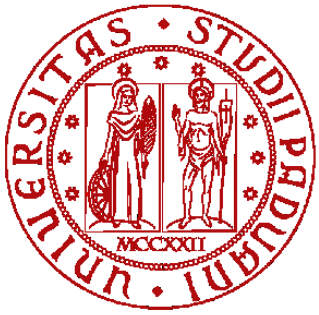


UNIVERSITÀ DEGLI STUDI DI PADOVA

CORSO DI LAUREA MAGISTRALE A CICLO UNICO IN MEDICINA E CHIRURGIA

DIPARTIMENTO DI SCIENZE CARDIO-TORACO-VASCOLARI E SANITÀ PUBBLICA
Direttore: Prof. Federico Rea

U.O.C. PATOLOGIA CARDIOVASCOLARE
Direttore: Prof.ssa Cristina Basso

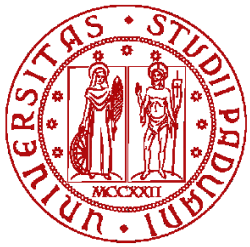
TESI DI LAUREA

Cardiac Amyloidosis and Endomyocardial Biopsy: Correlation of Extent and Pattern of Deposition with Amyloid Immunophenotype by Immunogold in a Single Institution

Relatore: Prof.ssa Stefania Rizzo
Correlatore: Dr.ssa Mila Della Barbera

Laureando: Cosimo Junior Di Mira

Anno Accademico 2022/23



UNIVERSITÀ
DEGLI STUDI
DI PADOVA

CORSO DI LAUREA MAGISTRALE A CICLO UNICO IN MEDICINA E CHIRURGIA

DIPARTIMENTO DI SCIENZE CARDIO-TORACO-VASCOLARI E SANITÀ PUBBLICA
Direttore: Prof. Federico Rea

U.O.C. PATOLOGIA CARDIOVASCOLARE
Direttore: Prof.ssa Cristina Basso

TESI DI LAUREA

Cardiac Amyloidosis and Endomyocardial Biopsy: Correlation of Extent and Pattern of Deposition with Amyloid Immunophenotype by Immunogold in a Single Institution

Relatore: Prof.ssa Stefania Rizzo
Correlatore: Dr.ssa Mila Della Barbera

Laureando: Cosimo Junior Di Mira

Anno Accademico 2022/23

Index

SUMMARY

Background: Amyloidosis is an infiltrative systemic disease characterized by the extracellular deposition of fibrillar proteins in different organs leading to tissue damage and dysfunction. Cardiac involvement by amyloidosis is common. The identification of the type of amyloid deposits is critical to the correct diagnosis and treatment. Several methods are currently used to characterize amyloid deposits.

Aim of the study: The aims of the present study were to (1) investigate the accuracy and reliability of light microscopy compared to immune-electron microscopy (IEM) study in diagnosis and characterization of cardiac amyloidosis on fixed paraffin-embedded tissues; (2) to study at light microscopy the association in endomyocardial biopsies (EMB) between the amyloid burden and its patterns of deposition (perimisial, interstitial nodular-like, perivascular) and the type of amyloid characterized by means of IEM; (3) to evaluate the diagnostic value of abdominal fat pad excisional biopsies (FPEB) vs endomyocardial biopsy (EMB) when both are performed, (4) and to find clinico-pathological correlations in cases of cardiac amyloidosis.

Methods: Consecutive EMBs, of patients with clinical evidence of hypertrophic-restrictive cardiomyopathy, suspected to be due to amyloidosis, submitted to our Pathology Unit over the last 5 years, were re-examined. Cases with FPEB besides EMB were also reviewed. All EMBs underwent to H&E, Congo Red and Thioflavin stains. IEM, performed with gold-labelled antibodies against light chains (AL, kappa light chains, lambda light chains), and transthyretin (TTR), was used to identify specific amyloid fibrils. Forty cases of the Hospital of Padua were re-examined to find clinical-pathological correlations.

Results: In the time interval 2017-2022, a series of 171 EMBs were collected (118 males, 69%; average age at diagnosis 66.6±11.5 years). A subgroup of 36 patients (21%) underwent also FPEB; in 27 (75%) of them, EMB was positive, 21 of which negative at FPEB. In 45 cases (26%) EMB amyloid deposits were not identified by means of H&E, Congo Red, and Thioflavin stains nor by IEM. In the remaining 126 EMB cases (74%), IEM was diagnostic, revealing even small amounts of amyloid fibrils, positive for AL in 82/126 (65%) and TTR in 44/126 (35%). Both

the interstitium and the vessels were affected, with different burden based on the type of amyloid. Cardiac structural damage and dysfunction is more relevant in ATTR amyloidosis.

Conclusion: IEM represents a sensitive, reliable, and low-cost method for amyloid typing. It can be used to test the reliability of light microscopy. EMB with IEM amyloid typing is crucial to establish diagnosis, prognosis, and appropriate treatment. Localized amyloidosis can only be diagnosed by biopsy of the affected organ or tissue. Choosing the correct tissue/organ to biopsy is essential to avoid false negatives and delays to diagnosis. The choice of a surrogate tissue should be discouraged.

RIASSUNTO

Presupposti dello studio. L'amiloidosi è una malattia sistemica infiltrativa caratterizzata dalla deposizione extracellulare di proteine fibrillari in diversi organi che porta a danni e disfunzioni tissutali. Il coinvolgimento cardiaco da amiloidosi è comune. L'identificazione del tipo di depositi di amiloide è fondamentale per la diagnosi e il trattamento. Diversi metodi sono attualmente utilizzati per caratterizzare i depositi di amiloide.

Scopo dello studio. Gli scopi del presente studio sono molteplici: (1) indagare l'accuratezza e l'affidabilità della immuno-elettro-microscopia (IEM) nella diagnosi e caratterizzazione dell'amiloidosi cardiaca su tessuti fissati in paraffina; (2) studiare l'associazione tra il carico di amiloide e i suoi pattern di deposizione (perimisiale, interstiziale simil-nodulare, perivascolare) valutati alla microscopia ottica su frammenti di biopsie endomiocardiche (EMB) e il tipo di amiloide caratterizzato mediante IEM; (3) valutare il valore diagnostico delle biopsie escissionali del tessuto adiposo addominale periombelicale (FPEB), in confronto con quello della EMB nella popolazione che ha eseguito entrambi i tipi di biopsia; (4) individuare correlazioni clinico-patologiche nei casi di amiloidosi cardiaca.

Materiali e metodi. Sono stati riesaminate le EMB consecutive di pazienti con evidenza clinica di cardiomiopatia ipertrofico-restrittiva, sospetta per amiloidosi, inviate alla nostra Unità di Patologia Cardiaca negli ultimi 5 anni. Sono stati esaminati anche i casi in cui, oltre ad essere stata eseguita una EMB, era documentata l'esecuzione di una FPEB. Tutte le EMB sono state sottoposte a colorazione H&E, Rosso Congo e Tioflavina. L'IEM, eseguita con anticorpi marcati con oro colloidale contro le catene leggere (kappa e lambda) e transtiretina (TTR), è stata utilizzata per identificare il tipo di proteina amiloidogenica. Quaranta casi dell'Azienda Ospedaliera di Padova sono stati riesaminati per trovare correlazioni clinico-patologiche.

Risultati. Nell'intervallo temporale 2017-2022 sono state raccolte una serie di 171 EMB (118 maschi, 69%; età media alla diagnosi 66,6±11,5 anni). Un sottogruppo di 36 pazienti (21%) sono stati sottoposti anche a FPEB; in 27 (75%) di questi,

l'EMB era positivo, 21 dei quali negativi al FPEB. In 45 casi (26%) i depositi di amiloide su EMB non sono stati identificati da IEM, confermando la negatività dell'esame di microscopia ottica con le colorazioni H&E, Rosso Congo e Tioflavina. Nelle restanti 126 EMB (74%), l'IEM era diagnostica, rilevando anche piccole quantità di fibrille di amiloide, positiva per AL in 82/126 casi (65%) e per ATTR in 44/126 casi (35%). Erano coinvolti sia l'interstizio che i vasi, con carico variabile in base al tipo di amiloide. Il danno strutturale e la disfunzione cardiaca erano più rilevanti nell'amiloidosi ATTR.

Conclusioni. La IEM rappresenta un metodo sensibile, affidabile e a basso costo per la tipizzazione dell'amiloide. Può essere utilizzata per testare l'affidabilità dell'analisi di microscopia ottica. L'esecuzione della EMB con tipizzazione dell'amiloide tramite IEM è fondamentale per stabilire diagnosi, prognosi e trattamento appropriati. Forme localizzate di amiloidosi possono essere diagnosticate solo mediante la biopsia dell'organo o del tessuto interessato. La scelta del tessuto corretto per la biopsia è essenziale per evitare falsi negativi e ritardi nella diagnosi. La scelta di un tessuto surrogato va disincentivata.

INTRODUCTION

AN OVERVIEW OF AMYLOIDOSIS

Epidemiology. Patient care has the objective to cover a large number of diseases: some very common, to which physicians pay more attention, and others that are rarer. However, we must take into account the relativity of the concept of rarity and the importance that diseases with low frequency have in our society. Taken together, they indeed have a weight that cannot be neglected. Among them is amyloidosis, which was born as a rare disease and, like many others, is completely unknown to the public and the media. Cardiac amyloidosis (CA) has long been included in the group of rare diseases [1]. However, the epidemiology of amyloidosis has been reconsidered due to recent advances in non-invasive diagnostic tools that facilitate diagnosis and update epidemiology. Moreover, the advances are not only diagnostic but also therapeutic: new strategies have been introduced and the clinical implications of CA diagnosis have changed [2].

Definition. Amyloidosis is a disease with many different forms.
It is characterized by the deposition of amyloidogenic proteins in the extracellular space. What defines an amyloidogenic protein is the loss of its natural folding: It rearranges itself in a cross-β-sheet structure [1].

Diagnosis. In some cases, particularly ATTR amyloidosis, we can make the diagnosis without biopsy; however, tissue biopsy and characterization remains the gold standard. In particular, if the patient presents plasma cell dyscrasia, the suspicion of AL amyloidosis is high and this diagnosis must be excluded. The other case is when the nuclear medicine findings and other techniques are inconclusive and thus the biopsy is the last resort to make sense of the clinical findings [3].

Typing. The complexity of the disease consists of the existence of many different proteins that can become amyloidogenic. This logically leads to the fact that many different forms of the disease exist.
Until today, more than 30 amyloidogenic proteins have been recognised. The most common amyloidogenic precursors of cardiac amyloidosis are immunoglobulin

light chains (AL) and transthyretin (TTR), which are responsible for AL and ATTR amyloidosis respectively [4]:

- AL amyloidosis is a systemic disease that affects almost every organ. For the fact of being so invasive, it has a poor prognosis, especially if the heart is one of the organs affected [5].
- ATTR amyloidosis is divided into two forms: Wild-type ATTR (ATTRwt) and ATTR variant (ATTRv). ATTRv is so named because there are point mutations in the TTR gene that are responsible for the higher incidence of amyloidosis.

Typing is essential for prognosis and treatment. In particular, the cases of amyloidosis with cardiac localization have the worse outcome. Also, in the context CA, the severity of cardiopathy, due to the myocardial damage, and the functional impairment, due to the stiffness of the heart, are related to the type of amyloidogenic protein. Furthermore, the prognosis is influenced not only by the type but also by the amount of amyloidogenic protein and thus by the extent of the deposits as well as by the pattern of expansion [5].
Amyloid typing, as expected, is critical for clinical management: there are some appropriate treatments available, and the identification of the causative protein is the only clue to recommend their use [6]. Consequently, the misidentification of the protein can lead to inappropriate treatments [6].

Pathology. Recent studies have identified several mechanisms, including fibrosis and inflammatory processes, which may play a significant role in myocardial dysfunction. The importance of scientific progress in this field will be emphasised, since it leads to a better understanding of the disease and more targeted treatments [7].

Problems. The diagnosis of amyloidosis hides under the complexity of the clinic of cardiomyopathy. For this reason, systemic disease is under-recognised, under-diagnosed. Therefore, of course, it may be natural that the prevalence is higher than assumed and that its epidemiology should be corrected. In particular, the prevalence may be significantly higher in elderly people, who are more frequently affected. Besides the problem of a complex diagnosis, the delay in diagnosis may also be caused by the lack of awareness of the disease. Last but not least, the heterogeneity

and complexity of symptoms at presentation are the biggest obstacle to overcome [3].

The aim of this discussion is to provide the elements for a deep understanding of the pathology, to explore its multiple causes that make amyloidosis a collective disease, consisting of a multitude of variants. The aim is also to deepen the meaning of its manifestations and to explore its clinical facets. With this work, the reader can face a questioning of the diagnostic procedures available today for the diagnosis and screening of this pathology with a difficult clinical classification. We also present the results of an analysis of a collection of data to clarify clinical suspicion.

DISCOVERY AND STUDY OF AMYLOIDS

The term “amyloid” was introduced in 1854 by Rudolph Virchow. He noticed a macroscopic, not microscopic, tissue abnormality. The tissues containing amyloids had a specific property: a positive reaction to staining with iodine [8]. He mistakenly interpreted it as a polysaccharide [9].

The iodine staining has not been uncommon in the scientific fields; it was used for example to distinguish the exterior chains of the mammalian glycogens from the invertebrate ones in 1988 [10].

After this macroscopic observation made by Virchow, even a microscopic finding came up. Indeed, using the light microscope, implemented with polarizing optics, the tissues under investigation showed a birefringence of amyloid deposits. The birefringence is a property typical of amyloid deposits and it sensibly increases after Congo red staining (instead of the iodine staining used by Virchow).

Birefringence is the optical property of a material having a refractive index. It can be imagined as a change in colour of the material. That depends on the polarization of light, and on the fact that it propagates in different direction in the specimen. Therefore, the birefringent (or birefractive) materials can be defined as optically anisotropic. The last pearl that could be shared about birefringence in this context is the way we quantify it: if a material has many refractive indices, birefringence is the maximum difference that can be found between them. However, why should some materials have this physical property instead of

others? Which property should amyloid deposits have in common with this group of materials? Well, the materials that often show birefringence are crystals with a non-cubic structures [11].

Then, in 1959, amyloidotic tissues, prepared and turned into ultrathin sections, were examined at the electronic microscope: it revealed the presence of fibrils, with a indeterminate length and a width going from 80 to 100 Å.

After these discoveries, the animal kingdom was revealed to be rich in amyloidosis cases: using the Congo red staining at the optic microscopy and the fibrillar morphology at the electronic one, 20 or more distinct forms of amyloid have been identified; and the very specific feature of them is that each is specifically associated with a unique clinical syndrome.
Fibrils, going from 80 to 100 Å, were isolated from tissues, using the following strategy:

- realising of the specimens
- using differential sedimentation of the particles in the homogenate
- using differential solubility of the particles in the homogenate

Then, another essential discovery drove the scientific community to the pathogenic pathway of the disease: X-ray diffraction analysis showed that all the fibrils had a specific conformation: the β-sheet one. Moreover, the observers found the so called "cross β structure", a term used to indicate that the direction of the polypeptide backbone was perpendicular to the fibril axis.

Nowadays, despite all the discoveries and the scientific progress in the argument, still some doubts can be found about amyloid. One of these, for instance, is the question about the behaviour of amyloid: is it the same *in vitro* and *in vivo*? This kind of discussion was born because if we took the fibrils extracted from an amyloid-laden tissue (turning it in an *in vitro* specimen) and we compared it with the amyloid fibrils in tissue sections (which is *in vivo* amyloid), we would find that the stain properties and the dimensions are quite similar and comparable between them. Nonetheless, they are not identical. Indeed, a difference could consist in the rapport between the fibrils of amyloid P component (AP) and the proteoglycans of

the tissue, which in case could be important to discover, in the future, new features of the pathogenic pathway of amyloidosis [8].

MOLECULAR MECHANISMS AND DEFINITION OF AMYLOIDOSIS

Protein structural levels

In nature, proteins have four structural levels: primary, secondary, tertiary, and quaternary. The primary structure is the protein backbone, i.e. the set of amino acids in their linear order. The secondary structure derives from all the hydrogen bonds between near amino acids. Three structures can be found at this stage: α helix, β-pleated sheet, and β-turn. The tertiary structure derives from the interaction between the protein backbone and the aqueous environment in the cell: hydrophilic residues will be exposed outside, in contact with water, while the hydrophobic ones will be pushed in a hidden position. Then, quaternary structure is the result of the interaction between two or more proteins. [12] It is possible, for protein that are normally in their tertiary structure, to convert in their secondary structure: this mechanism has been recognised as the first step in the amyloidogenic pathway. Then, the secondary structure makes it possible for the protein to aggregate, forming deposits, the amyloids. The reason why it happens is that proteins have an innate tendency to change their structural levels [13].

This phenomenon is connected with different diseases [14], e.g. Alzheimer disease, Parkinson diseases and type II diabetes [13]; they are referred to as misfolding diseases, or protein conformational diseases [14]. Amyloidosis is the largest group of misfolding diseases [14].

Pathogenesis

The change from the soluble tertiary structure to the secondary structure is only the beginning of the pathogenic cascade [13].

The secondary structure consists of a β-pleated sheet shape. A peptide in this conformation induces, by hydrogen bonds, other circulating peptides to assume its same shape, stabilising it under a thermodynamic aspect. The result is a higher grade of aggregation, called “cross-beta super-secondary structure” or just “amyloid” [14]. They are homopolymers, polymers made by identical monomers. Intermediate oligomers can also give organ damage or dysfunction [14]. Once formed, amyloid cannot be removed: the conformation that peptides assume prevents its proteolysis and it is resistant to clearance. [15].

The process that leads to amyloidosis is complex. There are intracellular predisposing factors, such as protein quality control checkpoints, and there are extracellular ones, such as defects in proteolysis and clearance of amyloidogenic peptides [14].

Functional amyloids

However, amyloids are not necessarily connected to a pathological process; they are also present in nature under physiological conditions. The characteristic that makes these proteins very favourable for assuming a fundamental function in many living species is their ability to switch from their soluble to their insoluble state, generating stable and resistant structures. There are some examples where amyloids are functional in mammals: the basic major protein of eosinophils and protein storage [16].

Definition

Amyloidosis is a group of misfolding diseases [14], characterized by the aggregation of amyloid deposits, in which proteins are arranged in a β-pleated sheet secondary structure. If the specimen undergoes polarized light microscopy, a typical green birefringence can be noticed with Congo red staining. In this context, the problem is the tissue deposition of amyloid, which leads to organ failure in different organs [1]. If the proteins invade specific organs, we speak of localised amyloidosis; if, on the other hand, the disease takes over the whole body, we speak of systemic amyloidosis. Basing on its causes, it is classified in:

- Primary, idiopathic;
- Secondary, caused by another disease, such as multiple myeloma;
- Hereditary, with a genetic mutation [17].

The most common amyloidosis syndromes manifest through the following pathogenic ways [18]:

- intrinsic tendency of proteins to change from a native structure to a misfolded amyloid structure: ATTR, AApoAI, AIns, AANF;
- excess production of a wild-type amyloidogenic protein: AA, AL, ACal;
- proteolytic digestion of a wild-type protein into an amyloidogenic fragment: AMed, AL;
- Mutations that alter the protein sequence: ATTR, AApoAI, ALys, AL.

"The amyloidoses differ in the protein precursor undergoing aggregation, the target organs involved and, consequently, in their clinical features" [14].

CLASSIFICATIONS: AMYLOIDOSIS NOMENCLATURE

It is not sufficient to diagnose amyloidosis by microscopic observation. To begin effective treatment, it is necessary to know the type of causative protein that affects the patient. In the following chapters, we will discuss amyloid typing and the different techniques available to complete the diagnosis. In this chapter, we review the amyloidogenic proteins nomenclature and we introduce the most common amyloidosis types.

Nomenclature

There are 30 amyloidogenic proteins in humans (6).
The current amyloid classification and nomenclature, excluding primary central nervous system amyloidosis, is summarized in the Table 1.

As can be seen, there are localized forms and systemic forms; there are also forms in between. In addition, it can be seen that some types of amyloidosis can be inherited, while most of them are acquired.

Table 1 Amyloidosis: nomenclature and classification (excluding primary central nervous system amyloid syndromes)

From *Amyloidosis: Insights from Proteomics* [18]

Nomenclature	Protein	Gene	Systemic (S) or localized (L)	Acquired (A) or hereditary (H)
AL	Immunoglobulin light chain	*IGK@* *IGL@*	S, L	A, H
AH	Immunoglobulin heavy chain	*IGH@*	S, L	A
AA	Serum amyloid A	*SAA1* *SAA2*	S	A
ATTR	Transthyretin	*TTR*	S	A, H
Aβ2M	β-2-microglobulin	*B2M*	S, L	A, H
AApoAI	Apolipoprotein A-I	*APOA1*	S	H
AApoAII	Apolipoprotein A-II	*APOA2*	S	H
AApoAIV	Apolipoprotein AIV	*APOA4*	S	A
Not assigned	Apolipoprotein C-III	*APOC3*	S	H
AGel	Gelsolin	*GSN*	S	A
ALys	Lysozyme	*LYZ*	S	A
ALECT2	Leukocyte chemotactic factor-2	*LECT2*	S	A
AFib	Fibrinogen A	*FIBA*	S	A
ACys	Cystatin C	*CST3*	S	H
ACal	Calcitonin	*CALCA*	L	A
AIAPP	Islet amyloid polypeptide	*IAPP*	L	A

AANF	Atrial natriuretic factor	*NPPA*	L	A
Apro	Prolactin	*PRL*	L	A
ASPC	Lung surfactant protein	*SFTPD*	L	A
AGal7	Galectin 7	*LGALS7*	L	A
Not assigned	Keratin 5/14	*KRT5, KRT14*	L	A
ACor	Corneodesmosin	*CDSN*	L	A
AMed	Lactadherin (Medin)	*MFGE8*	L	A
Aker	Kerato-epithelin	*TGFBI*	L	A, H
ALac	Lactoferrin	*LTF*	L	A
AOAAP	Odontogenic ameloblast–associated protein	*ODAM*	L	A
ASem1	Semenogelin 1	*SEMG1*	L	A
AIns	Insulin (synthetic)	*INS*	L	A
AEnf	Enfuvirtide (synthetic, HIV1 gp41 mimic)	*Env_HIV1*	L	A

AL amyloidosis

Amyloid light chain (AL), together with AA and ATTR amyloidosis, is one of the most common and severe of the amyloidosis syndromes.

Underlying the aetiology of the disease is the presence of a plasma cell clone, which proliferates at a low rate and produces immunoglobulin free light chains (FLCs). There are two types of FLCs, kappa and lambda; indeed, it can be notices in the table the presence of two genes responsible for the disease. FLCs are free to invade tissues and give rise to organ failure [19].

AL amyloidosis has the widest range of organ involvement and most commonly affects the kidneys (46%) and heart (30%) [20] [21]. The frequent renal involvement is due to the high nephrotoxicity of the FLCs: the most common clinical presentation is proteinuria and the disease is often diagnosed by renal biopsy [22]. When the disease affects the heart, mortality is increased: patients may die of either heart failure or arrhythmia [23]. Interestingly, some clinical signs are almost pathognomonic for this form of disease: Factor X deficiency and macroglossia [21].

Congo red staining is used for diagnosis and electron microscopy allows sub-classification

There are certain parameters that can be controlled in follow-up and are predictors of disease activity and survival:

- Troponin I (TnI),
- Troponin T (TnT),
- N-terminal prohormone of brain natriuretic peptide (NT-proBNP),
- Serum free light chains (FLC) [24].

AH amyloidosis

AH amyloidosis results from the deposition of immunoglobulin heavy chains. Compared to AL amyloidosis, it is a rare disease: only a few cases where reported. As well as the AL amyloidosis, AH forms is typical of elderly people and it is caused by a lymphoproliferative process driven by plasma cells or B cells [22].

Amyloid fibrils deposit in various organs, most commonly in the kidneys, resulting in renal dysfunction, and sometimes leading to nephrotic syndrome and end stage renal disease. Cardiac, liver and nerves involvement has also been described [25].

The diagnosis consist of the biopsy of the most involved organ [22].

AA amyloidosis

AA amyloidosis results from the deposition of an acute phase protein, the serum amyloid A protein (SAA) which is why it is a systemic form of amyloidosis. Like all other acute phase proteins, SAA increases in quantity under conditions of systemic inflammation. This explains why AA amyloidosis is a complication of many chronic inflammatory disorders.

The most commonly affected organs are the kidneys, leading to nephrotic syndrome. Some other organs are:

- The gastrointestinal tract
- Adrenals
- The reticuloendothelial system.

Some organs are affected only at a very late stage of disease:

- Autonomic system
- The heart [26].

ATTR amyloidosis

Transthyretin (ATTR) amyloidosis is caused by an increase in circulating transthyretin (TTR). The TTR is a carrier of retinol-binding protein-vitamin A complex and it is a minor transporter of thyroxine in blood [27]. The disease can occur in the presence of a mutation of the TTR gene; in this case we speak of ATTR variant (ATTRv) amyloidosis. The mutation is autosomal dominant and, from it, various pathological forms of amyloidosis take origin:

- Familial amyloid polyneuropathy,
- Familial amyloid cardiomyopathy,
- Familial leptomeningeal amyloidosis.

Alternatively, wild-type TTR may also behave in an amyloidogenic manner, especially in the elderly; in this case we speak of wild-type ATTR (ATTRwt).

