AMILOIDOSE POR FIBRINOGÉNIO:
FENÓTIPO, GENÓTIPO, TRATAMENTO E PROGNÓSTICO

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PREFACE

In 1939, Corino de Andrade introduced the theme of amyloidosis in Portugal. Later, in 1952, when he published his findings on the familial amyloidotic polyneuropathy in Portuguese patients, worldwide attention was brought to the hereditary forms of systemic amyloidoses. In this context, amyloid and amyloidoses ceased to be a medical curiosity of only academic interest to become subject of interest to diverse clinicians and basic scientists such as biophysicists, protein biochemists, biologists, pathologists, nephrologists, hematologists, neurologists, endocrinologists, radiologists, veterinarians and public health.

The pathogenesis of amyloidosis is a complex process that is still under intense investigation and that allows the identification of an ever-expanding number of amyloid fibril proteins. The knowledge of the molecular mechanisms underlying protein misfolding, fibril formation and aggregation toward amyloid, have led to the development of therapies targeting the source of amyloid fibril protein and specific steps on the amyloid cascade.

Early accurate diagnosis is the key to effective therapy. Unequivocal identification of the amyloidogenic protein may require advanced technologies and expertise. Epidemiologic knowledge of hereditary systemic amyloidoses is useful because it increases the degree of suspicion thus contributing to the anticipation of their diagnosis.

Hereditary systemic amyloidoses are rare, but some forms are characteristic in specific geographic areas. Hereditary transthyretin amyloidosis was the only form of hereditary systemic amyloidoses recognized in Portugal until the beginning of the 21st-century. It was in this context that I started working with Professor Luisa Lobato in 2001, after being challenged by Dr. Elísio Carvalho. At that time, I was far from imagining that the research team, headed by Professor Luisa Lobato, in which I had the privilege of being included, would identify the endemic foci of fibrinogen amyloidosis in northern Portugal and that this would be one of the largest in the world. This is undoubtedly one of the major contributions of this doctoral research work.

This thesis presents a review of the state-of-the-art in the field of amyloid and amyloidoses and a series of original papers about fibrinogen amyloidosis. It starts with Introduction which is a comprehensive review about amyloid, amyloidoses and fibrinogen Aα chain. The objectives and methodology used in the research work are presented in the Aims and Methods sections, respectively. The original research is described in the Results section which is divided into Epidemiology, Natural history and treatment, Genetics and genealogy. The discussion and main conclusions of this thesis are presented in the Discussion section.

Throughout this project we have proved that the lack of knowledge about fibrinogen amyloidosis and the absence of routine kidney biopsy for immunohistochemistry typing with an antibody panel that includes antibodies
directed against fibrinogen contribute to the underdiagnosis of this disease. On the other hand, the nonspecific nature of its major complications contributes to the misdiagnosed of fibrinogen amyloidosis as chronic kidney disease attributable to hypertensive nephrosclerosis or unknown etiology.

At the end of this work, many questions remain unanswered, some of which have emerged along this research and as such are innovative and perhaps will allow a new approach in the diagnosis of patients with fibrinogen amyloidosis. Despite of that, I hope that this thesis contributes to increase the degree of the disease suspicion, at least in Portugal.
ACKNOWLEDGMENTS

It was a long and hard journey that I went through unpretentiously and during which I had to face several challenges with the support of many researchers to whom I present my gratitude. The work we have been doing has been an amazing and rewarding experience. None of this would have been possible without the support of Professor Luisa Lobato and Professor João Paulo Oliveira. I had the privilege of being supervised by them through this project. They are outstanding physicians and medical science researchers, recognized worldwide, that taught me a lot. I will be eternally grateful to them.

I also had the opportunity to meet amazing researchers that worked alongside with me and to whom I am very thankful. In particular, I thank Professor Paulo Pinheiro Costa from Instituto Nacional de Saúde Pública Doutor Ricardo Jorge for his vital and decisive collaboration throughout the course of the research work, namely in the immunohistochemical evaluation of all renal biopsies, molecular diagnosis of subjects carrying the FGA p.Glu545Val variant, in the study of haplotypes, and in the reviews of articles and this thesis. Very special thanks to Doctor Luciana Moreira and Dr. Pedro Lacerda from Instituto Nacional de Saúde Pública Doutor Ricardo Jorge for their laboratory works that supported the immunohistochemical and molecular study present in this thesis. It was also a pleasure to meet and work with Doctor Mârcia Oliveira and Doctor Rosário Almeida from Centro de Genética Médica Dr. Jacinto Magalhães, Centro Hospitalar do Porto.

I counted on with the invaluable collaboration of many health professionals to whom I would like to thank. In particular, I thank Professor Manuel Pestana, past and present medical and nursing staff from the Department of Nephrology, Centro Hospitalar de São João, for allowing me to develop this project and for creating conditions for its performance. Medical staff from the Department of Nephrology, Hospital de Santo António, Centro Hospitalar do Porto, for their collaboration in diagnosis and follow-up of patients with fibrinogen amyloidosis. To Dr. Abel Rua and past medical staff from the Department of Internal Medicine from Hospital de São Marcos, Braga, where I worked for 4 years. To Dr. Alfredo Loureiro from the Department of Nephrology, Instituto Português de Oncologia do Porto, Dr. Carlos Soares and Dr. Sofia Rocha from the Department of Nephrology, Hospital de Braga and to Dr. Ana Marta Gomes from the Department of Nephrology, Centro Hospitalar de Gaia, for their collaboration in the diagnosis and referral of patients with fibrinogen amyloidosis.

The screening study in the hemodialysis units was only possible due to the exceptional availability and support given to us for whom I thank Dr. António Castro Henriques, medical and nursing staff from the Nephrocare-Braga; Professor Joaquim Pinheiro, medical and nursing staff from the Nephrocare-Fafe; Dra. Susana Sampaio, medical and nursing staff from the Hemoatlântico-Vila Verde.
It will be forever in my memory the trips I made to the hemodialysis units with Laurinda Teixeira from Instituto Nacional de Saúde Pública Doutor Ricardo Jorge. Those were days of hard work, in the evaluation and selection of patients for study, and in the collection of blood samples.

Very special thanks to Dra. Joaquina Coelho and Dra. Helena Amado from Centro de Saúde Vieira do Minho, Braga, for their collaboration in the study of families with fibrinogen amyloidosis.

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All this work would not have been possible without a grant awarded by the Portuguese Society of Nephrology to whom I thank.

I am deeply grateful to Professor Andreia Coroas and to Dr. Berta Carvalho for their unconditional support. Both possess a singular intelligence and discernment, they never let me give up and tried always to show me the best way helping me to solve some challenges.

To my family, my husband Nuno and my children Pedro and Carolina. My unavailability during the development of this project was the reason for my absence in some fundamental stages of their lives, yet they have never abandoned me or held any signs of sadness. Thank you for your unconditional support.

Finally, to the patients and their families who are the source of my inspiration and to whom I dedicate this thesis.
CONTENTS

CATEDRÁTICOS DA FACULDADE DE MEDicina DA UNIVERSIDADE DO PORTO..............................................5
JÚRI DA PROVA DE DOUTORAMENTO.................................................................................................7
PREFACE ................................................................................................................................................9
ACKNOWLEDGMENTS ..........................................................................................................................11
CONTENTS.............................................................................................................................................13
LIST OF FIGURES ...............................................................................................................................14
LIST OF TABLES .....................................................................................................................................14
ABBREVIATIONS ..................................................................................................................................15
AUTHOR CONTRIBUTIONS ................................................................................................................17
ABSTRACT .................................................................................................................................................19
RESUMO ..................................................................................................................................................21
I – INTRODUCTION ..............................................................................................................................23
1. AMYLOID AND AMYLOIDOSES ......................................................................................................25
   1.1. Overview .......................................................................................................................................25
   1.2. Definition .......................................................................................................................................26
   1.3. Nomenclature ...............................................................................................................................27
   1.4. Epidemiology ...............................................................................................................................29
   1.5. Pathogenesis ..................................................................................................................................30
   1.6. Diagnosis .......................................................................................................................................33
   1.7. Therapy ..........................................................................................................................................35
2. FIBRINOGEN Aα CHAIN ...................................................................................................................39
   2.1. Structure and function ................................................................................................................39
   2.2. Pathogenesis of hereditary fibrinogen amyloidosis.......................................................................41
   2.3. The starting point for this original study and the obscurity of AFib amyloidosis in Portugal........43
II – AIMS ................................................................................................................................................45
III – METHODS .......................................................................................................................................49
IV – RESULTS .........................................................................................................................................53
1. EPIDEMIOLOGY ..................................................................................................................................55
   1.1. Renal amyloidosis: classification of 102 consecutive cases.........................................................55
   1.2. Fibrinogen Aα chain amyloidosis: a non-negligible cause of chronic kidney disease in dialysis patients ...............................................................65
2. NATURAL HISTORY AND TREATMENT .........................................................................................69
   2.1. Unrecognized fibrinogen Aα chain amyloidosis: results from targeted genetic testing... 69
   2.2. Long-term follow-up of patients with hereditary fibrinogen Aα chain amyloidosis......81
   2.3. Renal amyloidosis in northern Portugal: the clinical differences in distinct forms........85
3. GENETICS AND GENEALOGY .......................................................................................................................... 91

3.1. Homozygosity for the E526V mutation in fibrinogen Aα chain amyloidosis: the first report ................................................................. 91

3.2. Haplotype analysis of newly diagnosed Portuguese and Brazilian families with fibrinogen amyloidosis caused by the FGA.p.Glu545Val variant ............................................................... 99

V – DISCUSSION .............................................................................................................................................. 121

VI – CONCLUSIONS ........................................................................................................................................... 131

VII – FUTURE WORK ........................................................................................................................................ 135

VIII – BIBLIOGRAPHY ..................................................................................................................................... 139

LIST OF FIGURES

Figure 1 | Amyloid identification and typing........................................................................................................... 33
Figure 2 | Immunofluorescence............................................................................................................................... 34
Figure 3 | Fibrinogen structure................................................................................................................................. 40
Figure 4 | Localization of amyloidogenic mutations in the fibrinogen gene cluster................................................. 40
Figure 5 | Study design and results......................................................................................................................... 65
Figure 6 | Abdominal fat involvement...................................................................................................................... 81
Figure 7 | Spleen involvement in AFibE526V (p.Glu545Val) amyloidosis............................................................... 81
Figure 8 | Ileum involvement in AFibE526V (p.Glu545Val) amyloidosis............................................................... 82
Figure 9 | Colon involvement in AFibE526V (p.Glu545Val) amyloidosis.............................................................. 82

LIST OF TABLES

Table 1 | Amyloid fibril proteins and their precursors in humans............................................................................ 28
Table 2 | Amyloidogenic mutations in fibrinogen Aα chain gene............................................................................. 42
Table 3 | Penetration of AFibE526V (p.Glu545Val) amyloidosis............................................................................. 69
ABBREVIATIONS

AApoAl  Amyloid fibril apolipoprotein A I  
APOA1  Apolipoprotein A I gene  
AApoAll  Amyloid fibril apolipoprotein A II  
APOA2  Apolipoprotein A II gene  
AApoCII  Amyloid fibril apolipoprotein C II  
APOC2  Apolipoprotein C II gene  
AApoCIII  Amyloid fibril apolipoprotein C III  
APOC3  Apolipoprotein C III gene  
Aβ  Amyloid fibril β-protein  
Aβ2M  Amyloid fibril β2-microglobulin  
ABri  Amyloid fibril Bri  
ACys  Amyloid fibril Cystatin C  
ADan  Amyloid fibril Dan  
AFib  Amyloid fibril fibrinogen Aα chain  
AGel  Amyloid fibril gelsolin  
AFibE526V (p.Glu545Val)  Fibrinogen amyloidosis due to substitution of glutamic acid by valine at position 526 (protein variant including 19 amino acids signal peptide)  
AIAPP  Amyloid fibril islet polypeptide  
AL  Amyloid fibril immunoglobulin light chain  
ALECT2  Amyloid fibril leukocyte chemotactic factor-2  
ALys  Amyloid fibril lysozyme  
APrP  Amyloid fibril prion  
ASOs  Antisense oligonucleotides  
ATTR  Amyloid fibril transthyretin  
ATTRwt  Amyloid fibril wild-type transthyretin  
B2M  β2-microglobulin gene  
CKD  Chronic kidney disease  
CLKT  Combined liver and kidney transplant  
CHSJ  Centro Hospitalar de São João  
CPHPC  (R)-1-[[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid  
CST  Cystatin C gene  
DNA  Deoxyribonucleic acid  
ESRD  End-stage renal disease  
GAGs  Glycosaminoglycans  
GSN  Gelsolin gene  
FGA  Fibrinogen Aα chain gene  
FGA  Fibrinogen Aα chain protein  
FGB  Fibrinogen Bβ chain gene  
FGG  Fibrinogen γ chain gene  
HSA/CHP  Hospital de Santo António/Centro Hospitalar do Porto  
IgG1  Immunoglobulin G1  
IHC  Immunohistochemistry  
ISA  International Society of Amyloidosis  
LECT2  Leukocyte chemotactic factor-2 gene  
LYZ  Lysozyme gene  
mAb 11-1F4  Monoclonal antibody 11-1F4  
mRNA  Messenger ribonucleic acid  
NCT number  Clinical Trials.gov identifier  
OLT  Orthotopic liver transplantation  
OMIM  Online Mendelian Inheritance in Man
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end-products</td>
</tr>
<tr>
<td>RNAs</td>
<td>Ribonucleic acids</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
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<tr>
<td>SAP</td>
<td>Serum amyloid P</td>
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<tr>
<td>siRNAs</td>
<td>Small interfering ribonucleic acids</td>
</tr>
<tr>
<td>TTR</td>
<td>Transthyretin gene</td>
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<tr>
<td>TTR</td>
<td>Transthyretin protein</td>
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AUTHOR CONTRIBUTIONS


ABSTRACT

Introduction: Fibrinogen Aα chain is the main polypeptide chain of fibrinogen and is encoded by the FGA gene. Several FGA variants have been found, either affecting fibrinogen quantity, quality or, in more rare conditions, resulting in amyloid formation with renal predominant deposition. Fibrinogen Aα chain (AFib) amyloidosis is an autosomal dominant disease described in 1993 that was reported for the first time in Portugal in 2004.

Aims: Evaluation of the possibility of unidentified or suspected cases of AFib amyloidosis in Portugal. Epidemiological characterization of the disease in northern Portugal. Definition of the natural history and outcomes of AFib amyloidosis associated with the FGA p.Glu545Val variant [AFibE526V (p.Glu545Val)]. Haplotype studies to investigate the possibility of a common ancestor.

Material and Methods: First, a total of 102 consecutive kidney biopsies, performed at the Department of Nephrology of Centro Hospitalar de São João, were retrospectively reviewed by immunohistochemistry staining using a panel of antibodies directed against serum amyloid A, κ and λ light chains, transthyretin, fibrinogen Aα chain, apolipoprotein A I and lysozyme. Second, a total of 267 prevalent hemodialysis patients from the district of Braga were evaluated and genetic testing for the FGA c.1634A>T (p.Glu545Val) mutation (rs121909612) was offered to patients with chronic kidney disease of unknown or presumed etiology or with unclassified amyloidosis. Third, evaluation of 50 subjects carrying the FGA p.Glu545Val variant, belonging to 13 families from the same district, in order to characterize the clinical phenotype of AFib amyloidosis. Finally, the FGA haplotype markers rs6050, an FGA missense variant in exon 5, and rs533633927, an FGA 3’ polymorphism, were genotyped in four Portuguese families and in one Brazilian family. The rs6050 [A] variant is a cutting site for the restriction enzyme Rsal (GT*AC) and the rs533633927 variant represents a 28 bp insertion that, when present, is recognized by the restriction enzyme TaqI.

Results: AFib amyloidosis was diagnosed in 4 (3.9%) out of 102 consecutive kidney biopsies being therefore the third most common cause of amyloid nephropathy diagnosed in northern Portugal, outside the reference centre for hereditary transthyretin amyloidosis. A total of 122 hemodialysis patients underwent genetic testing. The FGA p.Glu545Val variant was identified in 12 (6 unclassified amyloidosis, 3 hypertensive nephrosclerosis, 3 with unknown etiology) as cause of stage 5 renal disease, corresponding to a disease prevalence of 4.5% in the district of Braga. Its natural history begins with hypertension in the sixth decade of life, followed by proteinuric chronic kidney disease after 6 years, eventually progressing to end-stage renal disease within 5 years of recognition of kidney involvement. There is an increasing prevalence of extrarenal manifestations in older patients, most typically resulting from amyloid deposition in the heart, liver, spleen and vascular vessels of abdominal fat, ileum and colon. AFib amyloidosis has an age dependent penetrance.
whose estimated mean renal survival was 65.4 (95% CI, 61.8-69.0) years, and estimated mean lifetime survival was 73.7 (95% CI, 70.5-76.8) years. AFib amyloidosis occurs in the heterozygous state but the first homozygous patient harboring the FGA p.Glu545Val variant was also identified in the district of Braga. Finally, a total of 9 Caucasian subjects, 7 Portuguese and 2 Brazilian heterozygous for the FGA c.1634A>T (p.Glu545Val) missense mutation shared allele A from FGA rs6050 marker, Rsal(+). Regarding the FGA rs533633927 marker, absence of the 28 bp insertion, Taql(−), was also found in all the subjects.

**Conclusions:** This work enabled the identification of endemic foci of AFibE526V (p.Glu545Val) amyloidosis in the district of Braga, northern Portugal. This is a systemic disease with age dependent penetrance, which always involves the kidneys and typically develops whole-body amyloid load with aging. The lack of knowledge about the disease and the nonspecific nature of its major complications contribute to the underdiagnosis of AFibE526V (p.Glu545Val) amyloidosis as chronic kidney disease inerable to hypertensive nephrosclerosis or unknown etiology. The study of five families, four Portuguese and one Brazilian, using two FGA haplotype markers showed that they shared the same phenotype and Rsal(+)/Taql(−) haplotype as northern European families, which may be the result of migration flux and strengthens the hypothesis of a common origin for AFibE526V (p.Glu545Val) amyloidosis.
RESUMO

Introdução: A cadeia Aα do fibrinogênio é a maior cadeia polipeptídica do fibrinogênio e é codificada pelo gene FGA. Foram identificadas várias variantes FGA, que afetam a quantidade ou a qualidade do fibrinogênio, e em situações mais raras, podem resultar na formação de amilóide que se deposita predominantemente no rim. A amiloidose da cadeia Aα do fibrinogênio (AFib) é uma doença autossômica dominante que foi descrita em 1993 e reportada pela primeira vez em Portugal em 2004.


Material e Métodos: Em primeiro lugar, procedeu-se à revisão retrospectiva de 102 biópsias renais consecutivas, realizadas no serviço de Nefrologia do Centro Hospitalar São João, por imuno-histoquímica usando um painel de anticorpos dirigidos contra a proteína sérica amiloide A, as cadeias leves κ e λ, transtirretina, cadeia Aα do fibrinogênio, apolipoproteína A I e lisozima. Em segundo lugar, foram avaliados 267 doentes prevalentes em hemodiálise no distrito de Braga e foi disponibilizada a pesquisa da mutação FGA c.1634A>T (p.Glu545Val), rs121909612, àqueles com doença renal crónica de etiologia desconhecida ou presumida ou com amiloidose não classificada. Terceiro, foram avaliados 50 indivíduos portadores da variante FGA p.Glu545Val e pertencentes a 13 famílias oriundas do mesmo distrito. Finalmente, os marcadores haplótipicos FGA rs6050, uma variante missense localizada no exão 5, e rs533633927, um polimorfismo da região 3', foram genotipados em quatro famílias portuguesas e numa família brasileira. A variante rs6050 [A] é um local de corte para a enzima de restrição Rsal (GT*AC) e a variante rs533633927 representa uma inserção de 28 pb que, quando presente, é reconhecida pela enzima de restrição TaqI.

Resultados: A amiloidose AFib foi diagnosticada em 4 (3,9%) de 102 biópsias renais consecutivas sendo, portanto, a terceira causa mais comum de nefropatia amilóide diagnosticada no norte de Portugal, fora do centro de referência para a amiloidose hereditária por transtirretina. Um total de 122 doentes em hemodiálise realizaram testes genéticos e a variante FGA p.Glu545Val foi identificada em 12 (6 com amiloidose não classificada, 3 com nefrosclerose hipertensiva, 3 de etiologia desconhecida) como causa de doença renal estádio 5, correspondendo a uma prevalência da doença de 4,5% no distrito de Braga. A história natural da amiloidose AFibE526V (p.Glu545Val) caracteriza-se pelo início de hipertensão arterial na sexta década de vida, seguida do desenvolvimento de doença renal crónica proteinúrica após 6 anos e, finalmente, evolução para doença renal terminal 5 anos mais tarde.

Existe uma prevalência cumulativa de manifestações extrarrenais em doentes mais
velhos, tipicamente resultantes da deposição de amilóide no coração, fígado, baço e vasos da gordura abdominal, ileum e cólon. A amiloidose AFib tem uma penetrância dependente da idade, uma sobrevida renal média estimada de 65,4 (IC 95%, 61,8-69,0) anos e uma sobrevida global média estimada de 73,7 (IC 95%, 70,5-76,8) anos. A amiloidose AFib ocorre predominantemente em heterozigotia, mas o primeiro caso de homozigotia para a variante FGA p.Glu545Val foi identificado no distrito de Braga. Finalmente, 9 indivíduos caucasianos, 7 portugueses e 2 brasileiros heterozigóticos para a mutação missense FGA c.1634A>T (p.Glu545Val), partilhavam o alelo A do marcador haplotípico FGA rs6050, Rsal(+/). Em relação ao marcador haplotípico FGA rs533633927, a ausência da inserção de 28 pb, TaqI(−), foi comum em todos os casos.

Conclusões: O trabalho de investigação apresentado nesta tese permitiu a identificação de um foco endémico de amiloidose AFibE526V (p.Glu545Val) no distrito de Braga, norte de Portugal. Esta é uma doença sistémica com penetrância dependente da idade, envolvimento renal universal e que tipicamente progride com o envelhecimento podendo envolver outros órgãos. A falta de conhecimento sobre a doença e a inespecificidade das suas principais características contribuem para que esta amiloidose não seja diagnosticada, ou então, seja mal diagnosticada como doença renal crónica atribuível a nefrosclerose hipertensiva ou de etiologia desconhecida. O estudo de cinco famílias, quatro portuguesas e uma brasileira, utilizando dois marcadores haplotípicos do FGA, mostrou que aquelas partilhavam o mesmo fenótipo e o haplótipo Rsal(+/)/TaqI(−) com famílias do norte da Europa, o que pode ser resultante de fluxos migratórios e favorece a hipótese de uma origem comum para a amiloidose AFibE526V (p.Glu545Val).
I – INTRODUCTION
1. AMYLOID AND AMYLOIDOSES

1.1. Overview

It is generally accepted that the first description of what we now consider to be amyloid and amyloidoses was made by Nicolaus Fontanous in 1639, in the report of the autopsy of a young man with ascites, jaundice and epistaxis who had an abscess in the liver and a large spleen filled with white stones [1, 2].

The term amyloid stems from the Latin amylum and from the Greek amylon for starch and was brought in the scientific literature by the German botanist Matthias Schleiden in 1838 to describe a normal amylaceous constituent in plants detected by the iodine-sulphuric acid test [2, 3]. In 1854, Rudolf Virchow applied the term amyloid in the medical literature for the first time because of the reaction of the corpora amylacea of the nervous system with the iodine-sulphuric acid test [4].

A new insight into the biochemical characterization of amyloid was presented in 1859 by August Kekule based on the observation of high proportion of nitrogen in organs infiltrated by amyloid [2, 5]. In 1875, André-Victor Comil from Paris, Richard Heschl from Vienna and Rudolph Jürgens from Berlin, independently reported the use of aniline dyes in the recognition of amyloid [6]. These metachromatic stains challenged Virchow’s iodine-sulphuric acid test used for decades, and were eventually replaced by Congo red which was introduced by Herman Bennhold in 1922 [7].

Paul Divry and Marcel Florkin introduced in 1927 the green birefringence when an amyloid plaque from the brains of patients with Alzheimer’s disease exhibited apple-green birefringence when stained with Congo red and viewed under polarized light [8]. In 1959, Alan Cohen and Evan Calkins investigated amyloid inclusions from histological preparations of various tissues and organs and showed that they were formed by filaments or fibrils of 75–100 Å in thickness and 1,000–16,000 Å in length when viewed under the electron microscope [9].

A significant step forward took place when Alan Cohen and Evan Calkins in 1964, and Mordechai Pras and colleagues in the late 1960s described the method to extract proteins from amyloid-Haden tissues using water [10, 11]. This has been used to extract almost all types of amyloid and enabled subsequent research about secondary structure of amyloid and biochemical nature of precursor proteins. Analysis of amyloid fibrils by X-ray diffraction and subsequently infra-red spectroscopy revealed an underlying cross β organization [12-14].

A major development was the recognition that amyloid fibrils, in previously called the primary and nowadays the AL amyloidosis, are fragments of immunoglobulin light chains [15]. Subsequently, several amyloid proteins have been identified. Amyloidosis associated with inflammation, previously called the secondary and nowadays the AA amyloidosis, was shown to be caused by the normal protein serum amyloid A (SAA), an acute phase protein [16]. In 1978, prealbumin (transthyretin) was found to be the protein constituent of amyloid deposits in
Portuguese amyloid polyneuropathy [17]. In 1980, transthyretin (TTR) was characterized as the amyloid protein also in senile cardiac amyloidosis [18], whose designation changed to senile systemic amyloidosis when it became clear that this is a systemic disease [19].

Developments in molecular genetics techniques have accelerated the identification of amyloid fibril proteins and its amyloidogenic variants. To date, several variant forms of fifteen different amyloid fibril proteins have been associated with clinically important hereditary amyloidoses [20]: transthyretin (ATTR) [21], apolipoprotein A I (AApoAI) [22, 23], Aβ protein (Aβ) [24], AbRII (AbRII) [25], ADanPP (ADan) [26], prion protein (PrP) [27], apolipoprotein A II (AApoAII) [28], lysozyme (ALys) [29, 30], fibrinogen Aα chain (AFib) [31, 32], cystatin C (ACys) [33], gelsolin (AGel) [34], β2-microglobulin (β2M) [35], immunoglobulin light chain (AL) [36], apolipoprotein C II (AApoCII) [37, 38], and apolipoprotein C III (AApoCIII) [39].

Wider availability of diagnostic tests, along with greater recognition of amyloidosis, has led to an ever-increasing number of new amyloid fibril proteins. In clinical grounds, amyloid typing has been routinely performed by immunohistochemistry (IHC) in paraffin sections [40-43]. Nowadays, mass spectrometry–based proteomics is a complementary useful tool for amyloid typing [44-47].

1.2. Definition

The term amyloid was introduced in the scientific literature by Matthias Schleiden in 1838 and since then its concept has been changing according to the trends in the development of amyloid biology. Earlier researchers considered amyloid as amorphous extracellular deposits formed in human and animal tissues [5, 48].

Subsequent structural studies characterized amyloid as non-branching protein fibrils consisting of monomers linked mainly due to hydrogen bonds between β strands of intermolecular β sheets arranged perpendicularly to the lateral axis of a fibril [12]. In addition to the major fibrillar protein component, immunohistological and chemical studies showed that amyloid frequently contains serum amyloid P (SAP) component, glycosaminoglycans (GAGs), proteoglycans, apolipoprotein E, laminin and forms of collagen [49-54]. Progress in biochemical purification and analysis showed that amyloid fibrils could be formed by several precursor proteins [10, 15, 17, 55].

The accumulation of experimental data allowed the development of research methods for detection of new amyloid fibrils at the proteome level giving rise to amyloidomics [56-59]. In this field, Technique for Amyloid Purification and Identification (TAPI) [60], and Proteomic Screening and Identification of Amyloidosis (PSIA) [58], are current methods applied for detection of amyloid fibril protein candidates, whose amyloid features need to be tested by additional experiments. For all this, there is no conventional concept for amyloid, but the most consensual
definition is that of a fibrillar protein aggregate with cross β diffraction pattern [20, 59].

Amyloidosis is not a single disease but a term for localized or systemic diseases in which circulating proteins misfold and self-aggregate into fibrillar polymers that precipitate mainly in the extracellular spaces of a range of organs and tissues [20, 61, 62], and represent a subset of a growing class of diseases [63].

1.3. Nomenclature

The chemical diversity of amyloid fibril proteins raised the need for a nomenclature system that was presented for the first time in 1974 at the II\textsuperscript{nd} Symposium on Amyloidosis [64]. Thereafter, the Nomenclature Committee of the International Society of Amyloidosis (ISA) has been publishing systematic nomenclature guidelines for amyloid fibril proteins and amyloidosis based on biochemical and biophysical characterization [20].

To be included in the official ISA Amyloid Fibril Protein Nomenclature list, an amyloid fibril protein must have been unambiguously characterized by protein sequence analysis when possible and described in a peer reviewed journal [20].

The amyloid fibril protein is designated protein A and followed by a suffix that is an abbreviated form of the parent or precursor protein [20]. For instance, when amyloid fibrils are derived from fibrinogen Aα chain the amyloid fibril protein is AFib and the disease is AFib amyloidosis.

