Renal Amyloidosis:
what you need to know and why

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Objectives

On completion of this activity, the participant should be able to:
1. explain the rationale behind the current classification of amyloidoses
2. identify special stains and techniques used to diagnose amyloidosis
3. contrast and compare the clinical features and kidney biopsy findings in minimal change disease, focal and segmental glomerular sclerosis, diabetes and amyloidosis
4. identify the most important prognostic factors in treatment of amyloidoses
5. evaluate amyloid typing options in renal pathology including advantages and limitations of each method

Disclosure:
- nothing to disclose
Outline:
1. Amyloid: what is it and why it forms
2. Classification and major types of amyloid
3. Management
4. Diagnosis: Congo red, Thioflavin, other?
5. Typing methods: IF, IHC, proteomics
6. Amyloid beyond disease
Amyloidoses – protein folding disorders

- hydrophobic
- insoluble
- sticky
- resistant to degradation…
Molecular events leading to amyloidosis

Intracellular protein quality control system

Amyloid precursor

Microenvironment of target organs

Proteolysis, metals

Matrix components GAGs, collagen

Extracellular chaperones

in vivo clearance

Matrix components

GAGs

SAP

oligomers

Amyloid fibrils

Organ dysfunction

Why amyloid forms?
1. Intrinsic instability, increased concentration, mutations, proteolytic cleavage, combination...
2. Pre-fibrillar species, fibrils
3. Oligomers in equilibrium with amyloid fibrils are believed to exert cytotoxic effect
4. Tissue factors (GAGs, SAP) – formation & persistence of deposits

mature fibrils – ALL amyloid protein types:
- affinity to Congo red with birefringence under polarized light
- fibrillar ultrastructure
Amyloidoses – protein folding disorders

- α helix
- β pleated sheet

Fibrillogenesis

Conformational shift to β-pleated sheet 2⁰ structure

- extracellular
- hydrophobic, insoluble
- non-functional
- sticky
- resistant to degradation…

protein quality control systems:
- intracellular (proteasomes),
- extracellular (macrophages)

intracellular (proteasomes), extracellular (macrophages)
Amyloid P [AP] component and amyloid:
- In vivo by serum AP [SAP] scintigraphy
- “In vitro” by immunohistochemistry [IHC]

IHC:
AP co-localizes with amyloid protein
aka “amyloid signature”
NOT present in
Light Chain Deposition Disease
AMYLOIDOSES:

1. >32 protein types, many more variants
2. Localized, systemic or systemic or/and localized
3. Specific organs, i.e. cerebral, endocrine organs...
4. Geographic areas, i.e. Icelandic
5. Most prevalent versus rare versus exceedingly rare
6. Treatable versus not-treatable, genetics...
Amyloidoses – protein folding disorders

- Increased concentration
- Mutations
- Intrinsic instability
- Proteolytic cleavage

Protein quality control systems:
- Intracellular (proteasomes)
- Extracellular (macrophages)

Clonal plasma cells – AL (amyloid light chain) protein
Chronic inflammatory reaction – AA amyloidosis
Mutations - hereditary amyloidoses (transthyretin variant)
Intrinsic instability – amino acid substitutions, “senile” amyloidoses ... combination...

- Precursor protein
- Misfolded protein
- Protofibril
- Mature fibrils
Cerebral amyloidoses

MAD COW DISEASE

CJD/vCJD
<table>
<thead>
<tr>
<th>Fibril protein</th>
<th>Precursor protein</th>
<th>Systemic &amp;/or Localized</th>
<th>Acquired or hereditary</th>
<th>Target organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL, AH</td>
<td>Immunoglobulin light or heavy chain</td>
<td>S, L</td>
<td>A (H)</td>
<td>KIDNEY, all, except CNS</td>
</tr>
<tr>
<td>AA</td>
<td>(Apo) Serum Amyloid A</td>
<td>S</td>
<td>A</td>
<td>KIDNEY, all, except CNS</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin, wild type variants</td>
<td>S</td>
<td>A</td>
<td>Cardiac, kidney some variants PNS, ANS, heart, eye, leptomeninges</td>
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<tr>
<td>AApoAl, All, C-III</td>
<td>Apolipoprotein variants (AI, All, C-II, C-III), wild type (AIV)</td>
<td>S, S</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>A</td>
<td>Heart, liver, KIDNEY, PNS... KIDNEY</td>
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<tr>
<td>AFib</td>
<td>Fibrinogen α, variants</td>
<td>S</td>
<td>H</td>
<td>KIDNEY primarily</td>
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<tr>
<td>ALECT2</td>
<td>Leukocyte chemotactic factor-2</td>
<td>S</td>
<td>A</td>
<td>KIDNEY primarily, liver</td>
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<td>Aβ2M</td>
<td>β2Microglobulin, wild type variant</td>
<td>L, S</td>
<td>A</td>
<td>Musculoskeletal ANS</td>
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<td>Cerebral: Aβ, ABri, ACys, APrP</td>
<td>Wild Variants, Wild</td>
<td>L, S</td>
<td>A</td>
<td>CNS</td>
</tr>
<tr>
<td>Endocrine</td>
<td>ACal (Pro)calcitonin, Islet amyloid polypeptide (Amylin), Atrial natriuretic factor, Prolactin</td>
<td>L</td>
<td>A</td>
<td>Thyroid (C-cell), Islets of Langerhans, atria, pituitary</td>
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<tr>
<td>Iatrogenic</td>
<td>Alns (insulin), AEnf (Enfurvitide)</td>
<td>L</td>
<td>A</td>
<td>Site of injection</td>
</tr>
<tr>
<td>other</td>
<td></td>
<td></td>
<td></td>
<td>Lung, skin, aorta, cornea...</td>
</tr>
</tbody>
</table>
AL – amyloid Light chain 
derived from immunoglobulin light chain