The TTR is a protein that normally bonds to form a tetramer. ATTRv amyloidosis is determined by the fact that the protein, instead of remaining in the form of a tetramer as in physiological conditions, can dissociate; at this point, the TTR is free to get misfolded and aggregate.

Diagnosis is made with genetic, biochemical and immunohistochemical tests [28].

ATTRwt most common manifestation is cardiomyopathy in the elderly, and the most significant initial sign of disease is carpal tunnel syndrome.

ATTRv amyloidosis gives signs of itself through the following manifestations:

- neuropathy,
- cardiomyopathy,
- oculo-leptomeningeal involvement [29].

CNS amyloidosis

The involvement of the central nervous system (CNS) is a distinct chapter in the classification of this pathology.

The diseases in which amyloid fibrils have been found on anatomopathologic observation are various:

- Familial and acquired forms of Alzheimer's disease;
- Down syndrome in patients over 40;
- Hereditary cerebral hemorrhage of the Dutch type
- Batten's disease.
- The spongiform encephalopathies [30]

The most represented is Alzheimer’s disease, in which several amyloids can be found. Among them, the most important is the amyloid β precursor protein (AβPP). This membrane protein gives rise, through cleavage, to another protein, Aβ, which

deposits, forming neuritic plaques. The same protein has been found in amyloid angiopathy, a disease in which Aβ deposits in vessels walls [31].

Another group of neurodegenerative disorders consists of the transmissible spongiform encephalopathies (TSEs), or prion diseases:

- kuru, the first prion disease described in humans, in 1957,
- Creutzfeldt-Jakob disease (CJD),
- Gerstmann-Sträussler-Scheinker (GSS) syndrome,
- fatal familial insomnia in men,
- natural scrapie in sheep, goats and mufflons,
- transmissible mink encephalopathy,
- chronic wasting disease of mule deer and elk,
- bovine spongiform encephalopathy or "mad cow disease" and its analogues in antelopes and felids,
- feline spongiform encephalopathy in domestic cats [32].

Some other kinds of amyloid can localize in the CNS:

- AL amyloidomas, caused by the central localization of proliferating plasma cells,
- Transthyretin,
- BR12,
- Gelsolin,
- Cystatin C in inherited amyloid angiopathy [31].

Certain forms of systemic amyloidosis can also secondarily affect the CNS. In this case, it is more probable that the amyloidogenic proteins invade the brain through the regions poor in blood-brain barrier (BBB) [31].

DEFINITION AND CLASSIFICATION OF CARDIAC AMYLOIDOSIS

Cardiac amyloidosis (CA) is a pathological condition characterized by the deposition of amyloid within the tissues of the heart. The need to distinguish between types of amyloidosis involving the heart and the others lies in the different course of the disease and in all the issues concerning diagnosis.

Among the 30 amyloidogenic proteins already presented, only 9 of them are capable of determining cardiac amyloidosis. These are the only ones that determine an invasion of the myocardium up to the determination of a clinical finding [33].

Some amyloidosis types are very rare:

- AApoAI,
- AApoAII,
- AApoAIV,
- Ab2M,
- AFib,
- AGel

AA amyloidosis was more frequent in the past, while nowadays it is not often encountered [34]. The reason why the epidemiology of AA amyloidosis has recently changed is to attribute to different causes:

- an increase in the median age at diagnosis;
- an increase in the frequency of primary AL amyloidosis in respect to the AA type;
- an important change in the epidemiology and in the management of the chronic inflammatory and infectious diseases, causes of AA amyloidosis [35].

Nowadays, more than 98% of diagnosed CA derive from only two categories of proteins:

- immunoglobulin free light chains (AL)
- transthyretin (ATTR), either in its hereditary (ATTRv) or acquired (ATTRwt) form [34]

The data concerning survival and extracardiac signs of cardiac amyloidosis can be find in the Table 2 [34].

Table 2 Amyloidosis: heart involvement, survival and signs [34]

Amyloidosis type	**Frequency of heart involvement**	**Median survival from diagnosis (months)**	**Usual extracardiac signs**
AL	70%	24 or 6 (if HF at diagnosis and not treated)	Nephropathy, proteinuria, autonomic dysfunction, polyneuropathy, macroglossia, spontaneous bruising, liver involvement
ATTRwt	100%	57	CTS, LSS, ruptured biceps tendon
ATTRv	30–100%	31 (Val142Ile) 69 (non-Val142Ile)	Polyneuropathy, orthostatic hypotension, vitreous opacities, gastrointestinal problems
AA	5%	133	Renal impairment (95%), proteinuria, hepatomegaly, gastrointestinal problems
AFib	Rare	180	Renal impairment, proteinuria
AApoAI	Rare	No data	Primarily renal impairment, proteinuria, hepatosplenomegaly, adrenal insufficiency, dysphonia due to laryngeal involvement
AApoAII	Rare	No data	Primarily renal impairment, proteinuria
AApoAIV	Unknown	79	Primarily renal impairment
Ab2M	80%	No data	Long-term dialysis, CTS, joint problems
AGel	5%	Near normal life expectancy	Corneal lattice dystrophy, cutis laxa, drooping eyelids, paresthaesia, proteinuria (rare)

DIAGNOSIS OF CARDIAC AMYLOIDOSIS

The diagnosis of cardiac amyloidosis consists of several phases. First, the clinical suspicion must be raised and, in this chapter, we will see what this is based on; then, from this starting point, various diagnostic investigations can be carried out, with the need for diagnostic tools specific for amyloidosis [34].

The exact moment of diagnosis corresponds to the discovery, within the myocardial tissue, of amyloid fibrils. Various diagnostic procedures, as we will see, make it possible to confirm the presence of amyloidogenic proteins; anyway, the gold standard for diagnosis is microscopy, followed by typing. As can be guessed, this entire diagnostic process is invasive, because it requires the presence of cardiac tissue, obtained through an endomyocardial biopsy (EMB). However, only for ATTR amyloidosis, non-invasive diagnostic criteria are available, which will be duly discussed [34].

When to suspect cardiac amyloidosis

This paragraph is dedicated to the in-depth study of clinical suspicion for amyloidosis. It is divided into red flags and clinical scenarios.

Red flags

By red flags we mean all the signs and symptoms that suggest the presence of amyloidosis. These are signs that can be found in many diseases and therefore do not have a high specificity for CA; however, the simultaneous presence of many of them should raise suspicion of the disease [34].

We divide the red flags into cardiac and extracardiac. This is crucial, because, when the clinicians encounter a picture suggestive of amyloidosis through systemic signs, with the addition of certain signs indicating cardiac distress, one may come to suspect cardiac involvement by systemic pathology [34].

To complete the suspicion of cardiac amyloidosis, red flags are not sufficient: it will be necessary the confirmation of cardiac imaging, suggestive of amyloidosis [34].

The extra-cardiac red flags are many [34]:

- Proteinuria (even mild) (AL, AA, AApoAI, AApoAII, AFib),
- Renal insufficiency (AL, AA, AApoAI, AApoAII, AApoAIV, Ab2M, AFib),
- Macroglossia (AL),
- Skin bruises (AL),
- Bilateral carpal tunnel syndrome (ATTR),
- Polyneuropathy (ATTRv, AL, AA, AGel),
- Dysautonomia (ATTR, AL),
- Skin discoloration (AApoAI),
- Cutis laxa (AGel),
- Deafness (ATTRwt),
- Ruptured biceps tendon (ATTRwt),
- Lumbar spinal stenosis (ATTRwt),
- Vitreous deposits (ATTRv),
- Corneal lattice dystrophy (AGel),
- Family history (ATTRv, AApoAI, AApoAII),

There are also various red flags at the cardiac level [34]:

1) Clinical
- Hypotension or normotensive if previous hypertensive (ATTR, AL),
2) ECG findings
- Pseudoinfarct pattern,
- Low/decreased QRS voltage to degree of LV thickness,
- AV conduction disease
3) Laboratory findings
- Heart failure (with high NT-proBNP values) that appears to be in disproportion to echocardiogram findings, such as normal ventricular and valvular function,
- Persisting elevated troponin levels (ATTR, AL)
4) Echocardiogram findings
- Granular sparkling of myocardium,

- Increased right ventricular wall thickness,
- Increased valve thickness,
- Pericardial effusion,
- Reduced longitudinal strain with apical sparing pattern

5) CMR findings

- Subendocardial late gadolinium enhancement,
- Abnormal gadolinium kinetics,
- Elevated native T1 values,
- Increased extracellular volume.

Clinical scenarios

Not only signs and symptoms, but also many actual pathological conditions related to the disease can point towards the suspicion of amyloidosis.

Extra-cardiac systemic condition can be various[34]:

- plasma cell dyscrasia,
- nephrotic syndrome,
- peripheral neuropathy,
- predisposing chronic systemic inflammatory condition.

Inside of a scenario, cardiac involvement must be demonstrated through imaging and some features of the patient can help rising the suspicion [34]:

- patients older than 65 years,
- Increased wall thickness, with the coexistence of a non-dilated left ventricle,
- heart failure with preserved ejection fraction,
- hypertrophic cardiomyopathy,
- severe aortic stenosis, undergoing transcatheter aortic valve replacement [36], [37][11–13].

As abovementioned, the presence of suggestive clinical scenarios is insufficient to sustain a diagnosis of amyloidosis. In the next paragraphs, we introduce both invasive and non-invasive diagnostic criteria for CA [34].

Invasive diagnostic criteria

Entering into the world of the diagnosis of amyloidosis, we find necessary to point out that all forms of the disease, irrespective therefore of the nature of the causative protein, can be supported by an anatomopathologic investigation. In fact, performing a biopsy provides unimpeachable proof of the presence of amyloid at the cardiac level. However, in some cases a biopsy can be avoided for the patient; in this case, a diagnosis can be made by another site biopsy [34].

Therefore, in order to confirm diagnosis of CA [34]:

- The EMB should be positive for amyloid
  Or the presence of endomyocardial amyloid should be demonstrated through a less invasive biopsy with:
- An extra-cardiac biopsy resulting positive for amyloid
- Echocardiography or MRI demonstrating cardiac involvement.

Endomyocardial biopsy (EMB)

The EMB must be positive for Congo red staining. Since it provides a tissue rich in amyloid, the causative protein must be identified, giving essential information for prognosis and treatment. The gold standard for amyloidosis typing is mass spectrometry. However, since this is not present in all laboratories, other techniques, which, as it will be explained, are equally valid, are used to conclude the diagnosis in highly specialized centres: immunohistochemistry, and immunoelectron microscopy [9].

Echocardiography and CMR

If the presence of amyloid is diagnosed and the protein is characterised by the usage of other tissues biopsies, the cardiac involvement must be demonstrated. The imaging techniques are echocardiography and cardiac magnetic resonance (CMR). Some reviews have recently list the echocardiographic and CMR criteria for non-invasive and invasive diagnosis of CA [34].

In echocardiography, an unexplained LV thickness (>_12 mm) is suggestive of amyloidosis if combined with the points 1 or 2 [34]:

1. Characteristic echocardiography findings (>_ 2 of a, b, and c have to be present):
   a. Grade 2 or worse diastolic dysfunction
   b. Reduced tissue Doppler s', e', and a' waves velocities (<5 cm/s)
   c. Decreased global longitudinal LV strain (absolute value < -15%).
2. Multiparametric echocardiographic score >_8 points:
   a. Relative LV wall thickness (IVSþPWT)/LVEDD >0.6 (3 points)
   b. Doppler E wave/e' wave velocities >11 (1 point)
   c. TAPSE <_19mm (2 points)
   d. LV global longitudinal strain absolute value <_ -13% (1 point)
   e. Systolic longitudinal strain apex to base ratio > 2.9 (3 points) [38].

The most characteristic CMR findings are three [34]:

a. Diffuse subendocardial or transmural LGE (essential for diagnosis)
b. Abnormal gadolinium kinetics (essential for diagnosis)
c. ECV >_0.40% (strongly supportive, but not diagnostic)

Non-invasive diagnostic criteria

ATTR amyloidosis is the only exception in which CA diagnosis can be made without the execution of a biopsy.

Non-invasive diagnostic criteria consist of the coexistence of two including and one excluding criteria [34]:

- Grade 2 or 3 cardiac uptake at diphosphonate scintigraphy;
- Echocardiography or MRI finding typical of amyloidosis;
- Negative serum FLCs, and negative serum and urine immunofixation.

Scintigraphy

In order to demonstrate a cardiac involvement, echocardiography and electrocardiogram are insufficient, because nonspecific [39]. CMR can better acknowledge the presence of amyloidosis, but no typing is feasible; moreover, it cannot be performed in presence of implanted devices and if the patient is

claustrophobic [40]. In this scenario, scintigraphy is a very sensitive solution, used because of its high sensitivity to detect amyloids. Nowadays, several radiotracers can be used for this purpose:

- 99mTc-pyrophosphate (PYP),
- 99mTc-3,3-diphosphono-1,2-propanodicarboxylic acid (DPD),
- 99mTc-hydroxymethylene diphosphonate (HMDP)

The reason why scintigraphy is so sensitive is still unexplained: the discovery that radionuclide bone scan with ^{99m}Tc-labeled bisphosphonates can underline through cardiac uptake the presence of amyloid, particularly ATTR, was anecdotic [41].

The positivity of this technique is established basing on a score, called "Perugini grading system" [42]. There are four grades, from 0 to 3:
Grade 0. No cardiac uptake and normal bone uptake;
Grade 1. Cardiac uptake, which is less intense than the bone signal;
Grade 2. Cardiac uptake with intensity similar or greater than bone signal;
Grade 3. Cardiac uptake with attenuated or absent bone signal.
Grades 2 and 3 are diagnostic [42].

Scintigraphy has been demonstrated to be very sensitive for the diagnosis of ATTR amyloidosis [43], but unfortunately it cannot be considered specific for ATTR, because it can be positive also in a small portion of patients with AL amyloidosis [44]. Therefore, other techniques become essential for the differential diagnosis. That is why, it is important to satisfy the excluding diagnostic criteria: in this case, the specificity of bone scintigraphy for ATTR CA rise to almost 100% [43].

In order to have a good exam resolution and to exclude that the uptake signal comes from the chambers, scintigraphy should include single photon emission computed tomography (SPECT), which gives the possibility to analyse a 3D image [34].

The excluding diagnostic criteria: negative serum FLCs, negative serum and urine immunofixation

In order to speak of ATTR amyloidosis, given that the diagnosis is made using non-invasive criteria and that therefore clinicians will not be provided with a tissue containing amyloid to analyse, the need to exclude AL amyloidosis arise. This step is done through the analysis of [34]:

- serum FLCs,
- serum protein immunofixation electrophoresis (SPIE),
- urine protein immunofixation electrophoresis (UPIE).

The rationale for this diagnostic pathway lies in the etiopathogenesis of AL amyloidosis, caused by a clonal dyscrasia of B cells [43]. The combination of all these exams has a sensitivity of 99% [45].

The reason why we read the word “immunofixation” next to the word “electrophoresis” lies in the necessity to increase the sensitivity of the exam, on which it is based not only a diagnosis of amyloidosis, but even a typing without biopsy. Therefore, electrophoresis sensitivity is augmented thanks to immunofixation, which is used to identify immunoglobulins [34].

Sometimes these tests result in low levels of monoclonal proteins or in a mild elevation of the FLC ratio (κ/λ). The interpretation is not easy, because these results can be caused not only by AL amyloidosis, but also by chronic kidney disease (CKD) and monoclonal gammopathy of undetermined significance (MGUS). In CKD, the high concentration of FLCs is determined by their reduced clearance [46].

Genetic testing

Once cardiac ATTR amyloidosis is diagnosed, the patient should undergo genetic counselling in order to differentiate between ATTRwt and ATTRv, by finding the typical ATTRv mutations. Genetic counselling is also recommended in elderly patients, because they have a higher probability to present TTR mutations [47].

Diagnostic algorithm

The results of these tests could lead to four scenarios [34]:

- Both scintigraphy and haematologic tests for FLCs are negative. Scintigraphy is a very sensitive technique and laboratory tests are excluding AL amyloidosis. Therefore, there is a very low probability of CA the clinician should consider an alternative diagnosis. However, since scintigraphy could miss some ATTRv mutations (since radiotracers uptake depends on TTR structure) and rare types of CA, if suspicion persists, CMR and biopsy should be performed. The biopsy can be a EMB or an extra-cardiac one, but their different sensitivity should be taken into consideration.

- Scintigraphy is positive and haematologic tests are negative. If cardiac uptake is grade 2 or 3, we can make diagnosis of ATTR amyloidosis. Next step is genetic counselling, in order to differentiate ATTRv and ATTRwt. If uptake is Grade 1, non-invasive diagnostic criteria are not satisfied and an invasive diagnosis through biopsy is required.

- Scintigraphy is negative and at haematologic tests are positive. AL amyloidosis is possible and cardiac involvement must be demonstrated through CMR. If CMR findings are not compatible with amyloidosis, the diagnosis is very unlikely. In the case that CMR findings are supportive or inconclusive, a biopsy should be performed.

- Both scintigraphy and haematologic tests are positive. A biopsy is required, because different possible scenarios are possible:
  - AL amyloidosis;
  - Transthyretin amyloidosis with concomitant MGUS or another blood cell dyscrasia;
  - Coexistence of both AL and ATTR amyloidosis.

TISSUE BIOPSY FOR THE DIAGNOSIS OF AMYLOIDOSIS

There are two ways through the diagnosis of amyloidosis: the biopsy and the imaging. A difference between these two diagnostic paths is that biopsy is the gold standard for both diagnosis and typing of amyloidosis [6]. Moreover, while the first one can lead to diagnosis in every type of amyloidosis, the imaging can be diagnostic only in ATTR CA.

Patterns of distribution

Involved organ biopsy

The biopsy cannot be performed randomly over an organ. The disease has a pattern of deposition and some organs are more interested than others are. If the clinic strongly suggests that an organ is involved, to sample that specific organ is the most sensitive method to make diagnosis. In addition, it makes it possible to exclude pathologies in differential diagnosis [48].

Biopsy in systemic amyloidosis

But then, why are we performing biopsies in other sites such as the periumbilical fat? The decision to sample a non-symptomatic tissue depends on the typology of amyloidosis. In the localized forms of amyloidosis, the gold standard consists in sampling the affected organ. Instead, in systemic amyloidosis, the amyloid is supposed to infiltrate more homogeneously the organism and other sites may be biopsied:

- The abdominal fat,
- The gastro-intestinal tract, including the rectum, which is easily accessible
- Bone marrow
- Minor salivary glands.

The weakness of this practice is that its success depends on the involvement of the surrogate site and on the subtype of amyloidosis [6].

The surrogate site

There is an exception to what we said. In a localised form of amyloidosis, the cardiac one, the biopsy of a surrogate site can substitute the organ biopsy, because this one is riskier [49]. “However, a negative result at a surrogate site does not exclude the diagnosis and if the clinical suspicion is high, the biopsy of the involved organ should be performed” [50] [51].

Abdominal fat biopsy

Abdominal fat biopsy was introduced by Westermark and Stenkvist in 1973; they used it to diagnose amyloidosis patients with a suspect of systemic AA amyloidosis [52]. This technique has great advantages: it is easy and cheap to perform, and it is not too risky or too stressful for the patient [6].

Is it diagnostic?

As mentioned, the periumbilical fat biopsy can be used in systemic amyloidosis or also as a surrogate site in CA [53]. A periumbilical fat biopsy, however, is not sufficient to diagnose CA: it must be associated with imaging features suggesting heart involvement [54].
The techniques are abdominal fat fine-needle biopsy [55] and abdominal fat pad excisional biopsy (FPEB) [56].

Sensitivity and specificity

In the Table 3 we show the data about sensitivity and specificity concerning these techniques [57].

Table 3 Sensitivity and specificity of fat biopsies [57]				
Suspect	**Fat fine-needle biopsy**		**Fat pad excisional biopsy (FPEB)**	
	Sensitivity	*Specificity*	*Sensitivity*	*Specificity*
Suspect of AL CA	84%	100%	50% if sample <700 mm3 100% if sample <700 mm3	
Suspect of ATTRv CA	<45%			
Suspect of ATTRwt CA	15%			

It must be noted that, even in the case of AL CA, fat biopsy may miss the diagnosis (16%) that cannot excluded in the case of a negative result [58]. It can be noticed that of FPEB sensitivity depends on the sample size. It is wise to take an at least 1400 mm3 sample in order to submit it to different investigations [56].

Diagnosis is not easy. On the pathologists reports it is often written that amyloid deposits are thin and dispersed. Sometimes we can even establish the localisation, which is usually peri-cellular, septal or vascular (a clear nodular pattern is rarer). However, more often it is difficult to establish even if the observed structures are amyloids. Nonetheless, an important issue to underline is specimen adequacy, which has been esteemed 11% in fine-needle aspirates. The difference, indeed, between the fine needle aspirates and the FPEB is that with the first one we cannot exhibit a visual inspection of the fragment to exclude its inadequacy [58]. Another problem comes during fixation, because fatty fragments float in formalin and thus they may be easily lost. In order to reduce this risk, since the removal from the fixative is a very critical moment, it is possible to refrigerate the samples just before this phase.

Another limit of the fine-needle aspiration is the tissue size, which could be sufficient to see the amyloid but not to perform the typing [59]. A solution could be the FPEB, which is not much more invasive and which provides more material [53].

Endomyocardial biopsy

Endomyocardial biopsy (EMB) is commonly performed in order to give an evaluation for different heart issues: for the monitoring of transplanted patients [60], myocarditis, heart damage driven by drugs, cardiomyopathies (CMP) [61], arrhythmias [62], for the diagnosis of cardiac tumours [63] and, topic of our interest, secondary cardiac involvement by systemic diseases, such as amyloidosis [64].

The reason why EMB is performed is not only a diagnostic need; EMB represents also a tool to establish the prognosis, the therapeutic probability of success [65] and to assess a pathogenic hypothesis [66]. In order to allow this and to ensure the utility of the EMB, the scientific community has to provide papers concerning the appropriateness of the cardiac biopsy for each concerned disease and to provide indications for the execution and the treatment of the specimen and normal standards which could be used as elements to compare to the pathological ones [67].

Logically, the choice of executing an EMB has to be integrated with the possibility to use many others techniques. The physician has to take into account the recent developments in imaging, electrophysiology, and genetics, and to understand how these techniques can fill a complementary role with the histopathological examination. The decision to send the patient to an anatomopathologic evaluation or to take another diagnostic path is not only taken by the clinicians, the imaging evaluations and the geneticists, but it is often essential to include in the multidisciplinary collaboration a pathologist, who can give an important professional opinion upon the best diagnostic strategy [68].

How can we limit the error? Precautions in the EMB execution

Given the complexity of the process, the pathologists are often called to offer their professional help. Nonetheless, the report written by pathologists should have a uniform language and an agreed standardised way of communicating, especially about:

- Degrees of diagnostic certainty (e.g. certain, definite, probable, possible, nonspecific);

- Biopsy sample adequacy evaluation: (e.g. optimal, suboptimal).

This is essential in order to reduce the EMB reporting variability.

To be able to perform a proper EMB examination, the pathologist needs a specific professional training [69], the specimen must be accurately processed [70], and new diagnostic supports such as histochemical, immunohistochemical (IHC), molecular, or ultrastructural tests must be applied [66].

False-negative results are possible. Especially in some cases, when a disease affects the heart in a localized way (or even in a multifocal way) it is logical to deduct that it is easier to commit a sampling error. When a sampling error is possible and it cannot be excluded, the pathologist takes always note of that on the report [71].

Some general rules can optimize diagnostic accuracy:
- the EMB should be executed with a proper timing for the considered disease [72];
- the sampling technique should be adequate and, in order to reduce the already mentioned sampling error, which is higher in the case of focal distribution of the amyloid, particularly in initial diseases [63], the clinician can take more specimens, from different sites. The imaging can be useful to guide this phase and to increase the probability to sample only the truly affected tissues [67].

Techniques

The execution of the sample for EMB consists into performing a percutaneous access through the heart. The probe, the sheath, is guided by a fluoroscopy or by ultrasound, which is a guarantor of a safe and a rapid operation. The act consists of the percutaneous insertion of a bioptome.
The bioptome is a small instrument that has the shape of a pincer, with the capability to cut and grasp the anatomical tissues; it is used for taking endomyocardial biopsy specimens; moreover, it facilitates obtaining multiple specimens. [73]

Bioptic samples can be taken from the right ventricle; in this case the taken paths the jugular, subclavian, and femoral veins. The samples can also be taken from the left ventricle; in this other case, the operators need to execute a trans-septal puncture

(if they want to have an access from the venous tree) or they can choose to have a direct access to the arterial system, picking a peripheral artery (the femoral or the brachial one) [74].

In order to avoid sample errors, the physician needs to have a better definition of the anatomical site. To meet this need, we can use techniques of cardiac imaging, such as gadolinium magnetic resonance imaging (MRI) which can be useful to target the interested region. Also, in order to ensure the safety of the procedure and to ensure that an accurate insertion of the bioptome is performed, echocardiography can be the instrument of choice, since it doesn't have a risk connected to radiations [75].

Complications

The main complications of EMB are:

- Hematoma in the insertion site,
- arterio-venous fistula,
- vasovagal reaction during the operation,
- pneumothorax,
- arrhythmia, due to the cardiac damage,
- heart block, due to the cardiac damage,
- infection, due to the direct access to the cardiovascular system,
- tricuspid valve damage, since the bioptome can touch and damage the valve,
- pulmonary embolism, during right ventricular biopsy, since the probe is not made of endogen tissues and it provides alterations in the circulation flow,
- systemic embolism, during left ventricular biopsy,
- wall perforation, with hemopericardium and tamponade [76].