Amyloid fibril protein variants are named according to the substitution, deletion or insertion in the mature protein that is involved in the disease. The Human Genome Variation Society recommends that observations should be reported using an appropriate reference sequence [65]. As such, a genomic deoxyribonucleic acid (DNA) or a protein sequence are the preferred reference, whether the mutations were found after sequencing the DNA or the protein, respectively.

The ISA Nomenclature Committee further recommends the use of the recently introduced Locus Reference Genomic sequence [66, 67], the single amino acid code and the sequence numbering of the mature protein when reporting studies on amyloid proteins [68]. According to these recommendations, the FGA p.Glu526Val (substitution of glutamate by valine at amino acid position 526) should be described as FGA p.Glu545Val (considering the translation initiation site as amino acid 1, instead of basing the numbering on cleaved protein) and the hereditary amyloidosis segregating with the FGA p.Glu526Val variant should be designated AFibE526V (p.Glu545Val) amyloidosis [69]. This nomenclature will be followed hereinafter.

Currently, 35 distinct proteins have been identified as amyloid fibril proteins in humans, giving rise to distinct amyloidosis syndromes (Table 1). The amyloid and amyloidosis chemically based nomenclature has been adopted by the World Health Organization [70] and is consistently recommended by the ISA Nomenclature Committee [69].
Table 1 | Amyloid fibril proteins and their precursors in humans

<table>
<thead>
<tr>
<th>Fibril protein</th>
<th>Precursor protein</th>
<th>Systemic and/or localized</th>
<th>Acquired or hereditary</th>
<th>Target organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Immunoglobulin light chain</td>
<td>S, L</td>
<td>A, H</td>
<td>All organs, usually except CNS</td>
</tr>
<tr>
<td>AH</td>
<td>Immunoglobulin heavy chain</td>
<td>S, L</td>
<td>A</td>
<td>All organs except CNS</td>
</tr>
<tr>
<td>AA</td>
<td>(ApoB100) Serum amyloid A</td>
<td>S</td>
<td>A</td>
<td>All organs except CNS</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin wild type</td>
<td>S</td>
<td>A</td>
<td>Heart, ligaments, tenosynovium</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin variants</td>
<td>S</td>
<td>H</td>
<td>PNS, ANS, heart, eyes, leptomeninges</td>
</tr>
<tr>
<td>Aβ2M</td>
<td>β2-microglobulin wild type</td>
<td>S</td>
<td>A</td>
<td>Musculoskeletal system</td>
</tr>
<tr>
<td>Aβ2M</td>
<td>β2-microglobulin variant</td>
<td>S</td>
<td>H</td>
<td>ANS</td>
</tr>
<tr>
<td>APOAI</td>
<td>Apolipoprotein A I variants</td>
<td>S</td>
<td>H</td>
<td>Heart, liver, kidney, PNS, testis, larynx (C-terminal variants), skin (C-terminal variants)</td>
</tr>
<tr>
<td>APOAI</td>
<td>Apolipoprotein A II variants</td>
<td>S</td>
<td>H</td>
<td>Kidney</td>
</tr>
<tr>
<td>APOAI</td>
<td>Apolipoprotein A IV wild type</td>
<td>S</td>
<td>A</td>
<td>Kidney medulla and systemic</td>
</tr>
<tr>
<td>APOCI</td>
<td>Apolipoprotein C I variants</td>
<td>S</td>
<td>H</td>
<td>Kidney</td>
</tr>
<tr>
<td>APOCI</td>
<td>Apolipoprotein C II variants</td>
<td>S</td>
<td>H</td>
<td>Kidney</td>
</tr>
<tr>
<td>AGEI</td>
<td>Gelsolin variants</td>
<td>S</td>
<td>H</td>
<td>PNS, cornea</td>
</tr>
<tr>
<td>Alys</td>
<td>Lysozyme variants</td>
<td>S</td>
<td>H</td>
<td>Kidney</td>
</tr>
<tr>
<td>ALECT2</td>
<td>Leukocyte chemotactic factor-2</td>
<td>S</td>
<td>A</td>
<td>Kidney, primarily</td>
</tr>
<tr>
<td>AβFib</td>
<td>Fibrinogen Aα chain variants</td>
<td>S</td>
<td>H</td>
<td>Kidney, primarily</td>
</tr>
<tr>
<td>ACys</td>
<td>Cystatin C variants</td>
<td>S</td>
<td>H</td>
<td>PNS, skin</td>
</tr>
<tr>
<td>ABri</td>
<td>ABriPP variants</td>
<td>S</td>
<td>H</td>
<td>CNS</td>
</tr>
<tr>
<td>ADarP**</td>
<td>ADarP variants</td>
<td>L</td>
<td>H</td>
<td>CNS</td>
</tr>
<tr>
<td>Aβ</td>
<td>Aβ protein wild type</td>
<td>L</td>
<td>A</td>
<td>CNS</td>
</tr>
<tr>
<td>Aβ</td>
<td>Aβ protein variant</td>
<td>L</td>
<td>H</td>
<td>CNS</td>
</tr>
<tr>
<td>At−Syn</td>
<td>α-Synuclein</td>
<td>L</td>
<td>A</td>
<td>CNS</td>
</tr>
<tr>
<td>ATau</td>
<td>Tau</td>
<td>L</td>
<td>A</td>
<td>CNS</td>
</tr>
<tr>
<td>APrP</td>
<td>Prion protein wild type</td>
<td>L</td>
<td>A</td>
<td>CJD, fatal insomnia</td>
</tr>
<tr>
<td>APrP</td>
<td>Prion protein variants</td>
<td>L</td>
<td>H</td>
<td>CJD, GSS syndrome, fatal insomnia</td>
</tr>
<tr>
<td>APrP</td>
<td>Prion protein variant</td>
<td>S</td>
<td>H</td>
<td>PNS</td>
</tr>
<tr>
<td>ACal</td>
<td>(Pro)Calcitonin</td>
<td>L</td>
<td>A</td>
<td>C-cell thyroid tumors</td>
</tr>
<tr>
<td>AIAAPP</td>
<td>Islet amyloid polypeptide***</td>
<td>L</td>
<td>A</td>
<td>Islets of Langerhans, insulinomas</td>
</tr>
<tr>
<td>AANF</td>
<td>Atrial natriuretic factor</td>
<td>L</td>
<td>A</td>
<td>Cardiac atria</td>
</tr>
<tr>
<td>APro</td>
<td>Prolactin</td>
<td>L</td>
<td>A</td>
<td>Pituitary prolactinomas, aging pituitary</td>
</tr>
<tr>
<td>Ahfns</td>
<td>Insulin</td>
<td>L</td>
<td>A</td>
<td>Iatrogenic, local injection</td>
</tr>
<tr>
<td>ASPC***</td>
<td>Lung surfactant protein</td>
<td>L</td>
<td>A</td>
<td>Lung</td>
</tr>
<tr>
<td>AGal7</td>
<td>Galectin 7</td>
<td>L</td>
<td>A</td>
<td>Skin</td>
</tr>
<tr>
<td>ACor</td>
<td>Corneodesmosin</td>
<td>L</td>
<td>A</td>
<td>Cornified epithelia, hair follicles</td>
</tr>
<tr>
<td>AMed</td>
<td>Lactadherin</td>
<td>L</td>
<td>A</td>
<td>Senile aortic, media</td>
</tr>
<tr>
<td>Aker</td>
<td>Kerato-epithelin</td>
<td>L</td>
<td>A</td>
<td>Cornea, hereditary</td>
</tr>
<tr>
<td>ALac</td>
<td>Lactoferrin</td>
<td>L</td>
<td>A</td>
<td>Cornea</td>
</tr>
<tr>
<td>AOAAP</td>
<td>Odontogenic ameloblast-associated protein</td>
<td>L</td>
<td>A</td>
<td>Odontogenic tumors</td>
</tr>
<tr>
<td>ASem1</td>
<td>Semenogelin 1</td>
<td>L</td>
<td>A</td>
<td>Vescula seminalis</td>
</tr>
<tr>
<td>AEnf</td>
<td>Enувiridte</td>
<td>L</td>
<td>A</td>
<td>Iatrogenic</td>
</tr>
</tbody>
</table>

Proteins are listed, when possible, according to relationship. Thus, apolipoproteins are grouped together, as are polypeptide hormones; *mainly in males; **Dan is the product of the same gene as ABri; ***Also called amylin; ****Not proven by amino acid sequence analysis. Abbreviations: A, acquired; ANS, autonomic nervous system; CJD, Creutzfeldt-Jakob disease; CNS, central nervous system; GSS, Gerstmann-Sträussler-Scheinker; H, hereditary; L, localized; PNS, peripheral nervous system; S, systemic. Adapted from Sipe JD, et al; Amyloid 2016[20].
It is of utmost importance to use the chemically based nomenclature in the diagnosis and treatment of amyloidosis, as the treatment plans for chemically distinct forms of amyloidosis differ markedly.

Despite the broadened concept of amyloid, there are some amyloid fibril proteins, such as Aβ and islet amyloid polypeptide (AIAPP) that play a pathologic role in neurodegenerative and endocrine diseases, respectively, but are not clinically classified as amyloidosis [20]. Intracellular protein inclusions such as neurofibrillary tangles, α-synuclein, Lewy and Huntington bodies had been considered to be “intracellular amyloid”, but they were not included in the formal list of characterized amyloid fibril proteins [20]. In addition, it has been suggested that some human structures, such as the p-mel framework in melanosomes and some polypeptide hormones when stored in secretory vesicles, have an amyloid fibril structure but are not related to amyloidosis [71].

1.4. Epidemiology

AL amyloidosis is the most prevalent type of amyloidosis in the developed world being diagnosed in 85% of patients with systemic amyloidoses [72, 73]. The primary organs involved are the heart and kidney. AL amyloidosis is almost always associated with an underlying clonal plasma cell proliferation. However, a true B-cell or plasma cell neoplasia is diagnosed in only 10–15% of AL patients [74].

AA amyloidosis is the second most prevalent type of amyloidosis [75]. This occurs as a complication of chronic inflammatory or infectious diseases. However, familial cases associated with a mutation in genes for non-amyloid fibril proteins, that play a permissive role in the development of amyloid, have also been increasingly recognized, for example in children in which the most common cause of AA amyloidosis is now autoinflammatory diseases. Among this group of diseases, the most frequent one throughout the world is familial Mediterranean fever [76].

A number of other monogenic autoinflammatory diseases have also been identified, particularly, cryopyrin-associated periodic syndromes, which are outstanding with its clinical features and the predilection to develop AA amyloidosis in untreated cases [76].

While AL and AA together affect about 90% of patients with systemic amyloidoses, the remaining 10% have other types, comprising diverse conditions, including hereditary, nonhereditary and even iatrogenic diseases. In addition, wild-type transthyretin can also be amyloidogenic and cause wild-type ATTR (ATTRwt) amyloidosis that primarily affects the heart [74].

Hereditary systemic amyloidoses comprise a group of rare monogenic diseases inherited in an autosomal dominant fashion with variable penetrance [77]. Most affected individuals are heterozygous although rare homozygous cases have been reported [78].

Hereditary amyloidoses can clinically present from early childhood upwards, but they are usually late-onset diseases. Currently, at least 181 variants of genes known
to cause hereditary amyloidosis have been reported: TTR variants account for 68%, APOA1 for 11%, FGA for 8%, LYZ for 5%, APOA2 for 2%, GSN for 2%, B2M for 0.8%, CST for 0.8%, k light chain gene for 0.8%, APOC2 for 0.8% and APOC3 for 0.8% [36, 38, 39, 79].

ATTR amyloidosis is the commonest type of hereditary amyloidoses. There are three known geographic hot spots (Portugal, Sweden and Japan), although the disease is known to occur worldwide. It usually presents with peripheral and autonomic neuropathy, but it can also present with cardiac or renal involvement [80-83]. It is estimated that about 4.0% of Afro-Caribbean population carry a TTR p.Val142Ile variant that is associated with late-onset cardiac amyloidosis in an undetermined proportion of cases [84]. Several other hereditary amyloidoses have also been discovered (AFib, AApoAl, AApoAll, among others) and are remarkably diverse with respect to age of onset, mode of presentation, pattern of organ distribution, rate of progression and prognosis.

1.5. Pathogenesis

The inability of a protein to adopt or maintain its native soluble conformation, generally referred to as protein misfolding, is the origin of amyloidosis [54]. Protein misfolding can cause disease by affecting bio-availability of essential proteins, rendering them susceptible to form harmful fibrillar aggregates with extensive β sheet structure [85].

Predisposing conditions

Protein misfolding, the initial step in amyloid fibril formation [86-88], can be triggered by: genetically altered proteins due to single base substitutions, insertions or deletions (as occurs in hereditary amyloidoses such as AFib amyloidosis [89]); specific post-translational proteolytic cleavage of the precursor protein (as in the case of Aβ in Alzheimer’s disease [90]); intrinsic protein propensity which becomes evident with aging (as in the case of wild-type TTR in patients with ATTRwt amyloidosis [91]); or by persistently high serum concentrations (as in the case of Aβ2M in patients undergoing long term hemodialysis [92]).

A particular case is that of leukocyte chemotactic factor-2 (ALECT2) amyloidosis, whose predisposing factor is presumed to be an increased production of ALECT2 induced by an acute phase protein [93]. In addition, no pathogenic mutations have been found in ALECT2 amyloidosis patients, although all cases have been homozygous for G/A polymorphism involving nucleotide 172 in exon 3 of LECT2 gene, located on chromosome 5q31.1–32, accounting for the presence of valine or isoleucine at position 40 in the mature protein [94].

The process of protein misfolding and aggregation

The process of protein misfolding and its relationship to aggregation and fibrillogenesis toward amyloid is driven thermodynamically by the very low free energy of the fibrillar state [87, 88, 95, 96]. This deep free energy minimum is derived
from the extensive formation of intermolecular hydrogen bonds, hydrophobic interactions and water exclusion from the inner core of the fibrils [95].

The folding of a newly synthesized polypeptide occurs in a rapid sequence of conformational modifications in the cytoplasm [87]. According to the “folding energy landscape theory” a polypeptide can acquire an alternative and relatively stable misfolded conformation at a minimum of energy like that reached by the native protein [87, 95]. In the case of systemic amyloidoses the pathway of amyloidogenesis begins with native globular proteins that circulate freely in plasma and diffuse into the interstitial space where, under a free energy minimum and alterations in microenvironment conditions such as increased temperature, reduction in pH, introduction of organic solvents, changes in the sequence of the normal protein or removal of chaperones, change the native protein conformation toward misfolded states [95, 97-100]. In addition, mutations may alter the minimum free energy of the native protein and their intermediate states, effectively altering the free energy landscape making it possible to proceed to a partially unfolded intermediate state. This intermediate state appears to be a key step in any further transformation and a crucial entity in amyloid conversion [88]. Misfolded or partially folded proteins tend to aggregate due to intermolecular contacts driven by hydrophobic forces. Aggregation can lead to the formation of highly ordered, fibrillar aggregates called amyloid.

**Determinants of amyloid deposition and distribution**

The remarkable diversity in the organ distribution of amyloid deposits remains one of the most important unsolved problems in amyloid research [87]. In addition to the intrinsic amyloidogenic potential of the pathogenic precursor protein, other factors may act synergistically regarding amyloid deposition: the protein precursor must reach a critical local concentration; microenvironment factors such as temperature, pH, shear forces, removal of chaperones and the presence of fibril seeds [87, 101]; and interaction with extracellular matrix components, such as SAP, apolipoproteins E, GAGs, proteoglycans, metalloproteinases, collagen, or cell surface receptors such as the receptor for advanced glycation end-products (RAGE) [54, 87, 102-104].

Amyloid deposition may occur at, or close to, the biosynthetic site of its amyloidogenic precursor, or far from its local of synthesis [88]. When amyloid deposition occurs at or near the site of biosynthesis, fibrillogenesis may take place within the cell or close to it extracellularly. One example is AβAPP in β-cells of the islets of Langerhans [105]. In this situation, intracellular synthesized protein experience conditions that alter the stability of the folded protein creating a microenvironment that favors the generation of conformational intermediates, oligomers and, ultimately, fibrils [88]. Similarly, AFib and AAPoAl are synthesized in the liver but are deposited in extra-hepatic sites. It appears therefore that the generation of unstable forms and early aggregates happens not close to the cell which synthesizes...
the precursor or in plasma but more likely in the microenvironment where these proteins are finally deposited [88].

There is still much to understand about the potential microenvironment factors, how are they generated and determine which organ is targeted by the different amyloidogenic proteins and, particularly, why different mutations in a specific amyloidogenic protein (e.g., ATTR, AApOl or Aβ2M) can determine which organs will be involved. The fact that single amino acid substitutions can change the tissue site of amyloid deposition suggests that it might be influenced by structural and functional features of the amyloidogenic protein itself [88].

Experimental studies examining fibrillogenesis on decellularized amyloid organs will represent an innovative approach to further investigate amyloid disease and address crucial issues, such as, the relationship between de extracellular matrix and amyloid fibrils and the possible effects of natural fibrils on protein oligomerization [106].

**Mechanisms of tissue damage and organ dysfunction**

Disruption of tissue architecture by the deposition of large amounts of fibrillar material had long been accepted as the underlying mechanism of organ dysfunction in the amyloidosis [86]. Organs infiltrated with large quantities of amyloid, deposited between blood vessels and parenchymal cells using the stromal architecture of the extracellular matrix, become rigid and this rigidity may affect their function, regardless of amyloid type [107]. This is believed to impair the transfer of nutrients, metabolic and functional products between parenchymal cells and to constrict the space around these cells affecting their physiological function [107].

Amyloid fibrils may also cause organ dysfunction by interacting with local receptors, such as RAGE [104, 108].

Direct cell toxicity mediated by prefibrillar species such as monomers and oligomers has also been proposed as a mechanism of disease manifestations in amyloidosis [109-111]. Indirect support for this mechanism is the rapid improvement in markers of organ dysfunction after treatment and reduced production of precursor protein. One example in renal amyloidosis is the rapid decrease of proteinuria after treatment that markedly reduces production of the amyloidogenic precursor protein. Indeed, in small series of patients with AL amyloidosis who underwent serial kidney biopsies, the extent of amyloid deposition seemed to be similar in biopsies that were performed before treatment and after treatment-induced resolution of proteinuria [86, 112]. Another interesting example is impaired cardiac function in AL amyloidosis and its positive correlation with the plasma level of free amyloidogenic light chains rather than the quantity of amyloid deposited in the heart [113]. However, by the time these patients become symptomatic, amyloid deposits are already detected in the tissues [107].
1.6. Diagnosis

The diagnosis of amyloidosis requires accurate characterization of amyloid fibril protein (Figure 1) [86, 114]. Congo red stain remains the gold standard for detection of amyloid deposits [115]. In light microscopy, Congo red stained amyloid typically have a salmon-pink color that by itself is useful for diagnosis [41]. Congo red stained slides must be examined under polarized light and only the presence of apple-green birefringent deposits, which result from the intercalation of the Congo red dye into the amyloid fibrils [86], is considered diagnostic of amyloid [41]. In addition, the experience of the observer, good fixation, a proper staining protocol (alkaline Congo red), appropriate optics, and thicker sections (5–10 μm) are required for a reliable diagnosis [41].

![Detection of Amyloid](image)

<table>
<thead>
<tr>
<th>Detection of Amyloid</th>
<th>Type of Amyloid</th>
<th>Role of Genetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue biopsy is still needed in all cases</td>
<td>Congo red remains the gold standard</td>
<td>Immunohistochemistry in routine clinical practice</td>
</tr>
<tr>
<td>Identification of amyloid protein within the deposits</td>
<td>Mass spectrometry-based proteomics for inconclusive results</td>
<td>Hereditary amyloidosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Familial amyloidosis</td>
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<tr>
<td></td>
<td></td>
<td>DNA studies</td>
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</tbody>
</table>

**Figure 1 | Amyloid identification and typing.**

Other stains or techniques, such as fluorescent dyes, thioflavin S and T, methyl violet and sulphonated Alcian blue, are less specific and at times also less sensitive, so confirmation with Congo red stain is required [41].

Although Congo red staining properties represent the gold standard for amyloid identification, small deposits, in thinner sections, may not be detectable in light microscopy. Also, false positive staining occurs for collagen using Congo red, so we must avoid interpreting collagens as amyloid [41].

Oligothiophene fluorescence is a new optically active conformation sensitive ligand that may turn out to be very helpful in the detection of amyloid deposits in the near future [20, 116].

Any tissue can be evaluated for Congo red positivity, and the yield is greatest from sites with clinical evidence of involvement [86, 114]. However, if amyloidosis is suspected, the diagnosis often can be confirmed with abdominal fat aspiration rather than with an invasive biopsy.

The sensitivity of Congo red staining in abdominal fat is approximately 80 to 90% and 65 to 75% in AL and AA amyloidosis, respectively, but substantially lower in
many of the hereditary amyloidoses [117]. In addition, many pathologists have encountered less than optimal results due to inadequate samples and technically poor smears, and misinterpretation of Congo red stains because of the autofluorescence of collagen under polarization microscopy [41]. Therefore, the absence of Congo red positivity in abdominal fat does not eliminate the diagnosis.

In recent years, fat has reemerged as an underused source of tissue not only for amyloid diagnosis but also for amyloid typing and staging [41]. With an adequate fat specimen, it is possible to perform not only a Congo red stain but also immunofluorescence, electron microscopy, and, if necessary, a Western Blot or other molecular studies for amyloid typing [41, 118].

Salivary gland and rectal biopsies are also used as relatively noninvasive methods for detection of amyloid in tissue [86].

Amyloid fibrils are visible by electron microscopy, a standard approach of the histological evaluation of kidney biopsies, reducing the likelihood of a missed diagnosis with the routinely used Congo red dye. By electron microscopy, the amyloid deposits are identified by the presence of rigid, non-branching fibrils of 7.5 to 10 nm in diameter [86, 87].

After detecting the presence of amyloid, the current standard techniques used routinely to type it are immunostaining, in particular, immunofluorescence on frozen sections (Figure 2) or IHC on paraffin embedded sections [41].

![Image](image-url)

Figure 2 | Immunofluorescence. Renal biopsy in fibrinogen Aα chain amyloidosis. Immunofluorescence shows a glomerulus with strong reactivity for fibrinogen (courtesy of Dr. Roberto Silva, Department of Pathology, CHSJ).

Accuracy of diagnosis depends on antibody panel directed against known amyloidogenic proteins, background stain, correlation between IHC staining and Congo red positive areas, positive and negative controls, experience and expertise of the operator [41].

The challenges of amyloid IHC include: lack of commercially available amyloid and amyloid-type-specific antibodies; antibody heterogeneity; serum contamination; and a lack of availability of adequate controls [119-121].
The most accurate method for identifying the amyloidogenic protein is by mass spectrometry-based proteomics or amino acid sequencing of proteins that are extracted from amyloid deposits. However, these should be considered complementary to IHC [74]. Current application of these techniques should be for: typing of amyloid deposits where routine Immunofluorescence or IHC is equivocal or negative; confirmation of the amyloid type; detection of less common or unusual amyloid types; and cases where there is an inadequate sample for immunofluorescence or IHC typing [43, 47, 72].

Molecular genetics may be associated with amyloidoses in several ways. It is involved in the identification of: mutations in non-amyloid proteins (familial amyloidosis); mutations involving amyloid proteins itself (hereditary amyloidoses); and in sporadic amyloidosis through the identification of susceptible polymorphic coding regions (e.g., SAA genotype in AA amyloidosis) [41, 83, 86, 122-125]. The first of these mechanisms occurs in patients with familial AA amyloidosis, which is associated with various periodic fever syndromes, of which familial Mediterranean fever is the best known [122, 126]. These patients have an inborn error of inflammatory response in the innate immune system and mutations in genes for non-amyloid fibril proteins play a permissive role in the development of amyloid.

The pathology of sporadic versus familial AA amyloidosis has also to be based on clinical grounds. However, the distinction between these has implications for treatment and prognosis and should also involve genetic counseling [122]. Genetic testing should always be complementary to other diagnostic techniques that allow unequivocal identification of the amyloid protein [41].

The importance of accurate identification of the amyloid type has implications for the establishment of the genotype/phenotype relationship, therapeutic orientation, prognosis and genetic counseling.

1.7. Therapy

The therapeutic approach of amyloidoses depends on whether it is acquired or inherited, localized or systemic. In addition, it also depends on the typing of amyloid fibril protein and the impact of amyloid deposition which might be life-threatening or merely an incidental finding.

Advances in knowledge of the molecular mechanisms underlying protein misfolding and aggregation, insoluble fibril formation and amyloid deposition have led to the identification of several therapeutic targets, including reducing the supply of amyloid fibril precursor protein and different key steps in the amyloidogenic cascade [127].

**Reduction in the supply of amyloid fibril precursor proteins**

In systemic amyloidoses, current therapeutic strategies are centered on reducing the supply of the respective amyloid fibril precursor protein whilst supporting or replacing the function of affected organs [128].
In AA amyloidosis, the mainstay of therapy is the control of the underlying inflammatory condition: steroids, disease-modifying and biological drugs, mainly anti-tumor necrosis factor treatment against chronic inflammatory arthritis; antibiotics for chronic infections; colchicine or interleukin-1-receptor antagonists for familial Mediterranean fever; and anti-tumor necrosis factor treatment and interleukin-1-receptor antagonists for other autoinflammatory syndromes [129-131].

In AL amyloidosis, chemotherapy is targeted towards the underlying B-cell dyscrasia thereby reducing production of amyloid-forming monoclonal immunoglobulin light chains [132]. Furthermore, high-dose of melphalan followed by autologous stem cell transplantation is the first-line treatment in selected low-intermediate risk AL amyloidosis patients [133].

Finally, in hereditary ATTR, AAp0Al and AFib amyloidosis, orthotopic liver transplantation removes the source of the genetically variant amyloidogenic protein and has been performed as a “surgical gene therapy” [128, 134-136].

**Blockade of protein translation and transcription**

Oligonucleotide-based therapies, including small interfering ribonucleic acids (siRNAs) and antisense RNAs, act by changing the translational level of the protein without becoming integrated into the human genome [137].

Knockdown of the amyloidogenic light chain messenger ribonucleic acid (mRNA) or of polyclonal light chain mRNA using antisense oligonucleotides (ASOs) or siRNAs has been successfully tested in pre-clinical models of AA and AL amyloidosis [138-140]. This approach may be particularly attractive for those cases of AA amyloidosis for which the underlying inflammatory condition cannot be identified and successfully treated [127].

Knockdown of both wild-type TTR and mutant ATTR through siRNAs or ASOs is now under full clinical development [127]. Delivery of a siRNA moiety against part of the 3’ untranslated region of TTR mRNA, conserved in both the wild-type and mutant alleles, and encapsulated into first (ALN-TTR01; Alnylam, Cambridge, MA) or second (ALN-TTR02, Alnylam, Cambridge, MA) generation formulations of lipid nanoparticles has been tested in two phase 1 trials in hereditary ATTR amyloidosis patients (NCT01148953) and healthy volunteers (NCT01559077), respectively [141]. A dose-dependent suppression of both mutated ATTR levels and serum wild-type TTR was observed [141].

A phase 2 trial with the siRNA patisiran (ALN-TTR02) in patients with familial amyloid polyneuropathy has been associated with significant reductions in serum TTR levels without serious adverse events [142].

A phase 3 trial of patisiran (APOLLO study; NCT01960348) in patients with hereditary ATTR amyloid polyneuropathy resulted in significant improvement in polyneuropathy relative to placebo and was generally well tolerated [143, 144]. However, a phase 3 trial (ENDEAVOUR study; NCT02319005) of the siRNA revusiran
(ALN-TTRSC) for cardiac amyloidosis was terminated prematurely due to increased mortality in the treatment group [145].

Anti-sense oligonucleotides are synthetic nucleotide sequences that bind and promote degradation of mRNA suppressing gene expression [146]. Promising results were obtained with IONIS-TTRx (Ionis Pharmaceuticals, Carlsbad, CA), an ASO moiety against TTR mRNA, which has been proven to be safe in healthy volunteers during the course of a phase 1 trial, with a sharp decline in TTR production [147].

A phase 3 trial of inotersen (NEURO-TTR study; NCT01737398) demonstrated significant benefit on neurological disease progression and quality of life in patients with hereditary ATTRamyloid polyneuropathy [148].

Stabilization of the fibril precursor proteins

Studies in ATTR amyloidosis support the hypothesis that small molecules bound by amyloid fibril precursors can increase the stability of these proteins and reduce their propensity to off-pathway folding [128]. Diflunisal and tafamidis have been studied as stabilizers of the TTR tetramer.

Diflunisal, an old non-steroidal anti-inflammatory drug, significantly reduced the rate of neurological progression and improved the quality of life of patients with ATTR amyloidosis [149, 150]. Tafamidis can stabilize the amyloid polyneuropathy and cardiomyopathy in most patients and is the only approved drug for the treatment of ATTR amyloidosis [151, 152]. Recently, tolcapone, an orally active drug used against Parkinson’s disease, has been shown to bind and stabilize TTR tetramers, also from mutation carriers, suggesting repurposing of tolcapone for ATTR amyloidosis [153]. Since tolcapone can cross the blood-brain barrier, it could be particularly useful to treat patients with TTR mutations forming leptomeningeal deposits [127].

