renal clearance with the non-PEGylated peptide. SPECT imaging indicated that, at 1 h pi, there was a higher concentration of ¹²⁵I-PEG-p5R+14 in WT mice as compared to ¹²⁵I-p5R+14 suggesting that the serum half-life was modestly extended. The serum bioavailability did not, however, enhance amyloid-targeting efficacy. Autoradiographic analyses suggested the two peptides exhibited similar amyloid-binding throughout all tissues.

- Peptide p5R+14 was the most effective at binding ApoA2c amyloid, but despite modifications its amyloid targeting abilities remained sub-optimal. To further investigate this limitation, an alternative method for serum half-life extension—the use of peptides containing non-natural, D- amino acids—led to the development and investigation of peptide p43, a variant of p5 with D-type AQA residues replacing GGG at the N-terminal. *Ex vivo* “pull down” binding assays using ¹²⁵I-p43 showed its AA amyloid-binding potential exceeded that of ¹²⁵I-p5, but much like p5, it was incapable of binding ApoA2c amyloid; thus, p5R+14 remained the

most effective ApoA2c amyloid-targeting agent. Based on our studies using AA and ApoA2c amyloid-peptide interactions we speculated that the composition of ApoA2c amyloid differs in nature relative to AA amyloid, e.g. the HSPG/fibril content may vary, which could influence successful peptide-amyloid interactions.

- Because p43 did not effectively bind ApoA2c amyloid *ex vivo*, we determined its *in vivo* characteristics instead in both AA amyloid-laden H2/IL-6 and WT mice. SPECT/CT imaging, tissue radioactivity biodistribution measurements and ARG at 1 h, 4 h and 24 h pi revealed that ^{125}I -p43, like ^{125}I -p5, specifically bound AA amyloid in numerous tissues. In WT mice, however, ^{125}I -p43 exhibited behavior unlike that previously observed with ^{125}I -p5 [293]. At 1 h pi, there was evidence of ^{125}I -p43 in the renal cortices, but at later time points pi, the renal activity rapidly diminished. Remarkably, no radioiodide was detected in the stomach or thyroid at any time point, which indicated that the radiotracer was not dehalogenated—an unexpected finding based on peptide p5 data.
- The lack of dehalogenation of p43 was directly related to the presence of the D-amino acids at the N-terminal rather than simply substitution of AQA amino acids. This was confirmed in studies with p50, a peptide with the same amino acid sequence as p43 but with all L-entantiomers. The *in vivo* reactivity of p50 in AA and WT mice showed behavior similar to peptide p5, which proved that the D-enantiomers were responsible for protecting p43 from dehalogenase activity.

- To further characterize p43 *in vivo*, dynamic PET/CT imaging was performed using live WT mice. Analysis of PET images revealed the peak uptake time in the kidneys was ^{124}I -p43 was 10 min longer than that seen for ^{124}I -p5 [416], likely indicating serum half-life extension. These studies also confirmed that ^{124}I -p43 was not dehalogenated as evidenced by accumulation of radioactivity in the bladder. Thus, p43 was characterized as a non-dehalogenable, amyloid-binding peptide with slightly enhanced serum bioavailability—it offered properties of amyloid-imaging that no other examined peptide could achieve.

Future Directions:

The use of biological agents as imaging radiotracers is not a novel approach to disease detection. The data presented in this thesis and recent publications, validate the use of peptides for the detection of amyloidosis—a devastating disease which would benefit from early detection to improve patient survival. Our data indicated that there were unique characteristics associated with each of the peptides evaluated, which may make them more suitable for the clinical detection of certain types of amyloid disease. For instance, the enhanced binding of peptides with an additional 4 positively charged amino acids (e.g., p5R+14 or p5+14) may allow detection of scant amyloid deposits *in vivo* where high-affinity targeting is required; such would be the case in early disease detection where amyloid deposits are relatively small and the likelihood of detection would increase with a high-affinity, amyloid-binding reagent. In addition, diseases where small amyloid deposits form such as in cases of type 2 diabetes or cerebral

amyloid angiopathy in the brains of the elderly may benefit from a high affinity peptide imaging agent that would not suffer from the binding site barrier effect. Furthermore, the lack of dehalogenation of peptides with N-terminal, D- amino acids neighboring the iodinated tyrosine, may enhance the likelihood of detecting pancreatic or gastric amyloid deposits in patients with type 2 diabetes or AL/AA amyloidosis, respectively. Without dehalogenation, there would not be uptake of free iodide in the stomach, which could hinder the visualization of amyloid deposits in organs in close proximity to the stomach. Although we did not identify a single peptide to address all unmet clinical needs for amyloid detection, the many characteristics identified during these studies can be exploited for the detection of certain amyloid-associated conditions.

Defining the utility of peptides with unique characteristics and optimizing them for appropriate amyloid detection strategies would be clinically invaluable. Since cardiac and renal amyloidosis are associated with the most critical conditions in patients, earlier detection of such deposits should be the focus of peptide optimization for imaging. Combining and exploiting certain properties that were identified in this thesis may yield a superior peptide for targeting these types of deposition; the serum plasma half-life and avoidance of dehalogenation associated with D-amino acid incorporation of the extended, high-affinity peptide p5R+14 could provide the characteristics needed for successful detection of amyloid in the heart and kidneys of patients with AL. This combination of biochemical properties may also be advantageous in pancreatic amyloid detection. Therefore, future experiments that focus on optimizing and combining certain peptide characteristics should be conducted to assess hypothetically improved amyloid-targeting agents.

In the long term, before peptides are translated into the clinic, additional pre-clinical studies would need to be performed to ensure there are no adverse issues associated with toxicology or immunogenicity in mice. Additionally, it may be valuable to evaluate certain of the newly developed peptides in another species. This could be possible working in conjunction with the UT College of Veterinary Medicine, i.e. type 2 diabetic cats could receive a non-dehalogenable peptide radiolabeled with ^{123}I ($T_{1/2} = 13$ h) to assess radiotracer efficacy in detecting pancreatic AIAPP. Once sufficient preliminary data has been generated to justify a human clinical trial, GMP grade material would be developed and tested prior to applying to the FDA for approval to translate the reagent into human clinical trials.

A dire need for disease detection and monitoring exists for patients with amyloidosis around the world since approved strategies are not universally available, do not provide information for all critical organs, or do not target both AL and ATTR amyloid deposition—the two types most commonly seen in patients. The use of peptides as imaging agents for these diseases has yielded promising results, and transitioning these reagents into clinical trials could provide more information about the amyloid burden in patients than has been generated with other clinically available reagents. As has been stated by many researchers and physicians in the field, earlier detection is the key to fighting this devastating disease.

LIST OF REFERENCES

1. Blancas-Mejia, L.M. and M. Ramirez-Alvarado, *Systemic amyloidoses*. Annu Rev Biochem, 2013. **82**: p. 745-74.
2. Westermark, P., *Aspects on human amyloid forms and their fibril polypeptides*. FEBS J, 2005. **272**(23): p. 5942-9.
3. Bosch, M.N., et al., *Dogs with cognitive dysfunction syndrome: a natural model of Alzheimer's disease*. Curr Alzheimer Res, 2012. **9**(3): p. 298-314.
4. Jucker, M. and L.C. Walker, *Self-propagation of pathogenic protein aggregates in neurodegenerative diseases*. Nature, 2013. **501**(7465): p. 45-51.
5. Stevens, F.J. and R. Kisilevsky, *Immunoglobulin light chains, glycosaminoglycans, and amyloid*. Cell Mol Life Sci, 2000. **57**(3): p. 441-9.
6. Virchow, R., *Zur Cellulosefrage*. Virchows Arch. Pathol. Anat. Physiol., 1854. **6**: p. 416-426.
7. Lindahl, B. and U. Lindahl, *Amyloid-specific heparan sulfate from human liver and spleen*. J Biol Chem, 1997. **272**(42): p. 26091-4.
8. Pepys, M.B., *Amyloidosis*. Annu Rev Med, 2006. **57**: p. 223-41.
9. Rocken, C. and A. Shakespeare, *Pathology, diagnosis and pathogenesis of AA amyloidosis*. Virchows Arch, 2002. **440**(2): p. 111-22.
10. Kumar, S. and J. Walter, *Phosphorylation of amyloid beta (Abeta) peptides - a trigger for formation of toxic aggregates in Alzheimer's disease*. Aging (Albany NY), 2011. **3**(8): p. 803-12.
11. Fandrich, M., *On the structural definition of amyloid fibrils and other polypeptide aggregates*. Cell Mol Life Sci, 2007. **64**(16): p. 2066-78.
12. Tycko, R., *Progress towards a molecular-level structural understanding of amyloid fibrils*. Curr Opin Struct Biol, 2004. **14**(1): p. 96-103.
13. Astbury, W.T., S. Dickinson, and K. Bailey, *The X-ray interpretation of denaturation and the structure of the seed globulins*. Biochem J, 1935. **29**(10): p. 2351-2360 1.
14. Eanes, E.D. and G.G. Glenner, *X-ray diffraction studies on amyloid filaments*. J Histochem Cytochem, 1968. **16**(11): p. 673-7.
15. Sunde, M. and C.C. Blake, *From the globular to the fibrous state: protein structure and structural conversion in amyloid formation*. Q Rev Biophys, 1998. **31**(1): p. 1-39.
16. Emsley, J., et al., *Structure of pentameric human serum amyloid P component*. Nature, 1994. **367**(6461): p. 338-45.
17. Merlini, G. and V. Bellotti, *Molecular mechanisms of amyloidosis*. N Engl J Med, 2003. **349**(6): p. 583-96.
18. Westermark, P., et al., *Amyloid fibril protein nomenclature -- 2002*. Amyloid, 2002. **9**(3): p. 197-200.
19. Abraham, R.S., et al., *Immunoglobulin light chain variable (V) region genes influence clinical presentation and outcome in light chain-associated amyloidosis (AL)*. Blood, 2003. **101**(10): p. 3801-8.
20. Halle-Wittenberg, M.-L.-U. *Amyloid fibrils and their implication*. 2014 5-2-2014 [cited 2014; Available from: <http://www.natfak2.uni-halle.de/forschung/verbund/sfbtrr102/concepedia/amyloids/?lang=>].
21. Buxbaum, J.N. and R.P. Linke, *A molecular history of the amyloidoses*. J Mol Biol, 2012. **421**(2-3): p. 142-59.
22. Harper, J.D. and P.T. Lansbury, Jr., *Models of amyloid seeding in Alzheimer's disease and scrapie: mechanistic truths and physiological consequences of the time-dependent solubility of amyloid proteins*. Annu Rev Biochem, 1997. **66**: p. 385-407.
23. Jarrett, J.T. and P.T. Lansbury, Jr., *Seeding "one-dimensional crystallization" of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?* Cell, 1993. **73**(6): p. 1055-8.