If the operator is skilled, the procedure is safe [77]: the overall complication rate goes from 1% to 2% [64]. Moreover, the risk of cardiac perforation, which is the main concern in this case, was estimated at 0.4% by a consistent report in 1980 [74] and at 0.12% in a more recent one [78].

Patient selection and clinical indications

As stated before, the EMB is not a procedure without risk, even though it is relatively low. With this premise, it results wise to carefully select the patients and to send them only if necessary to a bioptic verification. This decision should be taken considering the cost–benefit ratio and the possible procedural risks for the specific patient. In order to guide this decision making process, the following criteria, dated 2011, can be used [68]:

- Consider the usage of an EMB in the context of a sequential diagnostic process. It will not be the first exam to be proposed to the patient, but it will be taken in consideration only after other appropriate clinical and instrumental tests of routine, in order to get nearer to the diagnosis, and to exclude the first diagnostic hypothesis [67];

- Consider EMB as an essential tool to [67]:
  - Obtain a precise diagnosis,
  - Establish the therapy and assess its utility,
  - Establish the prognosis,
  - Monitor the disease and check if the therapy is sufficient,
  - Propose the right diagnosis and exclude wrong hypothesis.

- Use EMB only in the cases in which its diagnostic potential has been demonstrated to be the highest [67]. The scenarios in which the EMB examination has highly been suggested are [61], [67]:
  - A new-onset and recent unexplained heart failure, going on from less than six months,
  - Bradyarrhythmias or tachyarrhythmias, in order to exclude the possibility that the arrhythmias come from the myocardial tissue, a situation which may occur in a myocarditis, or in the arrhythmogenic cardiomyopathy, or even in sarcoidosis,
  - Chronic heart failure, which may occur in amyloidosis; the most worrisome phenotypes to evaluate in a myocardial diseases are the dilated and hypokinetic one or hypertrophic and restrictive one, typical of amyloidosis,
  - An acute exacerbation of a known chronic cardiomyopathy,

- The investigation of a cardiac masse in order to exclude the presence of a tumour,
- The follow up of heart-transplanted patients.

The clinic is the fundamental premise

After an EMB is executed, the pathological analysis takes place. The study and the evaluation of the specimen is not simple. The process requires standard protocols and a specialised formation [79]. Moreover, since it is not easy to start the evaluation without a wider view of the patient and since every finding in the EMB examination should be connected with the general picture of the patient conditions, the pathologists are provided with all the necessary clinical information. Indeed, the clinic guides the diagnosis as well as the choice of the most appropriate techniques.

How should we get and triage a specimen

In order to reduce the sample error probability, the pathologists need at least three, or even four, endomyocardial fragments. The sample size should be 1-2 mm [61] each. In addition, the fragments should be immediately fixed in specific solutions: in order to send the specimen to the light microscopic examination, we need a formalin solution (10% formalin buffer solution) and, in this case, the room temperature is the one that matches better. Formalin-fixed and paraffin-embedded (FFPE) samples have the unique advantage that they can be stored at room temperature and, at the same time, they produce higher quality histological sections. For these reasons, FFPE specimens are the most common format in clinical practice [80].

If the clinical data arise the suspicion of focal myocardial lesions (which are linked with a higher probability of sampling error), additional sampling is recommended [61] [68].

If indicated, for further examinations one or two specimens can be frozen in liquid nitrogen and stored at −80°C: this process allows the execution of molecular tests or some specific stains. In alternative, fragments may be stored at room temperature in a solution that prevents RNA degradation, called RNA-later [68].

In the case of amyloidosis, the fragments also undergo ultrastructural tests. In this case, in order to perform an electronic microscopic examination, we need to fix the fragments (usually just one) in 2.5% glutaraldehyde or in Karnovsky solution [68].

In addition, if differential diagnosis with myocarditis is an open question, a sample of 5–10 ml of peripheral blood is taken and it is processed in EDTA or citrate. This allows the execution of molecular testing in order to verify that the viral genome sought in the blood sample is the same of the one found in the myocardial tissue. The same procedure is used in differential diagnosis with genetic CMPs, in order to perform genetic tests [68].

Pathological analysis

The examinations that the pathologists can perform are many. They include light microscopy, molecular tests and transmission electron microscopy.

Light microscopic examination is the most frequently performed one. It needs a fixation in formalin and embedding in paraffin. The possible following steps are:

- The realisation of hematoxylin–eosin sections, that should be high in number and numerated;
- The realisation of additional stains, such as histochemical or even IHC stains; in order to perform them, the fragments need paraffin-embedding (or frozen sactions can also be used in limited cases).

Among the molecular tests, quantitative or qualitative polymerase chain reaction (PCR), and reverse transcriptase-PCR can be performed. In order to be able to realise a PCR examination, the fragments must be frozen in liquid nitrogen or stored in RNA-later.

Pathologists often perform transmission electron microscopy examination. In this case, glutaraldehyde or Karnowsky fixations are needed, as well as resin-embedding. Before performing an ultrastructural analysis, pathologists examine the

semithin sections with a toluidine blue staining, which could add more informations [68].

EMB in cardiac amyloidosis

The evidence of recommendation for EMB in cardiac amyloidosis is strong: the scientific community completely supports its utilisation [68].

EMB diagnostic potential is widely recognized as one of the most important modalities in the evaluation of patients with cardiac amyloidosis.

Even though the Congo red staining is the most useful in cardiac amyloidosis, some considerations can be made about its aspect in hematoxylin–eosin staining. The amyloid is slightly eosinophilic and appears as an amorphous substance; there is no order in its appearance except for the fact that it infiltrates the tissues, retaining their structure and distributing homogeneously (Fig. 1, A, B, D) .

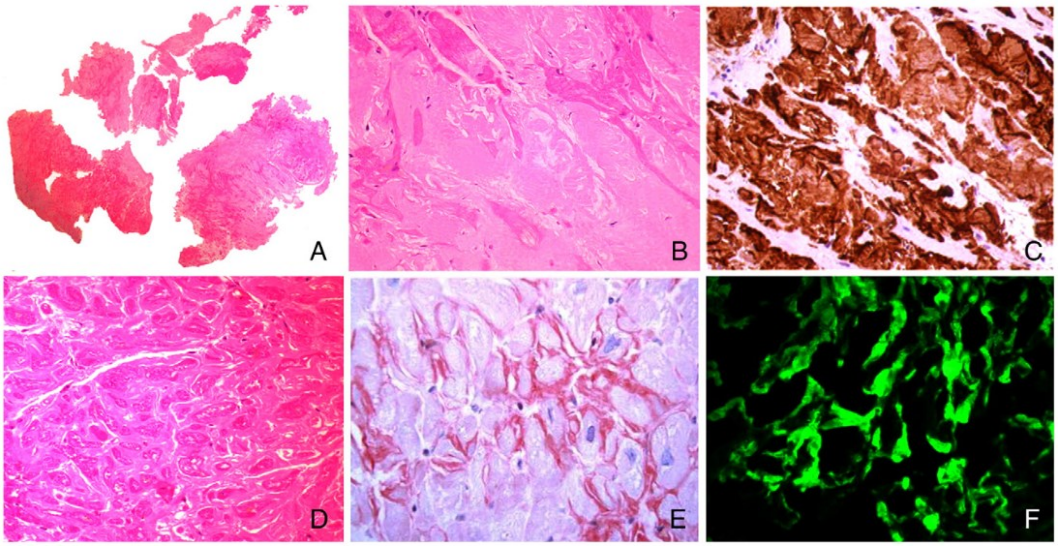


Figure 1 *(A–C) ATTR case in a 62-year-old man. A: diffuse interstitial deposits (25×), (H&E). B: amyloid appears as a homogeneous, amorphous, and eosinophilic substance (400×), (H&E). C: (IHC with anti-TTR Ab) diffuse and intense brown positivity (400×). (D–F) ATTR in 84-year-old man (400×). D: H&E. E: Congo red stain shows interstitial amyloid deposition. F: subtyping of the amyloid by immunofluorescence shows specific staining for transthyretin [68].*

However, in the case of amyloidosis, the recommended staining are modified sulfated alcian blue (Fig. 2), thioflavin T (Fig. 1, F), and Congo red (Fig. 1, C).

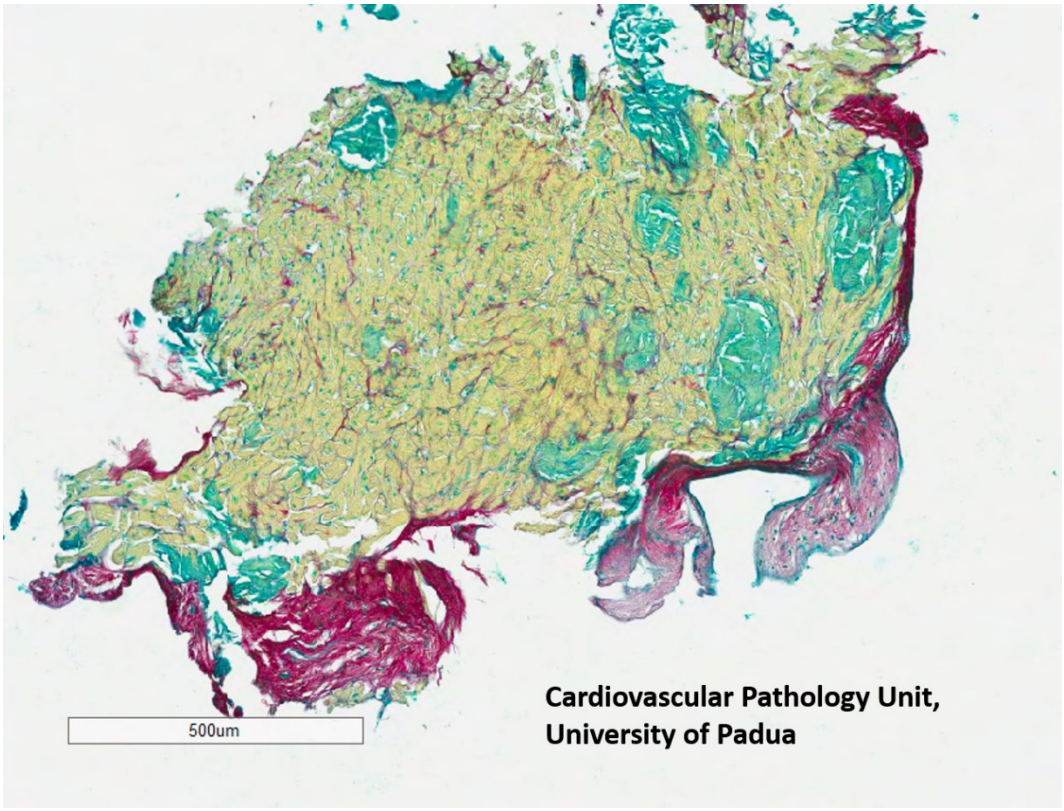


Figure 2 *ATTR amyloidosis in a 76-years-old man. Nodular prevalent pattern of deposition, highlighted by alcian blue stain.*

Congo red staining has the typical characteristic of highlighting the amyloid birefringence, which assumes an apple-green colour under polarized light.

Even immunofluorescence examination is possible and it requires, as stated before, that the specimen is frozen [68]

IHC and molecular aspects EMB has been proven useful for establishing the type of cardiac amyloid using techniques such as. Thus, it is recommended that efforts be made to subtype amyloid deposits in EMBs. Subclassification of amyloid deposits is nontrivial, and the misclassification of amyloid in pathologic specimens has been reported (reviewed by Collins et al. [81]).

Besides providing a diagnosis of amyloidosis, EMB is used to complete the diagnostic path with the typing. This is a very delicate phase: mistyping can have catastrophic effects, since for different types of amyloids there are very different prognosis and very specific treatments [39]. Amyloid subtyping should be performed in a centre with a proven expertise. The useful techniques are:

- Immune-histo-chemistry [82],
- immunofluorescence [81],
- immune-electron microscopy [83],
- protein sequencing [84],
- mass spectrometry [85].

Other biopsy sites

Besides the abdominal fat, other tissues can be used for the diagnosis of systemic amyloidosis or as surrogate sites in CA.

Since we are talking about systemic amyloidosis, whose deposition along the biopsy sites is unpredictable, the diagnostic yield depends on the extension of the disease.

For alternative biopsy sites, the following statistics have been presented:

- With the suspect of AL amyloidosis, a bone marrow biopsy should be performed (sensitivity of 50–60%) [86];
- With the suspect of AL amyloidosis the removal of one minor salivary gland has a sensitivity of 86% [87];
- With the suspect of AL amyloidosis, in elderly male patients the gastrointestinal biopsy gives easier a positive results (the sensitivity increases with age and sex) [88];
- The upper gastrointestinal tract is more sensible for AL κ+ and AA forms;
- The colorectal tract is more sensible for AL λ+ and ATTR forms [88];
- A rectal biopsy should not be performed at the beginning of a diagnostic process, since its sensitivity is high only in patients already positive at the abdominal fat biopsy [89];
- Gingival and skin biopsies are not sensitive enough for diagnosis [6].

HISTOLOGY

The aspect of amyloidosis is peculiar at histologic observation. Moreover, it can suggest some characteristics of the amyloid.

At the haematoxylin-eosin (HE) staining (Fig. 3), the amyloid deposits, if they are big enough, can be recognised as eosinophilic and amorphous aggregates. Instead, thin and initial amyloid deposits are more subtle: they can have an interstitial localization; otherwise, they can be searched even in the walls of the myocardial vessel. The deposition can also be nodular.
If the localization is interstitial, the amyloid surrounds the myocytes; these cells will probably be atrophic, which is a consequence of their progressive loss driven by the disease [48].

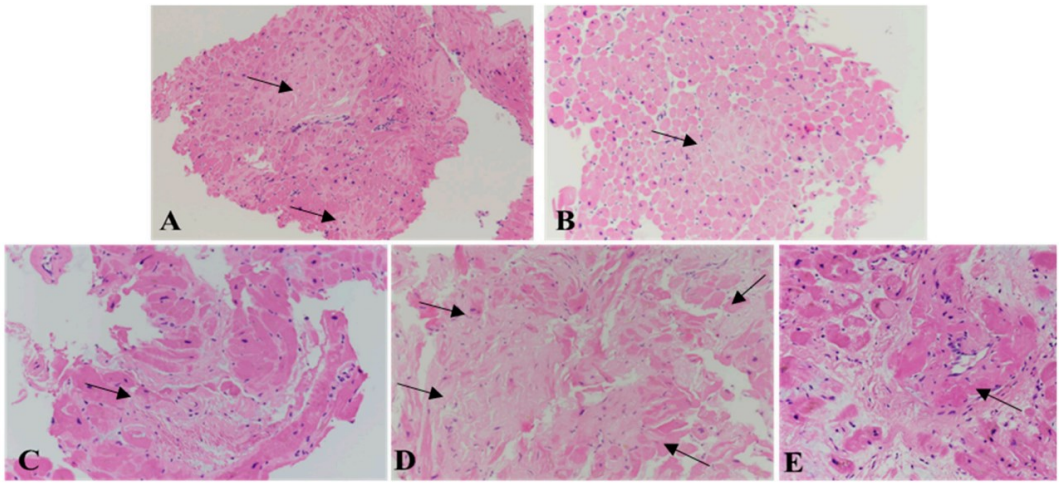


Figure 3 *H&E stain. A, B: (4×) initial forms of focal amyloid deposits formation (arrows). C, D: (4×) diffuse, interstitial and nodular forms (arrows). E: (10×) deposits in the wall of intra-myocardial vessels (arrow), with progressive loss of myocytes [68].*

Even though the light microscopic observation doesn't provide adequate information to establish the amyloid subtype [90], some morphological differences can be described.

In AL amyloidosis, the amyloid infiltrates more than 40% of myocardium, and its deposits are mainly pericellular and reticular. Since this disease is linked with an immune system alteration, we can also find inflammatory infiltrates, consisting of

T-cells and macrophages. This is a very peculiar characteristic of AL amyloidosis, in which inflammatory cells could contribute to the organ damage [7],[91].

In ATTR amyloidosis, the amyloid infiltrates are more irregular. There are two main patterns of deposition, which depend on the length of the fibrils [4]:
Type A. In this case, there are two kinds of fibrils, the full-length TTR proteins, which are long, and the C-terminal fragments of TTR, which are shorter. The presence of the shorter components makes the difference between *type A* and *type B*, because their smallness allows them to infiltrate the tissue with higher efficacy. Moreover, they will not manage to align well. Therefore, we find a diffuse interstitial pattern, with the addition of nodular deposits. This pattern can be seen in either ATTRv or ATTRwt amyloidosis.
Type B. In this case, the fibrils are longer, because there is only the full-length TTR component. Their length allows them to align better, but also to infiltrate less the tissue, characteristic that makes it a more subtle form: the deposits are thin. The deposition is vascular and interstitial. This form can be seen in ATTRv amyloidosis [92].

HISTOCHEMISTRY

Congo red staining

On light microscopy, the observation of amyloid deposits by using HE staining can lead to the suspect of amyloidosis, because of the peculiar morphology. However, this is not enough to reach the diagnosis. In order to be able to speak of amyloidosis, the deposits must be demonstrated by a specific staining. Here it comes the concept of histochemistry, in which the staining is specific for the target disease. The most used histochemical staining for amyloid highlighting is Congo red staining: the deposits assume a red or salmon-pink aspect, with the typical apple-green birefringence under polarized light (Fig. 4) [93]. The reason why polarized light is so useful to observe the birefringence phenomenon is that there are some structures (like collagen, fibrin and red blood cells) that show a white birefringence, while the apple-green birefringence is typical of amyloid fibrils.

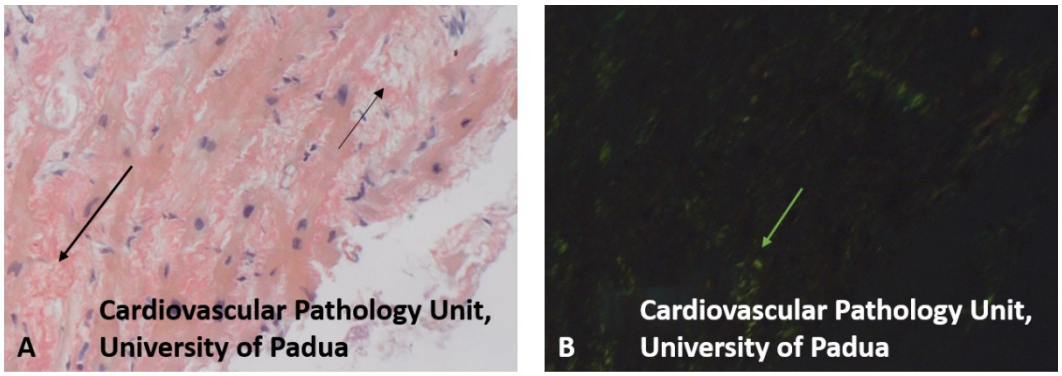


Figure 4 *AL amyloidosis in a 42-years-old woman. A: Congo-Red under light microscopy (100x); amyloid is visible as red deposits (arrows). B: Congo-Red under polarized light microscopy; amyloid can be identified as green-apple spots (arrow).*

In order to increase the sensitivity we must perform the test on sections of 8–9 μm of thickness. In order to make a better distinction, the pathologists have often a positive Congo red-stained control of amyloid on the same slide of the examined tissue.

The distribution of Congo red positivity gives information about the amyloid distribution, which can be "interstitial and/or nodular, focal or diffuse, perivascular" and also vascular, with deposits inside of the vessel walls

Masson's trichrome staining

Congo red staining is not the only one with the ability to highlight amyloid. There is also the Masson's trichrome staining, much less used. Its features are very different: the amyloid assumes a blue-violet colour, while the fibrous tissue assumes an intense blue colour. This characteristic is used to make a distinction between amyloid and fibrosis when they are not well identified using other means of staining [50].

Thioflavin T

Thioflavin T (ThT) is a kind of staining used to identify *in vitro* amyloid deposits. Among the thioflavins, thioflavin S is also capable of detecting amyloid but does

not produce a clear shift between the absorption and emission spectra. This results in a high background fluorescence signal, which makes quantification of amyloid fibrils impossible. For this reason, the ThT is used more frequently [94]. Thioflavin T is a benzothiazole salt obtained by the methylation of dihydrothiotoluidine with methanol in the presence of hydrochloric acid [95]. When it binds the amyloid, if the operator properly excite it at 450 nm, it emits a fluorescence signal, which reaches high intensity. The typical wave length is of 482 nm [96].

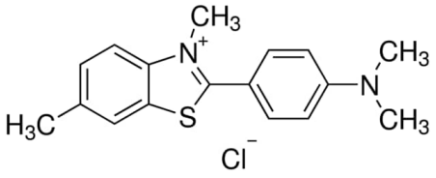


Figure 5 ThT molecular structure

In the central position of the molecule (Fig. 5) there is a C-C bond connecting the benzothiazole (on the left) and the aniline ring (on the right). The mechanism of fluorescence is determined by the fact that the bond between ThT and amyloid prevents the rotation of this bond [97].

ThT can be used not only for identification but also for quantification of amyloid deposits. This is made possible by the fact that ThT needs a well-defined amount of 4 beta-sheets to bind amyloid [98].

The problem of this technique is that ThT staining is not a standardized procedure. The ideal concentration of ThT to use has not yet been established [94].

Sodium sulphate-Alcian Blue

The Sodium sulphate-Alcian Blue (SAB) staining (Fig. 7) is a group of polyvalent basic dyes and it gives the amyloid a green colour. The signal is clear so that the amyloid can be identified even at low magnification (x3). Amyloidogenic proteins are not the only ones that are stained, but the risk of this leading to false positives is low because the other structures identified by the SAB are easily distinguished by their morphological features. The simplicity of this stain makes it is convenient to use and easy to reproduce. In addition, it allows us to specifically visualize other phenomena besides amyloidosis:

- Fibrosis,
- Basophilic myofibre degeneration,
- Tissue mast cells degeneration
- Mucoid degeneration of valves [99].

IMMUNOHISTOCHEMISTRY

Until now, we talked about the morphological and the histochemical recognition of amyloidosis. This chapter will instead talk about the technique that was firstly used to type the disease: immunohistochemistry. It consists of using different antibodies, each one with a high specificity for the subtype-determining protein.

Even nowadays, we can commonly use immunohistochemistry for the typing of amyloidosis, but there are critical points and precautions to take:

- Since the preparation of the sample and the evaluation of the biopsy require a precise expertise, the pathologists should receive a specific training;
- Specific antibodies should be available to recognize all the major amyloidogenic proteins;
- In order to avoid misleading results, every phase of the tissue processing should be standardized [6],[100];
- The endomyocardial antigens should be successfully unmasked, in order to let a proper antigen-antibody interaction;
- The antibodies should have a high quality, otherwise they will not properly interact with the antigen [100]

False-negative results

We are interested in reducing the risk of misleading results. In particular, false-negative results can slow down the diagnostic process, but there are strategies to cut them down. Two main elements can lead to a false negative result:

- Inappropriate experimental conditions;
- The characteristics of the specific antisera/antibodies.

That is why we should always test the specific antisera/antibodies on the myocardial tissue, in order to see if it properly works in our experimental conditions [100].

False-positive results: fixation problems

The main element that can lead to false-positive results is the over-fixation: an excess of interaction between formalin and the sampled tissue can alter the antigenicity of the proteins. The tissue becomes more reactive and the interaction with the antibody will give the impression of a misleading positive result. Two main strategies can help to avoid this process error:

- The quality of the antibodies and of the tissue antigens must be high, especially if the exam is performed on formalin-fixed and paraffin-embedded (FFPE) biopsies.
- In addition, the pathologists avoid the over-fixation by using formalin for only a few hours. This means that they will need to process the biopsy in the same day of fixation. This should give the idea of the importance to cross in the best way the needs of a well-performed exam and the established routine processes exhibited by pathologists [100].

Available techniques

A very qualitative technique that allows reproducible protocols and results is the automated immunostaining, that guarantee higher staining quality in comparison with manual staining [101].

The most used diagnostic technique in clinical practice is the immunoperoxidase staining, a sub-class of immunohistochemical exam. It is based on the so-called immunoperoxidase reaction. The procedure consists of the amyloidogenic proteins identification by using antibodies, which are linked to peroxidase, an enzyme that will catalyse a colour releasing reaction only if the antibody recognises its target.
The first step in immunoperoxidase staining is the binding of the specific (primary) antibody to the amyloidogenic protein. The detection of the primary antibody can be then accomplished *directly* (case 1) or *indirectly* (case 2 and 3).
Case 1. The primary antibody is directly linked with peroxidase, which will soon catalyse a chemical reaction and generate a coloured product.

Case 2. The primary antibody is linked with a small molecule A. A molecule B, which is linked to peroxidase, recognises the molecule A, activating its peroxidase. This method can be used to amplify the signal.
Case 3. A first untagged primary antibody binds the amyloidogenic proteins. Then, it is recognised by a secondary antibody, which recognises all the antibodies originating from the same animal species of the primary one. The secondary antibody is linked with peroxidase.

AL and ATTR subtyping

In order to economise the process of typing amyloidosis, we should take in consideration that lambda light chains represent the most common amyloidogenic precursors in AL amyloidosis. Therefore, it makes sense to search first lambda light chains with immunostaining. The process of firstly excluding AL λ+ amyloidosis by specifically searching it is called monotypic restriction [102],[54],[103].