Epigallocatechin-3-gallate, the main anti-oxidant polyphenol of green tea, has been shown to inhibit the formation of aggregates, thereby inhibiting amyloidogenesis in AL and ATTR amyloidosis [154, 155].

Recognizing that the relative instability of fibril precursor proteins is a key factor in amyloid fibrillogenesis indicates that precursor stabilization might be applicable to several amyloid types [128].

Inhibition of fibrillogenesis

Heparan and dermatan sulfate proteoglycans containing highly sulfated GAGs chains are a universal constituent of amyloid deposits and are thought to contribute to promoting or maintaining the conformational changes associated with amyloidogenesis. Inhibiting the interaction between GAGs and amyloid fibrils remains a promising therapeutic approach in all types of amyloidosis [128].

Eprodisate is a low molecular weight, negatively charged, sulfonated molecule structurally similar to heparin sulfate. It competitively binds to GAGs-binding sites on SAA, thereby inhibiting fibril polymerization and amyloid deposition in a mouse model of AA amyloidosis [156]. In a phase 2/3 clinical trial involving 180 patients, 2-year treatment with eprodisate (800–2400 mg/day based on creatinine clearance) led
to reduced disease worsening and reduced risk and speed of creatinine decline compared with placebo, but no significant effect on proteinuria or death [157].

**Fibril disruption**

Doxycycline was shown to disaggregate amyloid fibrils in vitro and reduce the deposits in a transgenic ATTR amyloidosis mouse model [158]. For ATTR amyloidosis, its therapeutic potential has been investigated in association with the anti-apoptotic drug tauroursodeoxycholic acid, with promising results in an open-label phase II study [159]. Doxycycline has been granted the designation of orphan drug for ATTR and dialysis-related amyloidosis [127].

**Enhancement of amyloid regression: targeting SAP component**

The nonfibrillar plasma glycoprotein SAP is universally present in amyloid deposits, reflecting its specific calcium-dependent binding to motifs present in all types of amyloid fibrils [160]. Furthermore, SAP persists within human amyloid deposits for prolonged periods and is completely unmodified with respect to circulating SAP [161]. SAP itself is highly resistant to proteolysis and the binding of SAP by amyloid fibrils in vitro protects them from degradation by phagocytic cells and proteolytic enzymes [162]. The possibility that SAP might contribute to the pathogenesis and/or persistence of amyloid deposits in vivo was strongly supported by inhibiting the production of experimental AA amyloidosis in SAP knockout mice [163]. In this context, a small molecule drug, miridesap [(R)-1-{6-[(R)-2-carboxy-pyrrolidin-1-yl]-6-oxo-hexanoyl]pyrrolidine-2-carboxylic acid], previously known as CPHPC, that inhibits the binding of SAP to amyloid fibrils was developed and is under investigation [164, 165].

**Immunotherapeutic clearance of amyloid**

As all amyloid fibrils share common structural motifs, an attractive strategy is the possibility that anti-amyloid antibodies might enhance clearance of amyloid deposits [128]. In this field, several immunotherapeutic drugs are under intense investigation.

Antibodies recognizing a generic fibril epitope have been raised [166] and, when passively administered, were shown to speed resolution of experimentally created AL amyloidomas in mice and to reduce amyloid burden in mice with AA amyloidosis [167, 168].

Prothera is developing NEOD001 as a potential first-in-class, disease modifying agent for the treatment of AL amyloidosis. NEOD001 is a monoclonal antibody designed to specifically target the amyloid that accumulates in patients with AL amyloidosis. Predilindrical studies suggest that this antibody recognizes light chain misfolded proteins and promotes phagocytes-mediated clearance of AL amyloid deposits [169].

There are two ongoing clinical trials of NEOD001 in patients with AL amyloidosis. A phase 2b trial (PRONTO study; NCT02632786) of previously treated patients with AL amyloidosis and cardiac dysfunction is evaluating best cardiac response over 12
months based on reduction of the cardiac biomarker NT-proBNP. A phase 3 trial (VITAL study; NCT02312206) of newly diagnosed, treatment naïve patients with AL amyloidosis and cardiac dysfunction is evaluating a composite endpoint of time to all-cause mortality or cardiac hospitalization. Improvements in some biochemical markers of cardiac function and reduction in proteinuria were reported in AL amyloidosis patients treated with NEOD001 which highlights its potential benefit [170].

PRX004 is an investigational monoclonal antibody that specifically targets disease-causing misfolded transthyretin in ATTR amyloidosis. Currently in preclinical development, Prothera plans to advance PRX004 into the clinic as a potential therapy for ATTR amyloidosis (NCT03336580).

The murine amyloid fibril-reactive monoclonal antibody 11-1F4 (mAb 11-1F4) recognizes an epitope on human light chain amyloid fibrils and initiates cell-mediated phagocytosis [171]. Improvement in both cardiac and renal function of patients with AL amyloidosis was demonstrated in a phase 1 trial (NCT02245867) of mAb 11-1F4 [171].

Combined therapy might enhance clearance of amyloid deposits. As previously mentioned, miridesap efficiently depletes SAP from the plasma but leaves some SAP in amyloid deposits. These can be specifically targeted by therapeutic monoclonal immunoglobulin G1 (IgG1) anti-SAP antibodies [165, 172].

Previous depletion of circulating SAP by miridesap followed by subsequent administration of the humanized monoclonal IgG1 anti-SAP antibody, dezamizumab, was safe (NCT01777243) and represents a novel and promising approach to the treatment of systemic amyloidoses [165, 173]. Dezamizumab binds to residual SAP in the amyloid deposits, activates the classical component pathway and opsonizes the deposits with fixed complement C3, attracting and engaging macrophages that fuse into multinucleated giant cells enabling amyloid removal [165].

Repeat cycles of miridesap followed by dezamizumab progressively removed amyloid from the liver, spleen and kidneys of patients with AL, ATTR, AApoAII and AFib amyloidosis [165]. This promising approach may alter the natural history of systemic amyloidoses in the near future.

2. **FIBRINOGEN Aα CHAIN**

2.1. **Structure and function**

Fibrinogen is a 340 kDa glycoprotein synthesized in hepatocytes [174] that circulates in plasma at a concentration of about 1.5 to 4.0 g/L and is essential for blood clotting, fibrinolysis, cellular and matrix interactions, wound healing, inflammation and angiogenesis [175-177]. The core structure consists of two outer D regions and a central E region (Figure 3).

The molecule exhibits a twofold axis of symmetry perpendicular to the long axis, consisting of two sets of three polypeptide chains, Aα, Bβ and γ, which assemble in a
hexameric structure \((A\alpha B\beta \gamma)_2\) joined in their amino terminal regions by disulfide bridges to form the E region [178-181].

The outer D regions contain the globular COOH-terminal domains of the B\(\beta\) and \(\gamma\) chains. Unlike these domains, the A\(\alpha\) chains are intrinsically unfolded and flexible and tend to be uncovalently tethered in the vicinity of the central E region [181].

Each A\(\alpha\) chain contains an NH2-terminal fibrinopeptide A sequence. Cleavage of these sequence by thrombin initiates fibrin assembly [176].

![Figure 3 | Fibrinogen structure.](image)

The individual chains, A\(\alpha\), B\(\beta\) and \(\gamma\), are blue, green and red, respectively; fibrinopeptides A and B (FpA and FpB) are magenta; the disulfide bonds are shown by black bars; triple arrows show proteolytic cleavages between the D and E regions, single arrows show cleavages resulting in the removal of the COOH-terminal regions of the A\(\alpha\) chains (\(\alpha\)C region), and NH2-terminal regions of the B\(\beta\) chains (\(B\Pi\)N region) [182]. Reuse with permission, license number 4307230015861, John Wiley and Sons.

Each polypeptide chain is encoded by a distinct gene, FGB (4q31.3) for B\(\beta\), FGA (4q31.3) for A\(\alpha\), and FGG (4q32.1) for \(\gamma\), ordered from centromere to telomere, with FGA and FGG transcribed from the reverse strand, in the opposite orientation to FGB (Figure 4) [180, 183]. Each gene is separately transcribed and translated to produce nascent polypeptides of 644 amino acids (A\(\alpha\)), 491 amino acids (B\(\beta\)), and 437 amino acids (\(\gamma\)) [181].

![Figure 4 | Localization of amyloidogenic mutations in the fibrinogen gene cluster.](image)

The three fibrinogen chains genes are shown ordered from centromere (cen) to telomere (tel), with the FGA and FGG genes transcribed in the opposite orientation to FGB. The FGB consists of eight exons, the FGA consists of 6 exons and the FGG consists of ten exons. All amyloidogenic mutations are clustered in exon 5 of FGA [180]. Reuse with permission, license number 4307221141479, John Wiley and Sons.

The FGA consists of six exons. Three different mRNA products are produced: the major mRNA species encoding the common A\(\alpha\) chain accounting for 90% of
transcripts is 2.2 kilobases (coding sequence 1.9 kilobases) and contains exons 1 to 5. An extended α-E isoform, accounting for only 1–2% of transcripts, splices out the last 15 codons of exon 5 and intron 5 and keeps exon 6. Unspliced bipartite transcripts with intact exon 5, intron 5 and exon 6 are produced encoding the common Aα chain [180, 184].

Mutations in FGB, FGA and FGG are commonly associated with autosomal or recessive bleeding or thrombotic disorders [185]. However, a small fraction of FGA variants is amyloidogenic and lead mainly to renal massive AFib amyloid deposition.

2.2. Pathogenesis of hereditary fibrinogen amyloidosis

Hereditary renal amyloidosis was formerly described by Benno Ostertag in 1932, due to the identification of a German family in which several members with hypertension and renal failure died with renal amyloidosis [186, 187]. However, the first clinical report of what was later determined to be AFib amyloidosis was published only in 1975 [188].

The underlying genetic cause of AFib amyloidosis (OMIM +134820) was originally demonstrated in 1993 in a Peruvian kindred, segregating with the FGA p.Arg573Leu variant, which is one of the rarest fibrinogen variants [31].

One year later, the FGA p.Glu545Val was first recognized as causative of AFib amyloidosis in American kindreds of Irish origin [189]; it has since become the most commonly reported FGA amyloidogenic variant worldwide after also being identified in a Canadian kindred of Polish origin [190] and in families from the United Kingdom [32, 120, 191, 192], Portugal [193], Australia [194], Austria [195], Italy [196], New Zealand [192], France [197-199], Germany [32, 123], United States [200-202], Brazil [203], and China [204].

Several mutations in the COOH-terminal region of the FGA have been associated with AFib amyloidosis (Table 2). In these cases, fibrinogen variants are functional in heterozygous patients because the fibrinogen activity is not decreased, but they are unstable and prone to form systemic amyloid fibrils leading to organ damage [32].

Protein sequencing of amyloid deposits isolated from renal and splenic tissues of patients with AFib amyloidosis has indicated that amyloid fibrils are composed of fragments of AFib that display green birefringence under polarized light when stained with Congo red and present a cross β structure in X-ray diffraction studies [31, 189].

The currently known 16 fibrinogen Aα chain variants (Table 2), clustered from residues 517 to 555 (536 to 574), have been identified as amyloid-prone in humans [79, 89, 205]. These fibrinogen variants are functional, but unstable and prone to form systemic amyloid fibrils which have a remarkable deposition in kidneys [206].

The reasons for deposition of these variants and their amyloidogenicity have not been completely elucidated.

Despite genetically altered proteins due to missense, deletion or insertion-deletion mutations, it is suggested that the αCOOH-terminal peptide of these
fibrinogen variants induces changes in the protein tertiary structure leading to an increased susceptibility to proteolysis of one or more degradation peptides that aggregate to form amyloid [89, 207].

Because early proteolysis of fibrinogen Aα chain occurs intravascularly, this may explain the targeting of the kidney for this type of amyloid fibril formation [207].

**Table 2 | Amyloidogenic mutations in fibrinogen Aα chain gene**

<table>
<thead>
<tr>
<th>FGAMutations</th>
<th>Protein variants</th>
<th>Geographic origin/ethnicity</th>
<th>References</th>
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<tbody>
<tr>
<td>Missense mutations</td>
<td></td>
<td></td>
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<tr>
<td>c.1627G&gt;A</td>
<td>Glu524Lys (p.Glu543Lys)</td>
<td>Scottish</td>
<td>[208]</td>
</tr>
<tr>
<td>c.1633G&gt;A</td>
<td>Glu526Lys (p.Glu545Lys)</td>
<td>Russian</td>
<td>[208]</td>
</tr>
<tr>
<td>c.1634A&gt;T</td>
<td>Glu526Val (p.Glu545Val)</td>
<td>US, Europe, Brazil, China</td>
<td>[32, 189, 193, 203, 204]</td>
</tr>
<tr>
<td>c.1670C&gt;A</td>
<td>Thr538Lys (p.Thr557Lys)</td>
<td>Chinese</td>
<td>[32]</td>
</tr>
<tr>
<td>c.1676A&gt;T</td>
<td>Glu540Val (p.Glu559Val)</td>
<td>German</td>
<td>[32, 209]</td>
</tr>
<tr>
<td>c.1712C&gt;A</td>
<td>Pro552His (p.Pro571His)</td>
<td>Afro-Caribbean</td>
<td>[32]</td>
</tr>
<tr>
<td>c.1718G&gt;T</td>
<td>Arg554Leu (p.Arg573Leu)</td>
<td>Mexico (Peruvian-Mexican), Europe, US (African-American)</td>
<td>[31, 210, 211]</td>
</tr>
</tbody>
</table>

| Deletion mutations |
|-------------------|-----------------|-----------------------------|------------|
| c.1611delA(488delA) | Gly519Glufs*30 (p.Gly538Glufs*30) | French                  | [208]      |
| c.1620delT(4895delT) | Phe521Leufs*28 (p.Phe540Leufs*28) | French                  | [212, 213] |
| c.1624_1627delAGTG (4899_4902delAGTG) | Ser523Argfs*26 (p.Ser542Argfs*26) | Japanese               | [214]      |
| c.1632delT(4907delT) | Thr525Thrfs*24 (p.Thr544Thrfs*24) | Chinese                 | [32]       |

| Insertion-deletion mutations |
|-------------------------------|-----------------|-----------------------------|------------|
| c.1606_1620del, 1619_1620insCA (4881_4895del, 4894_4895insCA) | Met517_Phe521delinsGlnSerfs*28 (p.Met536_Phe540delinsGlnSerfs*28) | Korean             | [216] |
| c.1720_1721delGGinsTT (5445_5446delGGinsTT) | Gly555Phe (p.Gly574Phe) | Norwegian           | [208]    |

Adapted from Mutations in Hereditary Amyloidosis [79] and Yazaki, M et al, Int J Mol Sci 2018 [217].

Fibrinogen amyloidogenic variants are synthesized in the liver but are deposited mainly in extra-hepatic sites. It appears therefore that if protein concentration, conformational instability and oligomers play the role proposed in vitro, it is more likely that the unstable forms and early aggregates are generated not close to the cell which synthesizes the precursor, or in plasma, but more likely in the microenvironment where these proteins are finally deposited [88].

Recently it was reported that at physiological pH, the wild-type FGA fragments remain monomeric, whereas its p.Glu559Val variant forms amyloid-like fibrils in vitro as observed by atomic force microscopy, while the p.Arg573Leu variant converts in vitro into globular β sheet aggregates, showing amyloid-like properties, and suggesting that FGA variants have increase amyloidogenicity [205].

Compelling evidence that VLITL confers amyloidogenic properties to Aα chain frameshift variants was recently reported [213]. This VLITL amyloid motif is
exclusively present at the COOH-terminal end of the amyloidogenic Aα chain frameshift sequences and is necessary for β sheet arrangement of the full-length Aα chain Phe540Leufs*28-peptide identified in AFib amyloid kidneys [213].

Despite the recent advances in the study of this disease, the underlying pathogenic mechanisms remain largely unknown.

### 2.3. The starting point for this original study and the obscurity of AFib amyloidosis in Portugal

In 2001, in Portugal, mutated transthyretin was the protein precursor usually associated with hereditary systemic amyloidoses. At that time, a joint study of Isabel Tavares and Luísa Lobato would be the lever for the further research that we have proposed. The characterization and immunohistochemical classification of a series of 56 consecutive native kidney biopsies, performed at the Department of Nephrology of CHSJ was the starting point for the current original work. In those biopsies, the diagnosis of amyloid nephropathy revealed that approximately 11% of the cases corresponded to forms of non-ATTR hereditary amyloidoses [218].

We thus found 6 cases of amyloid nephropathy whose IHC staining was negative for SAA, light chains κ and λ, and TTR. One case was of a patient who had undergone a renal biopsy in 1990 due to nephrotic syndrome. However, it was only in 2004 that the molecular genetic analysis of this case allowed the diagnosis of AFib amyloidosis. In the same year, we identified the second case of AFib amyloidosis, corresponding to a patient with chronic kidney disease (CKD) and proteinuria that had undergone a renal biopsy in 1994, which documented amyloid nephropathy.

The identification of these two cases of AFib amyloidosis, with origin in the same geographical region but from apparently unrelated families, was the beginning of the research work that underlies this thesis.
II – AIMS
In the scope of AFib amyloidosis in northern Portugal, the aims of the research study underlying this thesis were:

1. Evaluation of the possibility of unidentified or suspected cases of AFib amyloidosis;
2. Epidemiological characterization of the disease;
3. Identification of the amyloidogenic variants in the FGA gene;
4. Definition of the natural history of AFib amyloidosis associated with the FGA p.Glu545Val variant;
5. Establishment of a correlation between the phenotype and the genotype;
6. Definition of the most appropriate treatment, regarding to transplantation options;
7. Assessment of prognosis.

In addition, haplotype study was performed to investigate the possibility of a common ancestor.
III – METHODS
The methodology applied in this research work integrates methods of epidemiological, clinical, histological and molecular biology research. The study plan was as follow:

1. Systematic recording of patients with proteinuria or chronic renal failure and amyloid deposition in kidney or other tissue biopsy specimens;

2. Typing the amyloid precursor protein by immunohistochemical staining using a broad panel of antibodies against SAA, light chains κ and λ, transthyretin, fibrinogen Aα chain, apolipoprotein A1 and lysozyme;

3. Identification of the amyloidogenic variant in all cases of AFib amyloidosis through DNA sequence analysis of the coding region of exon 5 of FGA gene;

4. Definition of the district of Braga as an area of interest for epidemiological study to assess the prevalence of AFibE526V (p.Glu545Val) amyloidosis among northern Portuguese patients undergoing hemodialysis;

5. Phenotypic characterization of those affected by AFib amyloidosis, including determination of age at onset of nephropathy, clinical manifestations with attention to the onset of hypertension and cardiovascular involvement, symptomatic evolution, through retrospective and prospective evaluation of morbidity and mortality after renal replacement therapy (RRT) including renal transplantation;

6. Probands were defined as the first chronic kidney patients with AFib amyloidosis identified within a family. For every kindred a detailed genogram was drawn and updated. Clinical records of symptomatic relatives were retrospectively analysed. Molecular diagnosis of FGA p.Glu545Val was provided to proband’s relatives either symptomatic or asymptomatic;

7. Regular observation of FGA p.Glu545Val asymptomatic carriers aiming the determination of an early marker of glomerular lesion;

8. Analysis of the haplotypes of the FGA p.Glu545Val variant using two intragenic polymorphisms, the FGA Rsal cutting site in exon 5 (rs6050) [219] and the TaqI cutting site in the 3’ untranslated region (rs533633927) [220];

9. All the participants had more than 18 years old, were informed about the disease and gave written informed consent.

All the work was conducted in accordance with the Ethical Principles for Medical Research Involving Human Subjects adopted by the World Medical Association [221], and its design and research protocol were reviewed and approved by Centro Hospitalar de São João Heath Ethics Committee (resolution no. 90/2012).
IV – RESULTS
1. EPIDEMIOLOGY

1.1. Renal amyloidosis: classification of 102 consecutive cases

A precise epidemiology of amyloidoses is difficult to define as the disease is often undiagnosed or misdiagnosed, due to the fact that IHC typing is not performed routinely and selection bias of data from tertiary centres becomes potentially unrepresentative.

In Portugal, ATTR amyloidosis is the most frequent form of hereditary systemic amyloidoses since its identification in 1939 [222]. However, no epidemiological information exists for the other types of systemic amyloidoses, mainly outside the reference centres for ATTR amyloidosis.

In 2001, the absence of knowledge about the type of amyloid identified in renal biopsies performed at the department of Nephrology of CHSJ combined with the importance of amyloid typing due to its therapeutic, prognostic and genetic counseling implications, led to the beginning of a systematic histological record of amyloid nephropathy cases diagnosed using the Congo red staining.

From May 1978 to September 2013, a total of 102 consecutive patients performed a native kidney biopsy at the department of Nephrology of CHSJ. These biopsies were retrospectively reviewed by immunohistochemistry staining using a panel of antibodies directed against SAA, κ and λ light chains, transthyretin, fibrinogen Aα chain, apolipoprotein A I and lysozyme.

In our series, AA amyloidosis was the most frequent type of systemic amyloidoses, followed by AL amyloidosis. A Fib amyloidosis was diagnosed in four (3.9%) of the patients, being therefore the third most common cause of amyloid nephropathy diagnosed in northern Portugal outside of the reference centre for ATTR amyloidosis.

A Fib amyloidosis is an autosomal dominant disease caused by a fibrinogen amyloidogenic variant. It is a rare disease that was described for the first time in Portugal in 2004 as a sporadic case [193]. However, in our study we identified four patients from apparently unrelated families, all from the same geographic region, the district of Braga in northern Portugal.

Our results favored the possibility that A Fib amyloidosis in Portugal was not sporadic. Also, was helpful for clinicians to decide for an early and more definitive diagnosis of the disease, given its impact on treatment, prognosis and genetic counseling.

Renal amyloidosis: classification of 102 consecutive cases

Amiloidose renal: classificação de 102 casos consecutivos

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ABSTRACT

Amyloidoses are a group of heterogeneous diseases classified according to the nature of their causative amyloid proteins. Commonly, paraffin-embedded tissue is used for the typing of amyloid by immunohistochemistry. DNA analysis should always be considered if hereditary amyloidosis is suspected. Since the kidneys are one of the organs that are most commonly involved in amyloid deposition in systemic amyloidoses, we screened 102 consecutive cases with biopsy-proven amyloid disease by immunohistochemistry. DNA analysis was performed to confirm a diagnosis of hereditary amyloidosis. Demographic characteristics, underlying disease and clinical data at the time of renal biopsy were obtained by retrospective review of medical records.

The amyloidosis type according to immunohistochemical amyloid protein identification was AA in 60 (58.8%) patients, AL in 21 (20.6%), AFib in four (3.9%), ATTR in two (2.0%), AAPoAL in one (1.0%), Alx in one (1.0%) and combined AL and AA in one (1.0%). The type of protein could not be classified in 12 (11.7%) patients: eight (7.8%) because of negative immunohistochemistry and four (3.9%) due to the lack of adequate tissue. DNA analysis confirmed AFib and ATTR cases by the identification of the point mutations FGA p.Glu55Val and TTR p.Met55Val, respectively. Mean age at diagnosis was 53.3 years (49.4 for AA, 63.0 for AL and 53.9 for AFib). Chronic infections were the most frequent disorder associated with AA amyloidosis, mainly tuberculosis, and only one patient had familial AA associated with Muckle-Wells syndrome. Nephrotic syndrome was the most frequent clinical manifestation, independently of the amyloid type.

In our series, AA amyloidosis is still the most frequent type of systemic amyloidoses. Six patients had unequivocal hereditary amyloidosis. Immunohistochemistry did not establish the precursor protein in almost 8% of patients; however, an improvement could be obtained using a wider panel of amyloid antibodies.

Key-Words: Amyloidosis; diagnosis; hereditary; immunohistochemistry; kidney.
RESUMO

As amiloidoses são um grupo heterogêneo de doenças classificadas de acordo com a composição das suas proteínas amiloidogênicas. Frequentemente, os tecidos preservados em parafina são usados para identificação imunohistoquímica. A análise de ADN deve ser sempre considerada se houver suspeita de amiloidose hereditária. Dado que os rins são um dos órgãos mais frequentemente envolvidos nas amiloidoses sistêmicas, procedemos à classificação imunohistoquímica de 102 casos consecutivos de doença amíloide confirmada por biópsia renal. A análise de ADN foi realizada para confirmar o diagnóstico de amiloidose hereditária. As características demográficas, doença subjacente e dados clínicos à data da biópsia foram obtidos pela revisão retrospectiva dos registos médicos.

O tipo de amiloidose obtido por identificação imunohistoquímica foi AA em 60 (58,8%) doentes, AL em 21 (20,6%), Aβ em quatro (3,9%), ATTR em dois (2,0%), APOA1 em um (2,0%), Alys em um (2,0%), e em um (2,0%) coexistiam os tipos AL e AA. Em 12 (11,7%) não foi identificado o tipo de amiloidose: oito (7,8%) por imunohistoquímica negativa e quatro (3,9%) devido a amostra insuficiente. A análise de ADN confirmou os casos Aβ e ATTR pela identificação das mutações pontuais p.Glu54Val e p.Met75Val, respectivamente. A média de idade à data do diagnóstico foi 53,1 anos (±9,4 para AA, ±3,0 para AL e ±5,9 para Aβ). As infeções crónicas foram a principal causa de amiloidose AA, sobretudo a tuberculose, e foi apenas identificada uma AA familiar associada a síndrome de Muckle-Wells. A síndrome nefrótica foi a manifestação clínica mais frequente, independentemente do tipo de amiloidose.

Na nossa série, a amiloidose AA continua a ser a amiloidose sistémica mais frequente. Seis doentes tiveram amiloidose hereditária inequívoca. A imunohistoquímica não identificou a proteína precursora em quase 8% dos doentes; contudo, a utilização de um painel de anticorpos mais alargado poderá melhorar o diagnóstico.

Palavras-chave: Amiloidose; diagnóstico; hereditário; imunohistoquímica; rim.

INTRODUCTION

Amyloidosis comprise a heterogeneous group of diseases that have in common tissue deposits of extracellular fibrillar proteins of similar structure but different chemical composition. Although they have been known since the time of Virchow, in the 19th century, until recently amyloidoses were considered a medical curiosity with only academic interest rather than clinically relevant diseases. However, recent advances in the treatment of systemic amyloidoses have changed this position and, hence, the importance of an early and correct diagnosis of the type of amyloid protein has gained relevance. The sample to be analysed must be of reasonable quality and quantity. Currently, amyloid deposits are identified on the basis of their apple-green birefringence under polarized light microscopy in Congo red stained histological preparations, the gold standard for amyloid detection, and the presence of rigid, non-branching fibrils 7.5 to 10 nm in diameter, on electron microscopy. Immunohistochemical identification of the chemical type of amyloid is still the first step in classifying amyloid. However, it must be performed and interpreted with caution and inconclusive results must be further evaluated using more sophisticated methods available in referral centres. Additional genetic testing should be performed if a hereditary form is suspected after amyloid protein typing. In cases in which DNA sequencing detects a mutant amyloid precursor, protein analysis is the definitive evidence. To date, more than 25 different proteins have been recognized as causative agents of amyloid diseases. The two most common types of systemic amyloidoses, commonly associated with renal involvement, are immunoglobulin-derived amyloidosis, secondary to plasma cell dyscrasias, and serum amyloid A derived amyloidosis (AA), which is typically associated with chronic inflammation. The deposits in immunoglobulin-derived amyloidosis in the vast majority of patients are composed of fragments of monoclonal immunoglobulin light chains (AL), but...
Renal amyloidosis: classification of 102 consecutive cases

seldom develop from fragments of heavy chains (AH)\(^{17-19}\). Other rare forms of amyloidosis with renal involvement are those derived from transthyretin (ATTR)\(^{20}\), gelsolin (Gel)\(^{21}\), apolipoprotein A-I (ApoAI)\(^{22}\), fibrinogen A \(\alpha\)-chain (Afib)\(^{23}\), lysozyme (Alys)\(^{24}\), apolipoprotein A-II (ApoAI)\(^{25}\), apolipoprotein A-IV (ApoAI)\(^{26}\), and from the leukocyte chemotactic factor 2 (Alect2)\(^{27}\). This newly described form of amyloidosis is mainly a renal disease from a clinical perspective, although not enough is known yet about Alect2 to draw conclusions about the distribution of amyloid deposits\(^{27}\). A precise epidemiology of amyloidosis is difficult to define as the disease is often undiagnosed or misdiagnosed. Selection bias of data from tertiary centres becomes potentially unrepresentative\(^{1,2}\). In Portugal, ATTR is the most frequent form of hereditary systemic amyloidosis. Since the identification of the disease as a new entity, in 1999, Hospital Santo Antônio is the reference centre for this disease. Apart from ATTR, there is not a vast knowledge about the type of systemic amyloidosis in Portugal. The aims of this study were: (1) to classify by immunohistochemistry the type of amyloidosis of a northern Portuguese outpatient referral centre; (2) to identify amyloidogenic variants in the hereditary forms; (3) to determine amyloidogenic macromolecules and laboratory findings at the time of kidney biopsy.