8.9 per million person-years reported incidence
~ 85% of systemic amyloidoses
B-cell/plasma cell neoplasia:
- plasma cell dyscrasia
- multiple myeloma 10-15%
Clinical manifestations due to clone:
- bone lesions
- hypercalcemia
- infections
- systemic symptoms

Clinical manifestations due to M-protein:
- light chain cast nephropathy
- Hyperviscosity
- Amyloidosis 10-15%

85% AL

AL, LCDD, LCPT (crystalline and non-organized deposits with end-organ damage):
- kidney, heart, liver failure, polyneuropathy, other

“a small but dangerous clone”
AL- λ > AL- κ, AH
λVI, 10 structure?
intact, truncated light chain, some V region only
Chemotherapy + stem cell transplantation with durable responses
Effective therapies available
Durable responses achievable

Late diagnosis = major obstacle!!!
AL – light chain amyloidosis
~ 85% of systemic amyloidoses

Clonal proliferation of plasma cells
Proteinuria/nephrotic syndrome

Liver involvement ~ 25%
Peri orbital purpura ~ 14%
Macroglossia ~ 14%
Submandibular swelling ~15%
Peripheral neuropathy ~14%
# AA amyloidosis

## Serum amyloid A derived amyloidosis

| Pathogenesis | - upregulated SAA (serum amyloid A protein), acute phase reactant, fibril precursor produced by liver
|              | systemic: Kidney, GI tract, Spleen, liver |
| Sporadic     | Chronic inflammatory conditions  
|              |   Inflammatory arthritis  
|              |   chronic infections, AIDS, etc…  
|              |   US? 5%, declining reporting?  
|              |   UK 18%  
|              |   Worldwide 45%, 2nd most common underdeveloped countries AA > AL |
| Familial     | Hereditary auto-inflammatory diseases: an inborn error of inflammatory response of the innate immune system  
|              |   monogenic – FMF (familial Mediterranean fever), other, YOUNGER AGE  
|              |   polygenic and complex – inflammatory bowel disease |
| Covert       | no identifiable disease 6% |
| Treatment    | Treatment of inflammation with reduction of SAA level increases survival  
|              | DMRADs (*disease modifying antirheumatic drugs*) |
Hereditary amyloidoses:
mutation in the precursor protein renders it amyloidogenic

AFib – amyloidosis derived
from fibrinogen mutant
primarily renal amyloidosis…
**AFib:** mutation in Fibrinogen A α chain

most frequent hereditary amyloidosis in N. Europe
with worldwide distribution

plasma protein essential for the final phase of blood coagulation
produced exclusively by the liver
several variants, usually no effect on fibrinogen function
(exception - deletion, frame shift mutation),
some patients have decreased fibrinogen levels

median age @ presentation: 55 y

NS + HTN, massive *glomerular amyloid*, ~ no extraglomerular deposits,
- some phenotypic variability depending on mutation, with involvement of
  other organs but renal failure dominates the clinical picture
- spleen involvement may lead to anemia and rupture
- Family history frequently missing
Hereditary: ATTR, AFib, AApoAI, All, C-III...
individually rare, but collectively ~10%