In ATTR CA, there is no point in exhibiting a monotypic restriction for immunoglobulin light chains; the only exception is the very rare case of the co-existence of ATTR and AL λ+ amyloidosis.
In order to make diagnosis of ATTR amyloidosis, the immunostaining should be intense, giving a strong positive results; it should also accord with Congo red staining results and it must be negative at the monotypic restriction for lambda light chains [104].

IMMUNOFLUORESCENCE AND CONFOCAL LASER SCANNING MICROSCOPY

Another technique making use of antibodies is the immunofluorescence. It is used especially if samples are intended to be analysed with confocal microscopy, a technique born in 1955 in order to reduce the non-focal light in biopsy analysis and to build, from data obtained, spot by spot, with a focal light, a three-dimensional image with molecular details [105].

The following image (Fig. 6) made by “Université Paris Sud” clarifies the functioning of this technique [106].

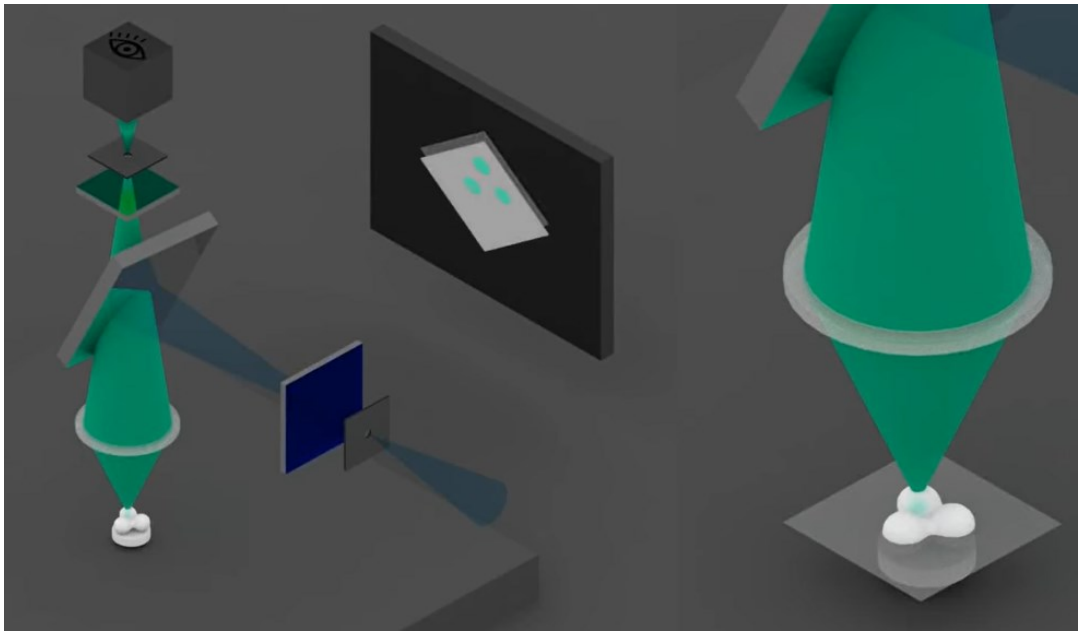


Figure 6 Confocal laser scanner microscope

The confocal microscopy can be used upon Congo red staining and upon immunofluorescence (Fig. 7). In the first one, we exploit the auto-fluorescence of the Congo red staining, improving its sensitivity and increasing the probability to reach an early diagnosis [79], while in the latter we use the fluorescence of specific antibodies or prepared anti-amyloid antisera bound to different fluorochromes [105].

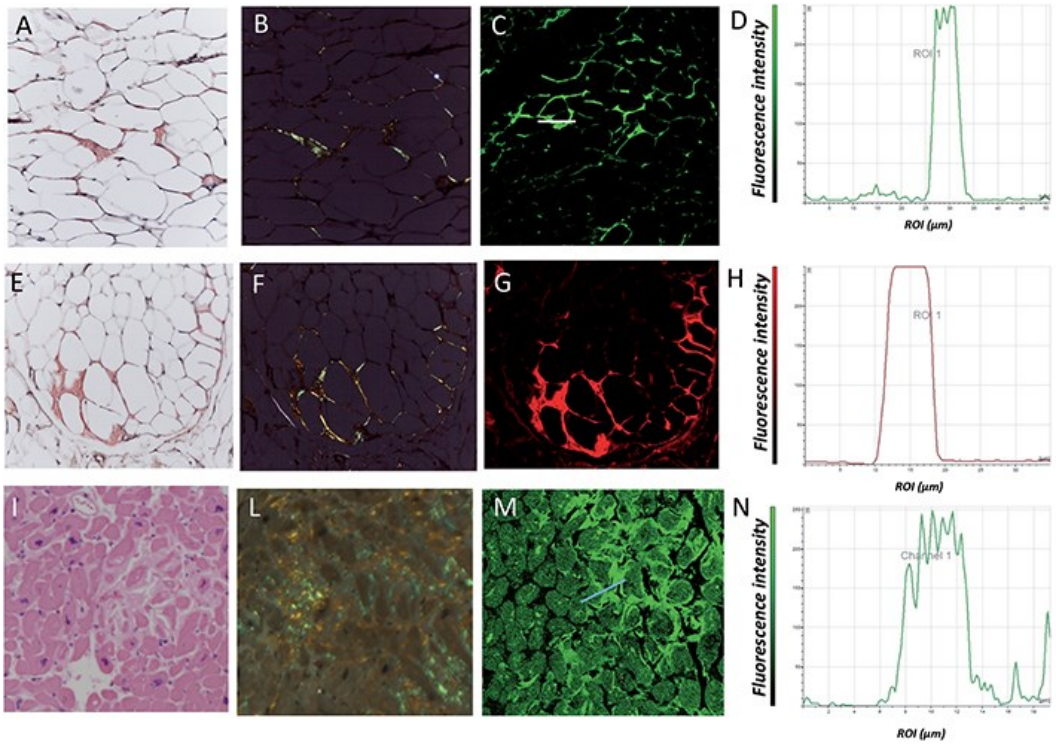


Figure 7 *CLSM. Fat tissue (A-H) and endomyocardial tissue (I-N) positive for amyloidosis. A, E: 20x Congo Red under LM. B, F: 20x Congo Red under polarized light results in apple-green, orange and yellow colours. C: 40x Congo Red under CLSM. D, H, N: fluorescence profile of ROI (region of interest).* ***G****: 40x Congo-Red staining under CLSM shows red fluorescence. I: 20x H&E under LM. L: 20x Cong-Red under polarized light.* ***M****: 20x ThT under CLSM [107].*

The most used confocal microscopy is the confocal laser scanning microscopy (CLSM). Thanks to this high-resolution technique, which allows seeing even sub-micrometric structures, pathologists can obtain three-dimensional biopsies images. The 3D nature of this exam makes it possible to observe the architecture of the amyloid in the context of the tissue structure, to better observe the localization of the deposits and their interaction with the para-physiological background [108].

MASS SPECTROMETRY-BASED PROTEOMIC ANALYSIS OF AMYLOIDOSIS

Mass spectrometry

Mass spectrometry is a tool that we use for measuring the mass-to-charge ratio (*m/z*) of molecules present in a sample. Every molecule has a mass and a

charge; the objective of this procedure is to measure their ratio, which is specific for each molecule and thus it allows their detection and recognition.

These measurements can even be used to calculate the exact molecular weight of the molecules as well. This is an essential characteristic of this technique, because it enables to make many investigation progresses:

- It can identify even unknown compounds via molecular weight determination;
- It can even quantify known compounds;
- It can determine the structure and some chemical properties of the molecules.

The most important components of every mass spectrometer are three:

- Ionization source
- Mass analyser
- Ion detection system

The ionization source converts molecules into gas-phase ions; thus, once ionized, the molecules will be able to move easier. We can use an electric and a magnetic field in order to start their motion: they will run according to their mass and charge.

Mass analyser is used to measure the mass-to-charge (*m/z*) ratios and it works at the same time with the ion detection system. Together, they give to the machine information about the localization and the abundance of the molecules. At the end *m/z* ratios are coupled on a graphic with their relative abundance, giving as results a mass spectrum. [109]

Mass spectrometry-based proteomics for amyloidosis subtyping

It is only in the recent years that mass spectrometry has been applied to amyloid deposits for the amyloid subtyping. We talk about mass spectrometry-based proteomic analysis or simply about mass spectrometry-based proteomics to refer to mass spectrometry analysis performed on proteins, in order to better investigate their nature [85].

The first time amyloid typing was performed using mass spectrometry-based proteomic analysis was in 2008 [110],[111].

The reason for the application of mass spectrometry in amyloidosis subtyping consists in the fact that, while for immunohistochemistry we largely talked about false-positive and negative results, this procedure does not have any bias [110]. In addition, the sample will not need to be processed with antibodies. The problem of antibodies is that we cannot have access to everyone, specific for each potential amyloidogenic protein; we often use a limited number of antibodies, according to the most recurrent amyloidogenic antigens reported in literature. Another problem of immunohistochemistry is that the antibody-antigen interaction takes place depending on the three-dimensional structure and conformation of both. Instead, mass spectrometry is free from this kind of concerns [18].

Techniques

Two main techniques can be used for the amyloid subtyping with mass spectrometry:

- Matrix-assisted laser desorption/ionization (MALDI);
- Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS).

The first one was used at the beginning, in the past. Indeed the first mass spectrometry amyloidosis subtyping was performed with this technique. It consisted of the separation of the proteins in the sample by using a gel (matrix); after that, proteins were loaded with laser energy and thus they were ionized [111].

Nowadays, we use in the majority of the cases the second technique: liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). This procedure is more advantageous because it is faster and it has a higher sensitivity [18].

“LC-MS/MS is a powerful qualitative and quantitative analytical technique with a wide range of clinical applications”.

We use the term “tandem mass spectrometry” because two mass spectrometers are coupled together in tandem. Between them, so between what we call the mass filters, there is a collision cell. The path taken by molecules is the following:

1. They are ionized in liquid phase (LC = liquid chromatography) using new ionization techniques such as electrospray ionization and atmospheric pressure chemical ionization

2. The firs mass spectrometer (MS) measures the ion mass of the proteins, that are called “precursor ions”

3. In the collision cell, the precursor ions collide with gas molecules and are fragmented into smaller ions that we call “product ions”;

4. The second MS measures the ion mass of the “product ions”; the sense in generating the smaller product ions consists of the fact that the fragmentation generates peptides in a predictable way, which is convenient if our objective is to identify the peptides.

The characteristics of this process make it perfect to analyse complex mixtures. MS/MS has superior selectivity for amyloidogenic proteins since it recognizes them by at least two parameters: the precursor and the product ion mass. The fact that MS/MS works in couple with LC generates another parameter that can help to recognise the protein, the retention time [112].

Mass spectrometry and samples

We can add a degree of complexity to the excursus, taking into account the substrate on which mass spectrometry-based proteomics is performed. The most common tissues for amyloidosis subtyping are the periumbilical fat pad excisional biopsy (FPEB) and the EMB. Literature papers also consider the abdominal fat fine-needle biopsy as a source of proteins to analyse, but as we said it is often leading to inconclusive results because of the fragility of the sample.

Of all the tissues from which we could extract proteins for analysis, the EMB is the best one in the case of CA. We know exactly that the heart is involved and that biopsy will be the most sensible; the same applies to other kind of organ biopsies, if we have clinical data suggesting the involvement of a specific organ.

In EMB, amyloid deposits are well defined, well visible, because they are consistent and high in number. The same cannot be said for the FPEB, where amyloid deposits are much lower in number and proteins distributes in a dispersed way; this is a problem, because it will be more difficult to isolate the amyloidogenic proteins, which means that the background of the rest of the sample will interfere with the result of the mass spectrometry-based proteomic analysis. Therefore, the diagnostic yield of a FPEB is very limited, especially for ATTRwt [113],[114].

Two problems can interfere with the result of the exam:

- Some blood remnants can be present on the sample. If there are some amyloidogenic proteins in the systemic circulation but not in the heart or the biopsy-undergoing organ, they will contaminate the tissue. The solution consist of a careful washing of the samples [115],[116].
- The amyloid deposits present themselves, on the sampled tissue, with a physiological background. In order to have a good sensibility, the healthy tissue should not be analysed by the mass spectrometer. Therefore, before the molecular analysis we should always isolate the proteins. We can firstly verify their presence with Congo red staining.
- In almost every case, the format of the biopsies is FFPE, because of the advantage of this preparation. Nonetheless, the methylene cross-links that prevent the tissue from degradation [80] are situated between the amine groups in proteins, even the proteins we are interested in. On the one hand, these modifications alter proteins structures, condition which should be taken into consideration in the moment of the mass spectrum analysis [117]. On the other hand, the protein are better linked to each other and this makes more difficult their isolation, which takes more time and more attention by the pathologists. A solution can be avoiding the over-fixation: the process will take only a few hours [118].

The procedure

For the execution of a LC-MS/MS, there is no standard procedure or protocol, because:

- There are different types of specimens, that should be treated in different ways;
- The availability of different machines is not the same from laboratory to laboratory;
- Data analysis is not a univocal process [119].

The first reason of concern is the variability in types of specimens. We clearly cannot treat an FFPE in the same way as an FPEB, because of the extreme fragility of the latter; in fact, periumbilical fat is usually processed without previous treatment: it will directly undergo solubilisation and digestion [114]. Instead, cardiac biopsies are usually in a FFPE format.

The procedure that is usually used to process and analyse a cardiac biopsy is long and complex:

A) Sample preparation.

1. The cardiac sample undergoes fixation and embedding in order to get higher stability for the sectioning. We obtain a FFPE specimen.
2. Sectioning. Several tissue sections can be obtained from a single block of cardiac tissue. This is fundamental, because it allows us to search the proteins in every section and the probability to find a consistent amyloid deposit to send to the MS increases.
3. Coloration. Congo red staining is used to highlight the deposits. This is essential for the following step of the procedure.

B) Laser-capture microdissection (LCM). In order to increase the sensibility of LC-MS/MS the normal tissue cannot follow the pathological one in the mass spectrometer. The presence inside of the sample of amyloid deposits areas and normal areas means that, if we analyse at LC-MS/MS the entire section, we will analyse both the amyloidogenic proteins and the ones normally present in the tissue. LCM is a technique that allow us to isolate the pathologic areas with a high precision laser cut. “Congo red-positive areas, confirmed by Congo red staining,

are selected and precisely cut by the instrument" .A section area, in order to be sufficient for the amyloid subtyping, should be 50,000 μm2 wide [85],[110].

C) Protein processing.

1. Protein extraction and tissue solubilisation. Since proteins are stuck in a FFPE environment, they should be released, by washing the sample with surfactants at warm temperatures for at least 90 minutes [85],[110]. The protein extraction process is different for FPEBs, that are just sonicated for 15-30 minutes [120].
2. The surfactant used for the protein extraction in FFPE biopsies is removed. For this purpose we can use dialysis [80] or molecular filters [121], in order to wash it away without losing proteins. During this process, we also change the buffer: instead of having surfactants, in contact with the sample substances there will be a buffer that will facilitate the proteolytic digestion.
3. Protein denaturation: reduction and alkylation. The objective of the following steps is the protein denaturation and digestion, in order to facilitate the LC-MS/MS analysis. The quaternary and tertiary structure of proteins are complex and the objective of the clinician is to alter it; an element of complexity is represented by the presence of disulphide bonds (S-S) inside of the protein structure. The perfect way to start protein denaturation is to break these bonds, by reduction reactions. Another way to aid protein denaturation is the alkylation. After these reactions, proteins will be more accessible by protease [122].
4. Tryptic digestion. We use proteases, usually trypsin, in order to start the protein digestion. The process runs at 37 ◦C for at least 2 h [122] to overnight [123] and it produces the so called "tryptic peptides".
5. Tryptic peptides purification. This process makes use of C18 solid phase extraction columns. The chromatography column is the main instrument used in the chromatography technique. It consists of two basic parts: the support and the packing. The support basically consists of the column itself, i.e. the cylinder in which the analysed molecules run. By packing, on the other hand, we mean the matrix inserted inside the column, a substance that allows us to separate the injected compounds. An example of a column is the so-called "C18". C18 is a molecule consisting of 18 carbon atoms that is used as a packing [124]
6. LC-MS/MS analysis. We can use C18 chromatography coupled with mass spectrometry in tandem [125] to obtain the mass spectrum.

7. Amyloidosis typing by Data-Dependent Analysis (DDA) [126].

As we said, there is not a univocal protocol for LC-MS/MS. “Interestingly, some protocols do not include protein reduction and alkylation [116], while some other protocols perform the reduction after the tryptic digestion” [122].

A visual description of LC-MS/MS preparation (Fig. 8) [50].

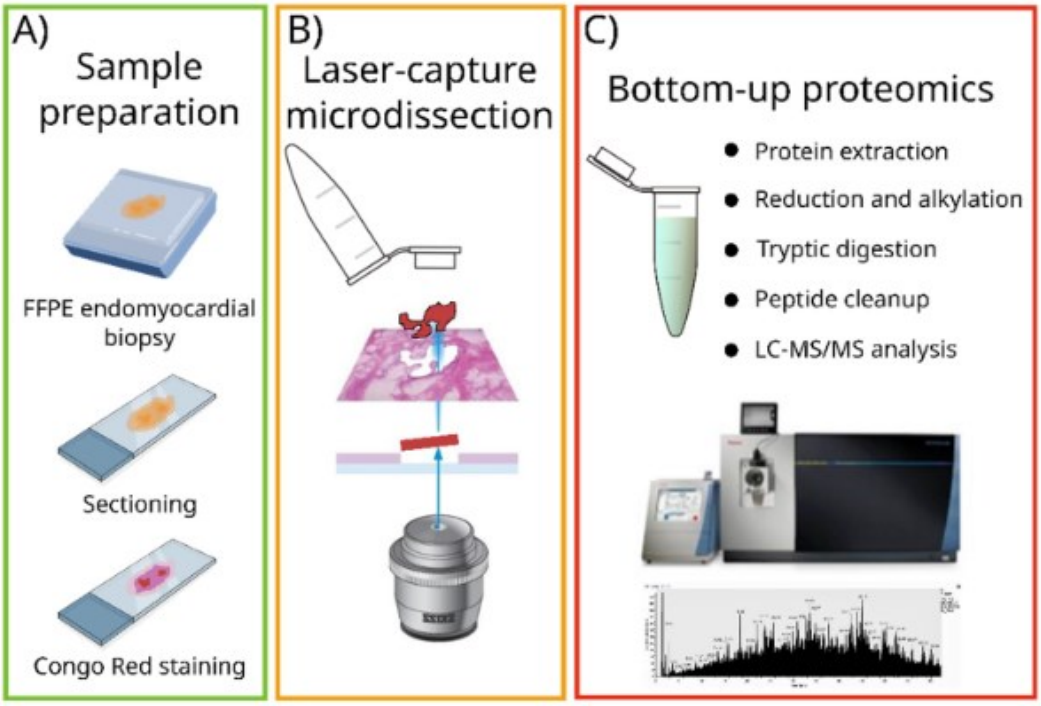


Figure 8 *LC-MS/MS preparation procedure*

LC-MS/MS open issues

In a DDA, there is the data of both the first and the second MS. Both are measuring the masses and the intensities of the peptides. For intensity, we mean the quantity of the peptides. The fact that masses and intensities of the peptides are measured even by the first MS before the definitive analysis is a crucial critical point.
The problem is that, for the next step, i.e. the fragmentation in the collision cell, only some ions are chosen. Then, the phase of the second MS analysis takes place, in order to generate a MS/MS spectrum, also referred to as M2 spectrum [126]; but the risk is that the M2 spectrum will be biased, because of the choice made by the machine to take one peptide or another one. This choice in not casual [50].

The problem can be seen in two different scenarios. To introduce them, it is useful to know that, when the LC-MS/MS experiment is taking place, we can let the machine work, without giving it a specification of which kind of peptides we are looking for. On the other hand, if we are looking for some specific peptides, the first MS will select them, under our indication, and their presence and identification will be confirmed by the second MS.

Scenario 1. The operator does not indicate a specification of peptide masses to select. In this case, the DDA will be able to find every protein, especially a not yet discovered one, because it will not apply a selection of the exact proteins in which the clinician is interested and it will be able to search and find some others. The whole proteome is potentially accessible. The reason why this scenario is problematic is that LC-MS/MS DDA will apply anyway a selection: "DDA preferentially selects peptide ions of higher intensity" [122]. The most abundant peptides will be selected by the first MS and they will undergo fragmentation for the following phase. Thus, it is easy to understand how problematic this can be if we are interested in finding an amyloidogenic protein, if it was not very abundant on the sample: here the quantity makes the difference. For instance, if we take into consideration a FPEB, since the deposits are dispersed and thin, it could be difficult to find them without specifically looking for them with a specification given to the MS. Another case in which the detection of proteins low in abundance could also be useful is of course the early diagnosis [119]. A possible solution can be to use modern LC–MS/MS instruments, which have a higher speed in scanning proteins and can select for fragmentation more than 50 peptides each second. In this way, only a little group of protein is not fragmented and the error risk is cut down [50].

Scenario 2. The operator indicates a specification of peptide masses to select. Since AL-κ+, AL-λ+ and ATTR amyloidosis are the most frequent forms, it makes sense to search only these proteins [122]. The advantage of this choice is that the laboratory will not need an expensive high scanning speed LC–MS/MS instrument; also, the protocol can be easier standardized. Anyway, with this technique it is impossible to see the wide range of peptides that we are not looking for. Our target list will not include new amyloidogenic proteins, nor some other mutant forms of the targeted proteins [50].

Blood contamination

It has already been mentioned that certain amyloidogenic proteins can contaminate the sample, altering the LC-MS/MS result. Such contamination has more consequences in a periumbilical fat sample, where deposits are rarefied and dispersed. One solution might be to compare the proteomic profile of a pathological sample with that of a series of control samples: in this way, the presence of certain circulating proteins in the healthy subject can be excluded as pathological if also present in a pathological sample [116].

Some of the proteins that extravasate infiltrating tissue may curiously be useful for diagnostic purposes. In particular Apo E, Apo A-IV and serum amyloid P-component (SAP), in the presence of an amyloidogenic protein, precipitate together with it in the tissues. This is why, even if we do not look for or find the amyloidogenic protein, but at the same time we come into these precipitated proteins, they must arouse a strong suspicion of amyloidosis, because they represent an indirect sign of the presence of disease. It has also been suggested that, in the case of a periumbilical fat biopsy, the search for these proteins is more sensitive than Congo red staining for diagnosis [121].

IMMUNO-ELECTRON MICROSCOPY

Another technique that can be used for the diagnosis and typing of amyloidosis is transmission electron microscopy (TEM). Also a immuno-electron microscopy can be performed, increasing the sensitivity and specificity of the test [55],[127].

The reason why TEM is a revolutionary tool for the diagnosis of amyloidosis lies in its ability to detect amyloid deposits even in the earliest stages of the disease. This is due to the high resolution of this kind of microscopy, which can clearly identify a protein in the ultrastructural world observation. Anyway, there is also a critical point to highlight: if deposits are focal and they distribute in an irregular way, if they are thin, rarefied and dispersed, it can be less probable to analyse an area rich in number of deposits, at the expense of the diagnostic yield of the technique [50].

Preparation

The preparation of the specimen consists of the following steps:

1. Collection of a sample. An advantage of this technique is that the sample does not require a lot of material: the TEM analysis can be performed with a very small sample.
2. Fixation. It takes place in a 2.5% glutaraldehyde solution or in a 2% Karnowsky solution. Karnowsky is a solution made up of 2% paraformaldehyde and 2% glutaraldehyde.
3. Post-fixation. Osmium tetroxide is used.
4. Embedding. We use a resin called Epon-Araldite.
5. Realization of ultrathin sections [55].

Morphological analysis

In order to perform a morphological analysis and to identify the amyloid by only its morphological features, the ultrathin section undergoes further treatments:

1. 5% uranyl acetate;
2. Reynold's lead citrate staining [55].

Typing

The typing is performed using a specific technique combined with TEM observation: immune-electron microscopy (IEM) by post-embedding immunogold technique.

Even in this case the ultrathin sections undergo further treatments:

1. Some specific antibodies are applied to the section; they are referred to as "primary antibodies" because they directly bind the amyloidogenic proteins. We do not use antibodies for every amyloid, but only the most frequent are targeted: TTR, λ and κ light chains.
2. The sample is washed in order to remove the antibodies that didn't link to a specimen structure.

3. Secondary antibodies are applied in order to detect the primary ones. They are anti-mouse or anti-rabbit antibodies, depending on the nature of the primary ones.
4. The pathologists identify the secondary antibodies, which are linked to gold particles of 5 to 15 nm diameter [55].

OUTCOME AND PROGNOSIS

Prognosis in CA

The attempts to generate a satisfying prognosis scoring systems in the last years were various and a miscellaneous group of prognostic factors is now available:

- Presence of congestive heart failure,
- β2-microglobulin,
- Presence of a peripheral neuropathy that dominates the presentation,
- Presence of a monoclonal light chain in serum or urine,
- Multiple myeloma,
- Hepatic involvement [128].

Nonetheless, the reliability of single factors is limited. Many authors proposed different solutions, basing on multi-parametric prognostic systems [129]–[133], which are summarized in the following table [34]. Different parameters are taken in consideration and they are measured at the patient presentation:

- FLCs levels;
- Troponin T;
- Troponin I;
- NT-proBNP;
- BNP;
- eGFR;
- Daily dose of furosemide;
- NYHA class.