SUBJECTS AND METHODS

The department of Nephrology of the Centro Hospitalar São João (CHSJ), in collaboration with the department of Renal Pathology of CHSJ, has a registry of native kidney biopsies with amyloid nephropathy diagnosed since 1978. The absence of amyloid type classification led the Nephrology department of CHSJ to start a systematic classification of all cases of amyloid nephropathy by immunohistochemistry. We conducted a retrospective review of 102 consecutive native kidney biopsies, from patients of northern Portugal, performed between May 1978 and September 2013. Five of those patients had one or two additional kidney biopsies. For those patients, only the first diagnostic biopsy was analysed. DNA studies were performed for confirmation of the amyloid type in suspected hereditary amyloidosis based on the protein identified in the deposits.

The study was reviewed and approved by the Health Ethics Commission of CHSJ.

Histology and Immunohistochemistry

All the immunohistochemistry slides were reviewed by a pathologist and a nephrologist with expertise in amyloid nephropathy. Congo red staining was performed on 6 \(\mu\)m thick formalin-fixed paraffin-embedded sections, and the presence of amyloid was analysed microscopically under polarized light. Immunohistochemical staining was performed on 2 \(\mu\)m thick formalin-fixed paraffin-embedded sections of the amyloid containing biopsies, using standard methods and a ready-to-use rabbit/mouse, peroxidase/diaminobenzidine (DAB) detection system (Dako Real EnVision). The monoclonal antibodies used were directed against serum amyloid A (Dako), apolipoprotein A-II (Abcam) and transthyretin (provided by one of the authors (PPC))\(^{22}\); polyclonal antibodies were used for \(\lambda\)-light chain, \(\kappa\)-light chain, fibrinogen A \(\alpha\)-chain, transthyretin, apolipoprotein A-I and lysozyme (all from Dako). For light chains and fibrinogen detection, sections were treated with 10 \(\mu\)g/mL proteinase K for 10 minutes at 37\(^\circ\)C and 10 minutes at room temperature. Blocking was done with 5\% bovine serum albumin/phosphate-buffered saline (BSA/PBS). The sections were incubated with the respective antibody for 2 hours at room temperature, diluted in 1\% BSA/PBS, as follows: monoclonal anti-TR was used directly; polyclonal anti-TR; anti-SAA 1:100; anti-kappa 1:100; anti-lambda 1:2000; anti-Tib 1:800; Anti-Lys 1:300; anti-Apo A-I 1:400; anti-Apo A-II 1:600.

DNA Sequence Analysis

We searched for DNA mutations in the genes coding for the proteins identified in the immunohistochemistry. DNA was isolated from peripheral blood leukocytes using the “Genomic DNA Purification Kit” (PureGene, Gentra Systems). The coding regions of the genes encoding transthyretin (exon 2), fibrinogen A \(\alpha\)-chain (exon 5), apolipoprotein A-I (exons 1 to 4), apolipoprotein A-II (exon 4), and lysozyme (exon 2) were amplified by polymerase-chain-reaction (PCR) using the primer pairs listed on Table I. PCR products were analysed by agarose gel electrophoresis, purified according to "High Pure PCR Purification Kit" (Roche) and sequenced with
Table I

<table>
<thead>
<tr>
<th>Gene</th>
<th>5' End</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTR</td>
<td>3</td>
<td>5'-CTGCTGATGCGACGATTC-3'</td>
<td>5'-CTGCGAAAGTGGAAATGA-3'</td>
</tr>
<tr>
<td>FGA</td>
<td>5</td>
<td>5'-GTGACAGCAGCCCTTCCCTG-3'</td>
<td>5'-CTGACAGCAGCCCTTCCCTG-3'</td>
</tr>
<tr>
<td>AP04</td>
<td>3</td>
<td>5'-GGGCGGAGTGGGCTCAGGGC-3'</td>
<td>5'-GGGCGGAGTGGGCTCAGGGC-3'</td>
</tr>
<tr>
<td>AP06</td>
<td>4</td>
<td>5'-GCAAGCACGCCCAGCTGTC-3'</td>
<td>5'-GCAAGCACGCCCAGCTGTC-3'</td>
</tr>
<tr>
<td>IGF2</td>
<td>2</td>
<td>5'-AGAAGCTTACCTGTGGTCTG-3'</td>
<td>5'-AGAAGCTTACCTGTGGTCTG-3'</td>
</tr>
</tbody>
</table>

The "BigDye Terminator v3.1 Cycle Sequencing Kit" (Applied Biosystems). Sequence results were analysed with ChromasPro and SeqManiager.

### Patients and Data Collection

A total of 182 consecutive patients with different types of amyloidosis were included. For each patient, data was obtained from retrospective review of medical records: date of birth, sex, underlying diseases, main clinical manifestation of renal involvement at the date of kidney biopsy.

The following clinical definitions were used: (1) nephrotic syndrome: nephrotic-range proteinuria (>3.0 g/d) with hypoalbuminemia (<3.5 g/d) and peripheral edema; (2) glomerular filtration rate estimation (GFRc) using 2009 CKD-EPI creatinine equation; (3) hypertension (HTN) was classified according to 2013 ESC/ESH guidelines.

### Statistics

All statistical analyses were performed in IBM SPSS version 20.0 for Windows. Descriptive statistics of nominal variables consisted on frequencies. Kolmogorov-Smirnov test was used to assess the normality of cardinal variables, and for this test the null hypothesis was rejected when p < 0.05. Despite the small size of some subsamples, means and standard deviations were used, as all the described cardinal variables presented normal distribution, and the use of medians and percentiles would not be more appropriate in such small groups.

Table II

<table>
<thead>
<tr>
<th>Amyloid type</th>
<th>Patients (%)</th>
<th>Males (%)</th>
<th>Age at diagnosis (yr)</th>
<th>HTN (%)</th>
<th>NS (%)</th>
<th>GFRc (ml/min/1.73m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>60 (60.8)</td>
<td>34.7</td>
<td>49.9 (49.9)</td>
<td>25.9</td>
<td>81.7</td>
<td>62.5 (62.5)</td>
</tr>
<tr>
<td>AL (AK)</td>
<td>25 (25.6)</td>
<td>42.9</td>
<td>63.9 (80.0)</td>
<td>30.1</td>
<td>75.2</td>
<td>51.7 (35.6)</td>
</tr>
<tr>
<td>ALX</td>
<td>14 (14.7)</td>
<td>42.9</td>
<td>65.2 (80.0)</td>
<td>30.1</td>
<td>89.7</td>
<td>51.7 (35.6)</td>
</tr>
<tr>
<td>ALZ</td>
<td>7 (6.9)</td>
<td>42.9</td>
<td>58.8 (6.9)</td>
<td>30.1</td>
<td>89.7</td>
<td>51.7 (35.6)</td>
</tr>
<tr>
<td>ANF</td>
<td>3 (3.0)</td>
<td>35.3</td>
<td>53.9 (15.0)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>ATAF</td>
<td>3 (3.0)</td>
<td>42.9</td>
<td>60.5 (15.9)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>Combined AL and AM</td>
<td>100</td>
<td>75.0</td>
<td>75.0 (100)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>ALZx</td>
<td>1 (1.0)</td>
<td>68.1</td>
<td>68.1 (1.0)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>AF/FOAM</td>
<td>1 (1.0)</td>
<td>65.7</td>
<td>65.7 (1.0)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>Unspecified</td>
<td>10 (10.2)</td>
<td>58.2</td>
<td>65.7 (10.2)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>Negative IQ</td>
<td>8 (8.7)</td>
<td>62.5</td>
<td>53.8 (6.9)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>Insufficient tissue</td>
<td>100</td>
<td>75.0</td>
<td>75.0 (100)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
<tr>
<td>Total</td>
<td>100 (100)</td>
<td>75.0</td>
<td>75.0 (100)</td>
<td>100</td>
<td>75.0</td>
<td>78.2 (22.8)</td>
</tr>
</tbody>
</table>

Abnormalities: YH, yaws; HTN, hypertension; NS, nephrotic syndrome; GFRc, glomerular filtration rate estimation according to CKD-EPI equation; AM, serum amyloid A amyloidic; AL, immunoglobulin light chain amyloidosis; ALX, lambda light chain amyloidosis; ALZ, kappa light chain amyloidosis; ALNF, Beta2-microglobulin amyloidosis; ATAF, transthyretin amyloidosis; ALXZ, monoclonal amyloidosis; 2AF, amloid P/P xH chain amyloidosis; ALB, immunoglobulin light chain amyloidosis.
RESULTS

Clinical and laboratory characteristics of the study patients, both overall and according to amyloid type are listed in Table III.

Amyloid Type Identification

According to immunohistochemical identification, the most prevalent type of amyloidosis was AA, corresponding to more than half of the patients (58.8%, n = 60), followed by AL in about one fifth (20.6%, n = 21). The ratio AA/AL was 2.9:1. The subtype of light chain in AL amyloidosis was \( \lambda \) in two thirds of patients. Two different types of amyloid deposits – light chain \( \lambda \) and serum amyloid A – were present in one patient. Immunostaining disclosed a predominant pattern for light chain \( \lambda \) deposition and small patchy deposits for serum amyloid A at different sites. AFib was the most frequent type of hereditary amyloidosis contributing to 3.9% (n = 4) of the cases. ATTR was identified in two (2.0%) patients, AApoA1 in one (1.0%), and ALys in one (1.0%). The type of amyloidosis remained unclassified in 12 (11.7%) patients, mostly due to negative immunohistochemistry.

Hereditary Forms and their Amyloidogenic Variants

Molecular diagnosis of hereditary forms disclosed the point mutations FGA p.Glu545Val in all AFib cases and TTR p.Met51Val in the two cases of ATTR. Sudden death of the ALys positive patient did not allow genetic study. In the AApoA1 case pathogenic changes were not detected in the encoding region (exons 2 to 4) and exon-intron respective transitions of the APOA1 gene.

Underlying Disease

In our series, systemic AA amyloidosis patients showed mainly chronic infectious complications of pulmonary tuberculosis (Table III). A familial AA associated to Muckle-Wells syndrome was unequivocal in one patient. AL amyloidosis was related to monoclonal gammapathy of undetermined significance (MGUS) in 20 (47.6%) and multiple myeloma in eight (18.2%) patients, whereas the diagnosis was unknown in three (14.3%). The combined AL and AA amyloidosis patient had multiple myeloma light chain lambda and a previous history of syphilis. One of the four patients with AFib and the AApoA1 patient also had MGUS. The ALys patient had rheumatoid arthritis.

Clinical Findings at Kidney Biopsy

The clinical features at kidney biopsy are listed in Table II. The 102 patients included 51 (50.0%) females and 51 (50.0%) males. The mean age at time of kidney biopsy for the entire group was 53.3 ± 14.9 years. AA patients were younger and had a better renal function than AL and AFib. CKD-EPI estimated glomerular filtration rate was 62.5 ± 43.9 mL/min/1.73m² for AA, 51.7 ± 34.6 mL/min/1.73m² for AL and 59.4 ± 22.8 mL/min/1.73m² for AFib patients.

Table III

<table>
<thead>
<tr>
<th>Underlying disorders in 60 patients with AA amyloidosis.</th>
<th>n (%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diseases</strong></td>
<td></td>
</tr>
<tr>
<td>Chronic infections</td>
<td>25 (41.7)</td>
</tr>
<tr>
<td>Pulmonary tuberculosis</td>
<td>13</td>
</tr>
<tr>
<td>Bursitis</td>
<td>4</td>
</tr>
<tr>
<td>Chronic cutaneous ulcers</td>
<td>3</td>
</tr>
<tr>
<td>Xanthogranulomatous pyelonephritis</td>
<td>1</td>
</tr>
<tr>
<td>Whipple's disease</td>
<td>1</td>
</tr>
<tr>
<td><strong>Inflammatory arthritis</strong></td>
<td>19 (31.7)</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>10</td>
</tr>
<tr>
<td>Juvenile rheumatoid arthritis</td>
<td>4</td>
</tr>
<tr>
<td>Arthritis</td>
<td>2</td>
</tr>
<tr>
<td>Psoriatic arthritis</td>
<td>2</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>1</td>
</tr>
<tr>
<td><strong>Inflammatory bowel diseases</strong></td>
<td>4 (6.6)</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>4</td>
</tr>
<tr>
<td><strong>Neoplastic diseases</strong></td>
<td>2 (3.3)</td>
</tr>
<tr>
<td>Non-Hodgkin's lymphoma</td>
<td>1</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>1</td>
</tr>
<tr>
<td><strong>Acquired immunodeficiencies</strong></td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>HIV/AIDS</td>
<td>1</td>
</tr>
<tr>
<td><strong>Hereditary diseases</strong></td>
<td>2 (3.3)</td>
</tr>
<tr>
<td>Muckle-Wells syndrome</td>
<td>1</td>
</tr>
<tr>
<td>Von-Hippel-Lindau disease associated with c-kit mutation</td>
<td>1</td>
</tr>
<tr>
<td><strong>Systemic vasculitides</strong></td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>Polymyalgia rheumatica</td>
<td>1</td>
</tr>
<tr>
<td><strong>Conditions predisposing to chronic infections</strong></td>
<td>3 (5.0)</td>
</tr>
<tr>
<td>Cyclic fibrosis</td>
<td>1</td>
</tr>
<tr>
<td>Dystrophic epidermolysis bullosa</td>
<td>1</td>
</tr>
<tr>
<td>Injected-drug use</td>
<td>1</td>
</tr>
<tr>
<td><strong>Undetermined disease</strong></td>
<td>5 (8.3)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>60 (100)</td>
</tr>
</tbody>
</table>
Nephrotic syndrome was the first sign of renal involvement in 83 (81.4%) patients with a similar frequency for all types of amyloidosis. The presence of hypertension was associated with AFib (100% for AFib, 38.1% for AL and 35% for AA).

The mean age of the patients at diagnosis of the underlying disorder was 36 ± 20 years for AA (data available for 53 patients) and 63 ± 9 years for AL (data available for 18 patients). The mean duration between the onset of the underlying disorder and the diagnosis of AA amyloidosis (data available for 53 patients) was 13.9 ± 12.3 years and for AL amyloidosis (data available for 18 patients) was 1.4 ± 1.5 years.

**DISCUSSION**

Here, we report the immunohistochemical classification, molecular diagnosis and clinical characterization of 102 northern Portuguese patients with kidney biopsy-proven amyloid disease, evaluated outside the referral centre for hereditary amyloidosis.

Diagnosis for amyloid diseases needs histological confirmation\(^\text{31}\). Immunohistochemistry is still the most frequent technique used in the identification of the amyloid fibril protein. However, it has limitations, mainly related with the low sensitivity of the technique, spectrum of amyloid antibody panel, low quality of the analysed tissue and observer experience\(^\text{32-35}\). These limitations are particularly evident in the cases of AL and hereditary amyloidosis. Twelve (11.7%) of our cases were unclassified, four (3.9%) due to the lack of adequate tissue and eight (7.8%) because of negative immunohistochemistry. Whenever this occurs, investigation should continue using a wider antibody panel or employing techniques, such as proteomics, that are currently performed only in highly specialized laboratories\(^\text{34,45-37}\), which may contribute to amyloid typing in more than 97% of renal amyloidosis cases\(^\text{38}\).

AA amyloidosis was the most frequent form of systemic amyloidosis in our series, similarly to what was reported in the largest series of renal amyloidosis in kidney biopsies described by Panizo\(^\text{39}\). On the other hand, Pinney and colleagues reported an epidemiological study about systemic amyloidosis in England and concluded that systemic AL amyloidosis was the most common type with an estimated minimum incidence of 0.3/100 000 population\(^\text{40}\).

Besides the long period of our study, the prevalence of AA amyloidosis remained stable from 1978 to 2013 (Fig. 1), although with recent advances in the treatment of chronic infectious diseases and autoimmune inflammatory processes a decline in the incidence of AA form would be expected\(^\text{41}\). Nevertheless, our findings agree with results of other series: a recent single-centre study detailing 20 years’ Florentine experience of AA amyloidosis showed that rheumatoid arthritis contributed to 45% of cases and 67% of patients had some form of renal involvement\(^\text{42}\). But, among us, chronic infectious disorders are still the most frequent cause of AA amyloidosis, mainly pulmonary tuberculosis, which is according to the fact that Portugal still has one of the highest tuberculosis incidence rates in European Union countries\(^\text{42}\). One of our AA patients had a Muckle-Wells syndrome, which is a familial auto-inflammatory disease, diagnosed when she was 16 years old. Auto-inflammatory diseases are now the most common cause of AA amyloidosis in children, and paediatric nephrologists should be aware that renal amyloidosis is potentially preventable in these conditions\(^\text{43}\). Clinically significant renal diseases may also arise in young adults with cystic fibrosis\(^\text{44}\). Our patient had cystic fibrosis since age 5 and AA amyloidosis was diagnosed at 21 years old. Early diagnosis and rapid control of the underlying inflammatory or infectious disease are of the utmost importance to prevent irreversible organ damage\(^\text{45}\). Our mean duration between the onset of the underlying disease and the diagnosis of AA amyloidosis presented a wide range, nonetheless this was a retrospective observational analysis. During such a long period of time, patient monitoring combined with adequate therapy of underlying disease and periodic search for subclinical amyloid deposits on abdominal fat aspiration, might help early diagnosis and alter the prognosis of the disease\(^\text{46}\). AA amyloidosis, in our series, affected younger individuals, which may be related to early beginning of most of the underlying conditions, mainly infectious and inflammatory diseases, and therapeutic failures at suppressing inflammation. However, genetic factors may also be involved on this namely SAA genotype\(^\text{47}\). A lesser degree of renal dysfunction in our patients may be related to an early diagnosis through repeated measurements of microalbuminuria and serum creatinine.

AL amyloidosis was our second most frequent form of systemic amyloidosis and these patients
were older than AA patients. These results should be interpreted cautiously since in older and unstable patients with monoclonal plasma cell disorders we may use less invasive diagnostic tests, such as abdominal fat aspiration or minor salivary gland biopsy for amyloid diagnosis as a way to minimize complications related to kidney biopsies.

Unequivocal hereditary amyloidosis contributed to 5.9% of our cases (3.9% AFib and 2.0% ATTR). DNA analysis is mandatory to confirm the diagnosis but it should always be complementary to other diagnostic techniques that allow unequivocal identification of amyloid protein. Genetic defects may be associated with amyloidosis either as a mutation in non-amyloid protein, as is the case of familial AA patients, where an inborn error of inflammatory response in the innate immune system plays a permissive role in the development of amyloid, or as a mutation involving amyloid protein itself, as is the case of AFib, ATTR, ALys, and AAPoAI patients. AFib is the most common type of hereditary amyloidosis in Europe. Our four AFib patients were from the same region and had the same amyloidogenic mutation, so haplotyping studies are necessary to conclude if they belong to the same family. Worst renal function at presentation in this small group may not be informative because of late diagnosis, in the absence of family history, due to variations in disease penetrance and progression. High prevalence of hypertension among AFib patients may be secondary to chronic kidney disease, but direct amyloid deposition in vascular walls may also be involved. ATTR amyloidosis was identified only in two cases, what was expected since this study was performed outside the referral centre for the disease in northern Portugal. For this reason, our results have no correlation with ATTR prevalence in our country. One of the patients performed kidney biopsy outside the referral centre because she had nephrotic syndrome and at the time of biopsy she denied knowing ATTR family history. The other patient was considered TTR p.Met54Val asymptomatic carrier that presented with nephrotic syndrome and haematuria. Kidney biopsy
disclosed IgA nephropathy and medullary amyloid deposition. Alys and APOA1 amyloidosis were diagnosed by immunohistochemistry, without molecular confirmation, so further studies are necessary to confirm amyloidosis type in both cases.

Said and colleagues reported the origin and clinical correlation of 474 recent cases of renal amyloidosis, based on immunohistochemistry and laser microdissection/mass spectrometry. They found immunoglobulin (Ig) amyloidosis in 85.6%; AA in 7%; leucocyte chemotactic factor 2 (LECT2) amyloidosis in 2.7%; Affib in 1.3%; ApoaA1, ApoaA1 or ApoAIV amyloidosis in 0.6%; combined AA/Ig heavy and light chain amyloidosis in 0.2%, and amyloidosis was undiagnosed in 2.3% patients. When we compare our results with those of our first conclusion is that we need to raise our spectrum of amyloid antibodies with the inclusion of anti-LECT2, anti-APO A-IV and anti-Ig heavy chain as a way to reduce uncategorised cases.

Improvements to understanding the pathogenesis of systemic amyloidosis, coupled with enhancements on diagnostic techniques, have led to the identification of therapeutic strategies that have already resulted in better outcomes for patients; so we should perform routine precise identification of the amyloid fibril protein on tissues containing amyloid deposits. Our results may prove helpful for clinicians in charge of patients with amyloidosis regarding the decision for a more definitive diagnosis of the disease.

Source of support in the form of grants, equipment, drugs, or all of these
This work was supported by a grant from the Portuguese Society of Nephrology and by the Multidisciplinary Unit for Biomedical Research (ICMOP-01124-FEDER/08/89).

Conflict of Interest Statement
None declared.

Acknowledgments
The authors gratefully acknowledge Dr. Pedro Ladeira from the National Institute of Health, INSA, Porto, for his contribution to the genetic study; Dr. Rui Pernhos from the Faculty of Nutrition and Food Sciences, University of Porto, for his contribution to the statistical analysis; all the colleagues that made this work possible by referring and caring for the patients.

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Renal amyloidosis: classification of 107 consecutive cases

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1.2. Fibrinogen Aα chain amyloidosis: a non-negligible cause of chronic kidney disease in dialysis patients

In the previous report, we found four patients with AFibE526V (p.Glu545Val) amyloidosis, all belonging to apparently unrelated families from the district of Braga, northern Portugal. The fact that this is a rare nephropathic disease and all the cases were identified in a circumscribed geographic region, led us to assess the prevalence of the disease among Portuguese patients undergoing hemodialysis in the same district, through genetic testing for the FGA p.Glu545Val variant.

A total of 267 prevalent hemodialysis patients were evaluated and genetic testing for the FGA p.Glu545Val variant was offered to patients with CKD of unknown or presumed etiology, or unclassified amyloidosis (Figure 5).

![Figure 5 | Study design and results.](image)

Tavares I et al, XVth International Symposium on Amyloidosis, Uppsala 2016 [223].

A total of 122 hemodialysis patients underwent genetic testing and the FGA p.Glu545Val variant was identified in 12 (6 unclassified amyloidosis, 3 hypertensive nephrosclerosis, 3 unknown etiology), corresponding to a disease prevalence of 4.5%. They belonged to 10 unrelated families that were unaware of their condition.

These data resulted from a study whose methodology, results and discussion were presented at the XVth International Symposium on Amyloidosis which took place in Uppsala in 2016. The work was awarded by the ISA as a Best Poster in the Clinical Area. The extended abstract was published after peer review in the journal *Amyloid*. Reference: Tavares I, Moreira L, Costa PP, Lobato L. Fibrinogen A alpha-chain amyloidosis: a non-negligible cause of chronic kidney disease in dialysis patients. *Amyloid* 2017; 24 (S1): 153-154, which follows.
Fibrinogen A alpha-chain amyloidosis: a non-negligible cause of chronic kidney disease in dialysis patients

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Background

Fibrinogen A alpha-chain (AFib) amyloidosis is a rare and late-onset disease, that result from amyloidogenic autosomal dominant mutations in the gene encoding AFib (FGA). Patients invariably develop chronic kidney disease (CKD), typically progressing to end-stage renal failure within 5 years of recognition of renal involvement [1]. In Portugal, four apparently unrelated patients with AFib amyloidosis were identified in the district of Braga, Northern Portugal. They all carried the FGA p.Glu545Val mutation, three were heterozygous and one homozygous [2,3]. This observation led us to assess the prevalence of AFibE526V (p.Glu545Val) amyloidosis among Portuguese patients undergoing hemodialysis in the same district, through genetic screening for the FGA p.Glu545Val mutation.

Materials and methods

A total of 267 prevalent hemodialysis patients, older than 18 years, treated at three outpatient dialysis centers across the district of Braga, were evaluated between 2005 and 2010. Genetic screening for the FGA p.Glu545Val mutation was offered to patients with CKD attributable to: (i) unclassified amyloidosis; (ii) unknown etiology; (iii) diabetic nephropathy in patients without diabetic retinopathy, hypertensive nephrosclerosis, glomerular and interstitial diseases, in the absence of histological confirmation. Exclusion criteria were primary glomerulonephropathies, diabetic nephropathy with diabetic retinopathy, secondary glomerulonephropathies with histological diagnosis, urinary tract disorders, polycystic and other identified hereditary disorders, and absence of informed consent. For each identified carrier, tissue samples were retrieved from the archives for histological evaluation and family trees were obtained. This study was carried out in accordance with the Declaration of Helsinki, and its design and research protocol were reviewed and approved by the Health Ethics Commission of Centro Hospitalar de São João. Written, informed consent was obtained from all patients.

Results

A total of 122 hemodialysis patients (aged 65.1 ± 14.8 years; 61.5% males) underwent genetic screening for the FGA p.Glu545Val mutation. Twelve (4.5%) patients were not previously diagnosed as having AFib amyloidosis were identified with the mutation at 67.4 ± 12.3 years. They had been diagnosed with unclassified amyloidosis (n = 6), hypertensive nephrosclerosis (n = 3), and unknown etiology (n = 3). Out 12 patients (6 males, 6 females) presented with hypertension at 52.6 ± 11.8 years and CKD stage 3 or worse at 59.7 ± 10.8 years. Proteinuria was universal, mainly in the nephrotic range (75%), and they started renal replacement therapy at 64.8 ± 11.2 years. Renal biopsies of 6 patients disclosed abundant amyloid deposition in the glomeruli, variable in arterioles and cortical interstitium, which stained with an antibody against fibrinogen. Furthermore, amyloid deposits were identified in surgical samples from spleen (n = 1), abdominal fat (n = 1), colon and ileum (n = 1). Two patients underwent renal transplantation and eight (66.7%) died at 75.7 ± 5.1 years. The 12 patients with the FGA p.Glu545Val mutation belonged to 10 unrelated families that were unaware of their condition. Family trees disclosed 5 deceased relatives who underwent hemodialysis in the same centers, likely to have had an unrecognized AFib amyloidosis.

Discussion and conclusions

This is the first study of the frequency of AFibE526V (p.Glu545Val) amyloidosis among hemodialysis patients from a circumscribed geographic region. Our findings, based on genotyping analysis, disclosed the diagnosis of AFibE526V (p.Glu545Val) in 12 patients. To date, 13 amyloidogenic FGA variants have been described [4], accounting for 8% of the hereditary amyloidoses [5]. The most common mutation is the FGA p.Glu545Val (p.Glu526Val, as previously reported) that
was identified for the first time in a Canadian kindred of Polish origin [6]. However, the great majority of cases are from the United Kingdom [1,7]. A fib526V (p.Gln545Val) amyloidosis was identified in 4.5% of hemodialysis Portuguese patients from the district of Braga. Most (75%) of these patients presented with nephrotic range proteinuria, nonetheless, half of them had been diagnosed with hypertensive nephrosclerosis or unknown etiology. These findings suggest that A fib amyloidosis may be undiagnosed among renal dialysis and/or transplant patients. In our case, unrecognized A fib amyloidosis, in a non-negligible number of dialysis patients, resulted also from lack of local physician’s awareness. A high index of suspicion should be reserved for Portuguese patients, from the district of Braga, with unexplained chronic kidney disease. Genetic testing for FGA p.Gln545Val mutation should be offered to subjects genetically at-risk in order to detect new cases and improve the prognosis of the disease through early diagnosis and treatment.

Declaration of interest
The authors report no conflicts of interest.

References
2. NATURAL HISTORY AND TREATMENT

2.1. Unrecognized fibrinogen Aα chain amyloidosis: results from targeted genetic testing

Previous epidemiological studies have provided a focus of AFibE526V (p.Glu545Val) amyloidosis, which we used for phenotypic, genotypic and prognostic evaluation of the disease.

We studied 13 families comprising 50 subjects with the same amyloidogenic variant, the FGA p.Glu545Val. The study of these families allowed us the characterization of the clinical phenotype of AFibE526V (p.Glu545Val) amyloidosis as a systemic disease with visceral involvement, especially renal. Its natural history can be portrayed as early-onset hypertension, followed by proteinuric CKD, eventually progressing to end-stage renal disease (ESRD), and increasing prevalence of extrarenal manifestations in older patients. In this late occurrence disease, the prevalence of cerebrovascular disease was high, estimated mean renal survival was 65.4 (95% CI, 61.8-69.0) years and estimated mean lifetime survival was 73.7 (95% CI, 70.5-76.8) years.

Five out of 13 probands with AFibE526V (p.Glu545Val) amyloidosis were not aware of a family history of ESRD or amyloidosis. Although it has been suggested by other investigators that AFib amyloidosis has a reduced penetrance [32], this was not the case in the Portuguese focus, where a high and age dependent penetrance was observed (Table 3).

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Penetration of AFibE526V (p.Glu545Val) amyloidosis</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>n/N</td>
</tr>
<tr>
<td>≤40</td>
<td>3/50</td>
</tr>
<tr>
<td>41-50</td>
<td>6/43</td>
</tr>
<tr>
<td>51-60</td>
<td>11/32</td>
</tr>
<tr>
<td>61-70</td>
<td>20/25</td>
</tr>
<tr>
<td>≥71</td>
<td>12/12</td>
</tr>
</tbody>
</table>

Penetration of kidney disease in AFib amyloidosis at prespecified years of age in carriers of the FGA p.Glu545Val variant. N indicates the number of subjects at risk (FGA p.Glu545Val carriers), n indicates the number of patients with AFibE526V (p.Glu545Val) amyloidosis. Abbreviation: CI, confidence interval.

