Analysis of the TTR gene in the investigation of amyloidosis: a 25 year single UK Centre experience

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Abstract

Transthyretin amyloidosis (ATTR) is caused by deposition of either wild-type (ATTRwt) or variant (ATTRm) transthyretin. ATTRwt presents with restrictive cardiomyopathy, whilst ATTRm displays a range of organ involvement. This retrospective analysis includes all patients referred to the single UK centre in the last 25 years for clinical and laboratory assessment of known or suspected amyloidosis who underwent TTR gene sequencing. 4459 patients were included in this study; 37% had final diagnosis of ATTR amyloidosis; 27% AL amyloidosis; 0.7% other types of amyloidosis; 21.3% had no amyloid and 14% had no data. TTR variants were found in 770 (17%) cases; the most prevalent were p.V142I, p.T80A and p.V50M identified in 42%, 25% and 16% respectively. The median age at referral in each group was: 76 (range 47-93); 66 (40-81) and 45 years (21-86) respectively. 42 rare or novel variants were identified. 42% of patients with ATTRm died at a median age of 73 years (33-89) with a median survival from diagnosis of 50 months. ATTRwt was the final diagnosis in 20% of patients undergoing genetic testing. Our findings of TTR variants in 17% of screened patients highlights the need for routine genetic testing in the evaluation of suspected ATTR amyloidosis.

Keywords: Transthyretin amyloidosis (ATTR); Wild-type (ATTRwt) transthyretin amyloidosis; Variant (ATTRm) transthyretin amyloidosis; TTR gene variants; Neuropathy; Cardiomyopathy.
Introduction

Transthyretin amyloidosis (ATTR) is caused by a deposition of fibrils which may be derived from either wild-type (ATTRwt) or variant transthyretin (ATTRm). Transthyretin (TTR) is synthesized primarily in the liver, but also in the choroid plexus and retina. In its native state, TTR is a homotetramer made of four monomers rich in beta sheet structure. Mutations in the TTR gene, strong biomechanical forces and proteolytic cleavage have all been reported to play a crucial role in destabilizing the TTR tetramer and release of a highly amyloidogenic 49-127 truncated protomer (Kelly, 2000; Marcoux, et al., 2015).

The TTR gene (MIM# 176300) (NCBI Reference Sequence NM_000371.3) is located on chromosome 18 and is known to be highly polymorphic with more than 140 variants of which 130 are amyloidogenic (Rowczenio, et al., 2014). With the exception of a valine residue deletion at position 142 and the recently reported 6 nucleotides duplication (c.212_217dupAGTCTG ) in exon 3 of the TTR gene (Klimtchuk, et al., 2018), all variants are nucleotide substitutions.

The possibly most common presenting phenotype associated with ATTRm (MIM# 105210) amyloidosis is familial amyloid polyneuropathy (FAP), in which amyloid deposition can affect any or all of major viscera including peripheral nerves, heart, gastrointestinal tract, vitreous, lungs and carpal ligament. Unlike FAP, in familial amyloid cardiomyopathy (FAC) ATTR amyloid accumulates predominantly in the heart causing progressive cardiomyopathy mainly characterized by diastolic dysfunction.

The earliest reported and most widely studied worldwide TTR variant is p.V50M. This was first described in 1952 in Portuguese kindreds with familial amyloid polyneuropathy (FAP) (Andrade, 1952). Subsequently large patient clusters in Portugal (Saraiva, et al., 1984), Japan (Araki, et al., 1980) and Sweden (Holmgren, et al., 1994; Olsson, et al., 2014; Sousa, et al., 1993) have been reported, with a considerable phenotypic variation in the age of onset, rate
of disease progression, involvement of different organ systems and penetrance. The possibly most prevalent TTR variant is p.V142I, which has been found at high frequency in patients of West African descent (Jacobson, et al., 2016). The clinical syndrome associated with this mutation is a late-onset restrictive cardiomyopathy and progressive heart failure with life expectancy up to five years from diagnosis (Ando, et al., 2013; Connors, et al., 2009). The estimated allele frequency among African Americans for p.V142I is 3.9% and cohort studies suggest allele carriers were at greater risk of heart failure even in the absence of overt amyloid deposits (Quarta, et al., 2015).