Follow-up revaluations do not change the prognostic score, but they supply the clinicians with useful updates about patients' status [34].

All the main prognosis scoring systems can be found in the Table 4 [34].

Table 4 Amyloidosis stage: scoring systems [34]

Kumar *et al.* (Mayo)		**Lilleness *et al.* (BU)**		**Grogan *et al.* (Mayo)**		**Gillmore *et al.* (NAC)**		**Cheng *et al.* (Columbia)**	
AL		**AL**		**ATTRwt**		**ATTRv and ATTRwt**		**ATTRv and ATTRwt**	
Staging parameters: FLC-diff ≥ 18 mg/dL Troponin T ≥ 0.025 ng/mL NT-proBNP ≥ 1800 pg/mL		**Staging parameters:** Troponin I > 0.1 ng/mL BNP > 81 pg/mL		**Staging parameters:** Troponin T > 0.05 ng/mL NT-proBNP > 3000 pg/mL		**Staging parameters:** eGFR < 45 mL/min/1.73 m^2 NT-proBNP > 3000 pg/mL		**Scoring parameters:** Mayo or NAC score (0–2 points) Daily dose of furosemide or equivalent: 0 mg/kg (0 points), >0–0.5 mg/kg (1 point), >0.5–1 mg/kg (2 points), and > 1 mg/kg (3 points) NYHA class I-IV (1 to 4 points)	
Stage	**5-year survival**	**Stage**	**Median survival**	**Stage**	**4-year survival/ median survival**	**Stage**	**Median survival**	**Score**	**Median survival**
Stage I (0 parameters)	68%	Stage I (0 parameters)	Not reached	Stage I (0 parameters)	57% 66 months	Stage I (0 parameters)	69.2 months	Score 1–3	90.5 months
Stage II (1 parameter)	60%	Stage II (1 parameter)	112.8 months	Stage II (1 parameter)	42% 40 months	Stage II (1 parameter)	46.7 months	Score 4–6	38.5 months (Mayo) 36 months (NAC)
Stage III (2 parameters)	28%	Stage III (2 parameters)	51.6 months	Stage III (2 parameters)	18% 20 months	Stage III (2 parameters)	24.1 months	Score 7–9	20.3 months (Mayo) 19.8 months (NAC)
Stage IV (3 parameters)	14%	Stage IIIb (2 parameters and BNP > 700 pg/mL)	12 months						

Follow-up of patients with CA

Some recommendations are available for the follow-up of patients with CA. Some differences between AL and ATTR follow-up are explained by the necessity to check, concerning AL CA, the haematologic picture, at the basis of the etiology of the disease; on the other hand, for what concern ATTR CA, it is also useful to monitor neurological and ophthalmological alterations [34].

For the follow-up of patients with AL amyloidosis, the following guidelines have been published [34]:

1) Every month, during initial haematological treatment:
- Complete blood count, basic biochemistry, NT-proBNP, and troponin
- Serum free light chain quantification
- Clinical evaluation by Haematology
- Evaluation by Cardiology if clinically indicated

2) Every 3–4 months, after completing initial haematological treatment:
- Complete blood count, basic biochemistry, NT-proBNP, and troponin

- Serum free light chain quantification
- Clinical evaluation by Haematology

3) Every 6 months:
- ECG
- Echocardiography/CMR
- Evaluation by Cardiology

4) Every 12 months:
- 24-h Holter ECG

For the follow-up of patients with ATTR amyloidosis, the following guidelines have been published [34]:

1) Every 6 months:
- ECG
- Blood tests including NT-proBNP and troponin
- Neurological evaluation (if ATTRv)
- 6-min walking distance (6MWD) (optional)
- Kansas City Cardiomyopathy Questionnaire (KCCQ) (optional)

2) Every 12 months:
- Echocardiography/CMR
- 24-h Holter ECG
- Ophthalmological evaluation (if ATTRv)

Genetic counselling and follow-up of TTR mutation carriers

If the patient has an inherited form of CA, even his family should undergo genetic test and counselling. For ethic reasons, since CA onset concerns the adult age, minors should not be tested. Young adults should be tested, in order to start a follow-up and a reproductive planning. If a family member results positive for genetic testing, follow up should be started 10 years before the age of onset. The

guidelines offered for the follow-up of mutation carriers is reported below [34], [134].

1) Yearly:
- ECG
- Blood tests including NT-proBNP and troponin
- Echocardiography
- Neurological and ophthalmological evaluation

2) Every 2 years:
- Holter ECG

3) Every 3 years or if any of above complementary tests is abnormal:
- Scintigraphy
- CMR

TREATMENT

Amyloidosis treatment is a complex process, requiring a multidisciplinary approach. Two categories of treatment can be distinguished [34]:

1. treatment and prevention of complications
2. stopping or delaying amyloid deposition by specific treatment.

Treatment of complications and comorbidities

Several complications and comorbidities accompany CA. In this chapter we introduce some possible solutions to the most common CA complications [135].

Aortic stenosis (AS)

As already mentioned, aortic stenosis is a common finding in echocardiographic and CMR CA examinations. The presence of a severe AS is a negative prognostic factor, especially when accompanied by AV block in patients with ATTRwt CA. A

reliable solution is represented by transcatheter aortic valve replacement, which improves the outcome.

Heart failure

In case of heart failure, haemodynamic stability must be restored, through fluid intake control and diuretics. Some drugs are contraindicated, such as β-blockers, ACEi and ARBs. Heart transplant is feasible for selected patients.

Atrial fibrillation (AF)

AF is a very common complication of CA. The preferred antiarrhythmic is amiodarone, while electrical cardioversion can lead to complications and AF relapses and it must be preceded by the exclusion of thrombosis.

Thromboembolism

Considered the frequency of atrial fibrillation (AF) in CA patients, it is understandable why thromboembolism is a common complication. If AF is present, patients will be treated with anticoagulants, which should also be considered independently of the heart rhythm and the CHADS-VASC score.

Conduction disorders

In case of conduction disorders, the most recommended treatment is the placement of a permanent pacemaker.

Ventricular arrhythmias

In patients with CA atrial arrhythmias are more often encountered, but rarely even ventricular ones are found. The placement of a transvenous implantable

cardioverter-defibrillator (ICD) is recommended, especially if used for secondary prevention.

Disease-modifying treatment

As already mentioned, many advances have been made regarding the discovery of amyloidosis aetiology and its pathogenic pathways. The progress in research is leading our community to the development of new drugs, able to target specific steps of the amyloid cascade [14]. When the treatment is able to stop or to delay the amyloid deposition, it is a disease-modifying treatment.

AL Amyloidosis

In AL CA, the disease is driven by B-cell clone, most commonly a plasma cell clone dyscrasia, which is the first element to treat in order to act against the *primum movens* of the pathogenic cascade: the haematologic one is the first disease-modifying treatment. However, this kind of patients is not easy to treat. The amyloidogenic FLCs spread, giving multi-organ damage, which makes the patient very vulnerable; careful approach to treatment toxicity should always be adopted. Before starting a treatment, the clinician will set up a risk assessment, in order to establish which kind of care the patient is able to tolerate; since the higher risk of death comes from heart involvement, this is the major factor to be taken into consideration in a risk evaluation process [34].

AL CA is treated with a multidisciplinary approach and patients should be cured in specialized centres, by many professional figures:

- Oncologists
- Haematologists, who will guide the treatment,
- Cardiologists [136],

a. for a precocious high risk of death evaluation before starting the haematologic treatment,
b. for the evaluation of the possibility to perform heart transplantation,
c. for monitoring of cardiac parameters during therapy.

Nowadays, many possibilities of treatment are available:

- autologous stem cell transplantation,
- autologous stem cell transplantation after conditioning, based on Bortezomib,
- Bortezomib, standard of care, used in patients who are not eligible for transplantation,
- A combination of Bortezomib and Daratumumab, which will probably become the new standard of care [137, p. 202].

The setup of a close follow-up, consisting of a monitoring of haematologic parameters, is essential in order to recognise non-responders and to lead them to another rescue treatment [137, p. 202].

ATTR Amyloidosis

ATTR amyloidosis is caused by an excess of TTR protein in the circulation. This premise is essential to understand the rationale of the treatment. An early diagnosis is of primary importance, as it allows reducing, through a treatment started with the right timing, the time in which the patient is subjected to high concentrations of amyloidogenic protein [34].

Some treatments are available, or still being approved, and they act at different levels of the pathogenic cascade:

- Liver transplantation reduces the production of TTR protein;
- Genetic silencers, i.e. antisense oligonucleotides and small interfering RNAs for suppression of variant and wild-type TTR synthesis, reduce the overall production of TTR protein [28];
- Stabilizers have the role to stabilize the TTR protein in a physiologic conformation, and to prevent their dissociation or cleavage into amyloidogenic fragments;
- Novel drugs capable to attack the amyloid deposits are still under investigation.

The treatment of choice for ATTRv amyloidosis is liver transplantation, because it solves the problem at its roots, suppressing the main source of variant TTR [28].

Before starting a therapeutic path, it is necessary to make differential diagnosis between ATTRv and ATTRwt amyloidosis:

In case of ATTRwt amyloidosis with cardiomyopathy (CA): treat with Tafamidis.
In case of ATTRv amyloidosis with

- Cardiomyopathy: treat with Tafamidis.
- Cardiomyopathy and polyneuropathy: combination treatment with both Tafamidis and Patisiran
- Polyneuropathy: combination treatment with Tafamidis, Patisiran and Inotersen.

As can be seen, Tafamidis are the up-front treatment, and they can be combined with other therapies in order to improve the outcome.

Tafamidis is a rationally designed benzoxazole derivative, which binds with high affinity and selectivity TTR. This bound confers stability to the tetramer, slowing monomer formation, which would lead to misfolding and amyloidogenesis. Tafamidis was the first drug approved for polyneuropathy in ATTRv amyloidosis [27].

Patisiran is new drug, capable to interfere with RNA. It is a small ribonucleic acid (ALN-18328), formulated in a lipid nanoparticle, and its mechanism of action consists of the inhibition of the TTR variant RNA silencing, in order to inhibit hepatic transthyretin protein synthesis [138].

Inotersen is a 2'- O-methoxyethyl-modified antisense oligonucleotide, which inhibits hepatic production of transthyretin [139].

ORGANIZATION OF PATIENT CARE

As mentioned above, the care of patients with CA is based on a multidisciplinary approach, focusing on disease-modifying strategies and the treatment of complications. The complexity of this diagnostic-therapeutic process requires the collaboration of various specialized centres, because both diagnostic and therapeutic techniques are not universally accessible. For these reasons, it has been

suggested to abandon the "hub-and-spoke" model, because patients need to be referred to centres of reference, capable of providing niche care [34].

AIMS OF THE STUDY

The aims of the present work were:

1. To investigate the accuracy and reliability of light microscopy with immune-electron microscopy (IEM) study in diagnosis and characterization of cardiac amyloidosis on fixed paraffin-embedded tissues.
2. To study at light microscopy in endomyocardial biopsies (EMB) the association between the amyloid burden and its patterns of deposition (perimysial, interstitial nodular-like, perivascular) and the type of amyloid characterized by means of IEM.
3. To evaluate the diagnostic value in cardiac amyloidosis of abdominal fat pad excisional biopsy (FPEB) vs EMB, when both are performed.
4. Clinical-pathological correlation in cases of cardiac amyloidosis studied in a single center, Azienda Ospedaliera-University of Padua

MATERIALS AND METHODS

1. **To investigate the accuracy and reliability of light microscopy with immune-electron microscopy (IEM) study in diagnosis and characterization of cardiac amyloidosis on fixed paraffin-embedded tissues.**

Study Population

We re-examined the EMBs of consecutive patients with clinical evidence of hypertrophic-restrictive cardiomyopathy, suspected to be due to amyloidosis, submitted to the Cardiovascular Pathology Unit, Azienda Ospedaliera-University of Padua, over the last 5 years (from January 2017 to December 2022). The samples were obtained from patients admitted to our hospital or from other hospitals throughout Italy. Patients underwent clinical examination, immunofixation of serum and urine for detection of monoclonal immunoglobulins, electrocardiography, echocardiography, cardiac magnetic resonance and bone 99mTc-biphosphonate scintigraphy, raising the suspicion for heart involvement by amyloid.

EMB protocol

EMB was performed by right ventricular sampling, and processed for histological and immunoelectron microscopy analysis. At least 3 endomyocardial biopsies were taken into consideration in order to minimize sampling errors and to ensure EMB adequacy. Each fragment corresponds to approximately $1mm^2$ of surface. The review of the slides for each patient of the studied population allowed us to provide the average number of fragments per patient. Thus, the study is based on a sufficient amount of data and a well-defined number of fragments to provide all the information.

Histopathological analysis

The samples (EMB and FPEB) were fixed in 10% formalin 0.5M phosphate bufferered solution, pH 7.2 followed by paraffin embedding and microtome sectioning. Three μm-thick serial sections were stained with haematoxylin–eosin,

Azan Mallory, Congo Red with typical green birefringence under cross-polarized light using a Zeiss microscope or a confocal laser microscopy Leica for both Congo Red stain [105] and thioflavin fluorescence for histological examination and diagnosis of amyloidosis, according to the literature [39]. Moreover, we evaluated the presence at HE of cytoplasmic vacuolization and necrosis of the cardiomyocyte in a qualitative manner and replacement type fibrosis at Azan-Mallory stain. To exclude inflammatory heart disease, paraffin tissue sections were treated with an avidin-biotin immuno-peroxidase method with application of the following monoclonal antibodies: CD3 (T-cells, Novocastra Laboratories, Newcastle, United Kingdom) and CD68 (macrophages, DAKO, Hamburg, Germany). Myocardial inflammation indicative of myocarditis was defined as the detection of 14 infiltrating leukocytes/mm^2 (CD3 T lymphocytes and/or CD68 macrophages) [140]. Additionally, electron microscopy for visualization of small amyloid deposits was always performed to rule out Congo red and thioflavin negative amyloidosis.

Amyloid characterization by post-embedding immunoelectron microscopy

All cardiac and abdominal fat specimens (with or without amyloid deposits at special stains) were selected by pathologists and underwent amyloid typing by IEM. The formalin-fixed and paraffin-embedded blocks underwent dewaxing, followed by Karnovsky's fixation (0.5% Karnovsky's solution - 0.5% glutaraldehyde, 2% paraformaldehyde in 0.1M phosphate buffer, pH 7.3) and then LR white resin (Agar Scientific Ltd, Stansyed, Essex, UK) embedding, in order to undergo post-embedding immunogold technique, as follows.

For dehydration of the samples, a graded ethanol series and propylene oxide step was the method of choice for embedding in LR White. For this purpose, a 1mm cube of tissue was infiltrated overnight at room temperature, followed by two short changes of resin. The most convenient way of achieving the polymerization step was to use gelatin capsules filled with the resin. Polymerization time and temperature are fundamental to the physical characteristics of the final block. We used a temperature of 45°C for a period of 24-48 hours. LR White has extremely good powers of penetration. After polymerization, trimming the block and cutting were performed with a diamond knive on the ultramicrotome (RMC Boeckeler,

Tucson, AZ, USA) obtaining semithin (1µm) sections, stained with toluidine blue and observed at histological level with a light microscope (Olympus BX51, Hamburg. Germany) to select the area with potential amyloid deposits. After that, ultrathin sections (90 nm) were collected on nickel grids and processed for post-embedding immunoelectron microscopy using primary antibodies for the most statistically represented forms of cardiac amyloidosis: anti-human TTR (dilution 1:10, BioLegend, Greenwood Pl, London, UK), anti-human kappa light chains, and anti-human lambda light chains (both dilution 1:100, Dako-Agilent, Santa Clara, CA, USA). The sections, incubated with the primary antibodies, were rinsed in the TBS buffer and then secondary antibody (anti-rabbit or antimouse IgG, dilution 1:50, Abcam, Cambridge, UK), conjugated to 10 nm colloidal gold particles as a revealing system, was applied. All the sections were stained using uranyl acetate 5% and observed with a Hitachi H7800 (Hitachi, Tokyo, Japan) transmission electron microscope. The staining results were evaluated as: (1) unable to localize amyloid fibrils; (2) amyloid fibrils localized by ultrastructure, with immunostaining negative; (3) amyloid fibrils localized by ultrastructure, with positive immunostaining..

2. **To study at light microscopy the association in endomyocardial biopsies (EMB) between the amyloid burden and its patterns of deposition (perimisial, interstitial nodular-like, perivascular) and the type of amyloid characterized by means of IEM.**

Pattern of depositions and burden of cardiac amyloidosis

Once put forward the diagnosis and subtype of cardiac amyloidosis, we evaluated the burden of amyloid deposits and the pattern of deposition by light microscopy (Leica, Germany) using H&E and Azan Mallory staining, and not by IEM, because the former allows the observation of a much larger field of tissue compared to the latter and therefore allows the analysis of different fragments of tissue within the same slide. In fact, while IEM analyses a surface of 0.1mm^2, optical microscopy allows the analysis of approximately 1mm^2 of endomyocardial tissue for each fragment, thus allowing a more homogeneous and more representative analysis of

the cardiac tissue. This allowed us to properly analyse the data concerning the extent of amyloidosis and its pattern of deposition.

The extent of amyloid was expressed in a semi-quantitative manner as percentage for each cardiac fragment by two independent observers (SR and CJDM). The presence of replacement-type fibrosis was evaluated similarly in each cardiac sample in a semi-quantitative manner and expressed as a percentage.

For each cardiac specimen, the localization of amyloid fibrils was determined, in terms of prevalent interstitial, perimysial or nodular, or prevalent vascular pattern. These patterns are not mutually exclusive and can therefore all three be present in the same fragment. The perivascular pattern was characterized by the involvement of the vessels, which appeared surrounded by amyloid. The perimysial pattern can be recognized due to the involvement of the interstitium around the cardiomyocytes: the myocyte cells are surrounded by amyloid and the tissue assumes a characteristic "amyloid network" appearance, in which the deposits, surrounding many contiguous cardiomyocytes, form a network, easily detectable by histochemical stains. The nodular pattern consisted of the presence of interstitial nodules of amyloid deposits. To make a distinction between a perivascular pattern with large deposits surrounding the vessels from a nodular pattern that has extended to the point of a vessel, we relied on the observation of the vessel:

- If it appears isolated and little affected by the pathological process, with some free walls, in a background in which there is instead amyloid all around, that will be a nodular pattern.
- If it is engaged by the pathological process, positioning itself in the center of the deposit and having an engagement of the walls and of the adjacent interstitium, that will be a perivascular pattern.

The amyloid burden and pattern of deposition were then compared with the amyloid subtype obtained by means of IEM.

3. **To evaluate the diagnostic value in cardiac amyloidosis of abdominal fat pad excisional biopsy (FPEB) vs EMB, when both are performed.**

Cases with FPEB besides EMB were also reviewed. FPEB consisted of excisional biopsy from abdominal skin, including subcutaneous adipose tissue.

The population of this part of the study consisted of the intersection of two sets of patients: those for whom an EMB was performed and those for whom an FPEB was performed. This part of the study aims to analyse the difference in sensitivity between the two techniques.
In particular, we tried to determine whether the FPEBs of patients with hypertrophic cardiomyopathy, suspected to be due to amyloidosis on the basis of clinical and imaging data, contained amyloid deposits, investigating for fibril immuno-characterization, thus concluding the diagnostic work-up for cardiac amyloidosis. As controls, we used the EMB from the same patients. FPEB were investigated (as for BEM) with Congo Red and thioflavin stains and immuno-electron microscopy, using the specific primary antibodies against TTR, κ light chains and λ light chains.

4. Clinico-pathological correlation in cases of cardiac amyloidosis studied in a single center, Azienda Ospedaliera-University of Padua

All patients with a diagnosis of amyloidosis made by IEM within our centre, Azienda Ospedaliera-University of Padua, were included in this part of the study. Data about echocardiography, collected by the cardiology ward of our center, were re-examined and correlated with pathological results.

Echocardiography

Echocardiography was performed using a Vivid 7 Ultrasound Machine. Inter-ventricular septum (IVS) thickness, posterior wall (PW) thickness, and left ventricle end-diastolic volume (LVEDV) were measured on extemporaneous pictures framing the mitral valve leaflet tips in their diastolic position. Left ventricle mass (LVM) was assessed using the American Society of Echocardiography/European Association of Cardiovascular Imaging (ASE/EACVI) -recommended formula. The biplane Simpson method was used to calculate the left ventricle ejection fraction (LVEF). Doppler echocardiography performed in the apical four-chamber view was used to evaluate the E wave (blood flow peak velocity in the early left ventricle diastole) and the A wave (blood flow peak velocity in late diastole due to

the atrial contraction), which were used to calculate the *E*/*A* ratio, index of diastolic function. Global longitudinal strain (GLS) was calculated [105].

STATISTICAL ANALYSIS

Continuous variables were expressed as median with 25th and 75th percentiles (Q1–Q3) and analyzed by Mann–Whitney test. Categorical variables were expressed as absolute numbers and percentages and were compared using the Fisher exact test and the $\chi 2$ test for categories of two and three groups, respectively. All statistical analyses were performed using IBM SPSS Statistics 27.0 package and Jamovi (version 2.3).

RESULTS

1. **To investigate the accuracy and reliability of EMB with immune-electron microscopy (IEM) study in diagnosis and characterization of cardiac amyloidosis on fixed paraffin-embedded tissues.**

In the time interval 2017-2022, a series of 171 EMBs from patients with clinical suspicion of cardiac amyloidosis were collected (118 males, 69%; average age at diagnosis 66.6±11.5 years, range 20-83) (data are summarized in Table 5). Identification numbers for both histopathological and ultrastructural analysis were used to create a database containing clinical and pathological data, in an anonymous manner.

In the first step, we reviewed all the EMBs to have the histochemical proof of amyloid deposits using Congo Red staining in polarized light or at confocal laser microscopy and thioflavin.

The second step was to identify the type of fibril protein by ultrastructural immunogold and, thereby, the probable underlying disease. At ultrastructure we looked for the presence of interstitial or perivascular non-branching linear fibrils of indefinite length with an approximate diameter of 10-12 nm (Fig. 9). We used IEM as a reference technique for establishing the reliability of results from light microscopy and typing the amyloid fibrils.

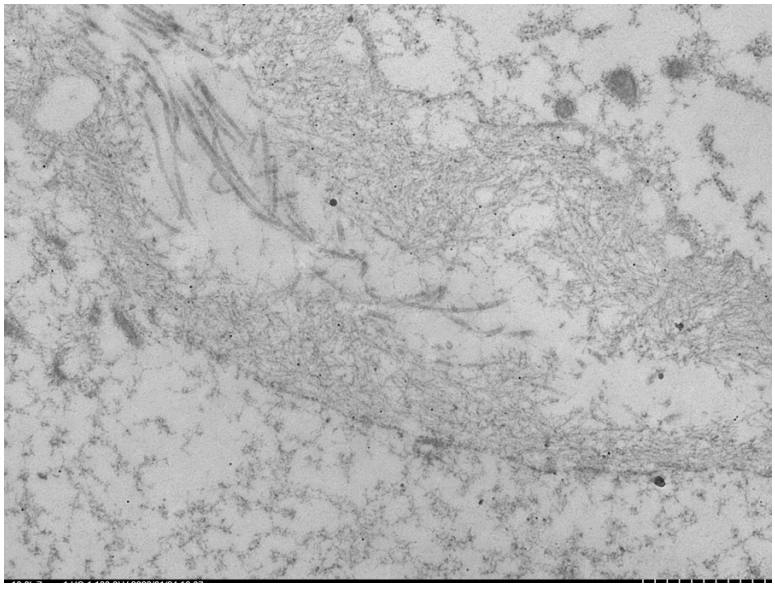


Figure 9 *Ultrastructure of amyloid fibrils, characterized by non-branching linear fibrils with an approximate diameter of 10-12 nm (scale bar 1 μm).*

In 45 cases (26%) EMB amyloid deposits were not identified by means of H&E, Congo Red, and Thioflavin stains nor by IEM. In the remaining 126 EMB cases (74%), IEM was diagnostic (6 only at IEM), revealing even small amounts of amyloid fibrils, positive for AL in 82/126 (65%) and TTR in 44/126 (35%) (Fig.10) (Table 6).

Table 5 *Study population*	
Variable	
n° patients	171
Sex, n° (%)	118 (69%)
Average age (years)	66.6±11.5 (range 20-83)
Amyloidosis +	126 (74%)
ATTR n° (%)	44 (35%)
AL	82 (65%)

Table 6 *Immunoelectron microscopy (IEM) vs histology (H)*			
	IEM -	**IEM +**	**Tot**
H -	45	6	
H +	0	120	
Tot	45	126	171

The analysis through IEM technique allows to measure reliability of means of light microscopy parameters:

- Sensitivity (91.3%),
- Specificity (88.9%),
- Positive Predictive Value (95.8%),
- Negative Predictive Value (78.4%).