Unrecognized Fibrinogen A α-Chain Amyloidosis: Results From Targeted Genetic Testing

Isabel Tavares, MD,1,2 João Paulo Oliveira, MD, PhD,2,3 Ana Pinho, MD,1 Luciana Moreira, PhD,4 Liliana Rocha, MSc,5 Josefina Santos, MD,6,7 Joaquim Pinheiro, MD, PhD,9 Paulo Pinho Costa, MD, PhD,6,4 and Luísa Lobato, MD, PhD6,4

Background: Fibrinogen A α-chain (AFib) amyloidosis results from autosomal-dominant mutations in the gene encoding AFib (FGA). Patients with this disorder typically present with proteinuria. Isolated cases of AFib amyloidosis, carrying the FGA p.Glu45Val variant, were identified in the district of Braga, in northwest Portugal. This observation led us to hypothesise that this disorder might be an unrecognized cause of kidney disease in that region and prompted us to carry out targeted genetic testing for the p.Glu45Val variant in the local haemodialysis population and family members of identified cases.

Study Design: Case series.

Setting & Participants: 3 groups of participants: (1) kidney biopsy registry, n = 122 of 267 patients; and (2) genetically at-risk individuals; n = 99 of 167 family members.

Outcomes: Kidney disease, kidney disease progression, and survival.

Results: The p.Glu45Val variant was identified in all 4 patients of the biopsy registry, 12 of 122 (9.8%) hemodialysis patients tested, and 34 of 69 (49%) relatives tested. These 50 cases belonged to 15 unrelated families with kidney disease or amyloidosis identified in 61% of probands. 35 individuals presented with hypertension at a mean of 51.0 ± 10.4 years. Of these, 30 developed kidney disease at a mean of 56.7 ± 12.0 years, and 21 initiated dialysis therapy at a mean of 61.4 ± 11.2 years. Heart, liver, spleen, colon, and lung were involved along the progression of the disease. Kidney disease was formerly attributed to hypertension in 25% of patients with AFib amyloidosis undergoing hemodialysis.

Limitations: Retrospective data collection for patients with amyloidosis previously diagnosed.

Conclusions: AFib amyloidosis appears to be an under-recognized disorder in Braga, Portugal, where we found a high frequency of the FGA p.Glu45Val variant. Due to the nonspecific nature of its major clinical features, the diagnosis of AFib amyloidosis should have a high index of suspicion, particularly in populations in which hypertension is prevalent.

INDEX WORDS: Amyloidosis; chronic kidney disease (CKD); fibrinogen A alpha-chain; genetic screening; hemodialysis; mutation; proteinuria; hypertension; FGA p.Glu45Val; Portugal; end-stage renal disease (ESRD).

Fibrinogen A α-chain (AFib) amyloidosis is a systemic disease caused by extracellular deposition of insoluble amyloid fibrils composed of abnormal fibrinogen, arising from autosomal-dominant mutations in the gene encoding AFib (FGA).1-3 Patients with AFib amyloidosis invariably develop chronic kidney disease (CKD), typically progressing to end-stage renal disease (ESRD) within 5 years of recognition of renal involvement.1 Diagnosis is based on the occurrence of proteinuria, positive family history, identification of amyloid deposits in affected tissues by immunohistochemistry or mass spectrometry, and detection of an FGA amyloidogenic genetic variant.1,6 Incomplete penetrance may complicate the diagnosis of AFib amyloidosis and should be taken into account in genetic counseling.

To date, 13 amyloidogenic FGA variants have been described,2 accounting for 8% of hereditary amyloidoses.3 Although AFib amyloidosis was originally described in 1993 in a Peruvian kindred, segregating with a mutation in FGA identified as p.Arg554Leu, indicating a substitution of arginine by leucine at amino...
acid 554), the most common FGA amyloidogenic variant reported worldwide is one described as p.Glu545Val (substitution of glutamate by valine at amino acid 526), having been identified in a Canadian kindred of Polish origin and in families from the United Kingdom, France, Germany, Brazil, United States, and China. According to recommendations of the Human Genome Variation Society, the p.Arg554Leu and p.Glu545Val variants should be described, respectively, as p.Arg573Leu and p.Glu545Val (i.e., considering the translation initiation site as amino acid 1, instead of basing the numbering on the cleaved protein). In line with this, the Nomenclature Committee of the International Society of Amyloidosis recommends that hereditary amyloidosis associated with the FGA p.Glu545Val variant should be designated AfibE526V (p.Glu545Val) amyloidosis, and this nomenclature will be followed hereinafter.

In Portugal, the first patient with AFib amyloidosis was reported in 2004, and the p.Glu545Val variant was found to be the causative mutation. Subsequently, 4 other apparently unrelated patients, including a woman with a homozygous mutation, were given a diagnosis of AFib amyloidosis by retrospective immunohistochemical characterization of 102 kidney biopsies showing amyloid nephropathy, retrieved from the archive of Centro Hospitalar de São João, a major university hospital in Porto, the northwest of Portugal. Because all 4 patients carried the p.Glu545Val variant and were from the same confined geographic area, we hypothesized that AfibE526V (p.Glu545Val) amyloidosis may be an underdiagnosed cause of ESRD in that region and carried out targeted genetic screening among the local outpatient hemodialysis population, offering cascade genetic screening to at-risk relatives of all identified cases.

We report demographic and clinical features of individuals carrying the FGA p.Glu545Val variant identified in this study, irrespective of their medical condition, and characterize the natural history and major outcomes of AfibE526V (p.Glu545Val) amyloidosis.

**METHODS**

**Study Design, Participant Ascertainment, and Data Collection**

Participants enrolled in this study were ascertained by 3 distinct sequential case-finding protocols (Fig 1), starting with the 4 patients who were retrospectively given diagnoses of AfibE526V (p.Glu545Val) amyloidosis, by review and immunohistochemical classification of archived kidney biopsies showing amyloid nephropathy (phase B).

![Figure 1](image-url)  
**Figure 1.** Study design showing setting, eligibility, and results of included cases. Targeted genetic testing led to the identification of 50 individuals with the FGA p.Glu45Val variant in the same geographical region. Abbreviations: Afib, fibrinogen A α-chain; ESRD, end-stage renal disease; FGA, fibrinogen A α-chain gene; NW, northwest.

236  
Molecular Analysis

Genomic DNA was extracted from peripheral blood samples with the Pellefry DNA Blood DNA Blood Kit (Pellefry Elmer) according to the manufacturer’s instructions. The p.Glu545Val variant, located on the coding sequence, was ascertained with polymerase chain reaction amplification followed by Sanger sequencing using forward primer 5’-CCCTACATG-CAGTCACAGGACATG-3’ and reverse primer 5’-GAGATTTAG-CATGGGCCTCTC-3’. Polymerase chain reaction amplification was carried out in a Veriti thermal cycler (Applied Biosystems) with the following conditions: 95°C for 5 minutes, 35 amplification cycles (40 seconds at 95°C for 1 minute, annealing at 61°C for 1 minute, and extension at 72°C for 1 minute), and final extension at 72°C for 10 minutes. Polymerase chain reaction products were analyzed with the QIAxcel system (Qiagen), purified with Agencourt AMPure XP PCR Purification (Beckman Coulter), and sequenced on an automated ABI PRISM TM 3.1 Genetic Analyzer (Applied Biosystems) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The National Center for Biotechnology Information reference sequences NM_000509.2 and NP_000509.2 were used as references for the FGA complimentary DNA and protein sequences, respectively.

Statistical Analyses

Categorical variables were expressed as number and percentage; continuous variables were expressed as mean with standard deviation or as median with interquartile range, depending on whether they were normally distributed. Individuals carrying the p.Glu545Val variant were grouped according to the case–control protocol in which they were identified, and the 3 cohorts were compared for relevant demographic and clinical data using Fisher exact test for categorical variables and 1-way analysis of variance or Kruskal-Wallis test for continuous variables, depending on goodness of fit to normal distribution. Prevalence, age at onset, and severity of hypertension were compared in p.Glu545Val carrier and noncarrier sibships, respectively identified in family screening. To minimize ascertainment bias, family probands (n = 10) were excluded from the analyses. The test or χ2 test, respectively, was used for comparing normally distributed continuous variables or categorical variables. The relationship between pancreatitis and eGFR in the 30 patients with CKD was analyzed by Pearson correlation coefficient, using the first available proteinuria value with protein excretion > 0.15 g/24 h. Decline in eGFR over time was modeled using mixed-effects models in the 20 patients with CKD who had at least 2 serum creatinine measurements obtained more than 1 year apart. Absolute risks for major adverse outcomes (e.g., ESRD and death) were estimated for the entire cohort (n = 50) using Kaplan-Meier curves to determine renal and overall mean survival, correcting for death in the case of ESRD. P < 0.05 was considered statistically significant. All statistical analyses were performed using the IBM SPSS, version 20.0, software package for Macintosh.

RESULTS

Study Population

As shown in Fig 1, a total of 195 individuals eventually underwent genetic testing for the FGA p.Glu545Val variant, including the 4 patients retrospectively given diagnoses of AFib–amyloidosis in phase I; all the eligible 122 hemodialysis patients enrolled in phase II; and 69 of 167 (41.3%) first-degree relatives of patients with AFib–amyloidosis (p.Glu545Val) amyloidosis who were identified in the 2 case-finding
protocols. All those individuals were of Portuguese ancestry, with family roots in the district of Braga. The diagnosis of AFibE526V (p.Glu545Val) amyloidosis was established by molecular analysis in all 4 patients enrolled in phase I and in 12 of 122 (9.8%) hemodialysis patients screened in phase II. Of the latter patients, 6 were given diagnoses of unclassified amyloid nephropathy, whereas 3 had received a diagnosis of hypertensive nephrosclerosis and the remaining 3 had been classified as having ESRD of undetermined cause. On family screening, 34 of 69 (49%) individuals were found to be heterozygous for the p.Glu545Val variant, including 14 (41%) patients with evidence of CKD, none of whom was unaware of their clinical condition. Overall, the p.Glu545Val variant was identified in 50 individuals from 13 apparently unrelated families. Notably, 7 individuals from 3 unrelated families had died while receiving regular hemodialysis treatment before the beginning of this study and therefore did not have a definite genetic diagnosis.

Characteristics of Individuals Carrying the FGA p.Glu545Val Variant

The relevant demographic and clinical characteristics of individuals with the p.Glu545Val variant diagnosed in this study, irrespective of clinical condition, are presented in Table 1. The data are summarized for the overall cohort and for participants grouped according to the case-finding protocol in which they were identified.

Mean age at genetic testing was 52.0 ± 16.8 years. Hypertension was detected in 35 (70%) individuals at a mean age of 51.0 ± 10.4 years and was present even in the absence of kidney disease. Thirty (60%) individuals developed CKD at a mean age of 56.7 ± 12.0 years, presenting with proteinuria with protein excretion >3 g/24 h and eGFR <60 mL/min/1.73 m² in more than half the cases, but only 17 (57%) such patients underwent a diagnostic kidney biopsy.

In phase III, a total of 20 of 34 (59%) individuals with the p.Glu545Val variant had no evidence of CKD at a mean age of 38.4 ± 9.7 years and were therefore classified as genetic carriers. These individuals were significantly younger than their 14 relatives with CKD diagnosed, with a mean age difference of 18.4 years. Five (25%) individuals with no evidence of CKD had high blood pressure diagnosed as essential hypertension.

Kidney Disease and Other Major Clinical Outcomes

All individuals with CKD presented with proteinuria. As shown in Fig 2, the severity of proteinuria associated inversely with eGFR (r = -0.426; P = 0.02), and mean change in eGFR was -4.39

<table>
<thead>
<tr>
<th>Table 1. Diagnostic Assessment and Main Clinical Characteristics of Individuals With the FGA p.Glu545Val Variant</th>
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<tr>
<td><strong>Assentainment</strong></td>
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<tr>
<td>Male sex</td>
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<tr>
<td>Age at genetic testing, y</td>
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<td>Hypertension</td>
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<td>Age at diagnosis, y</td>
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<tr>
<td>&gt;3 g/24 h</td>
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<tr>
<td>eGFR</td>
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<tr>
<td>&lt;60 mL/min/1.73 m²</td>
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<tr>
<td>&gt;60 mL/min/1.73 m²</td>
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<tr>
<td>Renal replacement therapy</td>
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<tr>
<td>Age at initiation, y</td>
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<tr>
<td>Dialysis vintage, mo</td>
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<td>Kidney transplantation</td>
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Note: Values for categorical variables are given as number (percentage); values for continuous variables, as mean ± standard deviation if normally distributed or median [interquartile range] if non-normally distributed. P values are from Fisher exact test for categorical variables and 1-way analysis of variance or Kruskal-Wallis for continuous variables, as appropriate.

Abbreviations: eGFR, estimated glomerular filtration rate; NA, not applicable.

*Patients ascertained by kidney biopsy registry and hemodialysis screening belonged to 13 unrelated families.

End of follow-up December 2014.

No applicability in this category.
Figure 2. (A) Scatterplot of proteinuria levels by estimated glomerular filtration rate (eGFR) for the 30 individuals with chronic kidney disease ascertained through a kidney biopsy registry (n = 4), hemodialysis patient screening (n = 12), and cascade family screening (n = 14), analyzed by Pearson correlation coefficient ($r = -0.426; P = 0.02$). (B) eGFR regression slope for 20 of the 30 patients with chronic kidney disease who had at least 2 serum creatinine measurements available for review.

(95% confidence interval [CI], $-3.65$ to $-5.14$ mL/min/1.73 m$^2$ per year. Ten men and 11 women progressed to ESRD, having initiated renal replacement therapy at 61.4 ± 11.3 years (Table 1); 2 women underwent kidney transplantation 6 years after the onset of hemodialysis therapy. Family history of kidney disease or amyloidosis was present in 61% of probands.

Table S1 (provided as online supplementary material) summarizes the most relevant comorbid conditions and severe medical events that have occurred in the 30 patients with CKD. Thirteen strokes were reported (6 events in 6 women and 7 events in 5 men), with the middle cerebral artery territory being the most commonly affected location. Mean age for first stroke was 66.4 ± 8.3 years. Amyloid deposits were also identified in surgical samples from hemodialysis patients (abdominal fat, n = 1; colon, n = 1; ileum, n = 1; and spleen, n = 1). Fourteen patients have died, at a mean age of 70.8 ± 9.6 years, and their reported immediate causes of death are also summarized in Table S1.

For the 50 patients with the p.Glu545Val variant (Fig 3), estimated mean renal survival was 65.4 (95% CI, 61.8-69.0) years, and estimated mean lifetime survival was 73.7 (95% CI, 70.5-76.8) years.

Systemic Hypertension

In individuals carrying the p.Glu545Val variant, hypertension was considerably more prevalent in those with CKD than in those without clinical evidence of kidney disease (97% vs 30%; $P < 0.001$).

In the unbiased comparison of individuals prospectively genotyped on family screening, the prevalence of hypertension was significantly higher in those harboring the p.Glu545Val variant than in their

Figure 3. Kaplan-Meier estimates of actuarial (A) renal and (B) overall survival from birth for the 50 individuals identified with the p.Glu545Val variant. Twenty-one patients developed end-stage renal disease and 14 died.
DISCUSSION

In this large-scale systematic study, we used 3 distinct but complementary case-finding protocols and identified the p.Glu545Val variant of AFib in 50 individuals belonging to 13 apparently unrelated families, all originating from neighboring villages in the district of Braga, northwestern Portugal. For this reason, possible confounding effects of environmental variation upon the phenotypic expression of the amyloidogenic p.Glu545Val variant are minimized in our cohort.

We have previously reported that AFibE526V (p.Glu545Val) amyloidosis accounted for 3.9% (4 of 102) of the cases of amyloid nephropathy, in a consecutive series of diagnostic native kidney biopsies performed over 36 years, at Centro Hospitalar de São João.\textsuperscript{11} This percentage does not significantly differ from the 1.7% (87 of 5,100) found in a much larger series from the National Amyloidosis Centre in the United Kingdom,\textsuperscript{12} which included tissue biopsies other than the kidney.

Results of the present study show that AFibE526V (p.Glu545Val) amyloidosis is a relatively common but frequently overlooked cause of ESRD in families living or originating from a circumscribed geographic area in the northwest of Portugal. The point prevalence of ESRD due to AFibE526V (p.Glu545Val) amyloidosis could be estimated at \textasciitilde 4.5\% among the local hemodialysis population, and it was the most common amyloid nephropathy, even considering the relatively high prevalence of hereditary transthyretin amyloidosis in that region.\textsuperscript{27-35}

The typical natural history of AFibE526V (p.Glu545Val) amyloidosis in our cohort was that of early-onset hypertension, followed by proteinuria, and eventually progressing to ESRD after 5 years. The pathophysiology of hypertension in AFib amyloidosis is unclear, although it has been regarded as secondary to CKD.\textsuperscript{1} Other investigators have proposed that vascular deposits of amyloid, leading to impaired endothelial function, might be the first step in the disease process.\textsuperscript{36-38} Our observation that the diagnosis of high blood pressure preceded any evidence of kidney involvement in most patients is in line with the latter hypothesis. However, the etiologic interpretation of the hypertensive disease in our patients, as well as assignment of the cause of CKD, is confounded by the high prevalence of essential hypertension in the Portuguese adult population, reaching 48.4\% in people aged 35 to 64 years.\textsuperscript{39,40} and the relatively high frequency of the diagnosis of hypertensive nephrosclerosis in the prevalent Portuguese dialysis population.\textsuperscript{41} In our cohort, hypertension had a higher prevalence than in the age-matched general population, and AFibE526V (p.Glu545Val) amyloidosis was misdiagnosed as hypertensive nephrosclerosis in 3 of 12 (25\%) patients on renal replacement therapy.

The rate of decline in kidney function observed in our patients was slower than that reported by Gillmore et al.\textsuperscript{1} in their, as well as in contrast to other types of amyloidosis,\textsuperscript{2} but it compares unfavorably with other more common causes of progressive CKD, such as diabetic nephropathy and hypertensive nephrosclerosis.\textsuperscript{33}

During follow-up, most of the patients with AFibE526V (p.Glu545Val) amyloidosis developed severe clinical manifestations attributable to extrarenal amyloid deposition in the heart, liver, spleen, colon, and ileum. Cardiac involvement has been documented in a patient who developed restrictive cardiomyopathy a few years after kidney transplantation.\textsuperscript{39} This is in line with genotype-phenotype correlation described for the p.Glu545Val variant\textsuperscript{13,35} and contrasts with the absence of extrarenal amyloidosis in

\begin{table}[h]
\centering
\caption{Demographic Characteristics and Prevalence of CKD and Hypertension in 7 Portuguese Families With AFibE526V (p.Glu545Val) Amyloidosis}
\begin{tabular}{lllll}
\hline
 & p.Glu545Val & & & \\
 & Negative & Positive & & \\
(n = 39) & (n = 31) & & & \\
\hline
Sex & & & & \\
Male & 14 (40) & 14 (45) & 0.7 & \\
Female & 26 (68) & 17 (55) & & \\
\hline
Age at genetic screening, y & 42.0 ± 14.3 & 48.8 ± 17.3 & 0.08 & \\
CKD & 1 (3) & 11 (36) & <0.001 & \\
Hypertension & 11 (31) & 19 (61) & 0.02 & \\
Age at diagnosis, y & 45.1 ± 12.7 & 50.4 ± 16.6 & 0.3 & \\
SBP, mm Hg & 160.8 ± 20.0 & 159.9 ± 17.4 & 0.9 & \\
DBP, mm Hg & 89.4 ± 8.3 & 85.0 ± 13.2 & 0.3 & \\
Antihypertensive therapy & 10 (91) & 14 (74) & 0.5 & \\
RAS inhibition & 8 (86) & 11 (85) & 0.5 & \\
Combination therapy & 5 (53) & 5 (59) & 0.4 & \\
\hline
\end{tabular}
\end{table}
AβIβR554L (p.Arg573Leu). The prevalence of cerebrovascular disease was high, with 11 of the 30 (37%) patients with AβIβR526V (p.Glu545Val) amyloidosis in this study reporting at least one stroke event after 55 years of age. The contribution of vascular fibrinogen amyloid deposits to the pathophysiology of cerebrovascular disease in our patients is confounded by the high incidence of stroke in northern Portugal and the increasing risk for stroke with CKD progression, which is substantially higher in patients with ESRD. About 39% of the 13 probands with AβIβR526V (p.Glu545Val) amyloidosis were not aware of a family history of ESRD or amyloidosis. Although it has been suggested by other investigators that AβIβ amyloidosis has a reduced penetrance, this might not be the case in our population because the proper interpretation of those data is confounded by the late occurrence of the most clinically severe manifestations of the disease and the much shorter life expectancy only a few generations ago. In 1940, for example, the life expectancy in Portugal was 49.1 years for men and 53.6 years for women, and complications of AβIβ amyloidosis might not have been recognized or have been misinterpreted as hypertensive.

Many of the first-degree relatives of the probands were living abroad (eg, Brazil, France, United States, United Kingdom, and Switzerland). This contributed to the slow progression of cascade heredity—a disease that is carried by <50% of the at-risk individuals, and to the observation of AβIβR526V (p.Glu545Val) amyloidosis in immigrant Portuguese communities, such as in France.

AβIβ amyloidosis is largely a kidney disease, and outcomes after kidney transplantation are fairly good (median transplant survival, about 6 years, limited primarily by recurrent renal deposition of amyloid). Notably, our patient who was homozygous for the variant lived for 16 years with a functioning kidney transplant. It has been demonstrated that combined liver-kidney transplantation eliminates the amyloidogenic variant, preventing additional amyloid deposits. However, the high risk for early perioperative mortality with combined liver-kidney transplantation should be properly taken into account because long-term benefits are yet to be shown.

Emerging immunotherapies that promote the clearance of amyloid deposits are potentially novel therapeutic agents for AβIβ amyloidosis. Our study has several limitations. First, the retrospectively collected data are susceptible to referral, selection, and indication biases, and criteria for the genetic testing of hemodialysis patients might have missed a few additional cases. Second, many of the patients and families, as well as their physicians, were not aware that they had or were at risk for a genetic condition; the lack of awareness might have contributed to underestimation of cases and delay of the diagnosis of affected individuals. Third, the requirement for molecular confirmation of the diagnosis led to exclusion from the study cohort a number of possibly affected deceased individuals with CKD stage 3 or worse.

In conclusion, we have characterized the clinical phenotype of AβIβR526V (p.Glu545Val) amyloidosis in a large cohort of affected patients from a circumscribed region in northwest Portugal, where the disease is particularly frequent. Its natural history can be portrayed as early-onset hypertension, followed by proteinuria and CKD, eventually progressing to ESRD, with increasing prevalence of extrarenal manifestations in older patients, most typically resulting from amyloid deposition in the heart, liver, and spleen. Due to the nonspecific nature of its major clinical complications, the diagnosis of AβIβR526V (p.Glu545Val) amyloidosis requires a high index of suspicion and can be easily overlooked. Particularly in populations like the Portuguese, for whom hypertension is highly prevalent, the cause of CKD in patients with undiagnosed AβIβ amyloidosis may be erroneously attributed to hypertensive nephrosclerosis. Increasing awareness of this disease among local primary care physicians and nephrologists is critical to minimize its under-recognition, and genetic screening of all at-risk relatives of affected individuals is the most effective approach to early diagnosis. This allows the timely institution of therapies directed at minimizing the cardiovascular disease, as well as to make more appropriate decisions about the indication for solid-organ transplantation. Furthermore, with the emergence of novel disease-modifying therapies, it will be greatly desirable for patients to start treatment before irreversible tissue injury or organ failure. Finally, couples at risk should be informed about the possibility of preimplantation or prenatal diagnosis for primary prevention of AβIβR526V (p.Glu545Val) amyloidosis in their progeny.

ACKNOWLEDGEMENTS

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We thank the patients and their relatives for their invaluable collaboration in this study. NephroCare-Porto, for having allowed the ascertainment of patients with AβIβ amyloidosis in the dialysis clinics of Braga and Fafe, Portugal; Hemodializar, for having allowed the evaluation of patients in the dialysis clinic of Vila Verde, Portugal; Dr Joaquim Coelho, from the Family and General Medicine, Centro de Saúde Vizela do Minho, Braga, Portugal, for collaboration in genologic evaluation; and Dr Pedro Rodrigues Pereira, from the Department of Pathology, Centro Hospitalar de São João, Porto, Portugal, for collaboration in histologic evaluation of kidney biopsies.

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Financial Disclosures: The authors declare that they have no other relevant financial interests.

Contributions: Research idea and study design: IT, LL; data acquisition: IT, LM, LR, JS, JP, PPC, LL; data analysis/interpretation: IT, JP, PPC, LL; statistical analysis: JP, AP; supervision or mentorship: LL. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved. IT and LL take responsibility that this study has been reported honestly, accurately, and transparently; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned have been explained.

Peer Review: Evaluated by 2 external peer reviewers, a Statistical Editor, a Co-Editor, and Editor-in-Chief Levey.

SUPPLEMENTARY MATERIAL

Table S1: Comorbidities and cause of death in those with CKD and the PFG p.Glu52Val variant.

Note: The supplementary material accompanying this article (http://dx.doi.org/10.1053/j.ajkd.2017.01.048) is available at www.ajkd.org

REFERENCES

Unrecognized Fibrinogen A α-Chain Amyloidosis


Table S1. Comorbidities and Cause of Death among Individuals Carrying the FGA p.Glu545Val Variant and with Chronic Kidney Disease

<table>
<thead>
<tr>
<th>Comorbidity</th>
<th>Number with condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular disorders</td>
<td></td>
</tr>
<tr>
<td>Ischemic stroke</td>
<td>11</td>
</tr>
<tr>
<td>Hemorrhagic stroke</td>
<td>1</td>
</tr>
<tr>
<td>Left ventricular hypertrophy, &gt;13 mm</td>
<td>7</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>5</td>
</tr>
<tr>
<td>Atrioventricular block</td>
<td>4</td>
</tr>
<tr>
<td>Pacemaker</td>
<td>1</td>
</tr>
<tr>
<td>Infiltrative amyloid cardiomyopathy</td>
<td>1</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>1</td>
</tr>
<tr>
<td>Severe aortic stenosis</td>
<td>1</td>
</tr>
<tr>
<td>Central retinal artery/vein thrombosis</td>
<td>1/1</td>
</tr>
<tr>
<td>Iliac deep venous thrombosis</td>
<td>1</td>
</tr>
<tr>
<td>Gastrointestinal disorders</td>
<td></td>
</tr>
<tr>
<td>Erosive gastriitis</td>
<td>4</td>
</tr>
<tr>
<td>Peptic ulcer</td>
<td>4</td>
</tr>
<tr>
<td>Chronic gastritis</td>
<td>2</td>
</tr>
<tr>
<td>Upper gastrointestinal bleeding</td>
<td>2</td>
</tr>
<tr>
<td>Erosive duodenitis</td>
<td>1</td>
</tr>
<tr>
<td>Erosive esophagitis</td>
<td>1</td>
</tr>
<tr>
<td>Acute colitis</td>
<td>1</td>
</tr>
<tr>
<td>Acute ileitis</td>
<td>1</td>
</tr>
<tr>
<td>Chronic colitis</td>
<td>1</td>
</tr>
<tr>
<td>Colon ulcer</td>
<td>1</td>
</tr>
<tr>
<td>Hepatomegaly</td>
<td>4</td>
</tr>
<tr>
<td>Splenomegaly</td>
<td>4</td>
</tr>
<tr>
<td>Spontaneous splenic rupture</td>
<td>1</td>
</tr>
<tr>
<td>Neurologic disorders</td>
<td></td>
</tr>
<tr>
<td>Lower-limb sensory peripheral neuropathy</td>
<td>4</td>
</tr>
<tr>
<td>Autonomic neuropathy</td>
<td>1</td>
</tr>
<tr>
<td>Carpal tunnel syndrome</td>
<td>3</td>
</tr>
<tr>
<td>Death</td>
<td>14</td>
</tr>
<tr>
<td>Cause unknown</td>
<td>6</td>
</tr>
<tr>
<td>Ischemic stroke</td>
<td>3</td>
</tr>
<tr>
<td>Hemorrhagic stroke</td>
<td>1</td>
</tr>
<tr>
<td>Mesenteric ischemia</td>
<td>1</td>
</tr>
<tr>
<td>Sepsis</td>
<td>2</td>
</tr>
<tr>
<td>Pneumonia</td>
<td></td>
</tr>
</tbody>
</table>

Note: All extra-renal comorbidity or death events were observed in 21 of 30 individuals carrying the FGA p.Glu545Val variant and with chronic kidney disease. Fourteen of them manifested two or more extra-renal comorbidities. None of the 20 individuals carrying the amyloidogenic variant but with no evidence of kidney disease manifested comorbidities or have died during the follow-up period.
2.2. Long-term follow-up of patients with hereditary fibrinogen Aα chain amyloidosis

Histological demonstration of amyloid deposition is an important tool in the characterization of amyloidosis. The systematic search for amyloid deposition in surgical biopsy specimens during long-term follow-up contributes to define the extent of the disease. In this sense, the systematic study of surgical biopsy specimens of patients with AFib amyloidosis allowed the identification of four sites of extrarenal amyloid deposition (abdominal fat, colon, ileum and spleen).