24. Naiki, H. and F. Gejyo, *Kinetic analysis of amyloid fibril formation*. Methods Enzymol, 1999. **309**: p. 305-18.
25. Kisilevsky, R., *Preparation and propagation of amyloid-enhancing factor*. Methods Mol Biol, 2005. **299**: p. 237-41.
26. Snow, A.D., et al., *A temporal and ultrastructural relationship between heparan sulfate proteoglycans and AA amyloid in experimental amyloidosis*. J Histochem Cytochem, 1991. **39**(10): p. 1321-30.
27. Solomon, A., et al., *Transgenic mouse model of AA amyloidosis*. Am J Pathol, 1999. **154**(4): p. 1267-72.
28. Mensua, C., et al., *Pathology of AA amyloidosis in domestic sheep and goats*. Vet Pathol, 2003. **40**(1): p. 71-80.
29. Prusiner, S.B., *Shattuck lecture--neurodegenerative diseases and prions*. N Engl J Med, 2001. **344**(20): p. 1516-26.
30. Xing, Y., et al., *Transmission of mouse senile amyloidosis*. Lab Invest, 2001. **81**(4): p. 493-9.
31. Cao, P., et al., *Islet amyloid: from fundamental biophysics to mechanisms of cytotoxicity*. FEBS Lett, 2013. **587**(8): p. 1106-18.
32. Eisele, Y.S., *From soluble abeta to progressive abeta aggregation: could prion-like templated misfolding play a role?* Brain Pathol, 2013. **23**(3): p. 333-41.
33. Haataja, L., et al., *Islet amyloid in type 2 diabetes, and the toxic oligomer hypothesis*. Endocr Rev, 2008. **29**(3): p. 303-16.
34. Mucke, L. and D.J. Selkoe, *Neurotoxicity of amyloid beta-protein: synaptic and network dysfunction*. Cold Spring Harb Perspect Med, 2012. **2**(7): p. a006338.
35. Zraika, S., et al., *Toxic oligomers and islet beta cell death: guilty by association or convicted by circumstantial evidence?* Diabetologia, 2010. **53**(6): p. 1046-56.
36. Katzman, R., *Alzheimer's disease*. N Engl J Med, 1986. **314**(15): p. 964-73.
37. Kuperstein, I., et al., *Neurotoxicity of Alzheimer's disease Abeta peptides is induced by small changes in the Abeta42 to Abeta40 ratio*. EMBO J, 2010. **29**(19): p. 3408-20.
38. Moreth, J., C. Mavoungou, and K. Schindowski, *Passive anti-amyloid immunotherapy in Alzheimer's disease: What are the most promising targets?* Immun Ageing, 2013. **10**(1): p. 18.
39. Terry, R.D., et al., *Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment*. Ann Neurol, 1991. **30**(4): p. 572-80.
40. Walsh, D.M., et al., *Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo*. Nature, 2002. **416**(6880): p. 535-9.
41. Iozzo, R.V., *Matrix proteoglycans: from molecular design to cellular function*. Annu Rev Biochem, 1998. **67**: p. 609-52.
42. Scott, J.E., *The nomenclature of glycosaminoglycans and proteoglycans*. Glycoconj J, 1993. **10**(6): p. 419-21.
43. de Beer, M.C., et al., *Structural prerequisites for serum amyloid A fibril formation*. J Biol Chem, 1993. **268**(27): p. 20606-12.
44. Kisilevsky, R. and P. Fraser, *Proteoglycans and amyloid fibrillogenesis*. Ciba Found Symp, 1996. **199**: p. 58-67; discussion 68-72, 90-103.
45. Kisilevsky, R., et al., *Arresting amyloidosis in vivo using small-molecule anionic sulphonates or sulphates: implications for Alzheimer's disease*. Nat Med, 1995. **1**(2): p. 143-8.
46. McCubbin, W.D., et al., *Circular-dichroism studies on two murine serum amyloid A proteins*. Biochem J, 1988. **256**(3): p. 775-83.
47. Li, J.P., et al., *In vivo fragmentation of heparan sulfate by heparanase overexpression renders mice resistant to amyloid protein A amyloidosis*. Proc Natl Acad Sci U S A, 2005. **102**(18): p. 6473-7.

48. Stenstad, T., J.H. Magnus, and G. Husby, *Characterization of proteoglycans associated with mouse splenic AA amyloidosis*. *Biochem J*, 1994. **303 (Pt 2)**: p. 663-70.
49. Smits, N.C., et al., *The heparan sulfate motif (GlcNS6S-IdoA2S)₃, common in heparin, has a strict topography and is involved in cell behavior and disease*. *J Biol Chem*, 2010. **285**(52): p. 41143-51.
50. Conrad, E., *Commentary on heparin-like glycosaminoglycans*. *Angiogenesis*, 1997. **1**(1): p. 16-9.
51. Clark, S.J., P.N. Bishop, and A.J. Day, *The Proteoglycan Glycomatrix: A Sugar Microenvironment Essential for Complement Regulation*. *Front Immunol*, 2013. **4**: p. 412.
52. Sarrazin, S., W.C. Lamanna, and J.D. Esko, *Heparan sulfate proteoglycans*. *Cold Spring Harb Perspect Biol*, 2011. **3**(7).
53. Gill, S., T.N. Wight, and C.W. Frevert, *Proteoglycans: key regulators of pulmonary inflammation and the innate immune response to lung infection*. *Anat Rec (Hoboken)*, 2010. **293**(6): p. 968-81.
54. Snow, A.D., et al., *Characterization of tissue and plasma glycosaminoglycans during experimental AA amyloidosis and acute inflammation. Qualitative and quantitative analysis*. *Lab Invest*, 1987. **56**(6): p. 665-75.
55. Nelson, S.R., et al., *Isolation and characterization of the integral glycosaminoglycan constituents of human amyloid A and monoclonal light-chain amyloid fibrils*. *Biochem J*, 1991. **275 (Pt 1)**: p. 67-73.
56. Hazenberg, B.P., et al., *Diagnostic performance of 123I-labeled serum amyloid P component scintigraphy in patients with amyloidosis*. *Am J Med*, 2006. **119**(4): p. 355 e15-24.
57. Pepys, M.B., et al., *Isolation and characterization of pharmaceutical grade human pentraxins, serum amyloid P component and C-reactive protein, for clinical use*. *J Immunol Methods*, 2012. **384**(1-2): p. 92-102.
58. Bickerstaff, M.C., et al., *Serum amyloid P component controls chromatin degradation and prevents antinuclear autoimmunity*. *Nat Med*, 1999. **5**(6): p. 694-7.
59. Noursadeghi, M., et al., *Role of serum amyloid P component in bacterial infection: protection of the host or protection of the pathogen*. *Proc Natl Acad Sci U S A*, 2000. **97**(26): p. 14584-9.
60. Tennent, G.A., L.B. Lovat, and M.B. Pepys, *Serum amyloid P component prevents proteolysis of the amyloid fibrils of Alzheimer disease and systemic amyloidosis*. *Proc Natl Acad Sci U S A*, 1995. **92**(10): p. 4299-303.
61. Benson, M.D., et al., *Kinetics of serum amyloid protein A in casein-induced murine amyloidosis*. *J Clin Invest*, 1977. **59**(3): p. 412-7.
62. Botto, M., et al., *Amyloid deposition is delayed in mice with targeted deletion of the serum amyloid P component gene*. *Nat Med*, 1997. **3**(8): p. 855-9.
63. Caspi, D., et al., *Imaging of experimental amyloidosis with 131I-labeled serum amyloid P component*. *Arthritis Rheum*, 1987. **30**(11): p. 1303-6.
64. Hawkins, P.N., J.P. Lavender, and M.B. Pepys, *Evaluation of systemic amyloidosis by scintigraphy with 123I-labeled serum amyloid P component*. *N Engl J Med*, 1990. **323**(8): p. 508-13.
65. Hawkins, P.N., et al., *Specific localization and imaging of amyloid deposits in vivo using 123I-labeled serum amyloid P component*. *J Exp Med*, 1988. **167**(3): p. 903-13.
66. Hawkins, P.N., et al., *Diagnostic radionuclide imaging of amyloid: biological targeting by circulating human serum amyloid P component*. *Lancet*, 1988. **1**(8600): p. 1413-8.
67. Bodin, K., et al., *Antibodies to human serum amyloid P component eliminate visceral amyloid deposits*. *Nature*, 2010. **468**(7320): p. 93-7.
68. Merlini, G., *Reducing the amyloid burden through immunotherapy: a major therapeutic advance*. *Nephrol Dial Transplant*, 2011. **26**(5): p. 1471-3.

69. Pepys, M.B., et al., *Targeted pharmacological depletion of serum amyloid P component for treatment of human amyloidosis*. *Nature*, 2002. **417**(6886): p. 254-9.
70. Gillmore, J.D., et al., *Sustained pharmacological depletion of serum amyloid P component in patients with systemic amyloidosis*. *Br J Haematol*, 2010. **148**(5): p. 760-7.
71. Smits, N.C., et al., *Phage display-derived human antibodies against specific glycosaminoglycan epitopes*. *Methods Enzymol*, 2006. **416**: p. 61-87.
72. Thompson, S.M., et al., *Heparan sulfate phage display antibodies identify distinct epitopes with complex binding characteristics: insights into protein binding specificities*. *J Biol Chem*, 2009. **284**(51): p. 35621-31.
73. Wall, J.S., et al., *SPECT imaging of peripheral amyloid in mice by targeting hyper-sulfated heparan sulfate proteoglycans with specific scFv antibodies*. *Nucl Med Biol*, 2012. **39**(1): p. 65-75.
74. Kyle, R.A., et al., *Incidence and natural history of primary systemic amyloidosis in Olmsted County, Minnesota, 1950 through 1989*. *Blood*, 1992. **79**(7): p. 1817-22.
75. Obici, L., et al., *Clinical aspects of systemic amyloid diseases*. *Biochim Biophys Acta*, 2005. **1753**(1): p. 11-22.
76. Aguzzi, F., et al., *Occurrence of monoclonal components in general practice: clinical implications*. *Eur J Haematol*, 1992. **48**(4): p. 192-5.
77. Comenzo, R.L., et al., *The tropism of organ involvement in primary systemic amyloidosis: contributions of Ig V(L) germ line gene use and clonal plasma cell burden*. *Blood*, 2001. **98**(3): p. 714-20.
78. Perfetti, V., et al., *Analysis of V(lambda)-J(lambda) expression in plasma cells from primary (AL) amyloidosis and normal bone marrow identifies 3r (lambdaIII) as a new amyloid-associated germline gene segment*. *Blood*, 2002. **100**(3): p. 948-53.
79. Prokaeva, T., et al., *Soft tissue, joint, and bone manifestations of AL amyloidosis: clinical presentation, molecular features, and survival*. *Arthritis Rheum*, 2007. **56**(11): p. 3858-68.
80. del Pozo Yauner, L., et al., *Influence of the germline sequence on the thermodynamic stability and fibrillogenicity of human lambda 6 light chains*. *Proteins*, 2008. **72**(2): p. 684-92.
81. Wall, J., et al., *Thermodynamic instability of human lambda 6 light chains: correlation with fibrillogenicity*. *Biochemistry*, 1999. **38**(42): p. 14101-8.
82. Helms, L.R. and R. Wetzel, *Specificity of abnormal assembly in immunoglobulin light chain deposition disease and amyloidosis*. *J Mol Biol*, 1996. **257**(1): p. 77-86.
83. Gambetti, P. and C. Russo, *Human brain amyloidoses*. *Nephrol Dial Transplant*, 1998. **13 Suppl 7**: p. 33-40.
84. Schroder, R., M. Deckert, and R.P. Linke, *Novel isolated cerebral ALlambda amyloid angiopathy with widespread subcortical distribution and leukoencephalopathy due to atypical monoclonal plasma cell proliferation, and terminal systemic gammopathy*. *J Neuropathol Exp Neurol*, 2009. **68**(3): p. 286-99.
85. Khurana, R., et al., *Partially folded intermediates as critical precursors of light chain amyloid fibrils and amorphous aggregates*. *Biochemistry*, 2001. **40**(12): p. 3525-35.
86. Khurana, R., et al., *A model for amyloid fibril formation in immunoglobulin light chains based on comparison of amyloidogenic and benign proteins and specific antibody binding*. *Amyloid*, 2003. **10**(2): p. 97-109.
87. Qin, Z., et al., *Structural characterization of the partially folded intermediates of an immunoglobulin light chain leading to amyloid fibrillation and amorphous aggregation*. *Biochemistry*, 2007. **46**(11): p. 3521-31.
88. Husby, G., *Immunoglobulin-related (AL) amyloidosis*. *Clin Exp Rheumatol*, 1983. **1**(4): p. 353-8.