ATTR = amyloidosis derived from transthyretin
- inherited mutation destabilizing TTR tetramer
- >95% produced by the liver; rest produced by choroid plexus, eye
- Transport of thyroxin & retinol (vitamin A)
- >100 mutations
- polyneuropathies, cardiac, gastrointestinal, some mutations with clinically significant kidneys involvement
  “hot spots” in Portugal, Sweden, Japan but also worldwide distribution
The most common hereditary amyloidosis in the US
3.9% of African Americans (TTR V122I)

wtATTR (wild type) in elderly causing cardiomyopathy

Other hereditary amyloidoses - essentially all affect kidneys
Variable penetrance
Late onset in some
Family history often missing
Can MIMICK AL – danger of misdiagnosis
Liver transplantation as “surgical gene therapy” for patients with clinically apparent amyloidosis ATTR as well as other hereditary amyloidoses AFib – amyloidosis derived from fibrinogen

ATTR: the presence of a mutant leads to formation of unstable tetramers with oligomers dissociating and undergoing fibrillogenesis
Old age – also wild type TTR tetramers unstable
Wild TTR (wtTTR) - contains large strips with β pleated conformation

Small molecules stabilizing tetramers
Clinical trials....

Issues: who and when should be treated?
- clinical disease
- carriers?
- wtATTR?
Hereditary amyloidoses – treatments

1. Liver transplantation – “surgical defective gene therapy”
2. Pharmacologic therapies – clinical trials for ATTR
3. Pre-emptive treatment? AFib
4. Carriers?

5. EARLY diagnosis, avoid misdiagnosis as AL...
ALECT2
amyloidosis derived from leukocyte chemotactic factor-2

- Mexican-American ethnicity
- etiology unknown*
- No effective treatment
- Not to be mistaken for AL!!!

- Mayo 2.7%
- Nephropath: all 9.6%, South West US 54%
- Egypt 31%
- Europe? Changing demographics?

- Kidney – 3rd most common amyloidosis type
  - typically slowly progressing renal failure
- Liver – 2nd most common amyloidosis type
  - typically not clinically apparent

* no mutation, \textit{LECT2} G/G genotype
digenic? older age?

\textbf{Primum non nocere – first do no harm!}

\textbf{avoid misdiagnosis as AL!}
Renal Amyloidoses

AL: ~85%

- IFE^ (serum, urine), sFLCh^^,
  bone marrow biopsy, cardiac assessment
- anti-plasma cell chemotherapy/ASCT^^^,
- kidney transplantation**

Non-AL: ~15%

AA:
- anti-inflammatory/anti-infectious
- autoinflammatory diseases? (younger age)
- kidney transplantation**

ALect2: avoid misdiagnosis as AL!
- no specific therapy
- kidney transplantation (recurrence)
- family history/renal function testing?
- regional differences in incidence

Other: avoid misdiagnosis as AL!
ATTR hereditary & wild type: underdiagnosed ?%

Other hereditary:
- genetic testing, family history frequently (-)
- liver transplantation
- clinical trials (transthyretin amyloidosis)
- heart/kidney transplantation**
- genetic counseling

Unknown/new type??? avoid misdiagnosis as AL!

^ - immunofixation electrophoresis
^^ - serum Free Light Chain assay
^^^ - autologous stem cell transplantation
** - heart/kidney transplantation
(combined with amyloid type specific therapy)
How to diagnose amyloidosis?

TISSUE DIAGNOSIS:
1. Detection
2. Typing
3. Staging: systemic versus localized, organ involvement
Congo red polarization - diagnostic

green
yellow or orange birefringence
aka anomalous colors
Anomalous colors

Apple green - under ideal optical conditions
Green is the most specific finding but other anomalous colors (yellow or orange) are also diagnostic
Anomalous colors appear/disappear owing to strain birefringence (yellow) and/or during uncrossing of the polarizer and analyzer (orange)
Current diagnostic criteria: green, yellow and orange
Congo red stain viewed in bright field is NOT DIAGNOSTIC

Congo red polarization:
- specific
- relatively less sensitive
- polarization “shadow” - only a portion of deposits exposed at any given time

Congo red fluorescence TRITC filter:
- increased sensitivity
- no polarization “shadow”
- useful for screening
- verification with polarization required

*Picken MM. AJKD blog, 2016*
Congo red stained slides interpretation:
- strong light source
- darkened room
- pupils accommodated
- proper optics...
- thicker sections are NOT an absolute requirement!!!