Abdominal subcutaneous fat from a surgical biopsy of a hemodialysis patient carrying the FGA p.Glu545Val variant showed amyloid deposition in the vascular walls (Figure 6).

![Figure 6 | Abdominal fat involvement.](image)

Vascular wall amyloid deposition demonstrated by Congo red stain in a patient with AFibE526V (p.Glu545Val) amyloidosis (courtesy of Prof. Luisa Lobato, Department of Nephrology, HAS/CHP).

Search for amyloid deposition in abdominal fat can be used as the first diagnostic approach in systemic amyloidoses. In addition, it can also be used for histological documentation of a diagnosis based on clinical criteria, family history and molecular genetics. Abdominal fat biopsy is also an important tool for amyloid histological screening in dialysis patients and during follow-up of carriers of an amyloidogenic variant.

Spontaneous rupture of the spleen might be a severe complication of AFib amyloidosis. One of our patients performed splenectomy in this context and histological evaluation of spleen fragments showed amyloid deposition (Figure 7).

![Figure 7 | Spleen involvement in AFibE526V (p.Glu545Val) amyloidosis.](image)

Randomly (A), septal (B) and arteriolar (C) spleen amyloid deposition demonstrated by Congo red stain (courtesy of Prof. Elsa Fonseca, Department of Pathology, CHSJ).
We also demonstrated vascular wall amyloid deposition in surgical specimens of ileum and colon of a hemodialysis patient with AFib amyloidosis. Histological evaluation of a fragment of ileal segment removed from a surgical specimen consisting of an ileal section of 53 cm, obtained through ileal resection due to mesenteric ischemia, showed the presence of amyloid substance in the wall of an artery (Figure 8).

![Figure 8](image)

Figure 8 | Ileum involvement in AFibE526V (p.Glu545Val) amyloidosis.
Vascular wall amyloid deposition demonstrated by Congo red stain (A, B) and apple green birefringence under polarized light (C), (courtesy of Dr. Wen Xiaong, Department of Pathology, CHSJ).

The same patient developed ischemic colitis and was submitted to segmental colectomy. Subsequent revision of paraffin embedded fragment of colon removed from an operative part of about 12 cm long showed amyloid deposition in colon wall vessels (Figure 9).

![Figure 9](image)

Figure 9 | Colon involvement in AFibE526V (p.Glu545Val) amyloidosis.
Vascular wall amyloid deposition demonstrated by Congo red stain (A, B, D) and apple green birefringence under polarized light (C, E), (courtesy of Dr. Wen Xiaong, Department of Pathology, CHSJ).

Beyond the kidney, the detection of amyloid deposition in spleen and vessels of the abdominal fat, ileum and colon confirms the systemic involvement of AFib amyloidosis.

These data resulted from a study whose methodology, results and discussion were presented at the XIth International Symposium on Amyloidosis which took place in Rome in 2010. The extended abstract was published after peer review in the journal *Amyloid*. Reference: Tavares I, Lobato L, Moreira L, Santos J, Lacerda P, Pinheiro J, Costa P. Long-term follow-up of patients with hereditary fibrinogen A alpha-chain amyloidosis. *Amyloid* 2011; 18 (Suppl1): 221-222, which follows.
Long-term follow-up of patients with hereditary fibrinogen A alpha-chain amyloidosis

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Abstract: Fibrinogen A alpha-chain amyloidosis (AFib) is an autosomal dominant condition with variable penetrance, usually of late onset. Progression to stage V chronic kidney disease is a consistent feature. There is a cluster of AFib in the district of Braga, Portugal, characterized as a systemic disease with a high penetrance.

Introduction: Fibrinogen amyloidosis due to mutations in the fibrinogen A alpha-chain gene (FGA), first described by Benson et al. in 1993 [1], is emerging as the most common type of hereditary renal amyloidosis in Europe [2,3]. The first Portuguese case was reported in 2004 [4]. Gilmore et al. defined for the first time the phenotype of AFib based on an evaluation of a large patient series (71 patients), as a renal disease characterized by variable penetrance, distinctive histological appearance, proteinuria, and progressive renal impairment [5]. Conversely, in a recent issue of Blood, Ntagou et al. extended the definition of the disease to a systemic phenotype through detailed findings in 22 patients [6]. In both publications family history was frequently absent.

For better understanding of the disease, we made a systematic study of Portuguese cluster patients with AFib, and report the clinical and long-term outcome of 25 patients who were followed for a period of 8 years.

Methods: We included in this study 25 patients (12 identified in an initial biopsy study and 13 identified in a subsequent screening study).

Biopsy study: Immunohistochemical assessment of amyloidosis in consecutive kidney biopsies led to the identification of 12 AFib patients. All were from the same geographical area (patients on dialysis, chronic renal failure or proteinuria followed by nephrologists, and some relatives) led to the identification of 13 AFib patients.

We evaluated clinical features through clinical data obtained from medical records and interviews with patients and their relatives, disease-related organ dysfunction by histological demonstration of amyloid deposits on specimens obtained from biopsies or surgery, and outcome.

Results and discussion: We identified 25 patients with AFib, 14 asymptomatic carriers and 8 hypertensive carriers. All from Braga district in north Portugal. The FGA E520V mutation was identified in all patients, 24 heterozygous and 1 homozygous. Renal biopsy specimens showed massive amyloid deposition in glomeruli, but arteriolar and cortical interstitium were also involved in contrast to previous reports. One patient had amyloid deposits on abdominal subcutaneous fat obtained from surgical biopsy (Figure 1). A family history of renal disease was present in 20 (80%) patients, contrary to published data [5,6]. Renal involvement led to diagnosis in all patients (14 females, 11 males), median age at presentation was 62 years (33–77 years). Hypertension was universal and diagnosed at a median age of 58 years (34–71 years). Twenty-two patients progressed to dialysis after a median time from presentation of 50 months.

After a median follow-up of 8 years, clinical significant extra-renal manifestations were mainly characterized by vascular and gastrointestinal diseases.

Eight patients had cerebrovascular events (2 hemorrhagic strokes, 6 ischemic strokes). Mean age at first stroke was 71 years. Two patients had severe coronary disease (1 was submitted to a coronary bypass) and peripheral arteriopathy. Echocardiographic findings consistent with infiltrative amyloid...
cardiomyopathy were present in only one patient. One patient had a pacemaker.

One patient was submitted to ileum resection in February 2004 (Figure 2) and colon resection in October 2004. He died in May 2007 of mesenteric ischemia. Amyloid deposits were observed in vascular walls of both specimens raising the possibility that they had contributed to disease process.

Seven patients had peptic ulcer disease. Spleen amyloid deposits (peri-arteriolar, septal and randomly) were observed in 1 splenectomy specimen removed after a spleen spontaneous rupture. Periperal neuropathy was present in four patients. Plasma fibrinogen levels were normal, except low levels in the homozygous.

Only the homozygous patient underwent kidney transplant. She received an isolated kidney transplant in 1990, after 6 years of hemodialysis. Fourteen years later, no clinical recurrence of amyloid was evident.

Seventeen patients died at median age of 75 years (46-84 years). Stroke was the leading cause of death (5 patients). The other causes were mesenteric ischemia (1 patient), pneumonia (1 patient), sepsis (1 patient) and was unknown in 8 patients.

Data from the 2009 Registration Office of the Portuguese Society of Nephrology reported a mortality rate of 14.01% for patients undergoing hemodialysis. Cardiovascular disease was the cause of death in 30% of the cases [7].

In conclusion, AFib in our cluster is characterized as a systemic disease with visceral, vascular, cardiac, and neurological involvement, with high penetrance. Besides kidney we observed amyloid deposits in spleen and vascular walls of abdominal fat, ileum, and colon. Vascular disease was an important cause of morbidity and mortality, raising the role of the variation in the fibrinogen gene FGA or the contribution of vascular amyloid deposition to atherosclerosis acceleration. Interestingly, there has been no evidence of recurrence of renal amyloid in the single recipient of a kidney transplant after 14 years. Considering the number of identified carriers further collaborative studies are needed.

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References

The online version first published on 14 July 2011 showed the incorrect page numbers of this article. They have been now been corrected on this version.

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2.3. Renal amyloidosis in northern Portugal: the clinical differences in distinct forms

In our cohort, AA was the most frequent type of amyloidoses. AA amyloidosis manifests in younger patients and is diagnosed earlier than AL or AFib amyloidosis.

Compared to AA and AL amyloidosis, the natural history of the renal decline in AFibE526V (p.Glu545Val) amyloidosis is slower than in AL amyloidosis, but is faster than in AA amyloidosis. Compared to other forms of late onset hereditary amyloidoses, as is the case of AApoAI amyloidosis, renal decline to ESRD of 5 years in AFib is substantially faster than in AApoAI amyloidosis, in which it is typically approximately 8 to 9 years [136, 224]. However, this different rate of progression should be viewed under preferential histopathological involvement, because AFib is typically a glomerular disease in which a progression is faster and AApoAI is typically a tubulointerstitial nephritis in which the progression of a renal disease is slower [32, 224].

Additional renal damage observed in any kind of amyloidoses might contribute and accelerate the rate of disease progression to ESRD. This is particularly important for AA and AL amyloidosis since morbidity related to the evolution of the underlying diseases and the adverse effects of therapy may have significant additional effects on the course of renal involvement. However, this was not evaluated in our study.

Despite the constraints related to the absence of therapy to diminish production of the amyloidogenic fibrinogen variant, mean patient survival from clinical presentation is more than 15 years in AFib amyloidosis, contrasting markedly with the shortest survival of patients with AL amyloidosis. The longest survival observed in AFib amyloidosis reflects the combination of the slower natural history of the renal disease and the lack of severe clinically extrarenal involvement at diagnosis, that are commonly the cause of death in systemic AL amyloidosis.

In conclusion, compared to AA and AL, AFib amyloidosis patients presented with worst renal function but had the best prognosis.

SAA and AA amyloidosis

Renal amyloidosis in Northern Portugal: Apart from ATTR, AA still dominates

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ABSTRACT

The main objective of our study was to determine the epidemiology of systemic amyloidosis in a centre located in the North of Portugal, but not the tertiary referral centre for ATTR. We conducted a systematic immunohistochemical typing of 111 consecutive systemic amyloid cases with native kidney biopsy performed between 1978 and 2013. Clinical data and outcome were retrospectively reviewed. AA amyloidosis was most prevalent and diagnosed in 61 (55.0%) patients, AL in 23 (20.7%), combined AL and AA in 1 (0.9%), AFF in 9 (8.1%), ATTR in 2 (1.8%), AApoAL in 1 (0.9%), ALys in 1 (0.9%) and 13 (11.7%) remained unclassified. Mean age at diagnosis was 54 ± 15.3 years. Nephrotic syndrome was the main renal manifestation. Hypertension was present in 43.2% of patients. Severity two (8.9%) patients required dialysis 26.1 ± 42.2 months after diagnosis. Mean survival after dialysis was 29.6 ± 49.1 months. In our experience, outside ATTR referral centre, AA is the most frequent type of amyloidosis in Portugal.

INTRODUCTION

A precise epidemiology of amyloidoses is difficult to define as the disease is often undiagnosed or misdiagnosed. Selection bias of data from tertiary centres becomes potentially unrepresentative (1, 2). In Portugal transthyretin amyloidosis is the most frequent form of hereditary systemic amyloidosis. Since the identification of the disease as a new entity in 1989, Hospital Santo António – Centro Hospitalar do Porto is the reference centre for hereditary amyloidosis (3). Apart from ATTR, few data are available from large population-based studies on different types of systemic amyloidoses in Portugal. The aims of this study were: (i) to classify by immunohistochemistry the type of amyloidosis of a Northern Portuguese series evaluated outside the referral centre; (ii) to identify amyloidogenic variants in the hereditary forms; (iii) to determine underlying disorder, clinical and laboratory findings at diagnosis.

MATERIALS and METHODS

One hundred eleven Northern Portuguese cases of native kidney biopsy-proven systemic amyloidosis diagnosed between May 1978 and September 2013, at department of Nephrology from Centro
Hospitalei São João (CHSJ) were retrospectively reviewed for immunohistochemistry typing. DNA studies in suspected hereditary amyloidosis and outcome analysis. All biopsies were reviewed by a pathologist and a nephrologist with expertise in amyloid nephropathy. Congo red staining was performed on 6 μm thick formalin-fixed paraffin-embedded sections and amyloid was microscopically analyzed under polarized light. Immunohistochemical staining was performed on 2 μm thick formalin-fixed paraffin-embedded sections of the amyloid containing biopsies using standard methods and a ready-to-use rabbit mouse, peroxidase/stain/diaminobenzidine (DAB) detection system (Dako Real Envision). Monoclonal antibodies used were directed against serum amyloid A (Dako), apolipoprotein A-I (Abcam) and transthyretin (4); polyclonal antibodies were used for κ-light chain, λ-light chain, fibrinogen Aα-chain, transthyretin, apolipoprotein A-I and lysozyme (all from Dako). The sections were incubated for 2 hours at room temperature, diluted in 1% BSA/PBS: monoclonal anti-TTR was used directly; polyclonal anti-TTR 1:500; anti-SAA 1:100; anti-κ 1:1000; anti-λ 1:2000; anti-Fib 1:600; Anti-Lys 1:300; anti-ApoAI 1:400; anti-ApoAII 1:800. We searched for DNA mutations in suspected hereditary amyloidosis. DNA was isolated from peripheral blood leukocytes using the "Genomic DNA Purification Kit" (Puregene, Gentra Systems). The coding regions of the genes encoding transthyretin (exon 2), fibrinogen Aα-chain (exon 5), apolipoprotein A-I (exons 1 to 4), apolipoprotein A-II (exon 4) and lysozyme (exon 2) were amplified by polymerase-chain-reaction (PCR). PCR products were analyzed by agarose gel electrophoresis, purified according to "High Pure PCR Purification Kit" (Roche) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence results were analyzed with ChromasPro and RibomTraceEdit. Demographic, clinical and laboratory data were obtained from retrospective review of medical records. Clinical definitions used: (i) nephrotic syndrome characterized by nephrotic-range proteinuria (≥3.5 g/day), hypalbuminemia (<3.5 g/dL) and peripheral edema; (ii) glomerular filtration rate estimation using 2009 CKD-EPI creatinine equation (5); (iii) hypertension classified according to 2013 ESC/ESH guidelines (6). The study was reviewed and approved by the Health Ethics Commission of CHSJ.

RESULTS

According to immunohistochemical classification, the most prevalent type of amyloidosis was AA, corresponding to more than half of the patients, followed by AL in about one fifth. The ratio AA:AL was 2.6 to 1. The subtype of light chain in AL amyloidosis was λ in two thirds of patients. Two different types of amyloid deposits – light chain λ and SAA – were present in one patient. Immunostaining disclosed a predominant pattern for light chain λ deposition and small patchy deposits for SAA at different sites. AFβ was the most frequent type of hereditary amyloidosis contributing to 9 (8.1%) of the cases, followed by ATTR in 2 (1.8%), AApοAI in 1 (0.9%) and Aλy in 1 (0.9%). Thirteen (11.7%) cases remained unclassified: 9 (8.1%) due to negative immunohistochemistry and 4 (3.6%) due to lack of adequate tissue. Prevalence of AA amyloidosis remained higher from 1978 to 2013. Molecular diagnosis of hereditary forms disclosed the point mutations FGA p.Glu54Val in all AFβ cases and TTR p.Met51Val in the 2 cases of ATTR. Sudden death of Aλy immunohistochemistry positive patient did not allow genetic study. In AApοAI case it was not detected pathogenic changes in the encoding region (exons 2 to 4) and exon-ntron respective transitions of the AApοAI gene. Chronic infections were the main cause of AA amyloidosis, particularly tuberculosis that contributed to one fifth of all cases. One patient had familial AA associated to Muckle-Wells syndrome. AL amyloidosis was related to multiple myeloma in 9 (39.1%) and to monoclonal gammopathy of undetermined significance in 11
SAA and AA amyloidosis

(47.6%) patients. Combined AL and AA amyloidosis patients had lambda light chain multiple myeloma and a previous history of syphilis. Patients mean age at diagnosis of the underlying disorder was 36 ± 20 years for AA and 62 ± 9 years for AL. Table 1 shows clinical and follow-up data of overall studied patients and by the most prevalent types of systemic amyloidosis. AA patients were the youngest and had the best renal function. Nephrotic syndrome was the first sign of renal involvement in 91 (62%) patients with a similar frequency for all types of amyloidosis. Hypertension was significantly associated to AFib which also had the best survival.

Table 1: Clinical data referred at diagnosis; outcome of total of studied patients and by amyloidosis type

<table>
<thead>
<tr>
<th></th>
<th>All (n=111)</th>
<th>AA (n=61)</th>
<th>AL (n=23)</th>
<th>AFib (n=9)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>64.0 (15.5)</td>
<td>49.0 (16.5)</td>
<td>62.0 (3.7)</td>
<td>57.0 (11.3)</td>
<td>0.005</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>57 (51.4)</td>
<td>32 (52.5)</td>
<td>9 (39.1)</td>
<td>6 (66.7)</td>
<td>n.a.</td>
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<tr>
<td>eGFR, ml/min/1.73 m²</td>
<td>56.4 (41.1)</td>
<td>61.7 (44.0)</td>
<td>55.2 (35.3)</td>
<td>30.2 (22.5)</td>
<td>0.121</td>
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<tr>
<td>Nephrotic syndrome, n (%)</td>
<td>91 (82.0)</td>
<td>49 (80.3)</td>
<td>18 (78.2)</td>
<td>8 (88.9)</td>
<td>n.a.</td>
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<tr>
<td>Proteinuria, g/day</td>
<td>6.6 (4.5)</td>
<td>6.4 (4.2)</td>
<td>7.4 (6.2)</td>
<td>7.6 (3.7)</td>
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<tr>
<td>Anemia, n (%)</td>
<td>64 (57.7)</td>
<td>37 (60.7)</td>
<td>10 (43.5)</td>
<td>7 (77.8)</td>
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<td>Hypertension, n (%)</td>
<td>48 (43.2)</td>
<td>21 (34.4)</td>
<td>8 (34.8)</td>
<td>9 (100.0)</td>
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<tr>
<td>Time to diagnosis³, yr</td>
<td>9.2 (11.4)</td>
<td>13.9 (12.3)</td>
<td>1.4 (1.5)</td>
<td>n.a.</td>
<td>&lt;0.001</td>
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<tr>
<td>Dialysis, n (%)</td>
<td>72 (64.9)</td>
<td>39 (63.9)</td>
<td>14 (60.9)</td>
<td>8 (88.9)</td>
<td>0.425</td>
</tr>
</tbody>
</table>

| Time from diagnosis to dialysis, months | 26.1 (42.2) | 33.3 (50.7) | 9.1 (12.3) | 15.6 (19.8) | 0.290 |
| Deaths, n (%)               | 74 (66.7)   | 43 (70.0)  | 10 (55.2)  | 0 (50.0)    | 0.480 |
| Overall survival², months   | 46.5 (63.0) | 53.6 (67.8)| 14.7 (11.6)| 84.2 (110.6)| 0.274 |
| Survival after dialysis, months | 26.6 (49.1) | 30.9 (44.4) | 7.1 (6.6)  | 71.2 (123)  | 0.320 |

Table 1: Age, proteinuria, eGFR, time to diagnosis, time from diagnosis to dialysis, overall survival and survival after dialysis are reported as mean (SD). Abbreviations: Yr, years; n.a., not applicable; eGFR, estimated glomerular filtration rate by CKD-EPI equation; IHC, immunohistochemistry. *Time between onset of the underlying disorder and diagnosis. †Time from diagnosis (kidney biopsy) to death.

DISCUSSION

We report the immunohistochemical classification, molecular diagnosis and clinical characterization of 111 Northern Portuguese patients with native kidney biopsy-proven amyloid disease, evaluated outside the referral centre for hereditary amyloidosis. AA was the most frequent form of systemic amyloidosis.
SAA and AA amyloidosis

in our series, similarly to what was reported in the largest series of renal amyloidosis in kidney biopsies (7). A recent single-centre study detailing 20 years experience of AA amyloidosis showed that rheumatoid arthritis contributed to 45% of cases and 67% of patients had some form of renal involvement (8). However, between us, chronic infectious disorders are still the most frequent cause of AA amyloidosis, mainly pulmonary tuberculosis, due to the fact that Portugal still has one of the highest tuberculosis incidence rates among European Union countries (9). Besides the long period of our study, the prevalence of AA amyloidosis remained stable from 1978 to 2013. Nevertheless, with recent advances in the treatment of chronic infectious diseases and autoimmune inflammatory processes a decline in the incidence of AA form would be expected. Indeed, Pinney and colleagues reported an epidemiological study about systemic amyloidosis in England and concluded that systemic AL amyloidosis was the most common type (10). AA amyloidosis, in our series, affected younger individuals which may be related to early beginning of most of the underlying conditions, mainly inflammatory diseases, and therapeutic failures at suppressing inflammation. A lesser degree of renal dysfunction in our patients may be related to an early diagnosis through repeated measurements of microalbuminuria and serum creatinine. AL amyloidosis was our second most frequent form of systemic amyloidosis. However, results should be cautiously interpreted since in order and unstable patients with monoclonal plasma cell disorders we may use less invasive diagnostic tests, such as abdominal fat aspiration or minor salivary gland biopsy as a way to minimize complications related to kidney biopsies. Uncommon hereditary amyloidosis contributed to 9.9% of our cases (8.1% ATTR and 1.8% ATTR). DNA analysis is mandatory to confirm the diagnosis but it should always be complementary to other diagnostic techniques that allow the identification of amyloid protein. ATTR is the most common type of hereditary amyloidosis in Europe (11). Our nine ATTR patients were from the same region and had the same amyloidogenetic mutation, so haplotyping studies are necessary to conclude if they belong to the same family. Worst renal function at presentation in this small group may not be informative since variations in disease penetrance and progression may cause an absence of family history and delay the diagnosis. Best survival, both overall and after dialysis, may be related to less aggressive systemic involvement. High prevalence of hypertension among ATTR patients may be secondary to chronic kidney disease, but direct amyloid deposition in vascular walls may also be involved (11). Thirteen (11.7%) of our cases remained unclassified mainly due to negative immunohistochemistry. Raising our spectrum of amyloid antibodies with the inclusion of anti-LECT2, anti-Apo-AIV and anti-immunoglobulin heavy chain might improve diagnostic capacity (12). In the authors' experience, outside ATTR referral centre, AA is the most frequent type of amyloidosis in Portugal. ALECT2 is missing. ATTR patients presented hypertension as a phenotypic marker and had the best survival.

**SOURCES of SUPPORT and ACKNOWLEDGMENTS**

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REFERENCES

3. GENETICS AND GENEALOGY


Gene sequencing raised the ability to detect and diagnose amyloid and amyloidoses, currently being the gold standard to detect variations such as substitutions, and small deletions and insertions at the nucleotide level. Sequencing is particularly useful in hereditary amyloidoses due to genetic heterogeneity (multiple genes being involved in a disease) and allelic heterogeneity (multiple mutations in the same gene being able to cause the disease).

The identification of the amyloidogenic variant is crucial due to the correlation between genotype and phenotype, implications in therapeutic approach and genetic counseling. In addition, their identification may contribute to clarify the pathogenic mechanisms underlying amyloidosis.

AFib amyloidosis is an autosomal dominant disease associated with mutations in the FGA. To date, 16 FGA variants have been described [79]. These variants result from missense, deletion or insertion-deletion mutations in the exon 5 of the FGA (Table 2). AFibE526V (p.Glu545Val) amyloidosis has been reported in northern Europe and in this thesis we describe the Portuguese endemic foci of the disease. The other missense variants were described in isolated kindreds from various parts of the world, while frameshift variants are private (each of them has been reported in only a single family) [32, 207, 214-216].

AFib amyloidosis has been characterized according to the inherited heterozygous FGA amyloidogenic variant and as such manifested as a dysfibrinogenemia. In this section I present the first homozygous case of AFibE526V (p.Glu545Val) amyloidosis, characterized by changes in the quality and also in the quantity of fibrinogen, and as such manifested as a hypodyssfibrinogenemia. The impact of homozygosity was also related to an early age of onset of amyloid nephropathy, but not to venous or arterial thrombosis, spontaneous spleen ruptures, painful bone cysts or intrahepatic inclusions which can complicate the clinical course of patients with quantitative fibrinogen disorders [206].

Renal transplantation in AFib amyloidosis has been associated with recurrence of amyloid in the graft with resultant kidney loss after a median of 6.7 years [32]. However, our homozygous patient had an isolated kidney allograft survival of 16 years. This prolonged survival raised questions about benefit of liver transplantation.

Case Report

Homozgyosity for the E526V Mutation in Fibrinogen A Alpha-Chain Amyloidosis: The First Report

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Systemic hereditary amyloidoses are autonomous dominant diseases associated with mutations in genes encoding ten different proteins. The clinical phenotype has implications on the therapeutic approach, but it is commonly variable and largely dependent on the type of mutation. Except for rare cases involving gelsolin or transthyretin, patients are heterozygous for the amyloidogenic variants.

Here we describe the first patient identified worldwide as homozgyous for a nephropathic amyloidosis, involving the fibrinogen variant associated with the fibrinogen alpha-chain E526V (p.Glu543Val) mutation. In 1989, a 44-year-old woman presented with hypertension, hypoproteinemia, nephrotic syndrome, and renal failure. She started hemodialysis in 1990 and 6 years later underwent isolated kidney transplantation from a deceased donor. Graft function and clinical status were unremarkable for 16 years, despite progressively increased left ventricular mass on echocardiography. In 2012, 4 months before death, the deterioration rapidly with severe heart failure, precipitated by Clostridium difficile enteritis and urosepsis. Afflicted family members developed nephropathy, on average, nearly three decades later, which may be explained by the gene dosage effect on the phenotype of E526V (p.Glu543Val) fibrinogen A alpha-chain amyloidosis.

1. Introduction

Hereditary fibrinogen A alpha-chain (A fibrin) amyloidosis is a systemic amyloid disease first characterized in 1993 in a Peruvian kindred [1-4]. It presents with proteinuria and features a progressive decline in kidney function to end stage renal failure (ESRF) within 5 years of diagnosis [3]. Nonetheless, there is a wide variability in disease onset, systemic involvement, and penetrance. Renal replacement therapy and transplantation are currently the mainstay of therapy. However, because the liver is the source of the amyloidogenic variant fibrinogen and there is no evidence that WT fibrinogen can be amyloidogenic, the only curative treatment is liver transplantation [5].

A fibrin amyloidosis appears to be more common worldwide than previously recognized [6]. The R554L (p.Arg573Leu) mutation was the first fibrinogen amyloidogenic variant identified [1]. To date, 13 amyloidogenic mutations have been reported in the fibrinogen alpha-chain gene (FGA) (http://amyloidosmutations.com/mut-affib.php), accounting for 8% of hereditary amyloidosis cases [7]. The most common variant mutation, E526V (p.Glu543Val), was identified heterozygously in kindred members of Irish, British, Polish, Portuguese, French, German, and Brazilian origin [2, 8-11]. Homozygosity has only been reported for hereditary gelsolin and transthyretin amyloidosis (Table 1). In gelsolin (AGel) amyloidosis, homozygotes have been reported to show earlier onset and more severe clinical manifestations.
than heterozygotes, explained by the lethal effects of the mutant genes [12, 13]. However, in transthyretin (TTR) amyloidosis, the underlying molecular mechanisms are largely unknown [14–23].

Here, we report the first homozygous patient with AFibE506V (p.Cys354Ser) amyloidosis, identified in 1998 and followed up for 23 years. In this reported kindred, the comparison of the clinical pictures of homozygote and heterozygote provides important information about the gene dosage effects on the phenotype of AFib amyloidosis.

2. Case Report

2.1. Probands. A 44-year-old Caucasian northern Portuguese woman who had suffered a single previous episode of upper gastrointestinal hemorrhage presented with hypertension, nephrotic syndrome, and renal failure in April 1998. Renal biopsy 1 year later revealed abundant glomerular amyloid deposition (Figure 1a); her serum creatinine was 4.0 mg/dL and proteinuria of 7.5 g/day was detected. In the absence of any underlying inflammatory disease and unawareness of family history, she was presumed to have immunoglobulin light chain (AL) amyloidosis. However, there was no evidence of cardiac amyloidosis, and neither a monoclonal immunoglobulin nor a plasma cell disorder was identified. She was treated with melphalan and corticosteroids but progressed rapidly to ESRF and started hemodialysis in December 1999. During hemodialysis, her functional status remained good.

Successful isolated renal transplantation (IXTx) was performed in August 1996. At the time, she had no other health problems. Physical examination revealed an enlarged liver and spleen. There was no anemia or thymocytopenia and liver function tests were normal. Blood coagulation tests revealed low fibrinogen levels. Echocardiography showed mild left ventricular hypertrophy without the typical speckle appearance. Abdominal ultrasonography showed hepatomegaly (175 cm), splenomegaly (14.5 cm), and bilateral kidney atrophy. A neuropathologic study of the lower limbs found mild sensory peripheral neuropathy. Induction and maintenance immunosuppression for transplantation involved corticosteroids, cyclosporine, and azathioprine.