89. LifeForce. *LifeForce Hospitals: Amyloidosis*. 1999 [cited 2014; Available from: <http://www.chemo.net/newpage1.htm>].
90. Falk, R.H., *Diagnosis and management of the cardiac amyloidoses*. *Circulation*, 2005. **112**(13): p. 2047-60.
91. Falk, R.H., *Cardiac amyloidosis: a treatable disease, often overlooked*. *Circulation*, 2011. **124**(9): p. 1079-85.
92. Guan, J., et al., *Current perspectives on cardiac amyloidosis*. *Am J Physiol Heart Circ Physiol*, 2012. **302**(3): p. H544-52.
93. Shah, K.B., Y. Inoue, and M.R. Mehra, *Amyloidosis and the heart: a comprehensive review*. *Arch Intern Med*, 2006. **166**(17): p. 1805-13.
94. Comenzo, R.L., et al., *Dose-intensive melphalan with blood stem cell support for the treatment of AL amyloidosis: one-year follow-up in five patients*. *Blood*, 1996. **88**(7): p. 2801-6.
95. Dember, L.M., et al., *Effect of dose-intensive intravenous melphalan and autologous blood stem-cell transplantation on al amyloidosis-associated renal disease*. *Ann Intern Med*, 2001. **134**(9 Pt 1): p. 746-53.
96. Palladini, G., et al., *A modified high-dose dexamethasone regimen for primary systemic (AL) amyloidosis*. *Br J Haematol*, 2001. **113**(4): p. 1044-6.
97. Jiang, X., J.N. Buxbaum, and J.W. Kelly, *The V122I cardiomyopathy variant of transthyretin increases the velocity of rate-limiting tetramer dissociation, resulting in accelerated amyloidosis*. *Proc Natl Acad Sci U S A*, 2001. **98**(26): p. 14943-8.
98. Johnson, S.M., et al., *The transthyretin amyloidoses: from delineating the molecular mechanism of aggregation linked to pathology to a regulatory-agency-approved drug*. *J Mol Biol*, 2012. **421**(2-3): p. 185-203.
99. Kelly, J.W., et al., *Transthyretin quaternary and tertiary structural changes facilitate misassembly into amyloid*. *Adv Protein Chem*, 1997. **50**: p. 161-81.
100. Serag, A.A., et al., *Identification of a subunit interface in transthyretin amyloid fibrils: evidence for self-assembly from oligomeric building blocks*. *Biochemistry*, 2001. **40**(31): p. 9089-96.
101. Tsuchiya-Suzuki, A., et al., *Wild-type transthyretin significantly contributes to the formation of amyloid fibrils in familial amyloid polyneuropathy patients with amyloidogenic transthyretin Val30Met*. *Hum Pathol*, 2011. **42**(2): p. 236-43.
102. Connors, L.H., et al., *Tabulation of human transthyretin (TTR) variants*, 2003. *Amyloid*, 2003. **10**(3): p. 160-84.
103. Colon, W., et al., *FAP mutations destabilize transthyretin facilitating conformational changes required for amyloid formation*. *Ciba Found Symp*, 1996. **199**: p. 228-38; discussion 239-42.
104. Lai, Z., et al., *Guanidine hydrochloride-induced denaturation and refolding of transthyretin exhibits a marked hysteresis: equilibria with high kinetic barriers*. *Biochemistry*, 1997. **36**(33): p. 10230-9.
105. Lashuel, H.A., Z. Lai, and J.W. Kelly, *Characterization of the transthyretin acid denaturation pathways by analytical ultracentrifugation: implications for wild-type, V30M, and L55P amyloid fibril formation*. *Biochemistry*, 1998. **37**(51): p. 17851-64.
106. Miroy, G.J., et al., *Inhibiting transthyretin amyloid fibril formation via protein stabilization*. *Proc Natl Acad Sci U S A*, 1996. **93**(26): p. 15051-6.
107. Nestle, F.O. and G. Burg, *Bilateral carpal tunnel syndrome as a clue for the diagnosis of systemic amyloidosis*. *Dermatology*, 2001. **202**(4): p. 353-5.
108. Park, M.A., et al., *Primary (AL) hepatic amyloidosis: clinical features and natural history in 98 patients*. *Medicine (Baltimore)*, 2003. **82**(5): p. 291-8.

109. Rapezzi, C., et al., *Phenotypic and genotypic heterogeneity in transthyretin-related cardiac amyloidosis: towards tailoring of therapeutic strategies?* Amyloid, 2006. **13**(3): p. 143-53.
110. Rapezzi, C., et al., *Transthyretin-related amyloidoses and the heart: a clinical overview.* Nat Rev Cardiol, 2010. **7**(7): p. 398-408.
111. Ando, Y., et al., *Guideline of transthyretin-related hereditary amyloidosis for clinicians.* Orphanet J Rare Dis, 2013. **8**: p. 31.
112. Benson, M.D. and J.C. Kincaid, *The molecular biology and clinical features of amyloid neuropathy.* Muscle Nerve, 2007. **36**(4): p. 411-23.
113. Harats, N., R.M. Worth, and M.D. Benson, *Hereditary amyloidosis: evidence against early amyloid deposition.* Arthritis Rheum, 1989. **32**(11): p. 1474-6.
114. Benson, M., *Genetics: Clinical implications of TTR Amyloidosis*, in *Recent Advances in Transthyretin Evolution, Structure and Biological Functions*, S.J. Richardson, Cody, V, Editor. 2009, Springer. p. 173-189.
115. Koike, H., et al., *Distinct characteristics of amyloid deposits in early- and late-onset transthyretin Val30Met familial amyloid polyneuropathy.* J Neurol Sci, 2009. **287**(1-2): p. 178-84.
116. Rapezzi, C., et al., *Disease profile and differential diagnosis of hereditary transthyretin-related amyloidosis with exclusively cardiac phenotype: an Italian perspective.* Eur Heart J, 2013. **34**(7): p. 520-8.
117. Hellman, U., et al., *Heterogeneity of penetrance in familial amyloid polyneuropathy, ATTR Val30Met, in the Swedish population.* Amyloid, 2008. **15**(3): p. 181-6.
118. Plante-Bordeneuve, V., et al., *Genetic study of transthyretin amyloid neuropathies: carrier risks among French and Portuguese families.* J Med Genet, 2003. **40**(11): p. e120.
119. Saporta, M.A., et al., *Penetrance estimation of TTR familial amyloid polyneuropathy (type I) in Brazilian families.* Eur J Neurol, 2009. **16**(3): p. 337-41.
120. Ohmori, H., et al., *Common origin of the Val30Met mutation responsible for the amyloidogenic transthyretin type of familial amyloidotic polyneuropathy.* J Med Genet, 2004. **41**(4): p. e51.
121. Soares, M.L., et al., *Haplotypes and DNA sequence variation within and surrounding the transthyretin gene: genotype-phenotype correlations in familial amyloid polyneuropathy (V30M) in Portugal and Sweden.* Eur J Hum Genet, 2004. **12**(3): p. 225-37.
122. Zaros, C., et al., *On the origin of the transthyretin Val30Met familial amyloid polyneuropathy.* Ann Hum Genet, 2008. **72**(Pt 4): p. 478-84.
123. Conceicao, I. and M. De Carvalho, *Clinical variability in type I familial amyloid polyneuropathy (Val30Met): comparison between late- and early-onset cases in Portugal.* Muscle Nerve, 2007. **35**(1): p. 116-8.
124. Jacobson, D.R., et al., *Revised transthyretin Ile 122 allele frequency in African-Americans.* Hum Genet, 1996. **98**(2): p. 236-8.
125. Yamashita, T., et al., *A prospective evaluation of the transthyretin Ile122 allele frequency in an African-American population.* Amyloid, 2005. **12**(2): p. 127-30.
126. Amyloidosis, M.i.H., *Hereditary Amyloidosis Mutations Database.* 2010.
127. Buxbaum, J., et al., *Significance of the amyloidogenic transthyretin Val 122 Ile allele in African Americans in the Arteriosclerosis Risk in Communities (ARIC) and Cardiovascular Health (CHS) Studies.* Am Heart J, 2010. **159**(5): p. 864-70.
128. Dharmarajan, K. and M.S. Maurer, *Transthyretin cardiac amyloidoses in older North Americans.* J Am Geriatr Soc, 2012. **60**(4): p. 765-74.
129. Motoyama, T., et al., *Interleukin 6-producing malignant mesothelioma.* Virchows Arch B Cell Pathol Incl Mol Pathol, 1993. **64**(6): p. 367-72.

130. Rocken, C., et al., *Rosai-Dorfman disease and generalized AA amyloidosis: a case report*. Hum Pathol, 2000. **31**(5): p. 621-4.
131. Gillmore, J.D., et al., *Amyloid load and clinical outcome in AA amyloidosis in relation to circulating concentration of serum amyloid A protein*. Lancet, 2001. **358**(9275): p. 24-9.
132. Malle, E., A. Steinmetz, and J.G. Raynes, *Serum amyloid A (SAA): an acute phase protein and apolipoprotein*. Atherosclerosis, 1993. **102**(2): p. 131-46.
133. Husby, G.M., G; Dowtor, B; Sletten, K; Sipe, J.D., *Serum amyloid A (SAA): Biochemistry, genetics and the pathogenesis of AA amyloidosis*. Amyloid, 1994. **1**(2): p. 119-137.
134. Kisilevsky, R. and L. Subrahmanyam, *Serum amyloid A changes high density lipoprotein's cellular affinity. A clue to serum amyloid A's principal function*. Lab Invest, 1992. **66**(6): p. 778-85.
135. Kluve-Beckerman, B., J. Manaloor, and J.J. Liepnieks, *Binding, trafficking and accumulation of serum amyloid A in peritoneal macrophages*. Scand J Immunol, 2001. **53**(4): p. 393-400.
136. Rocken, C.K., R, *Binding and endocytosis of murine high density lipoprotein from healthy (HDL) and inflamed donors (HDL SAA) by murine macrophages in vitro. A light and electron microscopic investigation*. Amyloid, 1997. **4**(4): p. 259-273.
137. Obici, L. and G. Merlini, *AA amyloidosis: basic knowledge, unmet needs and future treatments*. Swiss Med Wkly, 2012. **142**: p. w13580.
138. Lundmark, K., et al., *Protein fibrils in nature can enhance amyloid protein A amyloidosis in mice: Cross-seeding as a disease mechanism*. Proc Natl Acad Sci U S A, 2005. **102**(17): p. 6098-102.
139. Lavie, G., D. Zucker-Franklin, and E.C. Franklin, *Degradation of serum amyloid A protein by surface-associated enzymes of human blood monocytes*. J Exp Med, 1978. **148**(4): p. 1020-31.
140. Skogen, B., L. Thorsteinsson, and J.B. Natvig, *Degradation of protein SAA to an AA-like fragment by enzymes of monocytic origin*. Scand J Immunol, 1980. **11**(5): p. 533-40.
141. Yamada, T., et al., *Accelerated amyloid deposition in mice treated with the aspartic protease inhibitor, pepstatin*. J Immunol, 1996. **157**(2): p. 901-7.
142. Yamada, T., et al., *In vitro degradation of serum amyloid A by cathepsin D and other acid proteases: possible protection against amyloid fibril formation*. Scand J Immunol, 1995. **41**(6): p. 570-4.
143. Westermark, G.T., U. Engstrom, and P. Westermark, *The N-terminal segment of protein AA determines its fibrillogenic property*. Biochem Biophys Res Commun, 1992. **182**(1): p. 27-33.
144. Arai, K., et al., *Transformation from SAA2-fibrils to AA-fibrils in amyloid fibrillogenesis: in vivo observations in murine spleen using anti-SAA and anti-AA antibodies*. J Pathol, 1994. **173**(2): p. 127-34.
145. Kisilevsky, R., et al., *During AA amyloidogenesis, is proteolytic attack on serum amyloid A a pre- or post-fibrillogenic event?* Amyloid, 1994. **1**: p. 174-183.
146. Yakar, S., et al., *Direct evidence for SAA deposition in tissues during murine amyloidogenesis*. Scand J Immunol, 1994. **40**(6): p. 653-8.
147. Gertz, M.A. and R.A. Kyle, *Secondary systemic amyloidosis: response and survival in 64 patients*. Medicine (Baltimore), 1991. **70**(4): p. 246-56.
148. Dubrey, S., *Amyloid heart disease*. The British Journal of Cardiology, 2009. **16**(1): p. 36-41.
149. van der Hilst, J., *Recent insights into the pathogenesis of type AA amyloidosis*. The Scientific World Journal, 2011. **11**: p. 641-650.
150. Hrnčić, R., et al., *Antibody-mediated resolution of light chain-associated amyloid deposits*. Am J Pathol, 2000. **157**(4): p. 1239-46.