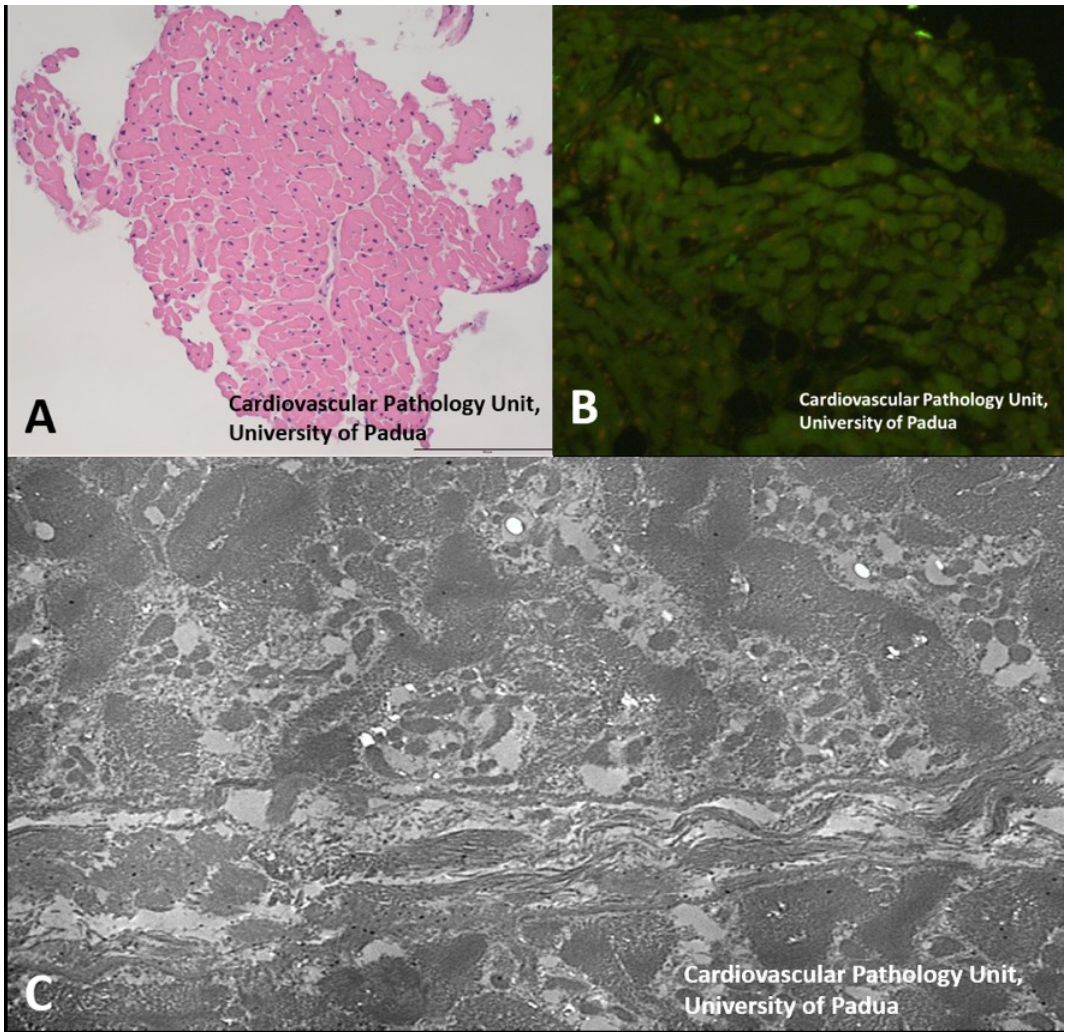


Figure 10 *73 years-old male, EMB with negative histology (A, H&E x100) and fluorescence (B, thioflavin x200) for amyloidosis. Under IEM, there were few amyloid fibrils in the myocardial interstitium positive for kappa light chains, visible as black little spots (arrows) (scale bar 2.0 µm)*

In addition, endocardium involvement by the pathologic process and the presence of necrosis and cytoplasmic vacuolization were studied (Table 7). Necrosis and vacuolization can be found in amyloidosis cases, but are not specific findings.

Table 7 *Endocardium involvement and the presence of necrosis and cytoplasmic vacuolization according to the type of amyloid*			
	Positive	**ATTR**	**AL**
Endocardium	25/126 (20%)	6/44 (14%)	19/82 (23%)
Necrosis	99/126 (78.5%)	37/44 (84%)	62/82 (76%)
Vacuolization	86/126 (68%)	33/44 (75%)	53/82 (65%)

2. **To study at light microscopy in endomyocardial biopsies (EMB) the association between the amyloid burden and its patterns of deposition (perimisial, interstitial nodular-like, perivascular) and the type of amyloid characterized by means of IEM.**

The EMBs consisted of an average of 4.8±2.2 samples with a range from one to 11 fragments, whose numerosity does not associate to a positive diagnosis (Fig.11). Nonetheless, among the positive fragments for each patient, there are 0.47±1.28 negative fragments.

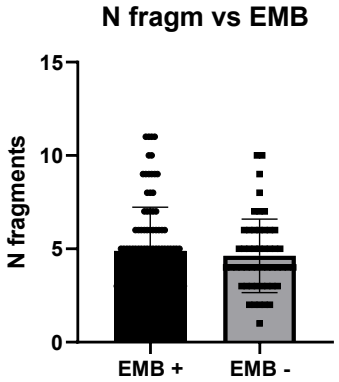


Figure 11 *Correlation between number of fragments and positivity of EMB*

At histology, among 120 patients positive at both LM and IEM, a comparison of vascular and interstitial involvement revealed vascular deposits in 49 cases (41%), distributed in a 32% of ATTR cases (14/44) and a 43% of AL cases (35/82).

Interstitial perimysial deposits were present in all cases (100%), with nodular deposits in 79 (66%) (Table 8). The vascular to interstitial ratio was of 1:2.18.

Table 8 *Pattern of amyloid deposition*	
Pattern	n° of cases
Vascular	49 (41%)
Perimysial	120 (100%)
Nodular	79 (66%)

Considering the prevalent pattern in each patient (Fig. 12-14), the majority of AL cases presents a perimysial pattern (70%), while ATTR ones have an equal distribution between the perimysial (46%) and the nodular (51%) patterns (Figg. 12-15); no particular considerations can be made about λ and κ subtypes. The vascular pattern is the less prevalent.

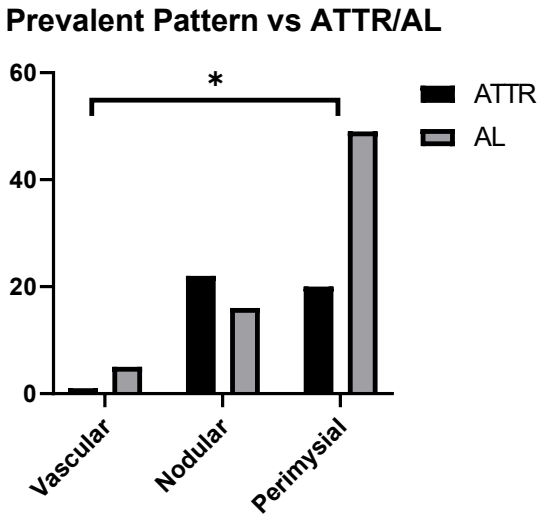


Figure 12 *Prevalent pattern of distribution in AL and ATTR amyloidosis.*

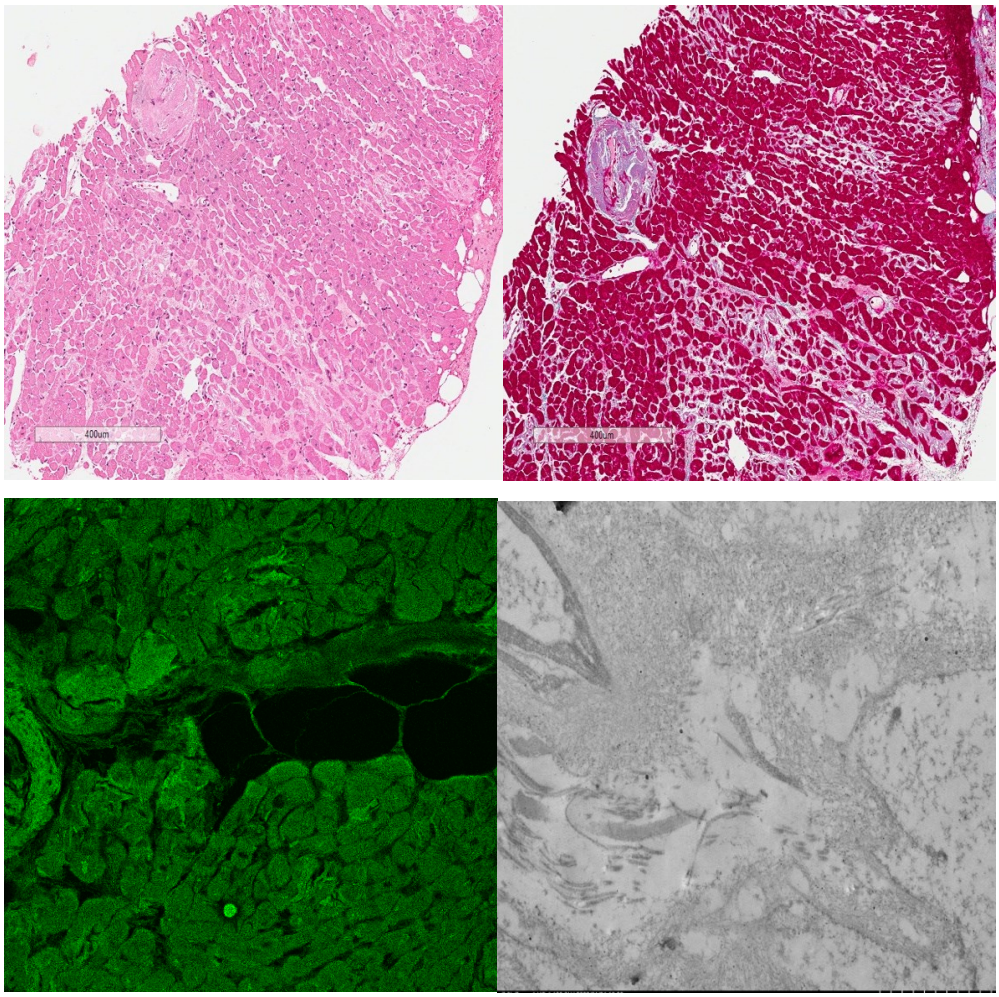


Figure 13 *Prevalent vascular pattern in a 63-years-old man with AL κ+. A,B: the vessel wall is thickened by amorphous eosinophil amyloid associated (A: H&E, 4x; B:Azan-Mallory, 4x). C: ThT highlights the vessel wall with a green fluorescence signal (10x). D: amyloid fibrils in a vessel wall (IEM, scale bar 2 µm)*

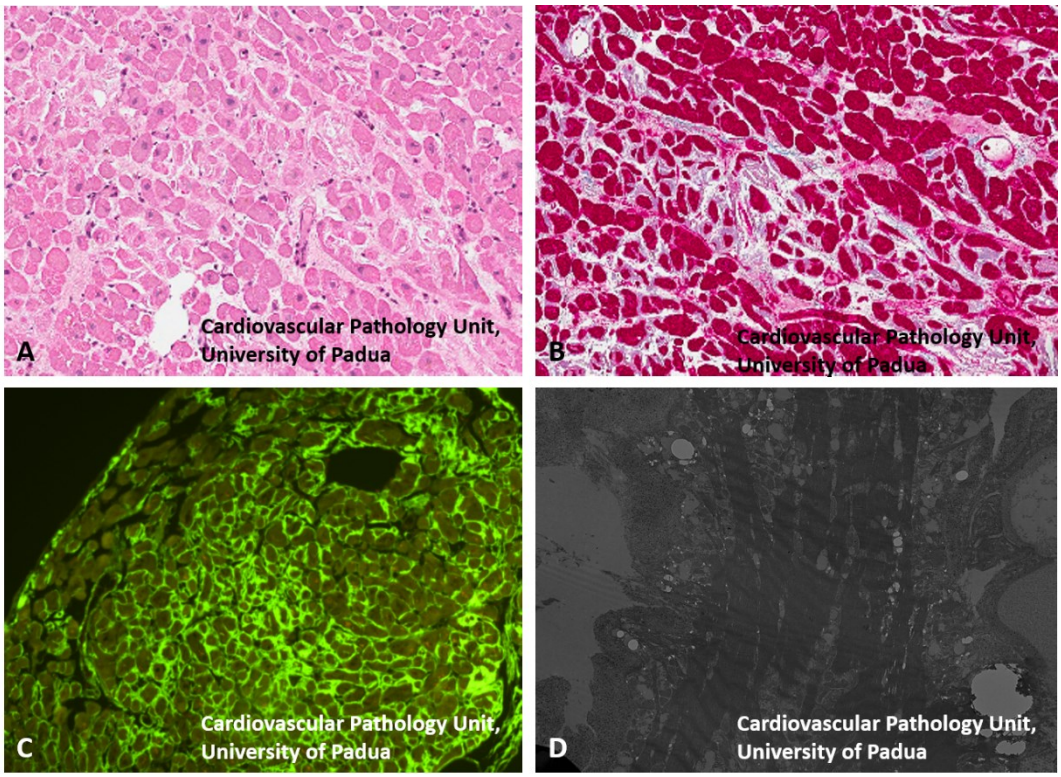


Figure 14 *AL λ+ amyloidosis in a 69-years-old woman. Perimysial prevalent pattern. A: in H&E amyloid appears as an eosinophil amorphous net surrounding the cardiomyocytes (4x). B: Azan-Mallory (4x). C: ThT clearly shows the amyloid net (2x). D: the interstitium is positive to anti- λ Ab surrounding the cardiomyocyte (IEM)*

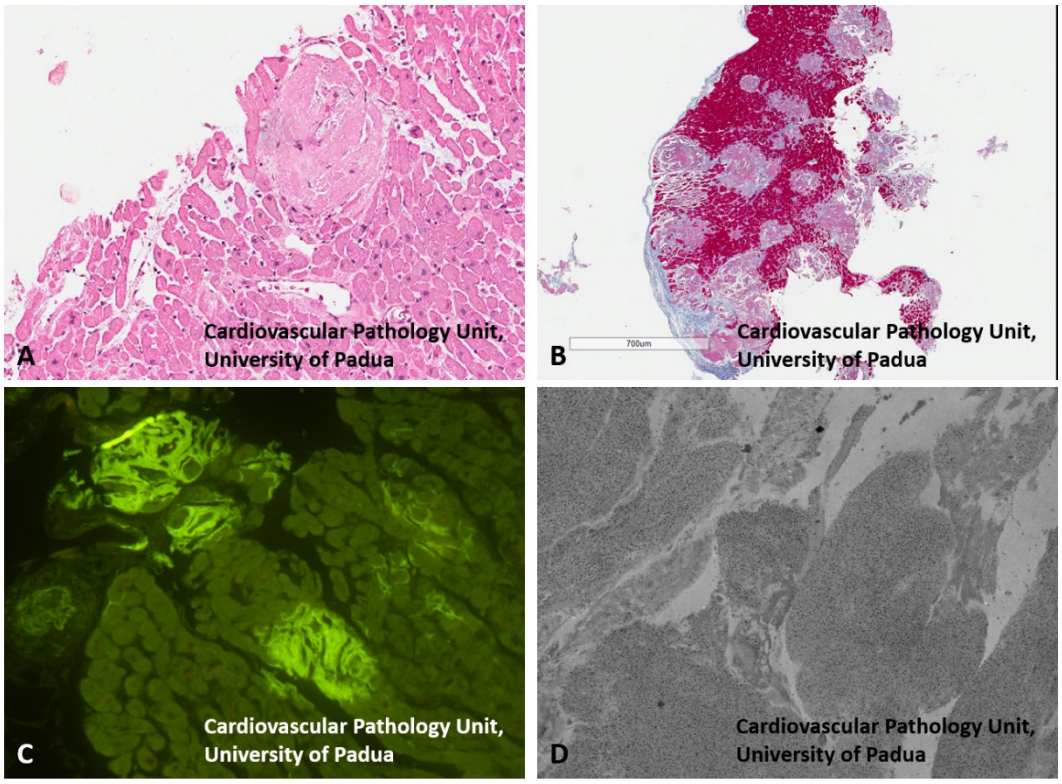


Figure 15 *ATTR amyloidosis in a 76-years-old man. Nodular prevalent pattern of deposition. A: under H&E the nodule appears as an irregular spheroidal deposit substituting the myocardial tissue (4x). B: a multitude of nodules is visible in Azan-Mallory even at small magnification (2x). C: ThT better highlights amyloid deposits in a nodular pattern with a green fluorescence signal (10x). D: nodules are positive for anti-ATTR Ab (IEM).*

No statistically significant difference was observed regarding burden and pattern of deposition comparing AL and ATTR cases of amyloidosis, but AL λ+ cases had a higher burden, with a median of 43% and a mean of 38%, than AL κ+ cases, with a median of 33% and a mean of 27%, determining a P value of 0.033 (Fig. 16) (<0.05). Difference between amyloid subtypes in replacement-type fibrosis burden and in endocardium involvement were not found.

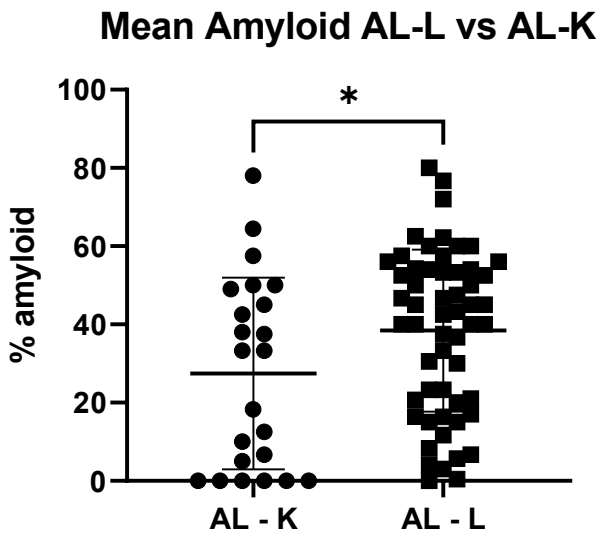


Figure 16 *Burden (percentage of amyloid within the tissue) in AL λ+ and AL κ+ cases*

3. **To evaluate the diagnostic value in cardiac amyloidosis of abdominal fat pad excisional biopsy (FPEB) vs EMB, when both are performed.**

In the cohort of 171 cases taken into consideration, 36 patients (21%) underwent also abdominal fat biopsy, before EMB. For the following results, IEM means are taken into account. In 27 (75%) cases, EMB was positive, 21 of them resulting false negative at FPEB, which was instead positive in 8 (22%) cases (Fig. 17), 2 of them escaping diagnosis performed on EMB, probably because of sampling error (Table 9).

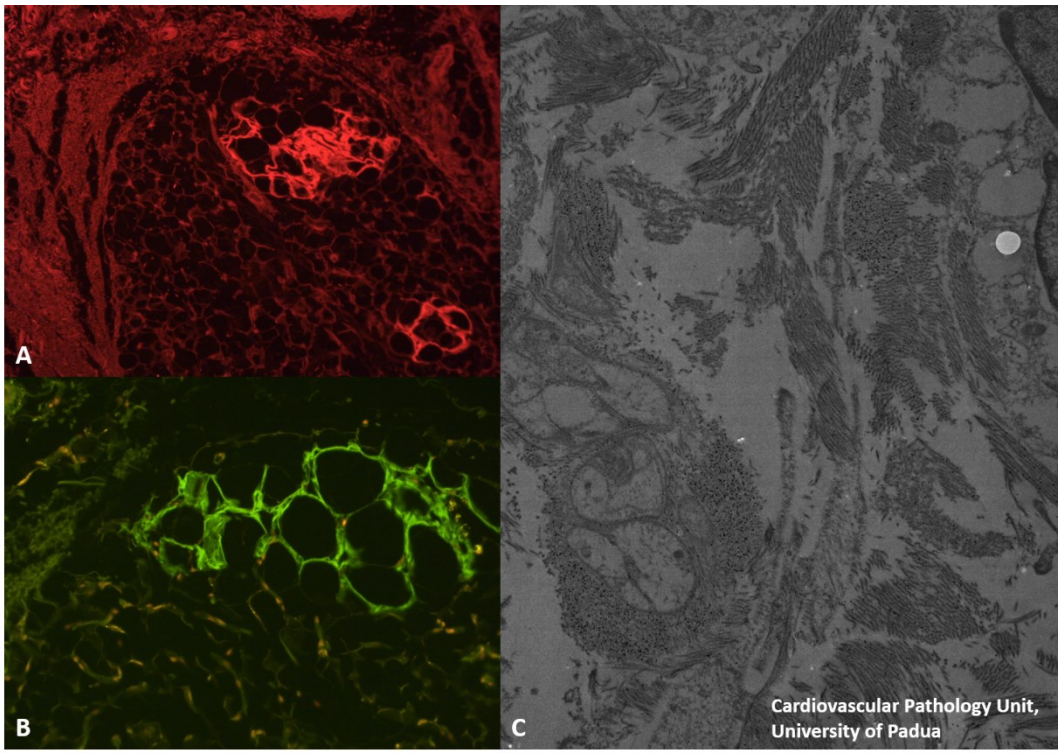


Figure 17 *FPEB positive for AL λ+ amyloidosis in a 71-years-old woman. A: Congo-Red is positive for amyloid deposits (red fluorescence in CLSM), stocked between adipocytes (4x). B: a green fluorescence signals the presence of amyloid (ThT, CLSM, 10x). C: amyloid deposits are positive to anti- λ Ab (IEM)*

Table 9 EMB vs FPEB			
	EMB -	**EMB +**	**Tot**
FPEB -	7	**21**	28
FPEB +	2	6	8
Tot	9	27	36

It follows that FPEB sensitivity (27.6%) was significantly inferior to the EMB one (93.1%). FPEB sensitivity was found resulting from different sensitivities in ATTR (12.5%) and AL (27.8%) cases (Fig.13).

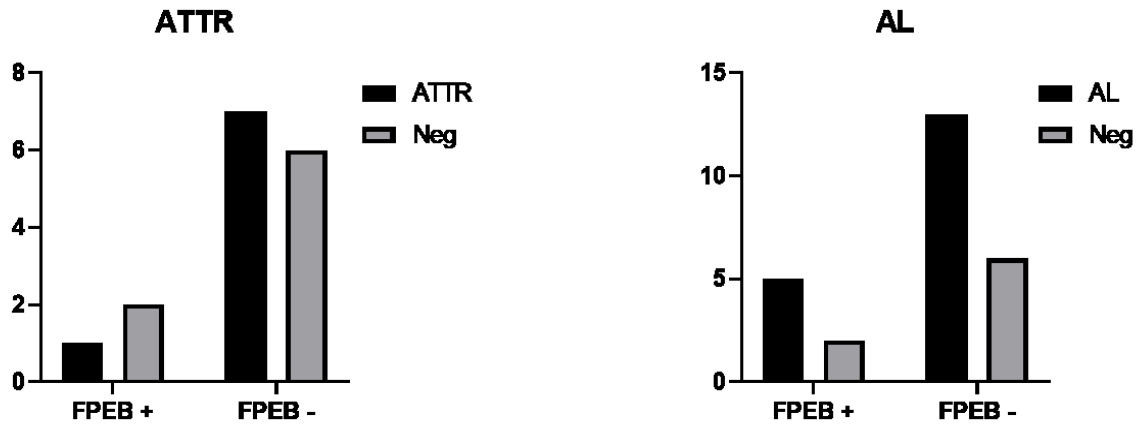


Figure 9 *FPEB diagnosis in ATTR and AL amyloidosis. The FPEB is negative for TN cases (in grey) and FN cases (in black), which are consistent. TP cases are more in AL amyloidosis, witnessing a higher (but still insufficient) sensibility for this type. FPEB positivity occurs in both positive and negative patients, so it is not a good indicator for making a diagnosis. Sensitivity and specificity are low.*

4. Clinical-pathological correlation in cases of cardiac amyloidosis studied at a single center, Azienda Ospedaliera-University of Padua

This retrospective study reviewed 40 patients from the archives of the Cardiology Unit, Azienda Ospedaliera-University of Padua, underwent to EMB for clinical suspicion of cardiac amyloidosis. Some statistically significant correlations were found. In a population of 19 patients that underwent coronarography, the presence of severe coronary artery disease, was more frequent in ATTR (21%) than in AL (11%) cases, with a P value of 0.046 (Fig.18).

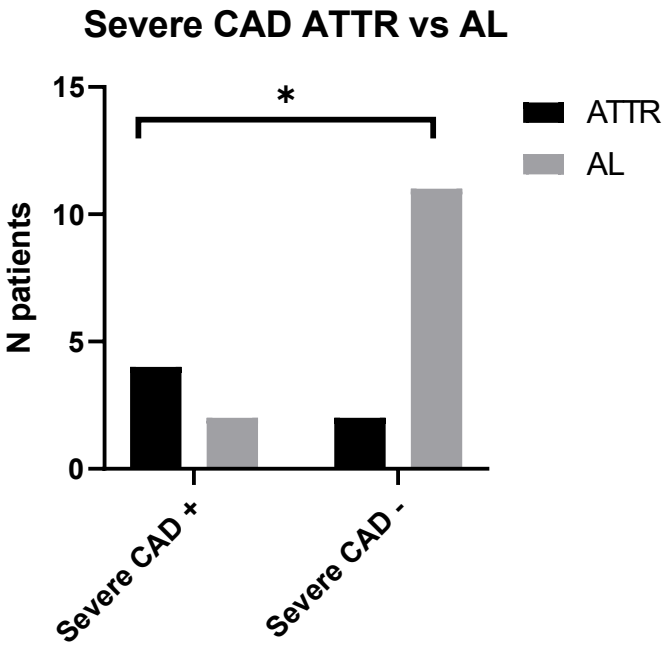


Figure 10 *Severe CAD distribution in ATTR and AL amyloidosis.*

In the of 31 patients in which left atrial volume was measured, its value is more altered in ATTR cases, with a mean of 57.0±14.7 ml/m^2, than in AL cases, with a mean of 42.0±12.1 ml/m^2 (P value of 0.017) (Fig. 19).