2.2. Histology and Immunohistochemistry. In 2008, we began an extensive investigation to determine the patient’s amyloid type. Congo red staining of 10 μm formalin fixed, paraffin embedded section of the native kidney biopsy confirmed abundant glomerular amyloid deposition and absence of vascular and interstitial involvement. Immunohistochemical staining was performed on 2 μm sections of amyloid-containing tissue using standard methods and a rabbit/mouse, peroxidase/diaminobenzidine detection system (REAL EnVision: Dako, Glostrup, Denmark). mAbs were directed against serum amyloid A (Dako), apolipoprotein A-ll (Abcam, Cambridge, UK), and transthyretin [24]; polyclonal antibodies were used for kappa light chain, lambda light chain, fibrinogen A alpha-chain (Fga), transthyretin, apolipoprotein A-ll, and lysozyme (Dako). For light chains and Fga, sections were treated with 10 μg/mL proteinase K for 10 min at 37°C and 10 min at room temperature. Blocking was performed with 5% bovine serum albumin/phosphate buffered saline (BSA/PBS). Sections were incubated with the antibodies for 2 h at room temperature and diluted in 1% BSA/PBS as follows: monoclonal anti-transthyretin, used directly; polyclonal anti-transthyretin, 1:500; anti-serum amyloid A, 1:1000; anti-kappa light chain, 1:1000; anti-lambda light chain, 1:2000; anti-IgG, 1:800; anti-lysozyme, 1:300; anti-apolipoprotein A-ll, 1:400; and anti-apolipoprotein A-ll, 1:600. Positive control tissues containing these amyloid proteins were also stained during each run. The glomerular amyloid deposits in the patient’s kidney biopsy reacted with the anti-serum to Fga (Figure 1b).
2.3. Genetic Evaluation. DNA was extracted from peripheral white blood cells obtained from whole blood. Exon 5 of the FGA gene was amplified by the polymerase chain reaction (PCR) with primers flanking the coding region (forward 5'-CCT TCT TCG ACA CTG CCT CAA CTG-3' and reverse 5'-TCG TCT GTT GTA ACT CGT GCT-3'), which amplified a fragment of 224 base pairs encompassing nucleotide 4827 to 5051. PCR products were analyzed by agarose gel electrophoresis, purified, and sequenced with Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in an Applied Biosystems 3700 sequencer. Sequences were analyzed using ChromasPro. A homozygous A to T transition at nucleotide 4899 in FGA was detected; this changes codon 526 of the mature protein (corresponding to position 545 of the unprocessed gene product), from GAG, encoding glutamic acid, to GTG, encoding valine (Figure 1b). Codons are numbered according to reference sequence NM_000508.3.

2.4. Proband Outcome. During 16 years of follow-up after RTx, there were no episodes of rejection and she was normotensive. Maximal proteinuria of 0.9 g/day was detected in 2009 and renal graft function remained good until 4 months before her death; thus, no renal allograft biopsy was performed. Deceptive ulcer due to Helicobacter pylori infection was diagnosed after an episode of upper gastrointestinal hemorrhage. Apparent progression of extrarenal amyloid disease was mainly cardiovascular and hepatic. Repeated echocardiography found sinus rhythm, normal intervals, and no criteria for hypertrophy. Serial echocardiography showed left atrial enlargement (53 mm), severely abnormal left ventricular hypertrophy (left ventricular mass index increased from 154 g/m² in 2006 to 170 g/m² in 2012, with a reference range of 43–95 g/m²), mild degenerative valvular disease, preserved systolic ventricular function, and moderate pulmonary hypertension. These findings were consistent with cardiac amyloidosis. Hepatic involvement was characterized by mild elevations of gamma-glutamyl transferase.

![Figure 1: Homozygous E526V (p.Glu545Val) mutation in the fibrinogen alpha chain gene (FGA) associated with fibrinogen A alpha chain amyloidosis in a Portuguese patient. (a) shows abundant glomerular amyloid deposition with typical apple-green birefringence (Congo red staining under polarized light, ×200, left). Immunohistochemical staining was positive with polyclonal anti-fibrinogen antibodies, (×200, right). (b) shows a partial sequence chromatograms of FGA. The mutation identified in the proband, which alters codon 545 (position 526 of the mature protein) from GAG (glutamic acid) to GTG (valine), is depicted in a circle. (c) shows the pedigree of the affected kindred. The homozygous patient (proband) is indicated by the arrow. The FGA p.Glu545Val mutation was identified heterozygously in family members III, III, IIIB, IV3, IV4, IV5, and IV6 (indicated by half-solid symbols). Obligatory heterozygotes IV2 and IV7 (indicated by question marks) did not perform genotyping because the former was abroad and the latter died at young age. Those with chronic renal failure who have not undergone histologic or genetic testing are indicated by a black column inside the symbol. Familiars whose genetic tests were negative were indicated by an N inside the symbol. Blank symbols indicate that tests have not been conducted and/or information is unavailable for these individuals. Shaded denote deceased members.](image-url)
and alkaline phosphatase with increasing hepatomegaly; the liver's ultrasound diameter reached 22.7 cm in September 2012. At that time, the spleen diameter was 9.4 cm and there were several calcifications. There was no sign of portal hypertension. Relevant laboratory data are listed in Table 2.

In August 2012, 4 months before her death, the patient was hospitalized for Clostridium difficile colitis, which was successfully treated with metronidazole. One month later, she was admitted for congestive heart failure with an N-terminal pro-B-type natriuretic peptide (NT-proBNP) level of 51 937 pg/ml; this was controlled with diuretic therapy. In November 2012, she was hospitalized for hypotension associated with effective circulating volume depletion. She died in December 2012 after admission for Klebsiella pneumoniae urosepsis, congestive heart failure with NT-proBNP 14 114 pg/ml, unresponsive to diuretic therapy, and acute kidney allograft injury. Autopsy was not performed.

Table 2: Laboratory data.

<table>
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<td>89</td>
<td>136</td>
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</tbody>
</table>

HbA1c: glycated hemoglobin; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; GGT: gamma-glutamyl transferase; iPTH: intact parathyroid hormone; NT-proBNP: N-terminal pro-B-type natriuretic peptide; CRP: C-reactive protein.

3. Discussion

Here, we describe the first Afib amyloidosis patient homozygous for the E526V (p.Glu545Val) mutation and her long term outcome after isolated RTx. The unexpected etiology and outcome of this case highlight important aspects of the clinical management of systemic amyloidosis in general. Our patient's prolonged survival without treatment and the absence of an identifiable monoclonal plasma cell disorder led us to question the diagnosis of AL amyloidosis. Retrospective finding that the amyloid deposits in her kidney biopsy...
were derived from FGA and the complete concordance between the presence of the ES26V (p.Glu54Val) and the development of amyloidosis indicated that this mutation was the cause of the disease in the proband's family. This approach ensured family screening.

Immunohistochemical classification of 102 northern Portuguese patients with amyloidosis diagnosed in native kidney biopsies disclosed 4 (3.9%) cases of A fibrs, including our proband. They were all from the same rural geographical area and belonged to apparently unrelated families [25]. In the case of our homozygous patient, both parents had CRF and consanguinity was not possible to prove, but they may share a common ancestor given the possibility of an endemic focus of the disease in their region. In this context, homozygosity proposal was based on DNA sequencing (Figure 1B).

Previously reported A fibr amyloidosis phenotypes result from the heterogeneous genotype. The effect of homozygosity on phenotype has been reported for patients with hereditary transthyretin [14-23] or gelsolin [12,13] amyloidosis (Table 1), but this is the first report for A fibr amyloidosis. Our patient presented at a relatively early age and during long term follow-up apparently developed a heavy disease burden with multi-system involvement. After RTX, serial echocardiography demonstrated increased wall thickness, despite normotension and normal graft function, consistent with cardiac amyloidosis. The main forms of amyloidosis that affect the heart are light chain and ATTR amyloidosis. Cardiac involvement of A fibr amyloidosis was described in a cohort of 22 A fibr patients [14], 52% had abnormal echocardiographic findings suggestive of amyloid cardiomyopathy, and 59% had parasympathetic dysfunction and risk of bradycardia. Coronary atherosclerosis was identified in 68%. In the present case, cardiac involvement in the setting of established A fibr amyloidosis, with left ventricular hypertrophy on echocardiography and a low voltage electrocardiogram, complicated by congestive heart failure refractory to standard medical therapy, was considered cardiac amyloidosis. Two of the family members developed atherosclerotic cardiovascular disease with no echocardiographic evidence of cardiac amyloidosis. Affecting family members developed nephropathy almost three decades later and five heterozygous carriers developed hypertension in their forties and fifties. Thus, the clinical phenotype of our homozygous patient was more severe (earlier onset of nephropathy, cardiac and hepatic involvement) than those of heterozygotes in the same family, consistent with gene dosage effects on the phenotype of A fibr amyloidosis. The follow-up of hypertensive heterozygous carriers will be helpful in the study of the pathogenesis of hypertension in A fibr amyloidosis.

In systemic amyloidosis, solid organ transplantation has been used to replace failing organ function [26-29]. Isolated RTX alone has been performed for ESRF in several patients with A fibr and probably remains appropriate when there is good evidence that amyloid deposition does not threaten the function of other vital organs. Gillmore et al. reported that RTX in A fibr is associated with recurrence of amyloid in the graft with resultant loss of transplanted kidneys after a median of 6.7 years [3]. CLKT in a patient with amyloidotic renal failure caused by the FGA p.Glu54Val mutation was first performed in 1995 [28]. Despite liver transplantation being the only currently available curative treatment for A fibr, it seems reasonable to propose it for younger and fitter patients, weighing the high risk of early perioperative death following CLKT against the elimination of the risk of recurrent amyloid disease in the allograft [5,29]. Transplantation in our patient aimed to replace her failing organ function, because in 1996 her phenotype was unknown; it was 5 years later that we made a retrospective diagnosis of hereditary fibrinogen amyloidosis. Despite isolated RTX, our patient's outcome was not unfavourable compared to the results with CLKT [3,4]. Her apparent cardiac and hepatic A fibr involvement progressed despite clinical absence of disease recurrence in the allograft. Mild proteinuria appeared 13 years after transplantation, but allograft function remained good until 4 months before the patient's death. At that time, cardiovascular symptoms were her principal problem, due to cardiac amyloidosis suggested by echocardiography. In patients with cardiac A fibr involvement, combined heart-liver, heart-kidney, or heart-liver-kidney transplantation could be discussed [30,31], but, given the high risk in our 65-year-old patient, she received recommended drug treatment only. She died 16 years after renal transplantation due to severe heart failure in the context of sepsis.

4. Conclusions

In conclusion, correct identification of amyloidogenic protein should always be pursued, even retrospectively, because it enables the choice of the most appropriate therapy, avoids unnecessary and potentially harmful treatments, and ensures family screening. This first report of a homozygous A fibrES26V (p.Glu54Val) amyloidosis expands our knowledge about the phenotype and the outcome of isolated renal transplantation and may be relevant for understanding the molecular mechanisms of dominance in hereditary amyloidosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Isabel Tavares and Luísa Lobato, the first and second authors, contributed equally to this paper.

Acknowledgments

The authors thank posthumously Dr. Elísio Carvalho of the Department of Nephrology and Renal Pathology of Centro Hospitalar de Sáo Joao, Porto, Portugal, for his contribution to this work. They thank Professor João Paulo Oliveira for his critical revision of the paper. This work was supported by the National Funds through the FCT, Fundação para a Ciência e Tecnologia (Portuguese National Funding Agency.
References


3.2. Haplotype analysis of newly diagnosed Portuguese and Brazilian families with fibrinogen amyloidosis caused by the FGA p.Glu545Val variant

In this thesis, we describe that hereditary fibrinogen amyloidosis is clustered in a limited geographic area from northern Portugal.

Among the 16 amyloidogenic variants identified in the FGA gene, the p.Glu545Val is the most common pathogenic variant and the only one identified in Portugal accounting for the disease in 17 families.

In AFib amyloidosis patients, renal phenotype is universal but age at onset varies. Hence, nephropathy is the predominant manifestation while striking differences in the age of onset are observed according to the mutation. Families with frameshift mutations have an early onset whereas late-onset expression is observed with missense mutations.

The FGA c.1634A>T (p.Glu545Val) mutation (rs121909612) was recognized as causative of AFib amyloidosis in American kindreds of Irish origin, Canadian kindred of Polish origin, and in families from the United Kingdom, Portugal, Australia, Austria, Italy, New Zealand, France, Germany, United States, Brazil and China.

The origin of the FGA p.Glu545Val variant among regions and its migration in the different populations is unclear. A better knowledge would contribute to understand the disease distribution.

A first study analysis of DNA polymorphisms in the FGA locus using the TCTT repeat region in intron 3, Rsal site in exon 5 [219] and TaqI site in the 3’ untranslated region [220] of the gene was performed in Irish-American and Polish-Canadian kindreds. This study showed that the mutated genes, in both kindreds with the AFibE526V (p.Glu545Val) amyloidosis, shared the haplotype B5-Rsal(+)TaqI(−) suggesting that they may have been derived from a single founder [190].

At this point of our research work, we aimed to refine the history of the FGA p.Glu545Val variant in one Brazilian (2 subjects) and four Portuguese (7 subjects) families affected with AFib amyloidosis. To this end we genotyped the haplotype markers rs6050 [A/G/T], an FGA missense variant [NM_000508.4:c.991A>G (p.Thr331Ala)], and rs533633927, an FGA 3’ polymorphism (NM_000508.4:c.*7_*34dup28). The rs6050 [A] variant is a cutting site for the restriction enzyme Rsal (GT*AC) and the rs533633927 variant represents a 28 bp insertion that, when present, is recognized by the restriction enzyme TaqI. All the subjects shared the allele A from FGA rs6050 marker, Rsal(+), and absence of the 28 bp insertion in the FGA rs533633927 marker, TaqI(−), which strengthens the hypothesis of a common origin of this amyloidogenic variant in the Portuguese and Brazilian populations.

These data resulted from a study whose methodology, results and discussion were described in an original paper submitted to the peer review journal Amyloid.
Title Page

Title
Haplotype analysis of newly diagnosed Portuguese and Brazilian families with fibrinogen amyloidosis caused by the FGA p.Glu545Val variant

Author’s first and last names
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**Running head**
Common haplotype in AFib amyloidosis

**Keywords**
Amyloid, ancestor, fibrinogen, hereditary amyloidosis, kidney biopsy, population genetics, proteinuria

**Abbreviations:** AFib, fibrinogen A alpha-chain; CKD, chronic kidney disease; DNA, deoxyribonucleic acid; FG4, fibrinogen A alpha-chain gene; Glu, glutamic acid; mRNA, messenger ribonucleic acid; RFLP, restriction fragment length polymorphism; TTR, transthyretin; VAL, valine

**Abstract**
*Background:* Fibrinogen A alpha-chain (AFib) amyloidosis is an autosomal dominant disease that typically presents with nephropathy and is caused by a fibrinogen amyloidogenic variant. The p.Glu545Val fibrinogen A alpha-chain gene (FG4) variant is the cause of AFib amyloidosis that was identified in Portugal and accounts for the disease in 17 families. We analysed the haplotypes of four newly identified unrelated Portuguese families and one Brazilian family to investigate the possibility of a common ancestry.
Methods: The FGA gene was analysed in 9 subjects carrying the p.Glu545Val variant; all the subjects were observed in the same centre. We used the RsaI site in exon 5 and the TaqI site in the 3' flanking region of FGA to perform haplotype analysis on DNA samples.

Results: All the subjects shared the RsaI(+)/TaqI(−) haplotype in association with the mutated allele p.Glu545Val independent of geographic origin. Eight of the subjects developed a common renal phenotype during the sixth decade.

Discussion and conclusions: Despite different origins in Portugal and a high degree of miscegenation in Brazil, the haplotype identities in our subjects led us to hypothesize the existence of one founder effect that could be explained by migratory flows. The same haplotype has been described in Polish, Irish and German families, which provides relevant data that support the probability of a single mutation event.

Introduction

Fibrinogen A alpha-chain (AFib) amyloidosis is an autosomal dominant disease that is mainly characterized by kidney involvement due to the extracellular deposition of amyloid fibrils of fibrinogen variants [1]. These variants result from a single base substitution or frameshift mutations in the coding region of the fibrinogen A alpha-chain gene (FGA) on chromosome 4q31.3. This is the only gene that is known to be associated with AFib amyloidosis [2]. To date, 15 amyloidogenic FGA variants have been described in different kindreds [3] and account for 8% of the hereditary amyloidosis cases [2].

The underlying genetic cause of AFib amyloidosis was originally described in 1993 in a Peruvian kindred and segregated with the FGA p.Arg554Leu variant [1]. One year later, the FGA p.Glu526Val was identified for the first time and became the most
common \textit{FGA} amyloidogenic variant reported worldwide [4]. According to the recommendations of the Human Genome Variation Society [5], the \textit{FGA} p.Arg554Leu and p.Glu526Val variants should be described as p.Arg553Leu and p.Glu545Val, respectively, according to the sequential numbering codons from the mRNA translation initiation codon. Accordingly, the Nomenclature Committee of the International Society of Amyloidosis [6] recommends that hereditary amyloidosis associated with the \textit{FGA} p.Glu545Val variant should be designated AFibE526V (p.Glu545Val) amyloidosis, and this nomenclature will be followed hereinafter.

The origin of the \textit{FGA} p.Glu545Val variant and its migration throughout different populations remains unclear, although a European common founder has been proposed since the first reports [7, 8]. In this study, we analysed the haplotypes of the \textit{FGA} p.Glu545Val variant using two intragenic \textit{FGA} polymorphisms in 9 newly identified subjects from four unrelated Portuguese families and one Brazilian family who were referred to a single centre to investigate the possibility of a common ancestry.

\textbf{Subjects and methods}

\textbf{Subject enrollment and clinical evaluation}

Eight patients with chronic kidney disease (CKD) and unclassified renal amyloidosis or a family history of AFib amyloidosis were referred to a single Portuguese reference centre for \textit{FGA} genotyping as a first-tier diagnostic approach to AFib amyloidosis. Relevant demographic and clinical data were collected, including the following: age at the onset of clinical manifestations; renal laboratory data; serum and urine monoclonal studies (protein electrophoresis, immunoglobulin and light chain measurements, and immunofixation); histories of vascular, gastrointestinal, and neurologic morbidities; and the subjects’ respective clinical outcomes.
Seven of the patients had a biopsy-proven kidney amyloid nephropathy that was reviewed at the same centre. Formalin-fixed paraffin-embedded sections were stained with Congo red and viewed under polarized light. Immunohistochemical staining was performed with antibodies directed against serum amyloid A, the kappa and lambda light chains, transthyretin (TTR), FGA, apolipoproteins A-I and A-II, and lysozyme, as described previously [9].

For every kindred, a detailed genogram capturing at least 3 generations was drawn by the same investigators to check for possible relatedness among the families and to identify genetically at-risk individuals. The latter were offered genetic counselling, and those who eventually accepted genetic testing underwent a comprehensive baseline clinical assessment that included blood pressure evaluation, plasma creatinine and proteinuria measurements, and kidney ultrasonography.

Follow-up medical care was offered to all participants in this study, and the relevant clinical events and their natural histories were prospectively collected until June 2017.

For the purpose of this study, the criteria for the diagnosis of CKD were a protein excretion of $\geq 0.15$ g/24 h or an estimated glomerular filtration rate of $< 60$ mL/min/1.73 m$^2$ for 3 or more months as calculated by the CKD-EPI (CKD Epidemiology Collaboration) creatinine equation[10].

This study was conducted in accordance with the Ethical Principles for Medical Research Involving Human Subjects adopted by the World Medical Association [11] and the institutional regulatory and ethical standards. Written informed consent was obtained from all participants.

DNA analyses
Genomic DNA was extracted from peripheral blood samples using a salting-out method. The \textit{FGA} c.1634A>T (p.Glu545Val) variant in exon 5 of the \textit{FGA} was assayed by polymerase chain reaction amplification followed by Sanger sequencing as previously reported [12]. The \textit{FGA} variants were named according to the sequence variant nomenclature of the Human Genome Variation Society [5]. The National Center for Biotechnology Information reference sequences NM_000508 [13] and NP_000499 [14] were used as the references for the \textit{FGA} cDNA and protein sequences, respectively.

**Haplotype analysis of the \textit{FGA} gene**

The \textit{FGA} \textit{RsaI} site in exon 5 and the \textit{TagI} site in the 3' flanking region were studied by restriction fragment length polymorphism (RFLP) analysis and sequencing as described previously [7, 15].

**Results**

A total of 9 Caucasian subjects, including 8 patients with CKD and an asymptomatic first-degree relative, underwent genetic testing for the \textit{FGA} p.Glu545Val variant. The subjects belonged to five unrelated families (Figure 1), including four Portuguese families (families 1 to 4) and one Brazilian family (family 5) with no known Portuguese family roots. The Portuguese families were from different northern municipalities of the country (Figure 2).

As presented in Table 1, eight subjects developed proteinuric kidney disease between the sixth and seventh decades. Seven of the patients underwent diagnostic kidney biopsies that revealed extensive glomerular amyloid depositions that stained specifically with antibodies to AFib. Amyloid deposits were also identified in a minor
salivary gland biopsy. Four men and two women progressed to end-stage renal disease, and initiated renal replacement therapy at 56.5 ± 3.5 years of age. A family history of renal disease was absent in only one kindred (Figure 1).

DNA analyses revealed that all the patients were heterozygous for the same single-base substitution (c.1634A>T), which results in the replacement of glutamic acid with valine at codon 545 of the mature AFib protein. Additionally, the asymptomatic first-degree relative was also found to be heterozygous for the same amyloidogenic variant. All the analyses exhibited co-segregation of the Rsal(+) and Trgl(-) polymorphisms, which indicated that the FGA c.1634A>T (p.Glu545Val) variant in these subjects had the same haplotype found in previous reports of AFibE526V (p.Glu545Val) amyloidosis [7, 8, 15].

During follow-up, patient III of family 2 had a retinal thrombosis at the age of 56 and underwent aortic valve replacement due to severe stenosis at the age of 58. Three patients underwent kidney transplantation (Table 1). Patient III of family 1 presented with a serum creatinine level of 2.1 mg/dl and an absence of proteinuria 2 years after transplantation. Patient III of family 2 underwent transplantation on post-transplantation day 8 due to uncontrolled bleeding associated with a small dehiscence of the arterial allograft anastomosis; the patient exhibited neither histological findings of acute rejection nor evidence of amyloid deposition in the allograft. Twenty days after returning to haemodialysis, this patient underwent urgent nephrectomy due to spontaneous native kidney rupture. There was massive amyloid deposition in the glomeruli with no interstitial, vascular or capsular involvement. Patient III of family 3 underwent a right coronary percutaneous intervention on post-transplantation day 3 due to acute coronary syndrome. Six months later, he underwent transplantation due to allograft obstructive uroeral hypertension and returned to haemodialysis.
Discussion

This study provided new data about the common origin of the \textit{FGA} p.Glu545Val variant in Portuguese and Brazilian carriers with AFib amyloidosis based on nine new subjects (seven Portuguese and two Brazilian). Eight of these subjects presented with the usual renal phenotype, with an age at onset between the sixth and seventh decades and kidney biopsies with characteristic glomerular amyloid depositions that stained specifically with antibodies to AFib. All the subjects were heterozygous for the \textit{FGA} p.Glu545Val variant, and a family history of renal disease was present in eight of the subjects. The Portuguese subjects were from different municipalities of northern Portugal, and the Brazilian patients had no known Portuguese family roots. All the subjects shared the \textit{RsaI(+)/TaqI(-)/c.1634A>T} haplotype, which strengthens the hypothesis of a common origin of this amyloidogenic variant in the Portuguese and Brazilian populations.

Hereditary renal amyloidosis was previously described by Oster tag in 1932 [16], but the first clinical report of what was later determined to be AFib amyloidosis was published in 1975 [17]. The underlying genetic cause of AFib amyloidosis was originally demonstrated in 1993 in a Peruvian kindred and segregated with the \textit{FGA} p.Arg573Leu variant, which is one of the rarest fibrinogen variants [1]. One year later, the \textit{FGA} p.Glu545Val was first recognized as causative of AFib amyloidosis in American kindreds of Irish origin [4]; it has since become the most commonly reported \textit{FGA} amyloidogenic variant worldwide (Table 2) after also being identified in a Canadian kindred of Polish origin [7] and in families from the United Kingdom [8, 18-20], Portugal [12, 15, 21], Australia [22], Austria [23], Italy [24], New Zealand [20], France [25-27], Germany [19, 28], the United States [29-31], Brazil [32], and China
[33]. Despite the widespread distribution of the disease, there is a major concentration of cases in Europe (Figure 3).

In attempts to identify the origin of the FGA p.Glu545Val variant, a few haplotype studies have been performed that have sought evidence of a common founder among the main AFib amyloidosis foci [7, 8, 15]. Two RFLPs (RsaI and TaqI) of the FGA gene have been reported in this setting, and these were used in our study. We found that the FGA p.Glu545Val variant co-segregated with the RsaI(+)TaqI(−) haplotype in both the Portuguese and Brazilian families. These results were expected given that the kindreds were clustered in a limited geographic area of northern Portugal with the exception of a case from São João da Pesqueira (Figure 2). Furthermore, Brazil’s Portuguese colonization dates from the 14th century, and at that time, Brazil was receiving migratory influxes from northern Portugal, which may have contributed to the widespread distribution of the FGA p.Glu545Val variant [34]. However, reported American kindreds of Irish origin, a Canadian kindred of Polish origin [7], and English and German kindreds [8] have no known Portuguese ancestries but have the same haplotype.

In view of the geographical lineage history of the northern Portuguese population, a common ancestor for the FGA p.Glu545Val variant is probably of European origin. The Portuguese foci of AFib amyloidosis is from a restricted area of the district of Braga, which has an area of 2,673 km² and 848,185 inhabitants [35]. Except for the unique case from São João da Pesqueira reported here, all the others were distributed within neighbouring areas of the district of Braga. Seven of sixteen kindreds identified in this region were from Vieira do Minho, which is one of the district’s counties (Figure 2). Vieira do Minho has an area of 218.5 km² and 12,697 inhabitants [35]. Geologically, Vieira do Minho is located in the Hesperic Massif, which is
characterized by a rugged relief and mountainous terrain and mainly comprises the
mountain of Cabreira and deep valleys that contributed substantially to its isolation [36].
In this territory, there are trace elements of the continuous occupation from the last
centuries before Christ. The Suevian-Visigoths, Romans and Medieval people also left
important trace elements. One example is the hill fort (Monte de Vieira) that exists in a
location high on the mountain of Cabreira and evidences occupation during the Iron
Age. Another example is the hill fort of Cantelhes named the castle of Vieira [37]. This
county is located in three hydrographic basins, and the most important are those of the
rivers Cávado and Ave, which might have contributed to the wide spread of AFib
amyloidosis from Vieira do Minho to Terras de Bouro, Amares, Braga and Barcelos
through the river Cávado and to Cabeceiras de Basto, Póvoa de Lanhoso and Fafe
through the river Ave (Figure 2). In addition to leaving material vestiges, the people
who passed through this region could have also left behind the genetic variant that leads
to AFib amyloidosis. However, the population of Vieira do Minho has strong traditions
of emigration and are mainly dispersed in Brazil, the United States, France, Germany,
Canada, Venezuela, Switzerland, Luxembourg, the United Kingdom, Spain, South
Africa, Oceania and Asia [38]. This migration flux might contribute to the wide spread
of the disease. However, we still do not know how the disease originated in Portugal.

Similar to what has been described for ATTR amyloidosis [39], our results
strengthen the epidemiologic evidence that Portuguese and Brazilian families with AFib
amyloidosis caused by the FGA p.Glu545Val might have their origins in northern
Portugal. This conclusion is further substantiated by the indistinguishable phenotypic
presentation, which supports the hypothesis that the amyloidogenic variant is the
primary cause of AFib amyloidosis and might have been derived from the same founder
rather than from independent sporadic variants.
Our experience with kidney transplantation in AFibE526V (p.Glu545Val) amyloidosis is limited and characterized by short-term post-transplantation complications that led to graft loss in two of three cases. In contrast, the progression of amyloid deposition may be the cause of serious complications, such as spontaneous renal rupture. Emerging therapies at the amyloidogenesis cascade level that seek to prevent the formation and promote the clearance of amyloid deposits are potentially novel agents, which might change the therapeutic approach to this disease in the future [40-42].

Our study has several limitations. First, we did not evaluate several members per family to perform studies of allelic segregation. Second, we used RFLPs that were not evaluated in the Portuguese population. The allelic frequency of Rsal was 0.759 in a randomly selected group of 110 individuals from California, and the allelic frequency for TaqI was 0.73 in 302 Caucasian British men [43-45]; however their frequencies in the Portuguese population have not been determined. Third, the analysis of the haplotype structure should have included more markers. Overall, our results might be explained by the fact that we did not sample enough haplotypes to observe recombinations. Finally, the estimation of the most recent common ancestor of the FGA e.1634A>T (p.Glu545Val) variant in different foci would clarify its origin and distribution.