Thioflavin T or S
- more sensitive but less specific than Congo red
- not permanent
- requirement for fluorescence microscope

Other stains:
- sulfated alcian blue = not specific, stains GAGs
- crystal violet = less sensitive, fading
- electron microscopy – small areas examined

Amyloid and legal issues: delayed diagnosis!!!
LM:
In H&E more advanced deposits of amyloid appear as eosinophilic, amorphous, “hyaline” material, always the same, regardless of the type of amyloid. Therefore Congo red stain must be examined to rule out amyloid and not just to confirm suspicion of amyloid based on light microscopy. In PAS stain amyloid is typically weakly positive. Exception: AH (amyloid derived from heavy chain) can be strongly PAS positive.
Subepithelial amyloid deposition - formation of irregular basement membrane “spicules”: amyloid deposits arranged in parallel arrays perpendicular to podocytes with loss of argyrophilia and fraying of the outer aspect of the basement membrane
Renal amyloidosis = systemic
Extra-renal genito-urinary amyloid = usually localized

- glomeruli, interstitium, extraglomerular blood vessels
- glomeruli: mesangium, extension into peripheral capillary walls

Segmental: small, discrete, confined to the mesangium, Differential Diagnosis: FSGS

Diffuse: mesangium expanded by weakly PAS-positive acellular deposits

Nodular: Differential Diagnosis: diabetic nephropathy, other forms of nodular glomerulosclerosis

Multinucleated giant cell reaction, rarely crescents - capillary wall rupture
Exclusively glomerular amyloid: typically in AFib, but also rarely in AL

Interstitial and peritubular deposits of amyloid in ~ 50% of cases; adjacent to blood vessels

Medullary amyloid deposits: around the vasa recta, loops of Henle, collecting ducts

Rare AA, mATTR, AApoAI, AApoAIV - amyloid limited to the interstitium and medulla

Scattered aggregates of lymphoplasmacytic cells may be present in AL

Arteriolar deposits = most frequent > arteries > peritubular capillaries > veins
Mimickers: hyalinosis, fibrinoid necrosis
Vascular deposits frequently coexist with glomerular amyloid

Rarely, only vascular deposits:
- AApo AII, vascular deposits with sparing of glomeruli
- Rare AA and AL amyloidosis with only vascular amyloid
Amyloid typing using frozen section immunofluorescence

Light chain restriction must be clear cut to be considered being diagnostic

- routinely used in renal pathology in North America
- first step in amyloid typing
- antibody panel success rate ~ 85%
- easy & fast
- clear background, high specificity
- high sensitivity, small deposits
- correlation with Congo red stain (co-localization)
- correlation with amyloid P component (intensity)
- detection of NON-AMYLOID PATHOLOGIES!!!
Amyloid typing using IHC (immunohistochemistry) in paraffin sections: Challenging!!!

- IHC of amyloid differs significantly from IHC in other areas of surgical pathology
- caution & experience necessary for its interpretation.

Challenges of amyloid IHC:
(i) serum contamination... (paraffin sections)
(ii) heterogeneity
(iii) lack of commercially available amyloid/amyloid-type-specific antibodies
(iv) controls
(v) rarity

Measures:
- frozen sections
- “comparative IHC” (panel of antibodies rather than a single antibody)
- antibodies to free light chains – “cleaner” stain
- KNOW THE LIMITS OF EACH METHOD
Comparative IHC:
apply a panel of several antibodies (rather than a single antibody) to find out which antibody gives the strongest reaction
Helpful in distinguishing specific versus non-specific stains

negative  nonspecific  Specific stain
Proteomics:

Rationale - relative abundance of amyloid protein in the tissue frequently the *dominant* protein

Diagnosis of amyloid by proteomic methods is based on the
- presence of large spectra numbers for the amyloidogenic protein
- in conjunction with apolipoprotein E and serum amyloid P component (aka “amyloid signature”)
To understand the relative strengths and weaknesses of IHC and mass spectrometry (MS)-based proteomics, it is helpful to compare these techniques with the differences that exist between fluorescence in-situ hybridization (FISH) and conventional cytogenetics.

**FISH**: paraffin sections are typically used, no extraction is required, and the information gained can be very precise, providing that one knows what to look for and that the corresponding probe is available.

**Conventional cytogenetics**: tissue must be harvested fresh and the cells grown in order to be subsequently spread for chromosomal evaluation. The final evaluation material is a picture of the entire (global) set of chromosomes, which may show expected as well as unexpected abnormalities, leading to a discovery of new data. However, an important limiting factor is the size of such abnormalities: while chromosomal deletions/translocation can be detected, gene abnormalities will not be seen in a conventional karyotype.
**FISH:**

- no sample preparation (paraffin section)
- need to know what to look for and need to have the appropriate probe.
- Can be very sensitive – detection of translocations of a single gene