151. Wright, J.R., et al., *Amyloid resorption: possible role of multinucleated giant cells. The apparent failure of penicillamine treatment.* Johns Hopkins Med J, 1972. **130**(5): p. 278-88.
152. Heegaard, N.H., *beta(2)-microglobulin: from physiology to amyloidosis.* Amyloid, 2009. **16**(3): p. 151-73.
153. Doherty, P.C. and R.M. Zinkernagel, *H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus.* J Exp Med, 1975. **141**(2): p. 502-7.
154. Paulsson, K.M., et al., *Distinct differences in association of MHC class I with endoplasmic reticulum proteins in wild-type, and beta 2-microglobulin- and TAP-deficient cell lines.* Int Immunol, 2001. **13**(8): p. 1063-73.
155. Germain, R.N. and D.H. Margulies, *The biochemistry and cell biology of antigen processing and presentation.* Annu Rev Immunol, 1993. **11**: p. 403-50.
156. Ljunggren, H.G., et al., *Empty MHC class I molecules come out in the cold.* Nature, 1990. **346**(6283): p. 476-80.
157. Cresswell, P., et al., *Immunological identity of the small subunit of HL-A antigens and beta2-microglobulin and its turnover on the cell membrane.* Proc Natl Acad Sci U S A, 1974. **71**(5): p. 2123-7.
158. Karlsson, F.A., L. Wibell, and P.E. Evrin, *beta 2-Microglobulin in clinical medicine.* Scand J Clin Lab Invest Suppl, 1980. **154**: p. 27-37.
159. Saito, A., et al., *Complete cloning and sequencing of rat gp330/"megalin," a distinctive member of the low density lipoprotein receptor gene family.* Proc Natl Acad Sci U S A, 1994. **91**(21): p. 9725-9.
160. Giorgetti, S., et al., *beta2-Microglobulin is potentially neurotoxic, but the blood brain barrier is likely to protect the brain from its toxicity.* Nephrol Dial Transplant, 2009. **24**(4): p. 1176-81.
161. Eichner, T. and S.E. Radford, *Understanding the complex mechanisms of beta2-microglobulin amyloid assembly.* FEBS J, 2011. **278**(20): p. 3868-83.
162. Garbar, C., et al., *Histological characteristics of sternoclavicular beta 2-microglobulin amyloidosis and clues for its histogenesis.* Kidney Int, 1999. **55**(5): p. 1983-90.
163. Jadoul, M., C. Garbar, and C. van Ypersele de Strihou, *Pathological aspects of beta(2)-microglobulin amyloidosis.* Semin Dial, 2001. **14**(2): p. 86-9.
164. Westermark, P., A. Andersson, and G.T. Westermark, *Islet amyloid polypeptide, islet amyloid, and diabetes mellitus.* Physiol Rev, 2011. **91**(3): p. 795-826.
165. Lukinius, A., et al., *Co-localization of islet amyloid polypeptide and insulin in the B cell secretory granules of the human pancreatic islets.* Diabetologia, 1989. **32**(4): p. 240-4.
166. Johnson, K.H., et al., *Immunolocalization of islet amyloid polypeptide (IAPP) in pancreatic beta cells by means of peroxidase-antiperoxidase (PAP) and protein A-gold techniques.* Am J Pathol, 1988. **130**(1): p. 1-8.
167. Karlsson, E., *IAPP as a regulator of glucose homeostasis and pancreatic hormone secretion (review).* Int J Mol Med, 1999. **3**(6): p. 577-84.
168. Jaikaran, E.T., M.R. Nilsson, and A. Clark, *Pancreatic beta-cell granule peptides form heteromolecular complexes which inhibit islet amyloid polypeptide fibril formation.* Biochem J, 2004. **377**(Pt 3): p. 709-16.
169. Janciauskiene, S., et al., *B cell granule peptides affect human islet amyloid polypeptide (IAPP) fibril formation in vitro.* Biochem Biophys Res Commun, 1997. **236**(3): p. 580-5.
170. Kudva, Y.C., et al., *A novel assay in vitro of human islet amyloid polypeptide amyloidogenesis and effects of insulin secretory vesicle peptides on amyloid formation.* Biochem J, 1998. **331** (Pt 3): p. 809-13.
171. Larson, J.L. and A.D. Miranker, *The mechanism of insulin action on islet amyloid polypeptide fiber formation.* J Mol Biol, 2004. **335**(1): p. 221-31.

172. Sellin, D., et al., *Suppression of IAPP fibrillation at anionic lipid membranes via IAPP-derived amyloid inhibitors and insulin*. Biophys Chem, 2010. **150**(1-3): p. 73-9.
173. Clark, A., et al., *Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes*. Diabetes Res, 1988. **9**(4): p. 151-9.
174. Konarkowska, B., et al., *The aggregation potential of human amylin determines its cytotoxicity towards islet beta-cells*. FEBS J, 2006. **273**(15): p. 3614-24.
175. Lorenzo, A., et al., *Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus*. Nature, 1994. **368**(6473): p. 756-60.
176. Lutz, T.A., *Control of energy homeostasis by amylin*. Cell Mol Life Sci, 2012. **69**(12): p. 1947-65.
177. Montane, J., et al., *Metabolic stress, IAPP and islet amyloid*. Diabetes Obes Metab, 2012. **14 Suppl 3**: p. 68-77.
178. Janson, J., et al., *The mechanism of islet amyloid polypeptide toxicity is membrane disruption by intermediate-sized toxic amyloid particles*. Diabetes, 1999. **48**(3): p. 491-8.
179. Carrillo-Mora, P., R. Luna, and L. Colin-Barenque, *Amyloid Beta: Multiple Mechanisms of Toxicity and Only Some Protective Effects?* Oxid Med Cell Longev, 2014. **2014**: p. 795375.
180. Gulli, G., L. Rossetti, and R.A. DeFronzo, *Hyperamylinemia is associated with hyperinsulinemia in the glucose-tolerant, insulin-resistant offspring of two Mexican-American non-insulin-dependent diabetic parents*. Metabolism, 1997. **46**(10): p. 1157-61.
181. Engel, M.F., *Membrane permeabilization by Islet Amyloid Polypeptide*. Chem Phys Lipids, 2009. **160**(1): p. 1-10.
182. Padrick, S.B. and A.D. Miranker, *Islet amyloid: phase partitioning and secondary nucleation are central to the mechanism of fibrillogenesis*. Biochemistry, 2002. **41**(14): p. 4694-703.
183. Klimek, A.M., et al., *Impaired proinsulin processing is a characteristic of transplanted islets*. Am J Transplant, 2009. **9**(9): p. 2119-25.
184. Rhodes, C.J., et al., *Stimulation by ATP of proinsulin to insulin conversion in isolated rat pancreatic islet secretory granules. Association with the ATP-dependent proton pump*. J Biol Chem, 1987. **262**(22): p. 10712-7.
185. Huang, C.J., et al., *Calcium-activated calpain-2 is a mediator of beta cell dysfunction and apoptosis in type 2 diabetes*. J Biol Chem, 2010. **285**(1): p. 339-48.
186. Masters, S.L., et al., *Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes*. Nat Immunol, 2010. **11**(10): p. 897-904.
187. Park, Y.J., et al., *Deletion of Fas protects islet beta cells from cytotoxic effects of human islet amyloid polypeptide*. Diabetologia, 2012.
188. Rivera, J.F., et al., *Human-IAPP disrupts the autophagy/lysosomal pathway in pancreatic beta-cells: protective role of p62-positive cytoplasmic inclusions*. Cell Death Differ, 2011. **18**(3): p. 415-26.
189. Subramanian, S.L., et al., *cJUN N-terminal kinase (JNK) activation mediates islet amyloid-induced beta cell apoptosis in cultured human islet amyloid polypeptide transgenic mouse islets*. Diabetologia, 2012. **55**(1): p. 166-74.
190. Westwell-Roper, C., et al., *IL-1 blockade attenuates islet amyloid polypeptide-induced proinflammatory cytokine release and pancreatic islet graft dysfunction*. J Immunol, 2011. **187**(5): p. 2755-65.
191. Zhang, S., et al., *Fibrillogenic amylin evokes islet beta-cell apoptosis through linked activation of a caspase cascade and JNK1*. J Biol Chem, 2003. **278**(52): p. 52810-9.

192. Zraika, S., et al., *Oxidative stress is induced by islet amyloid formation and time-dependently mediates amyloid-induced beta cell apoptosis*. Diabetologia, 2009. **52**(4): p. 626-35.
193. Cao, X. and T.C. Sudhof, *A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60*. Science, 2001. **293**(5527): p. 115-20.
194. Haass, C., et al., *Trafficking and proteolytic processing of APP*. Cold Spring Harb Perspect Med, 2012. **2**(5): p. a006270.
195. Borchelt, D.R., et al., *Familial Alzheimer's disease-linked presenilin 1 variants elevate Abeta1-42/1-40 ratio in vitro and in vivo*. Neuron, 1996. **17**(5): p. 1005-13.
196. Citron, M., et al., *Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice*. Nat Med, 1997. **3**(1): p. 67-72.
197. Benzinger, T.L., et al., *Propagating structure of Alzheimer's beta-amyloid(10-35) is parallel beta-sheet with residues in exact register*. Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13407-12.
198. Luhrs, T., et al., *3D structure of Alzheimer's amyloid-beta(1-42) fibrils*. Proc Natl Acad Sci U S A, 2005. **102**(48): p. 17342-7.
199. Petkova, A.T., et al., *A structural model for Alzheimer's beta -amyloid fibrils based on experimental constraints from solid state NMR*. Proc Natl Acad Sci U S A, 2002. **99**(26): p. 16742-7.
200. Perry, V.H., J.A. Nicoll, and C. Holmes, *Microglia in neurodegenerative disease*. Nat Rev Neurol, 2010. **6**(4): p. 193-201.
201. Hardy, J., *The amyloid hypothesis for Alzheimer's disease: a critical reappraisal*. J Neurochem, 2009. **110**(4): p. 1129-34.
202. Hardy, J. and D.J. Selkoe, *The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics*. Science, 2002. **297**(5580): p. 353-6.
203. Thies, W., L. Bleiler, and A. Alzheimer's, *2013 Alzheimer's disease facts and figures*. Alzheimers Dement, 2013. **9**(2): p. 208-45.
204. Mavoungou, C. and K. Zimmerman, *Immunotherapy with Anti-Abeta Monoclonal Antibodies in Alzheimer's Disease: A Critical Review on the Molecules in the Pipelines with Regulatory Considerations*. Frontiers in Clinical Drug Research--Alzheimer Disorders, 2013. **1**: p. 3-85.
205. Brown, P., et al., *Human spongiform encephalopathy: the National Institutes of Health series of 300 cases of experimentally transmitted disease*. Ann Neurol, 1994. **35**(5): p. 513-29.
206. Collinge, J., *Prion diseases of humans and animals: their causes and molecular basis*. Annu Rev Neurosci, 2001. **24**: p. 519-50.
207. Senatore, A., E. Restelli, and R. Chiesa, *Synaptic Dysfunction in Prion Diseases: A Trafficking Problem?* Int J Cell Biol, 2013. **2013**: p. 543803.
208. Jeong, B.H. and Y.S. Kim, *Genetic Studies in Human Prion Diseases*. J Korean Med Sci, 2014. **29**(5): p. 623-632.
209. Imran, M. and S. Mahmood, *An overview of human prion diseases*. Virol J, 2011. **8**: p. 559.
210. Ghetti, B., et al., *Prion protein amyloidosis*. Brain Pathol, 1996. **6**(2): p. 127-45.
211. Goldfarb, L.G., et al., *Fatal familial insomnia and familial Creutzfeldt-Jakob disease: disease phenotype determined by a DNA polymorphism*. Science, 1992. **258**(5083): p. 806-8.
212. Jansen, C., et al., *Prion protein amyloidosis with divergent phenotype associated with two novel nonsense mutations in PRNP*. Acta Neuropathol, 2010. **119**(2): p. 189-97.