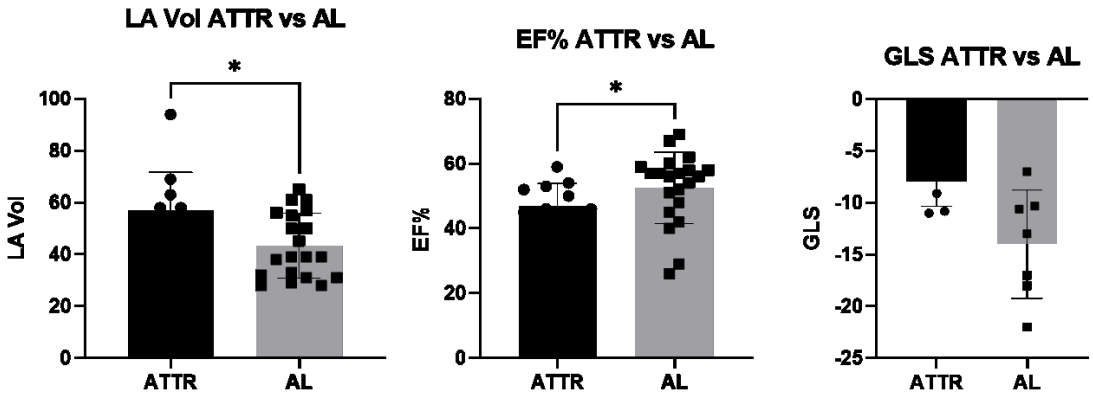


Figure 19 *LA volume, LVEF and GLS distribution in ATTR and AL amyloidosis. The presence of ATTR is more associated with structural damage (LA volume) and with functional impairment (EF and GLS).*

The left ventricle ejection fraction was measured in 32 patients and it was more reduced in ATTR cases, with a mean of 47.0±7.0%, than in AL cases, with a mean of 53.0±11.2% (P value of 0.043) (Fig. 19). Left ventricle GLS, considered in a population of 14 patients, was instead worse in AL cases, with a mean of -14.0±5.0%, than in ATTR cases, with a mean of -8.0±2.0% (P value of 0.037). Statistically significant and non-significant correlations are shown in the Table 10.

Table 10 Clinical-pathological correlations

Variable	n° cases	ATTR	AL	P value
Severe CAD	19	21%	11%	0.046
LA vol	31	57 ml/m^2	42 ml/m^2	0.017
LVEF	32	47%	53%	0.043
LV GLS	14	-8%	-14%	0.037
TnI	16	0,14 µg/l	0.12 µg/l	0.739
BNP	10	443 pg/ml	1189 pg/ml	0.309
NTproBNP	26	6353 pg/ml	7214 pg/ml	0.910
NYHA	13	2	2	0.684
ICD	18	17%	22%	0.326
AF	21	29%	29%	0.159
HTN	21	29%	24%	0.064
DM	18	6%	6%	0.490

DISCUSSION

Amyloidosis is characterized by local, organ-limited or generalized, proteinaceous fibrillar deposits of autologous origin.
Three different proteins, responsible for the 98% of all cases of CA, are AL (kappa and lambda light chains), and ATTR (transthyretin). On the basis of a timely clinical suspicion, diagnosis and classification of amyloidosis require prompt histological proof and typing for appropriate treatment.

Light microscopy and IEM

The first aim of this study was to summarize and demonstrate our experience in the recognition and typing of cardiac amyloidosis on EMBs, and to identify diagnostic pitfalls and future requirements. IEM is an excellent and reliable technique. IEM is considered, instead of classic histology and IHC, a diagnostic gold-standard, besides LC–MS/MS; both have high sensitivity and specificity. In the literature [127], they are considered compatible means of diagnosis, since where the sensibility of the one is lightly lower, the other can compensate, reaching a sensitivity near to 100%. IEM itself is capable of providing sensitivity and specificity values, near to 100%. These values were optimal because of the combined use of stains specific for amyloid, which improves the diagnostic sensibility. In fact, a critical topic in clinic-pathological practice is the false negative diagnosis of amyloid. The sensitivity of histopathology examination can be increased by the combined application of Congo red and thioflavin stains and immunogold examination to the same specimen.
In addition, in our study, IEM has proven to be an excellent typing technique, establishing the type of amyloidosis in 100% of cases. Although many methods of amyloid typing are available (immunofluorescence, immunogold, mass spectrometry), in our experience IEM represents the most reliable technique for the diagnosis and the characterization of cardiac amyloidosis.

The number of fragments

In the clinical practice, a variable number of fragments is taken from patients. There is no statistically significant association between the number of fragments on biopsy and a positive diagnosis. This means that it is not necessary to take a large number of cardiac samples to examine, as this does not increase the diagnostic sensitivity. These data are relevant to change clinical practice. Nonetheless, performing too few fragments would lead to false negative results; we recommend the sampling of 3-4 fragments for each patient.

Patterns of distribution

Many data have been collected about the burden of amyloidosis and the patterns of distribution. These data, although statistically significant, are not diagnostic. For instance, the rarest distribution pattern was found to be vascular. However, it does not allow differentiation between the two main types of CA, although they are more common in the AL forms. Similarly, the demonstration of the presence of prevalent distribution patterns based on the type of amyloidosis does not point towards typing, which necessarily occurs via IEM.

FPEB

The third diagnostic objective was to demonstrate the different performance of FPEB and EMB in early detection of CA. The importance of this part of the study lies in the high number of FPEB performed by many institutions throughout Italy instead of EMB, due to the less invasiveness in case of suspicion of cardiac amyloidosis. In the literature, for these reasons, the most suitable biopsy site is said the umbilical fat, which is said to be positive in all patients suspected to have cardiac amyloidosis and useful to characterize the disease through means of IEM in 100% of cases [55]. Instead, in our study, showing that FPEBs have a significantly lower sensitivity than EMBs, we demonstrated how dangerous it can be to trust FPEB as a surrogate tissue; is a strategically inferior technique compared to the affected organ sampling. Screening patients with clinical suspicion through FPEB (and probably other surrogate tissues) would involve an important risk of false negative

results, with serious consequences on patients' health, due to the diagnostic delay and therefore the lack of timely treatment.
We also showed that, even though the sensitivity of FPEB for AL is 2 times greater than for ATTR, it remains very low (27.8%).
Based upon the results of this study, it appears more reasonable to recommend the execution of EMB to all patients with suspected cardiac amyloidosis, in order to avoid a high number of false negative results. Choosing the correct tissue to biopsy is crucial to avoid false negatives and delays to diagnosis.

Clinical-pathological correlation

The fourth aim demonstrated that the appropriate classification of cardiac amyloid syndrome could only be achieved by collaboration between clinician and pathologist. Amyloidosis associates with some specific clinical features that have made it easier for clinicians to raise a suspicion of disease.
The rather high frequency (74%) of IEM-positive patients selected on the basis of clinical suspicion, suggests that the latter plays a fundamental role in the diagnostic pathway.
It can be noted that ATTR is more often associated with greater structural (left atrial volume) and functional (LVEF and GLS) cardiac damage than AL. Noteworthy is also the concordance between LVEF and GLS, both indices of left ventricle systolic function and both more severe in case of ATTR amyloidosis.
Although significant differences in the distribution of amyloid type by sex are not reported in literature [141], in our centre we have a significantly higher prevalence of disease in males, mainly characterised by ATTR, whereas in females an AL lambda type pattern predominates.

FUTURE DIRECTIONS

A possible future study could consider all the clinical information and perform all the diagnostic tests rising the suspicion of cardiac amyloidosis (bio-humoral tests, immunofixation, ultrasound, magnetic resonance and scintigraphy imaging), in order to better detect the associations between them and a diagnosis performed with IEM applied to EMB. It could be possible to elaborate a clinical score, capable of establishing clinical suspicion with high sensitivity and specificity. For example,

the presence of a patient with severe coronary artery disease could lead to a more accurate clinical suspicion of ATTR, but this requires many other parameters that contribute to the same result in order to give a diagnosis indication. A hypothesis is that a combination of this kind of results could lead to a high quality clinical score for amyloidosis suspicion. The presence of a strong clinical suspicion is essential in order to perform on time assessment tests and therefore to reach an early diagnosis of amyloidosis, fundamental for treatment and prognosis of the disease.

CONCLUSIONS

IEM can be used to diagnose and characterize or rule out cardiac amyloidosis with good sensitivity and excellent specificity in a clinical routine setting. During the EMB process, it is not necessary to sample a large number of fragments to examine, as this does not increase the diagnostic sensitivity. At light microscopy analysis, which should be performed with a combination of stains specific for amyloidosis, the patterns of distribution are vascular and interstitial (perimysial and nodular); they can be useful to recognize amyloidosis, but not to suspect the type. Localized amyloidosis can only be diagnosed by biopsy of the affected organ or tissue. Choosing the correct tissue to biopsy is crucial to avoid false negatives and delays in the diagnostic process. FPEB sensibility is low even in AL cases.
Appropriate classification of cardiac amyloidosis syndrome can only be achieved by collaboration between clinicians and pathologists.

REFERENCES

[1] M. Giampaolo and B. Vittorio, “Molecular Mechanisms of Amyloidosis,” *N. Engl. J. Med.*, 2003.

[2] A. Aimo *et al.*, “RNA-targeting and gene editing therapies for transthyretin amyloidosis,” *Nat. Rev. Cardiol.*, vol. 19, no. 10, pp. 655–667, Oct. 2022, doi: 10.1038/s41569-022-00683-z.

[3] M. S. Maurer *et al.*, “Expert Consensus Recommendations for the Suspicion and Diagnosis of Transthyretin Cardiac Amyloidosis,” *Circ. Heart Fail.*, vol. 12, no. 9, p. e006075, Sep. 2019, doi: 10.1161/CIRCHEARTFAILURE.119.006075.

[4] O. B. Suhr, E. Lundgren, and P. Westermark, “One mutation, two distinct disease variants: unravelling the impact of transthyretin amyloid fibril composition,” *J. Intern. Med.*, vol. 281, no. 4, pp. 337–347, Apr. 2017, doi: 10.1111/joim.12585.

[5] A. V. Kristen *et al.*, “Cardiac Amyloid Load,” *J. Am. Coll. Cardiol.*, vol. 68, no. 1, pp. 13–24, Jul. 2016, doi: 10.1016/j.jacc.2016.04.035.

[6] B. Wisniowski and A. Wechalekar, “Confirming the Diagnosis of Amyloidosis,” *Acta Haematol.*, vol. 143, no. 4, pp. 312–321, 2020, doi: 10.1159/000508022.

[7] C. S. Siegismund *et al.*, “Intramyocardial inflammation predicts adverse outcome in patients with cardiac AL amyloidosis,” *Eur. J. Heart Fail.*, vol. 20, no. 4, pp. 751–757, Apr. 2018, doi: 10.1002/ejhf.1039.

[8] J. D. Sipe and A. S. Cohen, “Review: History of the Amyloid Fibril,” *J. Struct. Biol.*, vol. 130, no. 2, pp. 88–98, Jun. 2000, doi: 10.1006/jsbi.2000.4221.

[9] J. J. Maleszewski, “Cardiac amyloidosis: pathology, nomenclature, and typing,” *Cardiovasc. Pathol.*, vol. 24, no. 6, pp. 343–350, Nov. 2015, doi: 10.1016/j.carpath.2015.07.008.

[10] S. A. Craig, A. M. McDonald, D. J. Manners, and J. R. Stark, “The iodine-staining properties and fine structure of some mammalian and invertebrate glycogens,” *Carbohydr. Res.*, vol. 179, pp. 327–340, Aug. 1988, doi: 10.1016/0008-6215(88)84129-6.

[11] “Light and Color - Optical Birefringence | Olympus LS.” https://www.olympus-lifescience.com/en/microscope-resource/primer/lightandcolor/birefringence/ (accessed Apr. 14, 2023).

[12] A. LaPelusa and R. Kaushik, “Physiology, Proteins,” in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2023. Accessed: May 09, 2023. [Online]. Available: http://www.ncbi.nlm.nih.gov/books/NBK555990/

[13] F. Chiti and C. M. Dobson, “Protein Misfolding, Amyloid Formation, and Human Disease: A Summary of Progress Over the Last Decade,” *Annu. Rev.*

Biochem., vol. 86, pp. 27–68, Jun. 2017, doi: 10.1146/annurev-biochem-061516-045115.

[14] G. Merlini, D. C. Seldin, and M. A. Gertz, "Amyloidosis: pathogenesis and new therapeutic options," *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.*, vol. 29, no. 14, pp. 1924–1933, May 2011, doi: 10.1200/JCO.2010.32.2271.

[15] M. A. Gertz and A. Dispenzieri, "Systemic Amyloidosis Recognition, Prognosis, and Therapy: A Systematic Review," *JAMA*, vol. 324, no. 1, pp. 79–89, Jul. 2020, doi: 10.1001/jama.2020.5493.

[16] D. Otzen and R. Riek, "Functional Amyloids," *Cold Spring Harb. Perspect. Biol.*, vol. 11, no. 12, p. a033860, Dec. 2019, doi: 10.1101/cshperspect.a033860.

[17] "Definition of amyloidosis - NCI Dictionary of Cancer Terms - NCI," Feb. 02, 2011. https://www.cancer.gov/publications/dictionaries/cancer-terms/def/amyloidosis (accessed May 09, 2023).

[18] A. Dogan, "Amyloidosis: Insights from Proteomics," *Annu. Rev. Pathol. Mech. Dis.*, vol. 12, no. 1, pp. 277–304, Jan. 2017, doi: 10.1146/annurev-pathol-052016-100200.

[19] R. Al Hamed, A. H. Bazarbachi, A. Bazarbachi, F. Malard, J.-L. Harousseau, and M. Mohty, "Comprehensive Review of AL amyloidosis: some practical recommendations," *Blood Cancer J.*, vol. 11, no. 5, p. 97, May 2021, doi: 10.1038/s41408-021-00486-4.

[20] R. H. Falk, R. L. Comenzo, and M. Skinner, "The systemic amyloidoses," *N. Engl. J. Med.*, vol. 337, no. 13, pp. 898–909, Sep. 1997, doi: 10.1056/NEJM199709253371306.

[21] S. Girnius, "Overview of systemic and localized amyloidosis," *Rev. Health Care*, vol. 4, no. 4, Art. no. 4, Oct. 2013, doi: 10.7175/rhc.v4i4.662.

[22] M. M. Picken, "Immunoglobulin light and heavy chain amyloidosis AL/AH: renal pathology and differential diagnosis," *Contrib. Nephrol.*, vol. 153, pp. 135–155, 2007, doi: 10.1159/000096765.

[23] S. W. Dubrey *et al.*, "The clinical features of immunoglobulin light-chain (AL) amyloidosis with heart involvement," *QJM Mon. J. Assoc. Physicians*, vol. 91, no. 2, pp. 141–157, Feb. 1998, doi: 10.1093/qjmed/91.2.141.

[24] "Serum cardiac troponins and N-terminal pro-brain natriuretic peptide: a staging system for primary systemic amyloidosis - PubMed." https://pubmed.ncbi.nlm.nih.gov/15365071/ (accessed May 11, 2023).

[25] I. U.-A. R. RESERVED, "Orphanet: AH amyloidosis." https://www.orpha.net/consor/cgi-bin/OC_Exp.php?lng=EN&Expert=442582 (accessed May 11, 2023).

[26] H. J. Lachmann *et al.*, "Natural history and outcome in systemic AA amyloidosis," *N. Engl. J. Med.*, vol. 356, no. 23, pp. 2361–2371, Jun. 2007, doi: 10.1056/NEJMoa070265.

[27] T. Coelho *et al.*, “Mechanism of Action and Clinical Application of Tafamidis in Hereditary Transthyretin Amyloidosis,” *Neurol. Ther.*, vol. 5, no. 1, pp. 1–25, Jun. 2016, doi: 10.1007/s40120-016-0040-x.

[28] Y. Sekijima, “Transthyretin (ATTR) amyloidosis: clinical spectrum, molecular pathogenesis and disease-modifying treatments,” *J. Neurol. Neurosurg. Psychiatry*, vol. 86, no. 9, pp. 1036–1043, Sep. 2015, doi: 10.1136/jnnp-2014-308724.

[29] H. Koike and M. Katsuno, “Transthyretin Amyloidosis: Update on the Clinical Spectrum, Pathogenesis, and Disease-Modifying Therapies,” *Neurol. Ther.*, vol. 9, no. 2, pp. 317–333, Dec. 2020, doi: 10.1007/s40120-020-00210-7.

[30] G. G. Glenner and M. A. Murphy, “Amyloidosis of the nervous system,” *J. Neurol. Sci.*, vol. 94, no. 1–3, pp. 1–28, Dec. 1989, doi: 10.1016/0022-510x(89)90214-1.

[31] F. J. Rodriguez, M. M. Picken, and J. M. Lee, “Amyloid Deposition in the Central Nervous System,” in *Amyloid and Related Disorders: Surgical Pathology and Clinical Correlations*, M. M. Picken, G. A. Herrera, and A. Dogan, Eds., in Current Clinical Pathology. Cham: Springer International Publishing, 2015, pp. 121–131. doi: 10.1007/978-3-319-19294-9_8.

[32] P. P. Liberski, “Historical overview of prion diseases: a view from afar,” *Folia Neuropathol.*, vol. 50, no. 1, pp. 1–12, 2012.

[33] M. D. Benson *et al.*, “Amyloid nomenclature 2018: recommendations by the International Society of Amyloidosis (ISA) nomenclature committee,” *Amyloid Int. J. Exp. Clin. Investig. Off. J. Int. Soc. Amyloidosis*, vol. 25, no. 4, pp. 215–219, Dec. 2018, doi: 10.1080/13506129.2018.1549825.

[34] P. Garcia-Pavia *et al.*, “Diagnosis and treatment of cardiac amyloidosis: a position statement of the ESC Working Group on Myocardial and Pericardial Diseases,” *Eur. Heart J.*, vol. 42, no. 16, pp. 1554–1568, Apr. 2021, doi: 10.1093/eurheartj/ehab072.

[35] D. Real de Asúa, R. Costa, J. M. Galván, M. T. Filigheddu, D. Trujillo, and J. Cadiñanos, “Systemic AA amyloidosis: epidemiology, diagnosis, and management,” *Clin. Epidemiol.*, vol. 6, pp. 369–377, Oct. 2014, doi: 10.2147/CLEP.S39981.

[36] E. González-López *et al.*, “Wild-type transthyretin amyloidosis as a cause of heart failure with preserved ejection fraction,” *Eur. Heart J.*, vol. 36, no. 38, pp. 2585–2594, Oct. 2015, doi: 10.1093/eurheartj/ehv338.

[37] T. Damy *et al.*, “Prevalence and clinical phenotype of hereditary transthyretin amyloid cardiomyopathy in patients with increased left ventricular wall thickness,” *Eur. Heart J.*, vol. 37, no. 23, pp. 1826–1834, Jun. 2016, doi: 10.1093/eurheartj/ehv583.

[38] M. Boldrini *et al.*, “Multiparametric Echocardiography Scores for the Diagnosis of Cardiac Amyloidosis,” *JACC Cardiovasc. Imaging*, vol. 13, no. 4, pp. 909–920, Apr. 2020, doi: 10.1016/j.jcmg.2019.10.011.

[39] R. H. Falk, “Diagnosis and Management of the Cardiac Amyloidoses,” *Circulation*, vol. 112, no. 13, pp. 2047–2060, Sep. 2005, doi: 10.1161/CIRCULATIONAHA.104.489187.

[40] S. Sachchithanantham and A. D. Wechalekar, “Imaging in systemic amyloidosis,” *Br. Med. Bull.*, vol. 107, pp. 41–56, 2013, doi: 10.1093/bmb/ldt021.

[41] A. Ali, D. A. Turner, S. W. Rosenbush, and E. W. Fordham, “Bone scintigram in cardiac amyloidosis: a case report,” *Clin. Nucl. Med.*, vol. 6, no. 3, pp. 105–108, Mar. 1981, doi: 10.1097/00003072-198103000-00003.

[42] D. F. Hutt *et al.*, “Prognostic utility of the Perugini grading of 99mTc-DPD scintigraphy in transthyretin (ATTR) amyloidosis and its relationship with skeletal muscle and soft tissue amyloid,” *Eur. Heart J. Cardiovasc. Imaging*, vol. 18, no. 12, pp. 1344–1350, Dec. 2017, doi: 10.1093/ehjci/jew325.

[43] J. D. Gillmore *et al.*, “Nonbiopsy Diagnosis of Cardiac Transthyretin Amyloidosis,” *Circulation*, vol. 133, no. 24, pp. 2404–2412, Jun. 2016, doi: 10.1161/CIRCULATIONAHA.116.021612.

[44] S. Bokhari, A. Castaño, T. Pozniakoff, S. Deslisle, F. Latif, and M. S. Maurer, “(99m)Tc-pyrophosphate scintigraphy for differentiating light-chain cardiac amyloidosis from the transthyretin-related familial and senile cardiac amyloidoses,” *Circ. Cardiovasc. Imaging*, vol. 6, no. 2, pp. 195–201, Mar. 2013, doi: 10.1161/CIRCIMAGING.112.000132.

[45] G. Palladini *et al.*, “Identification of amyloidogenic light chains requires the combination of serum-free light chain assay with immunofixation of serum and urine,” *Clin. Chem.*, vol. 55, no. 3, pp. 499–504, Mar. 2009, doi: 10.1373/clinchem.2008.117143.

[46] B. Sprangers *et al.*, “Comparison of 2 Serum-Free Light-Chain Assays in CKD Patients,” *Kidney Int. Rep.*, vol. 5, no. 5, pp. 627–631, Jan. 2020, doi: 10.1016/j.ekir.2020.01.019.

[47] Á. López-Sainz *et al.*, “Clinical profile and outcome of cardiac amyloidosis in a Spanish referral center,” *Rev. Espanola Cardiol. Engl. Ed*, vol. 74, no. 2, pp. 149–158, Feb. 2021, doi: 10.1016/j.rec.2019.12.020.

[48] A. Pucci *et al.*, “Amyloid Deposits and Fibrosis on Left Ventricular Endomyocardial Biopsy Correlate With Extracellular Volume in Cardiac Amyloidosis,” *J. Am. Heart Assoc.*, vol. 10, no. 20, p. e020358, Oct. 2021, doi: 10.1161/JAHA.120.020358.

[49] P. Mollee, P. Renaut, D. Gottlieb, and H. Goodman, “How to diagnose amyloidosis: How to diagnose amyloidosis,” *Intern. Med. J.*, vol. 44, no. 1, pp. 7–17, Jan. 2014, doi: 10.1111/imj.12288.

[50] V. Musetti *et al.*, “Tissue Characterization in Cardiac Amyloidosis,” *Biomedicines*, vol. 10, no. 12, p. 3054, Nov. 2022, doi: 10.3390/biomedicines10123054.

[51] J. D. Gillmore *et al.*, “Guidelines on the diagnosis and investigation of AL amyloidosis,” *Br. J. Haematol.*, vol. 168, no. 2, pp. 207–218, Jan. 2015, doi: 10.1111/bjh.13156.

[52] “A New Method for the Diagnosis of Systemic Amyloidosis | JAMA Internal Medicine | JAMA Network.” https://jamanetwork.com/journals/jamainternalmedicine/article-abstract/581915 (accessed Apr. 24, 2023).

[53] A. Aimo, M. Emdin, V. Musetti, A. Pucci, and G. Vergaro, “Abdominal Fat Biopsy for the Diagnosis of Cardiac Amyloidosis,” *JACC Case Rep.*, vol. 2, no. 8, pp. 1182–1185, Jul. 2020, doi: 10.1016/j.jaccas.2020.05.062.

[54] C. Fernández de Larrea *et al.*, “A practical approach to the diagnosis of systemic amyloidoses,” *Blood*, vol. 125, no. 14, pp. 2239–2244, Apr. 2015, doi: 10.1182/blood-2014-11-609883.

[55] E. Arbustini, L. Verga, M. Concardi, G. Palladini, L. Obici, and G. Merlini, “Electron and immuno-electron microscopy of abdominal fat identifies and characterizes amyloid fibrils in suspected cardiac amyloidosis: Original Articles,” *Amyloid*, vol. 9, no. 2, pp. 108–114, Jan. 2002, doi: 10.3109/13506120208995243.

[56] Y. Garcia, A. B. Collins, and J. R. Stone, “Abdominal fat pad excisional biopsy for the diagnosis and typing of systemic amyloidosis,” *Hum. Pathol.*, vol. 72, pp. 71–79, Feb. 2018, doi: 10.1016/j.humpath.2017.11.001.

[57] N. M. Fine *et al.*, “Yield of Noncardiac Biopsy for the Diagnosis of Transthyretin Cardiac Amyloidosis,” *Am. J. Cardiol.*, vol. 113, no. 10, pp. 1723–1727, May 2014, doi: 10.1016/j.amjcard.2014.02.030.

[58] K. Hummel, H. Meawad, W. T. Gunning, and A. F. Gohara, “Negative Fat Pad Biopsy in Systemic AL: A Case Report Analyzing the Preferred Amyloidosis Screening Test,” *Diseases*, vol. 9, no. 2, p. 40, May 2021, doi: 10.3390/diseases9020040.