**Cytogenetics:**

- need to prepare the tissue culture
- do not need to know what to expect - GLOBAL picture and potential for discovery of unsuspected abnormalities but with lesser sensitivity – fragment of a chromosome instead of a single gene
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Mass spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Routinely used to identify proteins</td>
<td>Newer technology</td>
</tr>
<tr>
<td>Need to have an antibody for each protein to identify</td>
<td>Global - entire proteome</td>
</tr>
<tr>
<td>Must know which protein to look for to get the correct antibody</td>
<td>Can seek to identify unknown proteins</td>
</tr>
<tr>
<td></td>
<td>Discovery of new markers</td>
</tr>
<tr>
<td>Antibodies are not available for most proteins</td>
<td>Not dependent on antibody for diagnosis</td>
</tr>
<tr>
<td>Antibody reactivity dependent on fixation, truncation, etc which may impact specificity</td>
<td>Identification dependent on enzyme cutting sites and informatics</td>
</tr>
<tr>
<td>No separation needed</td>
<td>Separation needed</td>
</tr>
<tr>
<td>Sensitive</td>
<td>Less sensitive for low abundance proteins</td>
</tr>
<tr>
<td>Cheap, fast</td>
<td>Time, accessibility</td>
</tr>
</tbody>
</table>
LCM: $10\mu$ thick sections stained with Congo red, viewed under fluorescence for amyloid
Sample = amyloid deposits identified and laser micro-dissected

Protein extraction, Trypsin digestion into peptides

HPLC: separation of peptides

ESI: peptides are ionized +++

Peptides sprayed into MS1:
- measures the parent mass of the peptide and
- selects the peptides for CID (collision-induced dissociation)

CID: upon collision with a neutral gas, the peptides are fragmented

MS2:
- measures the size of each fragment derived from the parent peptide mass
- these measurements are used to predict the amino acid sequence

Fragmenting Peptides

It is difficult to make large proteins “fly” hence proteins are fragmented (twice in MS/MS)

1. proteins analyzed in the first component of the tandem mass spectrometry (MS/MS)
2. peptides selected and dissociated into fragments that the second component can analyze
   (fragmenting proteins can be also done in cyberspace with a program that predicts the way that peptides fragment)

Results:
- displayed as spectra of the relative abundance of detected ions as a function of the mass-to-charge ratio
- molecules in the sample can be identified by correlating known masses to the identified masses
  or through a characteristic fragmentation pattern analogous to using fingerprints to identify a person

Protein identification:
- matching the identifying features of the peptides to a database of proteins
  - more believable if based on matching mass spectra from several peptides
MS/MS advantages:
1. Global identification of proteins
2. Discovery of unsuspected proteins/biomarkers, also variants

MS/MS limitations:
1. It may be difficult to detect low abundance proteins/peptides as signals from these peptides may be buried among massive amount of information obtained from more abundant proteins, and MS simply may not be able to scan them
2. a given protein can only be identified if peptide fragments with appropriate size for MS can be generated after enzymatic digestion
3. Reliance on computational predictive algorithms to a reference human genome obtained from publicly available databases
Amyloid typing by MS

Current application:

- typing of amyloid deposits where routine IF/IHC equivocal or negative
- confirmation of type
- detection of less common/unusual types: AFib, ALECT2, AGel, AApoAI, AI, ATTR, IgD, etc
- in cases with inadequate sample for immunofluorescence typing
The big picture - where does amyloid fit?
Images of amyloid
Functional amyloid

Amyloid in nature for functional purposes
- high-density packing of amino acids
- unique mechanical or chemical properties
- highly adapted use

bacterial biofilm, natural adhesives, fungal spore production, melanosome production in the skin, hormone packing in the secretory granules of the endocrine system

adhesive and cohesive strength in both temporary and permanent natural adhesives
adhesives that cure in different environments (moist surfaces of terrestrial habitats, fully submerged in seawater)

Marine life: Barnacle (marine crustacean) stick with amyloid adhesive

*amyloid-based natural adhesives, biomimetic adhesives – application in surgery*

Amylome

= the universe of proteins that are capable of forming amyloid-like fibrils
Amyloidosis and the clinicians...
Focus on early diagnosis
Improved screening
Legal issues: delayed diagnosis, misdiagnosis of the amyloidosis type with inappropriate treatment

Amyloidosis: Primary; Secondary; Hereditary...
Thank you
Questions?

Clinical suspicion...
Pathologic suspicion...
Patients’ perspective...
Awareness and education

Patients Support Group

INTERNATIONAL SOCIETY OF AMYLOIDOSIS
Selected references:


Picken MM. Renal amyloidosis – what we need to consider, practical tips. AJKD blog, January 21, 2016. https://ajkdblog.org/?s=Picken


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