213. Knight, R.S. and R.G. Will, *Prion diseases*. J Neurol Neurosurg Psychiatry, 2004. **75 Suppl 1**: p. i36-42.
214. Biasini, E., et al., *Prion protein at the crossroads of physiology and disease*. Trends Neurosci, 2012. **35**(2): p. 92-103.
215. Chiesa, R. and D.A. Harris, *Fishing for prion protein function*. PLoS Biol, 2009. **7**(3): p. e75.
216. Westergard, L., H.M. Christensen, and D.A. Harris, *The cellular prion protein (PrP(C)): its physiological function and role in disease*. Biochim Biophys Acta, 2007. **1772**(6): p. 629-44.
217. Poggiolini, I., D. Saverioni, and P. Parchi, *Prion Protein Misfolding, Strains, and Neurotoxicity: An Update from Studies on Mammalian Prions*. Int J Cell Biol, 2013. **2013**: p. 910314.
218. Capellari, S., et al., *Genetic Creutzfeldt-Jakob disease and fatal familial insomnia: insights into phenotypic variability and disease pathogenesis*. Acta Neuropathol, 2011. **121**(1): p. 21-37.
219. Caughey, B. and P.T. Lansbury, *Protofibrils, pores, fibrils, and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders*. Annu Rev Neurosci, 2003. **26**: p. 267-98.
220. Haass, C. and D.J. Selkoe, *Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide*. Nat Rev Mol Cell Biol, 2007. **8**(2): p. 101-12.
221. Murakami, T., N. Ishiguro, and K. Higuchi, *Transmission of systemic AA amyloidosis in animals*. Vet Pathol, 2014. **51**(2): p. 363-71.
222. Solomon, A., et al., *Amyloidogenic potential of foie gras*. Proc Natl Acad Sci U S A, 2007. **104**(26): p. 10998-1001.
223. Meyer-Luehmann, M., et al., *Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host*. Science, 2006. **313**(5794): p. 1781-4.
224. Aguzzi, A. and J. Falsig, *Prion propagation, toxicity and degradation*. Nat Neurosci, 2012. **15**(7): p. 936-9.
225. Watts, J.C., A. Balachandran, and D. Westaway, *The expanding universe of prion diseases*. PLoS Pathog, 2006. **2**(3): p. e26.
226. Johnson, K.H., et al., *Amyloid proteins and amyloidosis in domestic animals*. Amyloid, 1996. **3**: p. 270-289.
227. Roertgen, K. and K. Johnson, *Amyloidosis*, in *Noninfectious diseases of wildlife.*, A. Fairbrother, L. Locke, and G. Hoff, Editors. 1996, Iowa State University Press: Ames, Iowa, USA.
228. Zschesche, W. and W. Jakob, *Pathology of animal amyloidoses*. Pharmacol Ther, 1989. **41**(1-2): p. 49-83.
229. Wu, C. and J.E. Shea, *Structural similarities and differences between amyloidogenic and non-amyloidogenic islet amyloid polypeptide (IAPP) sequences and implications for the dual physiological and pathological activities of these peptides*. PLoS Comput Biol, 2013. **9**(8): p. e1003211.
230. Butler, A.E., et al., *Diabetes due to a progressive defect in beta-cell mass in rats transgenic for human islet amyloid polypeptide (HIP Rat): a new model for type 2 diabetes*. Diabetes, 2004. **53**(6): p. 1509-16.
231. Hayden, M.R., et al., *Longitudinal ultrastructure study of islet amyloid in the HIP rat model of type 2 diabetes mellitus*. Exp Biol Med (Maywood), 2007. **232**(6): p. 772-9.
232. Matveyenko, A.V. and P.C. Butler, *Islet amyloid polypeptide (IAPP) transgenic rodents as models for type 2 diabetes*. ILAR J, 2006. **47**(3): p. 225-33.
233. Chakraborty, S., B. Chatterjee, and S. Basu, *A mechanistic insight into the amyloidogenic structure of hIAPP peptide revealed from sequence analysis and molecular dynamics simulation*. Biophys Chem, 2012. **168-169**: p. 1-9.

234. Khurana, R., et al., *Is Congo red an amyloid-specific dye?* J Biol Chem, 2001. **276**(25): p. 22715-21.
235. Caughey, B., et al., *Binding of the protease-sensitive form of PrP (prion protein) to sulfated glycosaminoglycan and congo red [corrected]*. J Virol, 1994. **68**(4): p. 2135-41.
236. Kagan, H.M., N.A. Hewitt, and C. Franzblau, *A microenvironmental probe of elastin. Properties of a solubilized Congo red-elastin complex*. Biochim Biophys Acta, 1973. **322**(2): p. 258-68.
237. Woody, A.Y., R.R. Reisbig, and R.W. Woody, *Spectroscopic studies of Congo Red binding to RNA polymerase*. Biochim Biophys Acta, 1981. **655**(1): p. 82-8.
238. Kuciel, R. and A. Mazurkiewicz, *Molten globule as an intermediate on the human prostatic phosphatase folding pathway*. Acta Biochim Pol, 1997. **44**(4): p. 645-57.
239. Buxbaum, J.N., *The systemic amyloidoses*. Curr Opin Rheumatol, 2004. **16**(1): p. 67-75.
240. Glenner, G.G., *Amyloid deposits and amyloidosis. The beta-fibrilloses (first of two parts)*. N Engl J Med, 1980. **302**(23): p. 1283-92.
241. Linke, R.P., et al., *Classification of amyloidosis: misdiagnosing by way of incomplete immunohistochemistry and how to prevent it*. Acta Histochem, 2006. **108**(3): p. 197-208.
242. Merlini, G. and P. Westermark, *The systemic amyloidoses: clearer understanding of the molecular mechanisms offers hope for more effective therapies*. J Intern Med, 2004. **255**(2): p. 159-78.
243. Pepys, M.B., *Pathogenesis, diagnosis and treatment of systemic amyloidosis*. Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1406): p. 203-10; discussion 210-1.
244. Pras, M., et al., *The characterization of soluble amyloid prepared in water*. J Clin Invest, 1968. **47**(4): p. 924-33.
245. Kaplan, B., et al., *Microextraction and purification techniques applicable to chemical characterization of amyloid proteins in minute amounts of tissue*. Methods Enzymol, 1999. **309**: p. 67-81.
246. Kaplan, B., et al., *Biochemical subtyping of amyloid in formalin-fixed tissue samples confirms and supplements immunohistologic data*. Am J Clin Pathol, 2004. **121**(6): p. 794-800.
247. Kaplan, B., et al., *Micro-method to isolate and purify amyloid proteins for chemical characterization*. Amyloid, 2001. **8**(1): p. 22-9.
248. Shtrassburg, S., et al., *Demonstration of AA-protein in formalin-fixed, paraffin-embedded tissues*. Am J Pathol, 1982. **106**(2): p. 141-4.
249. Layfield, R., et al., *Extraction and protein sequencing of immunoglobulin light chain from formalin-fixed cerebrovascular amyloid deposits*. J Pathol, 1996. **180**(4): p. 455-9.
250. Linke, R.P., et al., *Identification of amyloid A protein in a sporadic Muckle-Wells syndrome. N-terminal amino acid sequence after isolation from formalin-fixed tissue*. Lab Invest, 1983. **48**(6): p. 698-704.
251. Arbustini, E., et al., *Electron and immuno-electron microscopy of abdominal fat identifies and characterizes amyloid fibrils in suspected cardiac amyloidosis*. Amyloid, 2002. **9**(2): p. 108-14.
252. Gallo, G.R., et al., *Characterization of tissue amyloid by immunofluorescence microscopy*. Clin Immunol Immunopathol, 1986. **39**(3): p. 479-90.
253. Linke, R.P., *Immunochemical typing of amyloid deposits after microextraction from biopsies*. Appl Pathol, 1985. **3**(1-2): p. 18-28.
254. Murphy, C.L., et al., *Characterization of systemic amyloid deposits by mass spectrometry*. Methods Enzymol, 2006. **412**: p. 48-62.
255. Olsen, K.E., K. Sletten, and P. Westermark, *The use of subcutaneous fat tissue for amyloid typing by enzyme-linked immunosorbent assay*. Am J Clin Pathol, 1999. **111**(3): p. 355-62.