[59] “Diagnostic value of fat aspirates for amyloidosis in 950 patients | Orphanet Journal of Rare Diseases | Full Text.” https://ojrd.biomedcentral.com/articles/10.1186/1750-1172-10-S1-P50 (accessed Apr. 24, 2023).

[60] M. E. Billingham *et al.*, “A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation,” *J. Heart Transplant.*, vol. 9, no. 6, pp. 587–593, 1990.

[61] J. E. Parrillo, H. T. Aretz, I. Palacios, J. T. Fallon, and P. C. Block, “The results of transvenous endomyocardial biopsy can frequently be used to diagnose

myocardial diseases in patients with idiopathic heart failure. Endomyocardial biopsies in 100 consecutive patients revealed a substantial incidence of myocarditis," *Circulation*, vol. 69, no. 1, pp. 93–101, Jan. 1984, doi: 10.1161/01.cir.69.1.93.

[62] C. Basso, D. Corrado, F. I. Marcus, A. Nava, and G. Thiene, "Arrhythmogenic right ventricular cardiomyopathy," *Lancet Lond. Engl.*, vol. 373, no. 9671, pp. 1289–1300, Apr. 2009, doi: 10.1016/S0140-6736(09)60256-7.

[63] A. Poletti, P. Cocco, M. Valente, G. Fasoli, R. Chioin, and G. Thiene, "In vivo diagnosis of cardiac angiosarcoma by endomyocardial biopsy," *Cardiovasc. Pathol. Off. J. Soc. Cardiovasc. Pathol.*, vol. 2, no. 1, pp. 89–91, 1993, doi: 10.1016/1054-8807(93)90018-W.

[64] J. P. Veinot, "Diagnostic endomyocardial biopsy pathology: secondary myocardial diseases and other clinical indications - a review," *Can. J. Cardiol.*, vol. 18, no. 3, pp. 287–296, Mar. 2002.

[65] G. M. Felker, W. Hu, J. M. Hare, R. H. Hruban, K. L. Baughman, and E. K. Kasper, "The spectrum of dilated cardiomyopathy. The Johns Hopkins experience with 1,278 patients," *Medicine (Baltimore)*, vol. 78, no. 4, pp. 270–283, Jul. 1999, doi: 10.1097/00005792-199907000-00005.

[66] A. Pawlak, E. Walczak, R. J. Gil, T. Wagner, J. Rzezak, and P. Seweryniak, "Is diagnostic myocardial biopsy useful in the XXI century?," *Kardiol. Pol.*, vol. 62, no. 4, pp. 360–371; discussion 371, Apr. 2005.

[67] O. Leone *et al.*, "[Consensus document on endomyocardial biopsy of the Associazione per la Patologia Cardiovascolare Italiana]," *G. Ital. Cardiol. 2006*, vol. 10, no. 9 Suppl 1, pp. 3S-50S, Sep. 2009.

[68] O. Leone *et al.*, "2011 Consensus statement on endomyocardial biopsy from the Association for European Cardiovascular Pathology and the Society for Cardiovascular Pathology," *Cardiovasc. Pathol.*, vol. 21, no. 4, pp. 245–274, Jul. 2012, doi: 10.1016/j.carpath.2011.10.001.

[69] G. Thiene *et al.*, "AECVP and SCVP 2009 Recommendations for Training in Cardiovascular Pathology," *Cardiovasc. Pathol.*, vol. 19, no. 3, pp. 129–135, May 2010, doi: 10.1016/j.carpath.2009.12.001.

[70] A. P. Burke, A. Farb, M. Robinowitz, and R. Virmani, "Serial sectioning and multiple level examination of endomyocardial biopsies for the diagnosis of myocarditis," *Mod. Pathol. Off. J. U. S. Can. Acad. Pathol. Inc*, vol. 4, no. 6, pp. 690–693, Nov. 1991.

[71] F. Calabrese and G. Thiene, "Myocarditis and inflammatory cardiomyopathy: microbiological and molecular biological aspects," *Cardiovasc. Res.*, vol. 60, no. 1, pp. 11–25, Oct. 2003, doi: 10.1016/s0008-6363(03)00475-9.

[72] A. Perkan, A. Di Lenarda, and G. Sinagra, "[Dilated cardiomyopathy: indication and role of endomyocardial biopsy]," *Ital. Heart J. Suppl. Off. J. Ital. Fed. Cardiol.*, vol. 3, no. 4, pp. 419–425, Apr. 2002.

[73] P. J. Richardson, “King’s endomyocardial bioptome,” *Lancet Lond. Engl.*, vol. 1, no. 7859, pp. 660–661, Apr. 1974, doi: 10.1016/s0140-6736(74)93204-8.

[74] A. M. From, J. J. Maleszewski, and C. S. Rihal, “Current Status of Endomyocardial Biopsy,” *Mayo Clin. Proc.*, vol. 86, no. 11, pp. 1095–1102, Nov. 2011, doi: 10.4065/mcp.2011.0296.

[75] C. Blomström-Lundqvist, A. M. Noor, J. Eskilsson, and S. Persson, “Safety of transvenous right ventricular endomyocardial biopsy guided by two-dimensional echocardiography,” *Clin. Cardiol.*, vol. 16, no. 6, pp. 487–492, Jun. 1993, doi: 10.1002/clc.4960160606.

[76] A. D. Osterhaus *et al.*, “Transmission of hepatitis B virus among heart transplant recipients during endomyocardial biopsy procedures,” *J. Heart Lung Transplant. Off. Publ. Int. Soc. Heart Transplant.*, vol. 17, no. 2, pp. 158–166, Feb. 1998.

[77] A. Yilmaz *et al.*, “Comparative evaluation of left and right ventricular endomyocardial biopsy: differences in complication rate and diagnostic performance,” *Circulation*, vol. 122, no. 9, pp. 900–909, Aug. 2010, doi: 10.1161/CIRCULATIONAHA.109.924167.

[78] M. Holzmann *et al.*, “Complication rate of right ventricular endomyocardial biopsy via the femoral approach: a retrospective and prospective study analyzing 3048 diagnostic procedures over an 11-year period,” *Circulation*, vol. 118, no. 17, pp. 1722–1728, Oct. 2008, doi: 10.1161/CIRCULATIONAHA.107.743427.

[79] J. R. Stone *et al.*, “Recommendations for processing cardiovascular surgical pathology specimens: a consensus statement from the Standards and Definitions Committee of the Society for Cardiovascular Pathology and the Association for European Cardiovascular Pathology,” *Cardiovasc. Pathol. Off. J. Soc. Cardiovasc. Pathol.*, vol. 21, no. 1, pp. 2–16, 2012, doi: 10.1016/j.carpath.2011.01.001.

[80] S. R. Sompuram, K. Vani, E. Messana, and S. A. Bogen, “A Molecular Mechanism of Formalin Fixation and Antigen Retrieval,” *Am. J. Clin. Pathol.*, vol. 121, no. 2, pp. 190–199, Feb. 2004, doi: 10.1309/BRN7CTX1E84NWWPL.

[81] A. B. Collins, R. N. Smith, and J. R. Stone, “Classification of amyloid deposits in diagnostic cardiac specimens by immunofluorescence,” *Cardiovasc. Pathol.*, vol. 18, no. 4, pp. 205–216, Jul. 2009, doi: 10.1016/j.carpath.2008.05.004.

[82] T. B. Crotty, C.-Y. Li, W. D. Edwards, and V. J. Suman, “Amyloidosis and endomyocardial biopsy: Correlation of extent and pattern of deposition with amyloid immunophenotype in 100 cases,” *Cardiovasc. Pathol.*, vol. 4, no. 1, pp. 39–42, Jan. 1995, doi: 10.1016/1054-8807(94)00023-K.

[83] “Light and electron microscopy immunohistochemical characterization of amyloid deposits: Amyloid: Vol 4, No 3.”

https://www.tandfonline.com/doi/abs/10.3109/13506129709014380 (accessed Apr. 24, 2023).

[84] M. D. Benson, J. Breall, O. W. Cummings, and J. J. Liepnieks, "Biochemical characterisation of amyloid by endomyocardial biopsy," *Amyloid Int. J. Exp. Clin. Investig. Off. J. Int. Soc. Amyloidosis*, vol. 16, no. 1, pp. 9–14, Mar. 2009, doi: 10.1080/13506120802676914.

[85] J. A. Vrana, J. D. Gamez, B. J. Madden, J. D. Theis, H. R. Bergen, and A. Dogan, "Classification of amyloidosis by laser microdissection and mass spectrometry–based proteomic analysis in clinical biopsy specimens," *Blood*, vol. 114, no. 24, pp. 4957–4959, Dec. 2009, doi: 10.1182/blood-2009-07-230722.

[86] N. Swan, M. Skinner, and C. J. O'Hara, "Bone marrow core biopsy specimens in AL (primary) amyloidosis. A morphologic and immunohistochemical study of 100 cases," *Am. J. Clin. Pathol.*, vol. 120, no. 4, pp. 610–616, Oct. 2003, doi: 10.1309/PFUG-HBX0-TY20-E08U.

[87] T. Suzuki *et al.*, "Labial salivary gland biopsy for diagnosing immunoglobulin light chain amyloidosis: a retrospective analysis," *Ann. Hematol.*, vol. 95, no. 2, pp. 279–285, Jan. 2016, doi: 10.1007/s00277-015-2549-y.

[88] S. Freudenthaler, U. Hegenbart, S. Schönland, H.-M. Behrens, S. Krüger, and C. Röcken, "Amyloid in biopsies of the gastrointestinal tract—a retrospective observational study on 542 patients," *Virchows Arch.*, vol. 468, no. 5, pp. 569–577, May 2016, doi: 10.1007/s00428-016-1916-y.

[89] C. D. L. Fritz and E. Blaney, "Evaluation and Management Strategies for GI Involvement with Amyloidosis," *Am. J. Med.*, vol. 135, pp. S20–S23, Apr. 2022, doi: 10.1016/j.amjmed.2022.01.008.

[90] E. I. Yakupova, L. G. Bobyleva, I. M. Vikhlyantsev, and A. G. Bobylev, "Congo Red and amyloids: history and relationship," *Biosci. Rep.*, vol. 39, no. 1, p. BSR20181415, Jan. 2019, doi: 10.1042/BSR20181415.

[91] M. A. Stats and J. R. Stone, "Varying levels of small microcalcifications and macrophages in ATTR and AL cardiac amyloidosis: implications for utilizing nuclear medicine studies to subtype amyloidosis," *Cardiovasc. Pathol.*, vol. 25, no. 5, pp. 413–417, Sep. 2016, doi: 10.1016/j.carpath.2016.07.001.

[92] J. Bergström *et al.*, "Amyloid deposits in transthyretin-derived amyloidosis: cleaved transthyretin is associated with distinct amyloid morphology," *J. Pathol.*, vol. 206, no. 2, pp. 224–232, Jun. 2005, doi: 10.1002/path.1759.

[93] A. J. Howie, "'Green (or apple-green) birefringence' of Congo red-stained amyloid," *Amyloid Int. J. Exp. Clin. Investig. Off. J. Int. Soc. Amyloidosis*, vol. 22, no. 3, pp. 205–206, 2015, doi: 10.3109/13506129.2015.1054026.

[94] C. Xue, T. Y. Lin, D. Chang, and Z. Guo, "Thioflavin T as an amyloid dye: fibril quantification, optimal concentration and effect on aggregation," *R. Soc. Open Sci.*, vol. 4, no. 1, p. 160696, Jan. 2017, doi: 10.1098/rsos.160696.

[95] A. I. Sulatskaya, M. I. Sulatsky, I. A. Antifeeva, I. M. Kuznetsova, and K. K. Turoverov, “Structural Analogue of Thioflavin T, DMASEBT, as a Tool for Amyloid Fibrils Study,” *Anal. Chem.*, vol. 91, no. 4, pp. 3131–3140, Feb. 2019, doi: 10.1021/acs.analchem.8b05737.

[96] H. Naiki, K. Higuchi, M. Hosokawa, and T. Takeda, “Fluorometric determination of amyloid fibrils in vitro using the fluorescent dye, thioflavin T1,” *Anal. Biochem.*, vol. 177, no. 2, pp. 244–249, Mar. 1989, doi: 10.1016/0003-2697(89)90046-8.

[97] A. Srivastava *et al.*, “Identifying the bond responsible for the fluorescence modulation in an amyloid fibril sensor,” *Chem. Weinh. Bergstr. Ger.*, vol. 16, no. 30, pp. 9257–9263, Aug. 2010, doi: 10.1002/chem.200902968.

[98] “Quantification of beta-sheet amyloid fibril structures with thioflavin T - PubMed.” https://pubmed.ncbi.nlm.nih.gov/10507030/ (accessed May 21, 2023).

[99] A. Pomerance, G. Slavin, and J. McWatt, “Experience with the sodium sulphate-Alcian Blue stain for amyloid in cardiac pathology,” *J. Clin. Pathol.*, vol. 29, no. 1, pp. 22–26, Jan. 1976, doi: 10.1136/jcp.29.1.22.

[100] A. Barreca *et al.*, “Immunohistochemical typing of amyloid in fixed paraffin-embedded samples by an automatic procedure: Comparison with immunofluorescence data on fresh-frozen tissue,” *PLOS ONE*, vol. 16, no. 8, p. e0256306, Aug. 2021, doi: 10.1371/journal.pone.0256306.

[101] S. Biesterfeld, H. L. Kraus, T. Reineke, L. Muys, A. M. Mihalcea, and C. Rudlowski, “Analysis of the reliability of manual and automated immunohistochemical staining procedures. A pilot study,” *Anal. Quant. Cytol. Histol.*, vol. 25, no. 2, pp. 90–96, Apr. 2003.

[102] G. Merlini *et al.*, “Systemic immunoglobulin light chain amyloidosis,” *Nat. Rev. Dis. Primer*, vol. 4, no. 1, p. 38, Oct. 2018, doi: 10.1038/s41572-018-0034-3.

[103] G. Merlini and G. Palladini, “Enlightening light chain deposition disease,” *Blood*, vol. 126, no. 26, pp. 2770–2771, Dec. 2015, doi: 10.1182/blood-2015-10-672626.

[104] G. Vergaro *et al.*, “Biopsy Evidence of Sequential Transthyretin and Immunoglobulin Light-Chain Cardiac Amyloidosis in the Same Patient,” *JACC Case Rep.*, vol. 3, no. 3, pp. 450–454, Mar. 2021, doi: 10.1016/j.jaccas.2020.12.047.

[105] C. Castellani *et al.*, “Application of confocal laser scanning microscopy for the diagnosis of amyloidosis,” *Virchows Arch.*, vol. 470, no. 4, pp. 455–463, Apr. 2017, doi: 10.1007/s00428-017-2081-7.

[106] “Quantum made simple.” http://toutestquantique.fr/en/ (accessed Apr. 25, 2023).

[107] A. Angelini *et al.*, “Cardiac amyloidosis: a review of the literature and a practical approach for the clinicians,” *Ital. J. Med.*, vol. 13, no. 2, Art. no. 2, Jun. 2019, doi: 10.4081/itjm.2019.1149.

[108] M. Scivetti, G. Favia, L. Fatone, E. Maiorano, and V. Crincoli, “Concomitant use of Congo red staining and confocal laser scanning microscopy to detect amyloidosis in oral biopsy: A clinicopathological study of 16 patients,” *Ultrastruct. Pathol.*, vol. 40, no. 2, pp. 86–91, Mar. 2016, doi: 10.3109/01913123.2016.1152339.

[109] “What is Mass Spectrometry?,” *Broad Institute*, Sep. 13, 2010. https://www.broadinstitute.org/technology-areas/what-mass-spectrometry (accessed Apr. 22, 2023).

[110] F. J. Rodriguez *et al.*, “Immunoglobulin derived depositions in the nervous system: novel mass spectrometry application for protein characterization in formalin-fixed tissues,” *Lab. Invest.*, vol. 88, no. 10, pp. 1024–1037, Oct. 2008, doi: 10.1038/labinvest.2008.72.

[111] F. Lavatelli *et al.*, “Mass spectrometry-based proteomics as a diagnostic tool when immunoelectron microscopy fails in typing amyloid deposits,” *Amyloid*, vol. 18, no. sup1, pp. 64–66, Jun. 2011, doi: 10.3109/13506129.2011.574354023.

[112] “Liquid Chromatography Tandem Mass Spectrometry | AACC.org.” https://www.aacc.org/cln/articles/2015/july/liquid-chromatography-tandem-mass-spectrometry (accessed Apr. 23, 2023).

[113] C. C. Quarta *et al.*, “Diagnostic sensitivity of abdominal fat aspiration in cardiac amyloidosis,” *Eur. Heart J.*, vol. 38, no. 24, pp. 1905–1908, Jun. 2017, doi: 10.1093/eurheartj/ehx047.

[114] M. D. Benson *et al.*, “Tissue biopsy for the diagnosis of amyloidosis: experience from some centres,” *Amyloid*, vol. 29, no. 1, pp. 8–13, Jan. 2022, doi: 10.1080/13506129.2021.1994386.

[115] F. Lavatelli and J. A. Vrana, “Proteomic typing of amyloid deposits in systemic amyloidoses,” *Amyloid Int. J. Exp. Clin. Investig. Off. J. Int. Soc. Amyloidosis*, vol. 18, no. 4, pp. 177–182, Dec. 2011, doi: 10.3109/13506129.2011.630762.

[116] F. Brambilla *et al.*, “Reliable typing of systemic amyloidoses through proteomic analysis of subcutaneous adipose tissue,” *Blood*, vol. 119, no. 8, pp. 1844–1847, Feb. 2012, doi: 10.1182/blood-2011-07-365510.

[117] D. Canetti *et al.*, “Misidentification of transthyretin and immunoglobulin variants by proteomics due to methyl lysine formation in formalin-fixed paraffin-embedded amyloid tissue,” *Amyloid*, vol. 24, no. 4, pp. 229–237, Oct. 2017, doi: 10.1080/13506129.2017.1385452.

[118] A. Tanca *et al.*, “Impact of fixation time on GeLC–MS/MS proteomic profiling of formalin-fixed, paraffin-embedded tissues,” *J. Proteomics*, vol. 74, no. 7, pp. 1015–1021, Jun. 2011, doi: 10.1016/j.jprot.2011.03.015.

[119] F. Lavatelli and G. Merlini, “How do we improve treatments for patients with amyloidosis using proteomics?,” *Expert Rev. Proteomics*, vol. 14, no. 7, pp. 561–563, Jul. 2017, doi: 10.1080/14789450.2017.1331737.

[120] F. Lavatelli, M. E. McComb, D. C. Seldin, G. Merlini, M. Skinner, and C. E. Costello, “Amyloidogenic and Associated Proteins in Systemic Amyloidosis Proteome of Adipose Tissue*□S”.

[121] J. A. Vrana *et al.*, “Clinical diagnosis and typing of systemic amyloidosis in subcutaneous fat aspirates by mass spectrometry-based proteomics,” *Haematologica*, vol. 99, no. 7, pp. 1239–1247, Jul. 2014, doi: 10.3324/haematol.2013.102764.

[122] M. Conti *et al.*, “A targeted proteomics approach to amyloidosis typing,” *Clin. Mass Spectrom.*, vol. 7, pp. 18–28, Jan. 2018, doi: 10.1016/j.clinms.2018.02.001.

[123] P. Mollee *et al.*, “Implementation and evaluation of amyloidosis subtyping by laser-capture microdissection and tandem mass spectrometry,” *Clin. Proteomics*, vol. 13, no. 1, p. 30, Dec. 2016, doi: 10.1186/s12014-016-9133-x.

[124] “Principle of Reversed-Phase Chromatography HPLC/UPLC (with Animation) | Analytical Chemistry | PharmaXChange.info,” Dec. 27, 2012. https://pharmaxchange.info/2012/12/principle-of-reversed-phase-chromatography-hplcuplc-with-animation/ (accessed Apr. 25, 2023).

[125] N. B. Palstrøm *et al.*, “Classification of Amyloidosis by Model-Assisted Mass Spectrometry-Based Proteomics,” *Int. J. Mol. Sci.*, vol. 23, no. 1, p. 319, Dec. 2021, doi: 10.3390/ijms23010319.

[126] H. Steen and M. Mann, “The ABC’s (and XYZ’s) of peptide sequencing,” *Nat. Rev. Mol. Cell Biol.*, vol. 5, no. 9, pp. 699–711, Sep. 2004, doi: 10.1038/nrm1468.

[127] N. Abildgaard *et al.*, “Immunoelectron microscopy and mass spectrometry for classification of amyloid deposits,” *Amyloid*, vol. 27, no. 1, pp. 59–66, Jan. 2020, doi: 10.1080/13506129.2019.1688289.

[128] M. A. Gertz and R. A. Kyle, “Amyloidosis: prognosis and treatment,” *Semin. Arthritis Rheum.*, vol. 24, no. 2, pp. 124–138, Oct. 1994, doi: 10.1016/s0049-0172(05)80006-x.

[129] S. Kumar *et al.*, “Revised prognostic staging system for light chain amyloidosis incorporating cardiac biomarkers and serum free light chain measurements,” *J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol.*, vol. 30, no. 9, pp. 989–995, Mar. 2012, doi: 10.1200/JCO.2011.38.5724.

[130] B. Lilleness, F. L. Ruberg, R. Mussinelli, G. Doros, and V. Sanchorawala, “Development and validation of a survival staging system incorporating BNP in patients with light chain amyloidosis,” *Blood*, vol. 133, no. 3, pp. 215–223, Jan. 2019, doi: 10.1182/blood-2018-06-858951.

[131] M. Grogan *et al.*, “Natural History of Wild-Type Transthyretin Cardiac Amyloidosis and Risk Stratification Using a Novel Staging System,” *J. Am. Coll. Cardiol.*, vol. 68, no. 10, pp. 1014–1020, Sep. 2016, doi: 10.1016/j.jacc.2016.06.033.

[132] J. D. Gillmore *et al.*, “A new staging system for cardiac transthyretin amyloidosis,” *Eur. Heart J.*, vol. 39, no. 30, pp. 2799–2806, Aug. 2018, doi: 10.1093/eurheartj/ehx589.

[133] R. K. Cheng *et al.*, “Diuretic Dose and NYHA Functional Class Are Independent Predictors of Mortality in Patients With Transthyretin Cardiac Amyloidosis,” *JACC CardioOncology*, vol. 2, no. 3, pp. 414–424, Sep. 2020, doi: 10.1016/j.jaccao.2020.06.007.

[134] I. Conceição *et al.*, “Assessment of patients with hereditary transthyretin amyloidosis - understanding the impact of management and disease progression,” *Amyloid Int. J. Exp. Clin. Investig. Off. J. Int. Soc. Amyloidosis*, vol. 26, no. 3, pp. 103–111, Sep. 2019, doi: 10.1080/13506129.2019.1627312.

[135] E. Muchtar, G. Lin, and M. Grogan, “The Challenges in Chemotherapy and Stem Cell Transplantation for Light-Chain Amyloidosis,” *Can. J. Cardiol.*, vol. 36, no. 3, pp. 384–395, Mar. 2020, doi: 10.1016/j.cjca.2019.11.032.

[136] G. Palladini and G. Merlini, “What is new in diagnosis and management of light chain amyloidosis?,” *Blood*, vol. 128, no. 2, pp. 159–168, Jul. 2016, doi: 10.1182/blood-2016-01-629790.

[137] G. Palladini, P. Milani, and G. Merlini, “Management of AL amyloidosis in 2020,” *Blood*, vol. 136, no. 23, pp. 2620–2627, Dec. 2020, doi: 10.1182/blood.2020006913.

[138] X. Zhang, V. Goel, H. Attarwala, M. T. Sweetser, V. A. Clausen, and G. J. Robbie, “Patisiran Pharmacokinetics, Pharmacodynamics, and Exposure-Response Analyses in the Phase 3 APOLLO Trial in Patients With Hereditary Transthyretin-Mediated (hATTR) Amyloidosis,” *J. Clin. Pharmacol.*, vol. 60, no. 1, pp. 37–49, Jan. 2020, doi: 10.1002/jcph.1480.

[139] M. D. Benson *et al.*, “Inotersen Treatment for Patients with Hereditary Transthyretin Amyloidosis,” *N. Engl. J. Med.*, vol. 379, no. 1, pp. 22–31, Jul. 2018, doi: 10.1056/NEJMoa1716793.

[140] A. L. P. Caforio *et al.*, “Current state of knowledge on aetiology, diagnosis, management, and therapy of myocarditis: a position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases,” *Eur. Heart J.*, vol. 34, no. 33, pp. 2636–2648, 2648a–2648d, Sep. 2013, doi: 10.1093/eurheartj/eht210.

[141] M. Zampieri *et al.*, “Sex-related differences in clinical presentation and all-cause mortality in patients with cardiac transthyretin amyloidosis and light chain amyloidosis,” *Int. J. Cardiol.*, vol. 351, pp. 71–77, Mar. 2022, doi: 10.1016/j.ijcard.2021.12.048.

ACKNOWLEDGEMENTS

I would like to thank all those who have made it possible for me to present my work today. First of all, it has been a pleasure to work with my team, friendly and professional, and to delve into such an interesting subject with them. I would like to thank my family; without their support, both human and pragmatic, this achievement would have been impossible. Last but not least, my friends and all the special people who have enriched my life during this journey. Thank you all.