Conclusion
This study indicates that the Portuguese foci of AFib amyloidosis most likely originated from Viana do Castelo in the district of Braga. Portuguese and Brazilian families share the same haplotype with English, Irish, German and Polish families, which may be the result of migration flux, although we still do not know how the disease originated in Portugal. Further studies of the extended FGA haplotype involving Irish individuals and
patients from different foet would be useful for investigating the founder effect and estimating the variant age and the most likely origin.

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Contributions: research idea and study design: IT, LL; data acquisition: IT, MO, LM, JS, LL; data analysis/interpretation: IT, MO, LM, PPC, LL; supervision or mentorship: LL. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of any the work are appropriately investigated and resolved. IT and LL take responsibility that this study has been reported honestly, accurately, and transparently; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned and registered have been explained.
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The authors have no financial conflict of interest to disclose that could have been construed to influence the results or the interpretation of their manuscript.
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Table 1. Demographic and clinical data of the subjects carrying the FGA p.Glu545Val variant associated with the Rsal(+)/Taql(−) haplotype.

<table>
<thead>
<tr>
<th>Family</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
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<td>Barcelos, Braga, Portugal</td>
<td>Vieira do Minho, Braga, Portugal</td>
<td>Amares, Braga, Portugal</td>
<td>Fortaleza, Ceara, Brazil</td>
</tr>
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<td>Male</td>
<td>Male</td>
<td>Male</td>
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<tr>
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<td>58</td>
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<td>No</td>
<td>Pepsic ulcer</td>
</tr>
</tbody>
</table>

Abbreviations: eGFR, glomerular filtration rate; HD, haemodialysis; NA, not available; PD, peritoneal dialysis; RRT, renal replacement therapy.
Table 2. Geographic distribution, ancestry, and clinical data of reported patients/families with AFibE526V (p.Glu545Val) amyloidosis.

<table>
<thead>
<tr>
<th>Geographic Location</th>
<th>Number of patients/families</th>
<th>Ancestry</th>
<th>Range of age (years)</th>
<th>Presenting feature(s)</th>
<th>Organ with pertain amyloid deposition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>United States</td>
<td>12/5</td>
<td>Irish</td>
<td>45-58</td>
<td>Nephrotic syndrome, hypertension,</td>
<td>Kidney, spleen, liver, lung</td>
<td>[8, 7, 46, 47]</td>
</tr>
<tr>
<td></td>
<td>1/1</td>
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<td>30-47</td>
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<td>[48]</td>
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<td>1/1</td>
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<td>Kidney</td>
<td>[50]</td>
</tr>
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<td>1/1</td>
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<td>NA</td>
<td>NA</td>
<td>[51]</td>
</tr>
<tr>
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<td>[52]</td>
</tr>
<tr>
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<td>Kidney, spleen, cerebral atrophy, nerve</td>
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<td>[51]</td>
</tr>
<tr>
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<td>Kidney</td>
<td>[51]</td>
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<td>Kidney</td>
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<td></td>
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<tr>
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<td>Kidney</td>
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</tr>
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<td>Portunese</td>
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<td>[51]</td>
</tr>
<tr>
<td></td>
<td>1/1</td>
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<td>[27]</td>
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<td>NA</td>
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<td>Proteinuria, renal failure, hypertension</td>
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<td>[51]</td>
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<td>France</td>
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<td>[51]</td>
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<td>1/1</td>
<td>Han</td>
<td>54</td>
<td>Proteinuria, hypertension</td>
<td>Kidney</td>
<td>[53]</td>
</tr>
</tbody>
</table>

Abbreviations: NA, not available.
Figure 1. Family trees of the four Portuguese families represented as 1, 2, 3, and 4 and the Brazilian family represented as 5. The probands are represented by arrows.

Figure 2. Geographic distribution of families with AFibE526V (p.Glu545Val) amyloidosis in Portugal (each circle represents a family: the black circles represent previously reported families, and the grey circles represent currently reported families).

Figure 3. The worldwide distribution of AFibE526V (p.Glu545Val) amyloidosis.
Figure 1
V – DISCUSSION
The identification of AFib amyloidosis in 3.9% of the cases of a series of a kidney biopsy proven amyloid disease from northern Portugal led us to the identification of 17 families with AFibE526V (p.Glu545Val) amyloidosis, through a systematic epidemiologic study. The typical natural history of the disease in our cohort was of early-onset hypertension, followed by proteinuric CKD, eventually progressing to ESRD after 5 years, with increasing prevalence of extrarenal manifestations in older patients, most typically resulting from amyloid deposition in the heart, spleen and vascular vessels of abdominal fat, colon and ileum. Notably, one of our patients, who was homozygous for the amyloidogenic variant, lived for 16 years with a functioning kidney transplant. Estimated mean renal survival was 65.4 (95% CI, 61.8-69.0) years, and estimated mean lifetime survival was 73.7 (95% CI, 70.5-76.8) years. Finally, Portuguese and Brazilian families share the same haplotype with English, Irish, German and Polish families, which may be the result of migration flux and strengthens the hypothesis of a common origin for AFibE526V (p.Glu545Val) amyloidosis.

**Epidemiology**

Hereditary AFib amyloidosis was first characterized in 1993 in a Peruvian kindred [31]. During several years, the definition of the disease was carried out with the publication of isolated kindreds, usually in the context of a novel amyloidogenic variant. In 2009, the first series of AFib amyloidosis, comprising 71 patients, was published [32]. It was then considered to be one of the most common types of hereditary amyloidoses in Europe [192, 225, 226].

The research work underlying this thesis allowed us to identify a marked geographic clustering of AFibE526V (p.Glu545Val) amyloidosis in northern Portugal, especially in the district of Braga. First, we reported that AFibE526V (p.Glu545Val) amyloidosis accounted for 3.9% of the cases of amyloid nephropathy, in a consecutive series of diagnostic native kidney biopsies performed over 36 years, at the CHSJ. This percentage does not significantly differ from the 1.7% found in a much larger series from the National Amyloidosis Centre in the United Kingdom, which included tissues other than the kidney [227]. Second, the finding that the initial cases were from the district of Braga led to the screening of the disease in the local hemodialysis population. In this way, we found that a point prevalence of ESRD due to AFibE526V (p.Glu545Val) amyloidosis could be estimated as 4.5% among the local hemodialysis population and it was the most common amyloid nephropathy, even considering the relatively high prevalence of ATTR amyloidosis in that region [82, 83, 228].

We have shown in this chapter that the late-onset of the AFibE526V (p.Glu545Val) amyloidosis may result in underdiagnosis of the disease, particularly in hypertensive patients, which would increase the real prevalence of the disease. Since 2004, 70 subjects from 17 families have been diagnosed with the FGA p.Glu545Val variant in the district of Braga (author unpublished data), which has an area of 2,673 km² and
848,185 inhabitants [229]. This makes the district of Braga the region of the world where AFibE526V (p.Glu545Val) amyloidosis is most prevalent.

**Natural history**

Hereditary systemic amyloidoses are not common, but some forms are characteristic in specific geographic areas. In northern Portugal, apart from ATTR amyloidosis we also found endemic foci of AFib amyloidosis, identified through the research work underlying this thesis. The FGA p.Glu545Val variant is the cause of AFib amyloidosis that was identified in our country and accounts for the disease in 17 families.

Clinical data on AFib amyloidosis are largely based on sporadic case reports or small case series and the natural history of the disease remains unknown. In this chapter, we showed that AFibE526V (p.Glu545Val) amyloidosis patients typically display early onset hypertension, followed by proteinuric CKD in the sixth decade of life, eventually progressing to ESRD after 5 years. For some time, AFib amyloidosis was considered predominantly a renal disease [32]. However, instead of a disease restricted to the kidney, we found an increasing prevalence of extrarenal manifestations in older patients, most typically resulting from amyloid deposition in the heart, spleen and vascular walls of abdominal fat, ileum and colon.

Compared to AA and AL amyloidosis, AFib amyloidosis patients in our cohort presented with worst renal function, but they had the best renal and overall survivals. Due to the nonspecific nature of its major clinical complications and frequent unawareness of a family history, the diagnosis of AFibE526V (p.Glu545Val) amyloidosis might be late. As we showed, the increasing awareness of this disease is critical to minimize its under recognition. Also, genetic screening of all at-risk relatives of affected individuals is the most effective approach to an early diagnosis. On the other hand, the absence of an underlying condition, and consequently a less aggressive systemic involvement, might contribute to the better prognosis.

AFib amyloidosis nephropathy has a distinctive histological appearance, characterized by striking glomerular enlargement with almost obliteration of the normal glomerular architecture by extensive amyloid deposition, but almost no amyloid in the vessels and renal tubular interstitium [32]. The factors beyond are complex and not well understood.

Many proteins have been shown to have multiple amyloidogenic variants and phenotypes may vary depending on those variants. Indeed, small differences in the amino acid sequence of an amyloidogenic protein can alter its tissue tropism [86], explaining the marked heterogeneity in tissue distribution of some hereditary amyloidosis such as ATTR amyloidosis [230-232]. Mutations in the NH2-terminal portion of an amyloidogenic protein are associated with kidney, liver, and occasionally heart involvement, whereas mutations in the COOH-terminal portion of an amyloidogenic protein seem to be associated with heart, skin, and often laryngeal involvement [233]. How the position of the mutation in the protein affects disease
manifestations is not clear. Renal pathology in AFibE526V (p.Glu545Val) amyloidosis is characterized by restriction of amyloid deposits to the glomeruli, mainly the mesangium [200]. This tropism might be related to: amino acid sequence of the amyloidogenic protein, as the position of the mutation in AFib amyloid deposits contain only peptides from the protease sensitive portion of the COOH-terminal portion [189]; specific uptake by mesangial cells, which has been demonstrated for light chains but does not seem to be a uniform requirement for amyloid deposition in the kidney [234, 235]; negative charge and high GAGs content of the glomerular basement membrane and the presence of certain proteases that could render a protein amyloidogenic or affect the stability of amyloid deposits [86]. Glycosaminoglycans promote fibrillogenesis by stabilizing or inducing conformational changes in amyloidogenic precursors, favoring fibril formation, and by providing protection from proteolysis during fibril formation and after tissue deposition [236, 237].

Disruption of tissue architecture by amyloid deposits had long been accepted as one of the underlying mechanism of organ dysfunction in amyloidosis [87]. However, several observations suggest that amyloid or prefibrillar oligomers are cytotoxic independently of the presence of amyloid deposits, and this cytotoxicity may as well contribute to the disease manifestations [86]. Findings that support the latter mechanism include: the lack of correlation between quantity of amyloid in the tissue and organ dysfunction [228, 238]; in vitro demonstrations of direct toxicity of amyloidogenic precursor proteins on cultured cells or tissues [104, 108, 239]; detection of amyloidogenic precursor proteins in tissues in the absence of amyloid [240]; and reduction of precursor protein production, as well as, rapid improvement in markers of organ dysfunction, after treatment [113].

In AFib amyloidosis, supporting evidence of a direct influence of the amyloid or prefibrillar oligomers in cell toxicity and renal disease comes from several observations such as: decrease of proteinuria in AFib amyloidosis after liver transplantation, due to removal of the source of production of the amyloidogenic protein [202]. Also, the resolution of proteinuria was observed in a case of preemptive liver transplant which suggests a possible reversal of the renal manifestations of amyloid deposition before the substantial degradation of existing amyloid deposits [87]. This is similar to in vitro demonstrations, in AL amyloidosis, of specific phenotype changes in cultured mesangial cells that are exposed to amyloidogenic light chains, changes that are not seen when the cells are exposed to non-amyloidogenic light chains [239].

Very little information is available regarding any changes in amyloidogenicity potential due to FGA variants. However, precise identification of those variants is important for establishing a phenotype. Lessons from ATTR amyloidosis taught us that the fact that amino acid variations can change the tissue site of amyloid deposition suggests that some of the information determining this deposition resides in the structural and functional features of the amyloidogenic protein itself.
For instance, wild-type TTR amyloid is seen mainly in the heart and in older individuals [241], the peripheral nervous system is the preference of TTR V30M (p.Val50Met) variant [242-245], in TTR V122I (p.Val142Ile) variant the amyloid deposition is almost exclusively in the heart [246] and in yet TTR T49P (p.Thr59Pro) it is apparently in the leptomeninges [247]. Insights from ATTR amyloidosis reveals that some ATTR variants show significant renal involvement as well [82, 83, 230, 248]. In contrast, AFib amyloidosis presents a common nephropathic phenotype regardless the amyloidogenic variant and gender. This might be related to the proximity of all the mutations which are clustered in the 5' end of exon 5 of the FGA gene and all the amyloidogenic fibrils subunits consists of residues 517 to 555 (536 to 574) [79, 217]. However, there are single amino acid substitutions, such as, p.Arg573Cys [249] and p.Arg573His [208] that despite being at position 517 to 555 (536 to 574), do not induce amyloid formation, suggesting that amyloid formation depends not only on the location of the variant but also on the type of replaced amino acids [217].

A particular feature of AFib amyloidosis is the significant difference in the age at onset of the disease related symptoms among patients who were diagnosed with frameshift mutations (median age 30 years) and those who had single amino acid substitutions (median age 59 years) [208]. The sequence of all FGA peptides arising from the frameshift mutations is a hybrid between a short fragment of normal sequence and a completely new COOH-terminal peptide [208]. Remarkably, the novel COOH-terminal has an almost identical amino acid sequence that terminates prematurely at position 548 (582) instead of the 610 (644) for the wild-type mature protein [207]. In addition, a recent study reported the importance of the COOH-terminal sequence composed of five amino acids (VLITL) in amyloidogenesis [213] which is a common feature among all the frameshift variants and might determine an early age at onset. Expected age at onset is critical to determine when amyloidosis testing should be requested.

Some proteolytic events after secretion from hepatocytes might also contribute to amyloid formation in AFib amyloidosis.

An important issue is the follow-up of FGA p.Glu545Val asymptomatic carriers. Current biomarkers for AFib amyloidosis nephropathy are late biomarkers of the disease, because when a carrier develops proteinuria, the glomerular amyloid deposition is extensive, and the kidney damage might be irreversible. The analysis of urine protein profiles, aiming to discover disease-specific biomarkers, is a challenging field in glomerular diseases [250]. Urine proteomics could be an early non-invasive and clinically available tool to discriminate between disease and carrier state. In addition, it also could be used in the early assessment of renal graft amyloid recurrence and renal response to potential new emerging therapies. However, clinical proteomics is still not available in most hospitals despite its huge application, ranging from the identification of amyloid proteins in the pathology laboratory, to a new generation of urinary biomarkers of CKD assessment and outcome prediction [251].
**Genetics and genealogy**

As previously mentioned, AFib amyloidosis is an autosomal dominant disease that is mainly characterized by kidney involvement due to the extracellular deposition of amyloid fibrils of fibrinogen variants [31]. These variants result from a single base substitution or frameshift mutations in exon 5 of the FGA on chromosome 4q31.3. This is the only gene that is known to be associated with AFib amyloidosis [77]. To date, 16 amyloidogenic FGA variants have been described in different kindreds [79].

AFib amyloidosis is typically associated to heterozygous FGA variants. However, in this chapter we presented the first case of homozygosity for the FGA p.Glu545Val variant, which was identified in a Portuguese patient and its impact in early-onset presentation.

About one third of our probands were not aware of a family history of ESRD or amyloidosis. This does not necessarily mean reduced penetrance as argued by some authors [32]. Instead, the penetrance of AFibE526V (p.Glu545Val) amyloidosis might be underestimated due to its late occurrence and underdiagnosis. The increase in life expectancy coupled with clinical awareness and suspicion about the disease might allow its redefinition as a disease with a high and age dependent penetrance.

A common founder of the AFibE526V (p.Glu545Val) amyloidosis kindreds was demonstrated in Portuguese, Brazilian, English, Irish, German and Polish families. The indistinguishable phenotypic presentation associated to common polymorphic markers and the migration fluxes, supports the hypothesis that the FGA p.Glu545Val amyloidogenic variant might have been derived from the same founder rather than from independent sporadic variants. In addition, the Portuguese foci of AFib amyloidosis were most likely originated from Vieira do Minho in the district of Braga, which may also be the result of migration flux, although we still do not know how the disease originated in Portugal.

**Therapy**

Ideally, early treatment of amyloid diseases should exploit synergizing approaches and strategies to reduce the precursor protein production, prevent misfolding and fibril formation and promote the reabsorption of amyloid deposits. However, for more advanced cases the replacement of failing organs by transplantation might be the only available therapeutic option.

Adequate treatment of AFib amyloidosis depends on the precise identification of the amyloid type, determination of the amyloidogenic variant, recognition of the liver as the source of production of the amyloidogenic variant and characterization of the extension of organ involvement.

As discussed previously, the precise diagnosis of AFib amyloidosis is based on tissue demonstration of the amyloid composed by a fibrinogen variant exhibiting typical glomerular immunofluorescence. Molecular genetic analysis is crucial for the identification of the fibrinogen variant. As for ATTR amyloidosis, there are differences
between phenotypes, including age at onset, associated with different variants of the same protein in AFib amyloidosis which have therapeutic implications [208]. In addition, replacement of failing organs depends on the extension of the organ involvement [199].

Treatment of AFib amyloidosis comprises supportive, disease modifying and novel therapeutic approaches. Supportive treatment directed mainly to renal disease comprises hypertension control, salt restriction, careful use of diuretics and angiotensin converting enzyme inhibitors or angiotensin receptor blockers, cholesterol control, nutritional status control and organ transplantation offered to patients with irreversible organ dysfunction.

Currently, the only disease modifying treatment to eliminate the source of amyloid production in AFib amyloidosis is liver transplantation. In systemic amyloidoses, solid organ transplantation has been used chiefly to replace failing organs [252]. However, unless the supply of the amyloid fibril precursor protein can be reduced, recurrent amyloid deposition in transplanted organs must be anticipated, although it may occur relatively slowly [191]. Orthotopic liver transplantation (OLT) for treatment of hereditary amyloidosis caused by an amyloidogenic mutation in a hepatically expressed gene was first performed in two Swedish patients with ATTRV30M (p.Val50Met) [134].

Fibrinogen Aα chain is synthesized only by the liver [174]. Since there is no evidence that wild-type FGA is amyloidogenic [174], OLT therefore results in complete replacement of variant amyloidogenic fibrinogen in AFib amyloidosis and thus halts amyloid accumulation altering the natural history of the disease [135, 253]. Combined liver and kidney transplant (CLKT) was first performed in 1995, in a patient with amyloidotic renal failure caused by AFibE526V (p.Glu545Val) amyloidosis [135]. Since then, several CLKT have been performed. However, the high risk of early perioperative mortality with this approach must be balanced against elimination of the risk of recurrent amyloid disease in the allograft [253].

AFib amyloidosis might not be associated with 100% penetrance, and therefore, liver transplantation should not be considered until the onset of amyloidosis has been documented by appropriate measures, specifically tissue biopsy with confirmation of AFib peptides in amyloid deposits [202]. Some authors recommend that preemptive liver transplantation should be considered before renal function has deteriorated to the point where major surgery may jeopardize adequate native renal function [202]. As such, they consider that liver transplantation should be recommended when a serum creatinine level reaches 1.5 mg/dl or there is greater than 2 gm/day of urinary protein loss [202].

We should adequate solid organ transplantation to this late-onset disease because CLKT, which by removing the source of the circulating amyloidogenic fibrinogen variant [174], prevents further amyloid deposition in the renal allograft or elsewhere [191]. However, the proposal for liver transplantation to late occurrence disorders that present with kidney disease at a mean age of 56.7 years, have an
estimated mean renal survival of 65.4 (95% CI, 61.8–69.0) years and an estimated mean lifetime survival of 73.7 (95% CI, 70.5–76.8) years, has several constraints. First, liver transplantation has an early high morbimortality; second, it may lead to anticipation of exposure to the adverse effects of immunosuppression; finally, there is lack of benefit demonstration in quality and quantity of life of AFibE526V (p.Glu545Val) amyloidosis patients undergoing this kind of transplantation. In addition, the experience with hemodialysis, although related to decreased survival, has a better outcome than for AA or AL amyloidosis [32].

Advances in the treatment of systemic amyloidoses, both at the source of the amyloidogenic precursor and at the level of amyloidogenic cascade, have revealed several new drug targets and therapeutic approaches with potential application to AFib amyloidosis [111, 127, 230, 254]. In this regard: (1) the synthesis of the amyloidogenic precursor may be eliminated by using chemotherapy in AL amyloidosis [111, 127], liver transplantation in AFib amyloidosis [202] or silencing by using RNA interference [141]; (2) inhibitors of proteases (secretase) and metal protein attenuating compounds are being evaluated in trials; (3) compounds that interfere with the binding of GAGs to the amyloid proteins (eprodise) have been successful in AA amyloidosis [157]; (4) small molecules capable of stabilizing the amyloid precursor and preventing its misfolding and aggregation (diflunisal, tafamidis) are being tested in ATTR amyloidosis [255]; (5) SAP can be cleared from amyloid deposits by using small palindromic drugs (e.g., miridesap) [160, 164]; (6) the clearance of amyloid deposits can be promoted and accelerated by specific antibodies through passive [256] and active immunotherapy [257], or by combining miridesap with anti-SAP antibodies [172, 173]. In AFib amyloidosis, miridesap demonstrated a reduction in proteinuria in four of five patients and renal survival was prolonged compared to historical control group [160]. Treatment with miridesap followed by an anti-SAP antibody (dezamizumab) was used in four patients with AFib amyloidosis although there was no evidence of change in amyloid load [165, 173]. These recent and substantial improvements in the understanding of the molecular underpinnings of systemic amyloidoses have unveiled different key steps in the amyloidogenic cascade, which can be valid therapeutic targets. Some of these treatments are currently being tested in animal models and clinical trials and will become available to the clinician in the near future [111]. If emerging treatments for amyloidoses will succeed, the best therapeutic approach for AFib amyloidosis might be a therapy towards the amyloidogenesis cascade, avoiding amyloid deposition, or a combination of this with transplantation of failing organs, avoiding graft recurrence or progressive extrarenal amyloidosis.

**Limitations**

The research work underlying this thesis has several limitations related to methodology.
First, antibody panel for amyloid typing by IHC should have included antibodies against LECT2 and inconclusive results should have been studied by proteomics to minimize the number of unclassified cases.

Second, the epidemiological study was based on retrospectively collected data which is susceptible to referral, selection, and indication biases.

Third, the criteria for the genetic testing of hemodialysis patients might have missed a few additional cases. Many of the patients and families, as well as their physicians, were not aware that they had or were at risk for a genetic condition which might have contributed to underascertainment of cases.

Fourth, screening of the FGA p.Glu545Val variant was limited to hemodialysis units from northern Portugal. The desirable extension of molecular screening to hemodialysis units from the rest of the country could help to characterize the prevalence of the disease in the national territory.

Fifth, the characterization of the homozygous patient would have benefited from renal graft biopsy and autopsy, but these were not performed.

Sixth, haplotype study should have used a more updated molecular approach to define the structure of FGA gene and appropriate statistical methods to formally test the “common founder” hypothesis. More than two polymorphisms in the FGA gene and the surrounding regions should have been analyzed, the number of patients should have been increased and a control group should also be included.

Finally, the number of patients undergoing liver transplantation is small and the follow-up time is still insufficient to demonstrate the benefit of this approach compared to the risk of major surgery in patients with cardiovascular risk.
VI – CONCLUSIONS
This thesis describes novel endemic foci of the AFib amyloidosis that was identified in 17 families from northern Portugal. The FGA p.Glu545Val variant is the cause of AFib amyloidosis that was identified and accounts for the disease in about 4.5% of hemodialysis patients from the district of Braga.

AFibE526V (p.Glu545Val) amyloidosis is a rare, late-onset, autosomal dominant condition related to the extracellular deposition of insoluble fibrils composed of FGA p.Glu545Val variant. This occurs in the heterozygous state but the first homozygous patient harboring the FGA p.Glu545Val variant was also identified in the Portuguese foci. Patients typically present with hypertension in the sixth decade of life, followed by proteinuric CKD after 6 years, eventually progressing to ESRD within 5 years of recognition of kidney involvement. This disorder is characterized by an age dependent penetrance with a rising in the prevalence of kidney involvement with age. With aging there is also an increase in the prevalence of extrarenal manifestations, most typically resulting from amyloid deposition in the liver, spleen, heart and vascular vessels of abdominal fat, colon and ileum. Estimated mean renal survival was 65.4 (95% CI, 61.8-69.0) years, and estimated mean lifetime survival was 73.7 (95% CI, 70.5-76.8) years.

There are no findings about potential benefits of conventional treatments that are used to slow the progression of CKD in AFib amyloidosis. Hemodialysis and peritoneal dialysis are good options for ESRD. Renal transplantation remains controversial due to the risk of graft loss from recurrent amyloid and progressive disease. The only currently available disease-modifying treatment to suppress precursor protein production is liver transplantation. However, it has a high risk of early perioperative morbidity and mortality. Furthermore, hepatic transplantation has not yet proven to be a beneficial long-term treatment option, and isolated renal transplantation does not necessarily imply clinical recurrence of amyloidosis even in the homozygous state.

The origin of the disease is unknown, but the Portuguese foci of AFibE526V (p.Glu545Val) amyloidosis most likely left from Vieira do Minho in the district of Braga and spread along the rivers Câvado and Ave. Portuguese and Brazilian families share the same Rsal(+)/TaqI(−)/c.1634A>T haplotype and phenotype with English, Irish, German and Polish families, which is consistent with the hypothesis of a single origin and spread worldwide through migration flux.

Due to the lack of awareness and to the nonspecific nature of its major clinical complications, the diagnosis of AFibE526V (p.Glu545Val) amyloidosis requires a high index of suspicion and can be easily overlooked or misdiagnosed as hypertensive nephrosclerosis. This thesis increases awareness about the disease, which enhances the chances of early diagnosis and appropriate genetic counseling. The Portuguese foci is an available source to study the emerging therapeutic approaches at the cascade level of amyloidogenesis.
VII – FUTURE WORK
This thesis led to the identification of the Portuguese foci of AFibE526V (p.Glu545Val) amyloidosis based on the systematic identification and characterization of the disease in the northern Portuguese population. Despite the contributions to the ever-expanding knowledge about the disease, there are several questions and challenges for the future.

Work is needed to establish the contribution of hypertension to the pathophysiology of nephropathy in AFib amyloidosis. This is challenging because hypertension usually precedes the development of any laboratory manifestation of CKD in AFib amyloidosis, which raises the hypothesis of being a primary manifestation of the amyloid disease. However, for many experts in the field, hypertension is considered secondary to CKD. A comprehensive description of the kidney pathology, with attention to hypertensive vascular lesions, might clarify the contribution of hypertension to CKD development and progression in AFib amyloidosis nephropathy.

The burden of atherosclerotic disease in AFib amyloidosis has also been linked to CKD. However, the contribution of amyloid vascular deposition in the pathogenesis of cardiovascular disease in AFib amyloidosis patients remains unclear. The use of proteomic technologies to study vascular biomarkers of atherosclerosis may be helpful to elucidate the pathogenic mechanisms and identify early biomarkers. In this regard, vascular protein expression analysis of fibrinogen, apolipoproteins or acute phase proteins may be helpful [258, 259].

Early diagnosis is crucial but lacks adequate biomarkers for disease detection, staging and prognosis. AFib amyloidosis nephropathy is a glomerular disease, so the urine seems to be the most appropriate sample for diagnostic biomarker discovery. Urinary proteome analysis has been extensively applied to biomarker discovery, diagnostics and prognostics in several diseases [260] and might also be useful for amyloid nephropathy. To define disease-specific urinary biomarkers I propose the comparison of urinary proteome profile of a disease group to that obtained only from normal healthy individuals [261]. Urine samples obtained from patients with other glomerular diseases such as diabetic nephropathy, membranous nephropathy, focal segmental glomerulosclerosis, minimal change disease and IgA nephropathy, must also be included. Finally, the urinary proteome profiles of FGA p.Glu545Val carriers, with and without nephropathy, would be evaluated aiming the identification of protein signatures that discriminate between the two stages. This inclusion would consider variations that may occur from differential stages of the disease. Besides early diagnosis, this might also be a useful tool for the assessment of renal response to potential new therapies.

I also propose the application of urinary proteomics to compare the early and late-onset AFib amyloidosis, frameshift and missense variants respectively, regarding factors associated with the expression of the disease. This might be useful to discover other mechanisms of tissue damage and organ dysfunction, as well as, new therapeutic targets in AFib amyloidosis.
It was demonstrated that urinary exosomes of patients with AL amyloidosis contained immunoglobulin light chain oligomers that were absent in patients with multiple myeloma [262, 263]. Urinary exosomes are members of the extracellular vesicle family that are excreted in the urine and were found to contain many disease-associated proteins [264]. Similarly, I hypothesize that the urinary exosomes analyzed by mass spectrometry might enable the identification of fibrinogen amyloid oligomers targeting early diagnosis, the assessment of renal response to the new emerging therapies and recurrence of the disease.

Importantly, the full spectrum of hereditary fibrinogen amyloidosis is still being discovered and the absence of any currently known amyloidogenic mutation does not rule out the disease associated with a new hitherto-unknown mutation.

Further studies of the extended FGA haplotypes involving Irish individuals and patients from different foci would be useful for investigating the founder effect and estimating the variant age and its most likely origin.

Finally, clarification of the pathogenesis of AFib amyloidosis is useful to the discovery of new therapeutic targets.
VIII – BIBLIOGRAPHY