256. Rocken, C., et al., *The classification of amyloid deposits in clinicopathological practice*. Histopathology, 1996. **29**(4): p. 325-35.
257. Kitamura, H., et al., *Pulmonary amyloidosis diagnosed by CT-guided transbronchial biopsy: a case report*. Jpn J Clin Oncol, 2001. **31**(5): p. 209-11.
258. Krishna, S.G., et al., *A first report of endoscopic ultrasound for the diagnosis of pancreatic amyloid deposition in immunoglobulin light chain (AL) amyloidosis (primary amyloidosis)*. JOP, 2013. **14**(3): p. 283-5.
259. Ostrow, L.W., et al., *Expanding the spectrum of monoclonal light chain deposition disease in muscle*. Muscle Nerve, 2012. **45**(5): p. 755-61.
260. Gillmore, J.D., et al., *Hereditary cardiac amyloidosis associated with the transthyretin Ile122 mutation in a white man*. Heart, 1999. **82**(3): p. e2.
261. Levin, C.S., *Primer on molecular imaging technology*. Eur J Nucl Med Mol Imaging, 2005. **32 Suppl 2**: p. S325-45.
262. Schoder, H., et al., *PET/CT: a new imaging technology in nuclear medicine*. Eur J Nucl Med Mol Imaging, 2003. **30**(10): p. 1419-37.
263. Bazanez-Borgert, M., *Basics of SPECT, PET and PET/CT Imaging*. 2006.
264. Adamson, K. and B. Walmsley, *Basics of CT*, in *Radionuclide and Hybrid Bone Imaging*, I. Fogelman, G. Gnanasegaran, and H. van der Wall, Editors. 2012, Springer Berlin Heidelberg. p. 135-148.
265. Dix, J.E., et al., *Accuracy and precision of CT angiography in a model of carotid artery bifurcation stenosis*. AJNR Am J Neuroradiol, 1997. **18**(3): p. 409-15.
266. Lev, M.H., et al., *Acute stroke: improved nonenhanced CT detection--benefits of soft-copy interpretation by using variable window width and center level settings*. Radiology, 1999. **213**(1): p. 150-5.
267. Webb, W., W. Brant, and C. Helms, *Introduction to CT of the abdomen and pelvis*, in *Fundamentals of body CT*, L. Bralow, Editor. 1991, Saunders: Philadelphia, PA. p. 137.
268. Lee, C.H., et al., *Radiation dose modulation techniques in the multidetector CT era: from basics to practice*. Radiographics, 2008. **28**(5): p. 1451-9.
269. Keat, N. *Report 05016 CT scanner automatic exposure control systems*. 2005 [cited 2014 February 19, 2014]; Available from: <http://www.impactscan.org/reports/Report05016>.
270. Kingman, A. and N.L. Pereira, *Cardiac amyloidosis*. J S C Med Assoc, 2001. **97**(5): p. 201-6.
271. Sawyer, D.B. and M. Skinner, *Cardiac amyloidosis: shifting our impressions to hopeful*. Curr Heart Fail Rep, 2006. **3**(2): p. 64-71.
272. Mitsuhashi, S., et al., *MRI analysis on a patient with the V30M mutation is characteristic of leptomeningeal amyloid*. Amyloid, 2004. **11**(4): p. 265-7.
273. Bornemann, A., et al., *Amyloidoma of the gasserian ganglion as a cause of symptomatic neuralgia of the trigeminal nerve: report of three cases*. J Neurol, 1993. **241**(1): p. 10-4.
274. Gandhi, D., R. Wee, and M. Goyal, *CT and MR imaging of intracerebral amyloidoma: case report and review of the literature*. AJNR Am J Neuroradiol, 2003. **24**(3): p. 519-22.
275. Banyersad, S.M., et al., *Updates in cardiac amyloidosis: a review*. J Am Heart Assoc, 2012. **1**(2): p. e000364.
276. Di Bella, G., et al., *MRI of cardiac involvement in transthyretin familial amyloid polyneuropathy*. AJR Am J Roentgenol, 2010. **195**(6): p. W394-9.
277. Maceira, A.M., et al., *Cardiovascular magnetic resonance in cardiac amyloidosis*. Circulation, 2005. **111**(2): p. 186-93.
278. Vogelsberg, H., et al., *Cardiovascular magnetic resonance in clinically suspected cardiac amyloidosis: noninvasive imaging compared to endomyocardial biopsy*. J Am Coll Cardiol, 2008. **51**(10): p. 1022-30.

279. Townsend, D.W., *Physical principles and technology of clinical PET imaging*. Ann Acad Med Singapore, 2004. **33**(2): p. 133-45.
280. Madsen, M.T., *Recent advances in SPECT imaging*. J Nucl Med, 2007. **48**(4): p. 661-73.
281. Melcher, C., *Perspectives on the future development of new scintillators*. Nuclear Instruments and Methods in Physics Research: Section A, 2005. **537**: p. 6-14.
282. Wilkinson, F., *Scintillators*, in *Emission Tomography: The Fundamentals of PET and SPECT*, M. Wernick and J. Aarsvold, Editors. 2004, Elsevier: San Diego, CA. p. 229-254.
283. Evans, R., *The Atomic Nucleus*. 1955, New York, USA: McGraw-Hill.
284. Ziegler, S., *PET and SPECT*, in *Small Animal Imaging*, F. Kiessling and B. Pichler, Editors. 2011, Springer Berlin Heidelberg. p. 231-236.
285. Yang, L., D. Rieves, and C. Ganley, *Brain amyloid imaging--FDA approval of florbetapir F18 injection*. N Engl J Med, 2012. **367**(10): p. 885-7.
286. Zanzonico, P. and S. Heller, *Physics, Instrumentation and Radiation Protection*, in *Clinical Nuclear Medicine*, H. Biersack and L. Freeman, Editors. 2007, Springer: Heidelberg. p. 1-33.
287. Cherry, S., J. Sorensen, and M. Phelps, *Physics in Nuclear Medicine*, in *Physics in Nuclear Medicine*, S. Cherry, J. Sorensen, and M. Phelps, Editors. 2003, Saunders: Philadelphia, PA. p. 325-359.
288. Zanzonico, P., *Principles of nuclear medicine imaging: planar, SPECT, PET, multi-modality, and autoradiography systems*. Radiat Res, 2012. **177**(4): p. 349-64.
289. Meikle, S.R., et al., *Small animal SPECT and its place in the matrix of molecular imaging technologies*. Phys Med Biol, 2005. **50**(22): p. R45-61.
290. Ma, K.H., et al., *Dual SPECT of dopamine system using [99mTc]TRODAT-1 and [123I]IBZM in normal and 6-OHDA-lesioned formosan rock monkeys*. Nucl Med Biol, 2002. **29**(5): p. 561-7.
291. Nakazawa, A., et al., *Usefulness of dual 67Ga and 99mTc-sestamibi single-photon-emission CT scanning in the diagnosis of cardiac sarcoidosis*. Chest, 2004. **126**(4): p. 1372-6.
292. Sanchez-Crespo, A., et al., *A novel quantitative dual-isotope method for simultaneous ventilation and perfusion lung SPET*. Eur J Nucl Med Mol Imaging, 2002. **29**(7): p. 863-75.
293. Wall, J.S., et al., *Comparative analysis of peptide p5 and serum amyloid P component for imaging AA amyloid in mice using dual-isotope SPECT*. Mol Imaging Biol, 2012. **14**(4): p. 402-7.
294. Weinmann, P., et al., *Clinical validation of simultaneous dual-isotope myocardial scintigraphy*. Eur J Nucl Med Mol Imaging, 2003. **30**(1): p. 25-31.
295. Antoch, G., et al., *Comparison of PET, CT, and dual-modality PET/CT imaging for monitoring of imatinib (STI571) therapy in patients with gastrointestinal stromal tumors*. J Nucl Med, 2004. **45**(3): p. 357-65.
296. Burger, I., et al., *PET/CT: diagnostic improvement in recurrent colorectal carcinoma compared to PET alone*. Radiology, 2002. **225**.
297. Mariani, G., et al., *A review on the clinical uses of SPECT/CT*. Eur J Nucl Med Mol Imaging, 2010. **37**(10): p. 1959-85.
298. Buck, A.K., et al., *Spect/Ct*. J Nucl Med, 2008. **49**(8): p. 1305-19.
299. Seo, Y., C. Mari, and B.H. Hasegawa, *Technological development and advances in single-photon emission computed tomography/computed tomography*. Semin Nucl Med, 2008. **38**(3): p. 177-98.
300. Johnson, K.A., et al., *Florbetapir (F18-AV-45) PET to assess amyloid burden in Alzheimer's disease dementia, mild cognitive impairment, and normal aging*. Alzheimers Dement, 2013. **9**(5 Suppl): p. S72-83.

301. Ferri, C.P., et al., *Global prevalence of dementia: a Delphi consensus study*. Lancet, 2005. **366**(9503): p. 2112-7.
302. Reitz, C., C. Brayne, and R. Mayeux, *Epidemiology of Alzheimer disease*. Nat Rev Neurol, 2011. **7**(3): p. 137-52.
303. Association, A., *Early-onset dementia: A national challenge, a future crisis*. 2006, Alzheimer's Association: Washington, D.C.
304. Hebert, L., et al., *Alzheimer's disease in the United States (2010-2050) estimated using the 2010 Census*. Neurology, 2013.
305. Camus, V., et al., *Using PET with 18F-AV-45 (florbetapir) to quantify brain amyloid load in a clinical environment*. Eur J Nucl Med Mol Imaging, 2012. **39**(4): p. 621-31.
306. Vallabhajosula, S., *Positron emission tomography radiopharmaceuticals for imaging brain Beta-amyloid*. Semin Nucl Med, 2011. **41**(4): p. 283-99.
307. Wong, D.F., et al., *In vivo imaging of amyloid deposition in Alzheimer disease using the radioligand 18F-AV-45 (florbetapir [corrected] F 18)*. J Nucl Med, 2010. **51**(6): p. 913-20.
308. Jacques, L., et al. *Proposed decision memo for beta amyloid positron emission tomography in dementia and neurodegenerative disease*. 2013 [cited 2014 May 25, 2014]; CMS.gov

Centers for Medicare and Medicaid Services]. Available from: <http://www.cms.gov/medicare-coverage-database/details/nca-proposed-decision-memo.aspx?NCAId=265>.
309. Scutti, S. *FDA approves vizamyl, 2nd brain imaging drug to detect Alzheimer's and dementia: Will it be approved for medicare?* 2013 [cited 2014 May 25, 2014]; Available from: <http://www.medicaldaily.com/fda-approves-vizamyl-2nd-brain-imaging-drug-detect-alzheimers-and-dementia-will-it-be-approved>.
310. Mitsis, E.M., et al., *A consecutive case series experience with [18 F] florbetapir PET imaging in an urban dementia center: impact on quality of life, decision making, and disposition*. Mol Neurodegener, 2014. **9**: p. 10.
311. Dorbala, S., et al., *Imaging cardiac amyloidosis: a pilot study using (1)(8)F-florbetapir positron emission tomography*. Eur J Nucl Med Mol Imaging, 2014. **41**(9): p. 1652-62.
312. Ikonomic, M.D., et al., *Post-mortem correlates of in vivo PiB-PET amyloid imaging in a typical case of Alzheimer's disease*. Brain, 2008. **131**(Pt 6): p. 1630-45.
313. Klunk, W.E., et al., *Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B*. Ann Neurol, 2004. **55**(3): p. 306-19.
314. Klunk, W.E., et al., *The binding of 2-(4'-methylaminophenyl)benzothiazole to postmortem brain homogenates is dominated by the amyloid component*. J Neurosci, 2003. **23**(6): p. 2086-92.
315. Leinonen, V., et al., *Assessment of beta-amyloid in a frontal cortical brain biopsy specimen and by positron emission tomography with carbon 11-labeled Pittsburgh Compound B*. Arch Neurol, 2008. **65**(10): p. 1304-9.
316. Lockhart, A., et al., *PIB is a non-specific imaging marker of amyloid-beta (Abeta) peptide-related cerebral amyloidosis*. Brain, 2007. **130**(Pt 10): p. 2607-15.
317. Vandenberghe, R., et al., *18F-flutemetamol amyloid imaging in Alzheimer disease and mild cognitive impairment: a phase 2 trial*. Ann Neurol, 2010. **68**(3): p. 319-29.
318. Rowe, C.C., et al., *Imaging of amyloid beta in Alzheimer's disease with 18F-BAY94-9172, a novel PET tracer: proof of mechanism*. Lancet Neurol, 2008. **7**(2): p. 129-35.
319. Antoni, G., et al., *In vivo visualization of amyloid deposits in the heart with 11C-PIB and PET*. J Nucl Med, 2013. **54**(2): p. 213-20.
320. Pauwels, E.K., et al., *A comparison between the diagnostic efficacy of 99mTc-MDP, 99mTc-DPD and 99mTc-HDP for the detection of bone metastases*. Eur J Nucl Med, 1983. **8**(3): p. 118-22.

321. Subramanian, G. and J.G. McAfee, *A new complex of 99mTc for skeletal imaging*. Radiology, 1971. **99**(1): p. 192-6.
322. Yano, Y., et al., *Technetium-99m-labeled stannous ethane-1-hydroxy-1 1-diphosphonate: a new bone scanning agent*. J Nucl Med, 1973. **14**(2): p. 73-8.
323. Mohty, D., et al., *Cardiac amyloidosis: updates in diagnosis and management*. Arch Cardiovasc Dis, 2013. **106**(10): p. 528-40.
324. Perugini, E., et al., *Noninvasive etiologic diagnosis of cardiac amyloidosis using 99mTc-3,3-diphosphono-1,2-propanodicarboxylic acid scintigraphy*. J Am Coll Cardiol, 2005. **46**(6): p. 1076-84.
325. Zannad, N., et al., *[Diagnosis of senile cardiac amyloidosis by (99m)Tc-DPD scintigraphy]*. Presse Med, 2009. **38**(4): p. 672-5.
326. Glaudemans, A.W., et al., *Bone scintigraphy with (99m)technetium-hydroxymethylene diphosphonate allows early diagnosis of cardiac involvement in patients with transthyretin-derived systemic amyloidosis*. Amyloid, 2014. **21**(1): p. 35-44.
327. Solomon, A., D.T. Weiss, and J.S. Wall, *Therapeutic potential of chimeric amyloid-reactive monoclonal antibody 11-1F4*. Clin Cancer Res, 2003. **9**(10 Pt 2): p. 3831S-8S.
328. Wall, J.S., et al., *Radioimaging of light chain amyloid with a fibril-reactive monoclonal antibody*. J Nucl Med, 2006. **47**(12): p. 2016-24.
329. Wall, J.S., et al., *Radioimmunodetection of amyloid deposits in patients with AL amyloidosis*. Blood, 2010. **116**(13): p. 2241-4.
330. Wall, J.S., et al., *Generation and characterization of anti-AA amyloid-specific monoclonal antibodies*. Front Immunol, 2011. **2**: p. 32.
331. Wall, J.S., et al., *AL amyloid imaging and therapy with a monoclonal antibody to a cryptic epitope on amyloid fibrils*. PLoS One, 2012. **7**(12): p. e52686.
332. Aprile, C., et al., *Cardiac and pleuropulmonary AL amyloid imaging with technetium-99m labelled aprotinin*. Eur J Nucl Med, 1995. **22**(12): p. 1393-401.
333. O'Nuallain, B., et al., *Localization of a conformational epitope common to non-native and fibrillar immunoglobulin light chains*. Biochemistry, 2007. **46**(5): p. 1240-7.
334. Rocken, C., et al., *Cathepsin protease activity modulates amyloid load in extracerebral amyloidosis*. J Pathol, 2006. **210**(4): p. 478-87.
335. Rocken, C., et al., *Proteolysis of serum amyloid A and AA amyloid proteins by cysteine proteases: cathepsin B generates AA amyloid proteins and cathepsin L may prevent their formation*. Ann Rheum Dis, 2005. **64**(6): p. 808-15.
336. Prothena. *Science. Targeting proteins in novel ways to resolve unmet medical needs in patients* 2014 [cited 2014 May 27, 2014]; Available from: <http://www.prothena.com/science>.
337. Campistol, J.M., et al., *Demonstration of plasma proteinase inhibitors in beta 2-microglobulin amyloid deposits*. Kidney Int, 1992. **42**(4): p. 915-23.
338. Eriksson, S., S. Janciauskiene, and G. Merlini, *The putative role of alpha-1-antitrypsin in the disaggregation of amyloid lambda fibrils*. J Intern Med, 1995. **237**(2): p. 143-9.
339. Aprile, C., et al., *Assessment of split renal function with 99mTc-aprotinin*. Eur J Nucl Med, 1986. **12**(1): p. 37-40.
340. Aprile, C., et al., *99mTc-aprotinin: comparison with 99mTc-DMSA in normal and diseased kidneys*. Nuklearmedizin, 1984. **23**(1): p. 22-6.
341. Bianchi, C., et al., *99mTc-aprotinin: a new tracer for kidney morphology and function*. Eur J Nucl Med, 1984. **9**(6): p. 257-60.
342. Janoki, G., et al. *Localization of the polypeptide aprotinin in the renal cortex: a new renal agent for scintigraphy*. in *Proceedings of the first international symposium on radiopharmacology*. 1978. Innsbruck, Austria.
343. Schaadt, B.K., et al., *99mTc-aprotinin scintigraphy in amyloidosis*. J Nucl Med, 2003. **44**(2): p. 177-83.

344. Shaw, A.D., et al., *The effect of aprotinin on outcome after coronary-artery bypass grafting*. N Engl J Med, 2008. **358**(8): p. 784-93.
345. Hawkins, P.N., R. Wootton, and M.B. Pepys, *Metabolic studies of radioiodinated serum amyloid P component in normal subjects and patients with systemic amyloidosis*. J Clin Invest, 1990. **86**(6): p. 1862-9.
346. Hawkins, P.N., et al., *Studies in vivo and in vitro of serum amyloid P component in normals and in a patient with AA amyloidosis*. Clin Exp Immunol, 1991. **84**(2): p. 308-16.
347. Hachulla, E., et al., *Prospective and serial study of primary amyloidosis with serum amyloid P component scintigraphy: from diagnosis to prognosis*. Am J Med, 1996. **101**(1): p. 77-87.
348. Hawkins, P.N., et al., *Scintigraphic quantification and serial monitoring of human visceral amyloid deposits provide evidence for turnover and regression*. Q J Med, 1993. **86**(6): p. 365-74.
349. Maulin, L., et al., *'Localized amyloidosis': 123I-labelled SAP component scintigraphy and labial salivary gland biopsy*. QJM, 1997. **90**(1): p. 45-50.
350. Rydh, A., et al., *Serum amyloid P component scintigraphy in familial amyloid polyneuropathy: regression of visceral amyloid following liver transplantation*. Eur J Nucl Med, 1998. **25**(7): p. 709-13.
351. Lovat, L.B., et al., *The liver in systemic amyloidosis: insights from 123I serum amyloid P component scintigraphy in 484 patients*. Gut, 1998. **42**(5): p. 727-34.
352. Bijzet, J. *Investigations. SAP Scintigraphy 2012* [cited 2014; Available from: www.amyloid.nl].
353. Otsubo, S., et al., *Characteristics of dialysis-related amyloidosis in patients on haemodialysis therapy for more than 30 years*. Nephrol Dial Transplant, 2009. **24**(5): p. 1593-8.
354. Munoz-Gomez, J., et al., *Synovial fluid examination for the diagnosis of synovial amyloidosis in patients with chronic renal failure undergoing haemodialysis*. Ann Rheum Dis, 1987. **46**(4): p. 324-6.
355. Noel, L.H., et al., *Tissue distribution of dialysis amyloidosis*. Clin Nephrol, 1987. **27**(4): p. 175-8.
356. Orfila, C., et al., *Unsuitable value of abdominal fat tissue aspirate examination for the diagnosis of amyloidosis in long-term hemodialysis patients*. Am J Nephrol, 1988. **8**(6): p. 454-6.
357. Sethi, D., et al., *Dialysis-associated amyloid: systemic or local?* Nephrol Dial Transplant, 1989. **4**(12): p. 1054-9.
358. Sole, M., et al., *Morphological and immunohistochemical findings in dialysis-related amyloidosis. An analysis of 16 cases*. Appl Pathol, 1989. **7**(6): p. 350-60.
359. Varga, J., et al., *Lack of amyloid in abdominal fat aspirates from patients undergoing long-term hemodialysis*. Arch Intern Med, 1987. **147**(8): p. 1455-7.
360. Floege, J. and M. Ketteler, *beta2-microglobulin-derived amyloidosis: an update*. Kidney Int Suppl, 2001. **78**: p. S164-71.
361. Floege, J., J. Schaffer, and K.M. Koch, *Scintigraphic methods to detect beta2-microglobulin associated amyloidosis (Abeta2-microglobulin amyloidosis)*. Nephrol Dial Transplant, 2001. **16 Suppl 4**: p. 12-6.
362. Haupt, C., et al., *Pattern recognition with a fibril-specific antibody fragment reveals the surface variability of natural amyloid fibrils*. J Mol Biol, 2011. **408**(3): p. 529-40.
363. Martin, E.B., et al., *Peptide p5 binds both heparinase-sensitive glycosaminoglycans and fibrils in patient-derived AL amyloid extracts*. Biochem Biophys Res Commun, 2013. **436**(1): p. 85-9.

364. Amdursky, N., Y. Erez, and D. Huppert, *Molecular rotors: what lies behind the high sensitivity of the thioflavin-T fluorescent marker*. *Acc Chem Res*, 2012. **45**(9): p. 1548-57.
365. Khurana, R., et al., *Mechanism of thioflavin T binding to amyloid fibrils*. *J Struct Biol*, 2005. **151**(3): p. 229-38.
366. Jameson, L., N. Smith, and S. Dzyuba, *Dye-binding assays for evaluation of the effects of small molecule inhibitors on amyloid (Abeta) self-assembly*. *ACS Chemical Neuroscience*, 2012. **3**(11): p. 807-819.
367. Morris, K.L. and L.C. Serpell, *X-ray fibre diffraction studies of amyloid fibrils*. *Methods Mol Biol*, 2012. **849**: p. 121-35.
368. Madine, J., et al., *Structural insights into the polymorphism of amyloid-like fibrils formed by region 20-29 of amylin revealed by solid-state NMR and X-ray fiber diffraction*. *J Am Chem Soc*, 2008. **130**(45): p. 14990-5001.
369. Tycko, R., *Solid-state NMR studies of amyloid fibril structure*. *Annu Rev Phys Chem*, 2011. **62**: p. 279-99.
370. Zhang, R., et al., *Interprotofilament interactions between Alzheimer's Abeta1-42 peptides in amyloid fibrils revealed by cryoEM*. *Proc Natl Acad Sci U S A*, 2009. **106**(12): p. 4653-8.
371. Wall, J.S., et al., *A binding-site barrier affects imaging efficiency of high affinity amyloid-reactive Peptide radiotracers in vivo*. *PLoS One*, 2013. **8**(6): p. e66181.
372. Skinner, M., et al., *Murine amyloid protein AA in casein-induced experimental amyloidosis*. *Lab Invest*, 1977. **36**(4): p. 420-7.
373. Snow, A.D. and R. Kisilevsky, *Temporal relationship between glycosaminoglycan accumulation and amyloid deposition during experimental amyloidosis. A histochemical study*. *Lab Invest*, 1985. **53**(1): p. 37-44.
374. Inoue, S. and R. Kisilevsky, *A high resolution ultrastructural study of experimental murine AA amyloid*. *Lab Invest*, 1996. **74**(3): p. 670-83.
375. Benson, M.D., et al., *Suppression of in vitro antibody response by a serum factor (SAA) in experimentally induced amyloidosis*. *J Exp Med*, 1975. **142**(1): p. 236-41.
376. Hawkins, P.N. and M.B. Pepys, *A primed state exists in vivo following histological regression of amyloidosis*. *Clin Exp Immunol*, 1990. **81**(2): p. 325-8.
377. Suematsu, S., et al., *Generation of plasmacytomas with the chromosomal translocation t(12;15) in interleukin 6 transgenic mice*. *Proc Natl Acad Sci U S A*, 1992. **89**(1): p. 232-5.
378. Wall, J.S., et al., *Quantitative tomography of early-onset spontaneous AA amyloidosis in interleukin 6 transgenic mice*. *Comp Med*, 2008. **58**(6): p. 542-50.
379. Kovalchuk, A.L., et al., *IL-6 transgenic mouse model for extraosseous plasmacytoma*. *Proc Natl Acad Sci U S A*, 2002. **99**(3): p. 1509-14.
380. Johan, K., et al., *Acceleration of amyloid protein A amyloidosis by amyloid-like synthetic fibrils*. *Proc Natl Acad Sci U S A*, 1998. **95**(5): p. 2558-63.
381. Wall, J.S., et al., *Quantitative high-resolution microradiographic imaging of amyloid deposits in a novel murine model of AA amyloidosis*. *Amyloid*, 2005. **12**(3): p. 149-56.
382. Ge, F., et al., *Amyloidosis in transgenic mice expressing murine amyloidogenic apolipoprotein A-II (Apoa2c)*. *Lab Invest*, 2007. **87**(7): p. 633-43.
383. Higuchi, K., et al., *Polymorphism of apolipoprotein A-II (apoA-II) among inbred strains of mice. Relationship between the molecular type of apoA-II and mouse senile amyloidosis*. *Biochem J*, 1991. **279** (Pt 2): p. 427-33.
384. Buxbaum, J., et al., *The pathogenesis of transthyretin tissue deposition: lessons from transgenic mice*. *Amyloid*, 2003. **10 Suppl 1**: p. 2-6.
385. Yamamura, K., et al., *Tissue-specific and developmental expression of human transthyretin gene in transgenic mice*. *Dev Genet*, 1987. **8**(4): p. 195-205.

386. Teng, M.H., et al., *Amyloid and nonfibrillar deposits in mice transgenic for wild-type human transthyretin: a possible model for senile systemic amyloidosis*. Lab Invest, 2001. **81**(3): p. 385-96.
387. Buxbaum, J.N., et al., *Why are some amyloidoses systemic? Does hepatic "chaperoning at a distance" prevent cardiac deposition in a transgenic model of human senile systemic (transthyretin) amyloidosis?* FASEB J, 2012. **26**(6): p. 2283-93.
388. Santos, S.D., R. Fernandes, and M.J. Saraiva, *The heat shock response modulates transthyretin deposition in the peripheral and autonomic nervous systems*. Neurobiol Aging, 2010. **31**(2): p. 280-9.
389. Teixeira, P.F., et al., *Endoplasmic reticulum stress associated with extracellular aggregates. Evidence from transthyretin deposition in familial amyloid polyneuropathy*. J Biol Chem, 2006. **281**(31): p. 21998-2003.
390. Yi, S., et al., *Systemic amyloidosis in transgenic mice carrying the human mutant transthyretin (Met30) gene. Pathologic similarity to human familial amyloidotic polyneuropathy, type I*. Am J Pathol, 1991. **138**(2): p. 403-12.
391. Gejyo, F., et al., *A new form of amyloid protein associated with chronic hemodialysis was identified as beta 2-microglobulin*. Biochem Biophys Res Commun, 1985. **129**(3): p. 701-6.
392. Gorevic, P.D., et al., *Beta-2 microglobulin is an amyloidogenic protein in man*. J Clin Invest, 1985. **76**(6): p. 2425-9.
393. Ivanova, M.I., et al., *An amyloid-forming segment of beta2-microglobulin suggests a molecular model for the fibril*. Proc Natl Acad Sci U S A, 2004. **101**(29): p. 10584-9.
394. Zhang, P., et al., *Mouse model to study human A beta2M amyloidosis: generation of a transgenic mouse with excessive expression of human beta2-microglobulin*. Amyloid, 2010. **17**(2): p. 50-62.
395. Solomon, A., D.T. Weiss, and M.B. Pepys, *Induction in mice of human light-chain-associated amyloidosis*. Am J Pathol, 1992. **140**(3): p. 629-37.
396. Solomon, A. and D.T. Weiss, *A perspective of plasma cell dyscrasias: clinical implications of monoclonal light chains in renal disease*, in *The Kidney in Plasma Cell Dyscrasias*, L. Minetti, G. D'Amico, and C. Ponticelli, Editors. 1988, Kluwer Academic Publishers: Dordrecht, The Netherlands. p. 3-18.
397. Ward, J.E., et al., *Doxycycline reduces fibril formation in a transgenic mouse model of AL amyloidosis*. Blood, 2011. **118**(25): p. 6610-7.
398. Teng, J., E.A. Turbat-Herrera, and G.A. Herrera, *An animal model of glomerular light-chain-associated amyloidogenesis depicts the crucial role of lysosomes*. Kidney Int, 2014.
399. DiBartola, S.P., et al., *Familial renal amyloidosis in Chinese Shar Pei dogs*. J Am Vet Med Assoc, 1990. **197**(4): p. 483-7.
400. Rivas, A.L., et al., *Inheritance of renal amyloidosis in Chinese Shar-pei dogs*. J Hered, 1993. **84**(6): p. 438-42.
401. Boyce, J.T., et al., *Familial renal amyloidosis in Abyssinian cats*. Vet Pathol, 1984. **21**(1): p. 33-8.
402. Niewold, T.A., et al., *Familial amyloidosis in cats: Siamese and Abyssinian AA proteins differ in primary sequence and pattern of deposition*. Amyloid, 1999. **6**(3): p. 205-9.
403. Betsholtz, C., et al., *Sequence divergence in a specific region of islet amyloid polypeptide (IAPP) explains differences in islet amyloid formation between species*. FEBS Lett, 1989. **251**(1-2): p. 261-4.
404. Johnson, K.H., et al., *The putative hormone islet amyloid polypeptide (IAPP) induces impaired glucose tolerance in cats*. Biochem Biophys Res Commun, 1990. **167**(2): p. 507-13.

405. Westermark, P., et al., *Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells*. Proc Natl Acad Sci U S A, 1987. **84**(11): p. 3881-5.
406. Habicht, G., et al., *Directed selection of a conformational antibody domain that prevents mature amyloid fibril formation by stabilizing Abeta protofibrils*. Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19232-7.
407. Haupt, C., et al., *Amyloid fibril recognition with the conformational B10 antibody fragment depends on electrostatic interactions*. J Mol Biol, 2011. **405**(2): p. 341-8.
408. van Kuppevelt, T.H., et al., *Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney*. J Biol Chem, 1998. **273**(21): p. 12960-6.
409. Wall, J.S., et al., *In vivo molecular imaging of peripheral amyloidosis using heparin-binding peptides*. Proc Natl Acad Sci U S A, 2011. **108**(34): p. E586-94.
410. Lee, S., J. Xie, and X. Chen, *Peptide-based probes for targeted molecular imaging*. Biochemistry, 2010. **49**(7): p. 1364-76.
411. Reubi, J.C. and H.R. Maecke, *Peptide-based probes for cancer imaging*. J Nucl Med, 2008. **49**(11): p. 1735-8.
412. Riccabona, G. and C. Decristoforo, *Peptide targeted imaging of cancer*. Cancer Biother Radiopharm, 2003. **18**(5): p. 675-87.
413. Schottelius, M. and H.J. Wester, *Molecular imaging targeting peptide receptors*. Methods, 2009. **48**(2): p. 161-77.
414. Jayaraman, G., et al., *Binding of a de novo designed peptide to specific glycosaminoglycans*. FEBS Lett, 2000. **482**(1-2): p. 154-8.
415. Zhang, Y., *I-TASSER server for protein 3D structure prediction*. BMC Bioinformatics, 2008. **9**: p. 40.
416. Martin, E.B., et al., *Dynamic PET and SPECT imaging with radioiodinated, amyloid-reactive peptide p5 in mice: A positive role for peptide dehalogenation*. Peptides, 2014. **60**: p. 63-70.
417. Wall, J.S., et al., *A novel method for quantifying peripheral tissue amyloid load by using the radiolabeled amyloidophilic peptide, p5*. Amyloid, 2013. **20**(1): p. 21-6.
418. Artimo, P., et al., *ExPASy: SIB bioinformatics resource portal*. Nucleic Acids Res, 2012. **40**(Web Server issue): p. W597-603.
419. Bailey, G.S., *Labeling of peptides and proteins by radioiodination*. Methods Mol Biol, 1994. **32**: p. 441-8.
420. Karonen, S.L., *Developments in techniques for radioiodination of peptide hormones and other proteins*. Scand J Clin Lab Invest Suppl, 1990. **201**: p. 135-8.
421. Hussain, A.A., et al., *Chloramine-T in radiolabeling techniques. II. A nondestructive method for radiolabeling biomolecules by halogenation*. Anal Biochem, 1995. **224**(1): p. 221-6.
422. Sobal, G., U. Resch, and H. Sinzinger, *Modification of low-density lipoprotein by different radioiodination methods*. Nucl Med Biol, 2004. **31**(3): p. 381-8.
423. Wall, J.S., et al., *Micro-imaging of amyloid in mice*. Methods Enzymol, 2006. **412**: p. 161-82.
424. Crandall, Y.M. and M.D. Bruch, *Characterization of the structure and dynamics of mastoparan-X during folding in aqueous TFE by CD and NMR spectroscopy*. Biopolymers, 2008. **89**(3): p. 197-209.
425. Rullo, A. and M. Nitz, *Importance of the spatial display of charged residues in heparin-peptide interactions*. Biopolymers, 2010. **93**(3): p. 290-8.
426. Araujo, R.L. and D.P. Carvalho, *Bioenergetic impact of tissue-specific regulation of iodothyronine deiodinases during nutritional imbalance*. J Bioenerg Biomembr, 2011. **43**(1): p. 59-65.

427. Baltz, M.L., R.F. Dyck, and M.B. Pepys, *Studies of the in vivo synthesis and catabolism of serum amyloid P component (SAP) in the mouse*. Clin Exp Immunol, 1985. **59**(1): p. 235-42.
428. Jevsevar, S., M. Kunstelj, and V.G. Porekar, *PEGylation of therapeutic proteins*. Biotechnol J, 2010. **5**(1): p. 113-28.
429. Levy, Y., et al., *Adenosine deaminase deficiency with late onset of recurrent infections: response to treatment with polyethylene glycol-modified adenosine deaminase*. J Pediatr, 1988. **113**(2): p. 312-7.
430. Ng, E.W., et al., *Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease*. Nat Rev Drug Discov, 2006. **5**(2): p. 123-32.
431. Serruys, P.W., et al., *Effect of an anti-PDGF-beta-receptor-blocking antibody on restenosis in patients undergoing elective stent placement*. Int J Cardiovasc Intervent, 2003. **5**(4): p. 214-22.
432. Fee, C. and J. Van Alstine, *PEG-proteins: Reaction engineering and separation issues*. Chemical Engineering Science, 2006. **61**: p. 924-939.
433. Gfeller, D., O. Michielin, and V. Zoete, *SwissSidechain: a molecular and structural database of non-natural sidechains*. Nucleic Acids Res, 2013. **41**(Database issue): p. D327-32.
434. Werle, M. and A. Bernkop-Schnurch, *Strategies to improve plasma half life time of peptide and protein drugs*. Amino Acids, 2006. **30**(4): p. 351-67.
435. Powell, M.F., et al., *Peptide stability in drug development. II. Effect of single amino acid substitution and glycosylation on peptide reactivity in human serum*. Pharm Res, 1993. **10**(9): p. 1268-73.
436. Reissmann, T., J. Engel, and B. Kutscher, *The LHRH antagonist cetrotorelix*. Drugs Future, 1994. **19**: p. 228-237.
437. Pinski, J., et al., *Evaluation of the in vitro and in vivo activity of the L-, D,L- and D-Cit6 forms of the LH-RH antagonist Cetrotorelix (SB-75)*. Int J Pept Protein Res, 1995. **45**(5): p. 410-7.
438. Hlavacek, J., et al., *Oostatic peptides containing D-amino acids: synthesis, oostatic activity, degradation, accumulation in ovaries and NMR study*. Amino Acids, 2012. **42**(5): p. 1715-25.

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