The phenotype of p.V142I ATTR amyloidosis clinically resembles that of the ATTRwt amyloidosis, as the late-onset restrictive cardiomyopathy is the cardinal feature in both. The latter however is caused by the deposition of wild-type transthyretin and predominantly affects elderly Caucasian males. The disease is most likely underdiagnosed, as an autopsy study revealed ATTRwt amyloid deposits were present in 25% of people over the age of 85 years (Tanskanen, et al., 2008), but the prevalence of ATTRwt amyloid deposits leading to clinical syndrome of ATTRwt cardiac amyloidosis has not yet been elucidated.

Interestingly, TTR tetramer comprising the non-pathogenic p.T139M TTR variant was found to be highly stable, and to play an inhibitory role in the ATTR fibrils formation (Marcoux, et al., 2015). In Portuguese families who were found compound heterozygotes for the p.T139M and p.V50M; the p.T139M attenuated the effects of the pathogenic p.V50M substitution, reducing the severity of the symptoms (Sebastiao, et al., 2001).

The UK National Amyloidosis Centre (NAC) is a national and international referral centre, which includes a long standing collaboration with Cyprus and it has been involved in diagnosis and management advisory service for patients with amyloidosis since 1990. In this retrospective review, we report the genetic testing results for patients with known or
suspected amyloidosis who underwent \textit{TTR} gene sequencing as part of their routine work-up over the last 25 years.

\textbf{Subject and Methods}

This retrospective analysis included 4459 patients referred to the single UK specialist centre between 1992 and 2017 for assessment of known or suspected amyloidosis who underwent \textit{TTR} gene sequencing as part of their routine work-up. Of these 3946 (88.5\%) had been assessed at the NAC and blood or DNA from the remaining cases was sent to NAC for a genetic review. The patients assessed at the NAC underwent additional clinical and laboratory investigations including histology with immunohistochemistry (Gilbertson, et al., 2012), laser micro dissection (LMD) of the amyloid deposits followed by liquid chromatography and tandem mass spectrometry (MS) (Vrana, et al., 2009), echocardiography (Quarta, et al., 2012), cardiac magnetic resonance (CMR) (Fontana, et al., 2015), \textsuperscript{123}I-SAP and \textsuperscript{99}Tc-DPD scintigraphy (Hawkins, et al., 1993; Hutt, et al., 2014) and measurements of cardiac biomarker NT-pro BNP (Wechalekar, et al., 2011). The presence of a monoclonal protein was sought by serum free light chain assay, conventional electrophoresis and immunofixation of serum or urine.

Patient survival from the time of diagnosis was analysed by Kaplan Meier analysis using GraphPad Prism v5.03 software.

Informed consent was provided by all subjects included in this study, and the ethical approval for the study was obtained from Royal Free Hospital and University College Medical School Research Ethics Committee (REC reference number 06/Q0501/42)

\textbf{DNA amplification and direct sequence analysis of} \textit{TTR} \textbf{gene}

Genomic DNA in all patients was extracted from whole blood treated with EDTA (Talmud, et al., 1991). The coding regions of the \textit{TTR} gene were amplified by polymerase-chain-
reaction assay and analysed by automated sequencing. Amplification was carried out with Ready-To-Go tubes (Amersham Pharmacia Biotech, Piscataway, NJ) with the use of primers, solutions, and cycling conditions that have been described previously (Booth, et al., 1995). The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Velno, The Netherlands) according to the manufacturer’s protocol and sequenced with the Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) following the procedures recommended by manufacturers. The sequence of the TTR gene was analysed on the ABI 3130x1 Genetic Analyser, using Sequencing Analysis Software version 5.4.

Results

Of the 4459 patients in whom the TTR gene was sequenced 37% had the final diagnoses of ATTR amyloidosis; 27% light chain (AL) amyloidosis; 0.7% other types of amyloidosis; 21.3% had no evidence of amyloid deposition and in 14% we have no data. Forty-five different TTR variants were identified in 770 (17%) of the 4459 individuals that underwent TTR gene testing between 1992 and 2017; 695 (92%) were assessed at the NAC. The list of TTR variants and number of cases associated with each variant are shown in Figure 1A. The most prevalent were: p.V142I identified in 323 subjects (42%, 72% of whom were male); p.T80A in 193 (25%, 66% of whom were male) and p.V50M in 121 (16%, 75% of whom were male). The demographics for these three variants are shown in Table 1. Nearly 50% of cohort with p.V142I had Afro-Caribbean ancestry, the remaining originated from different regions of Africa, mostly from the West. The TTR p.T80A was identified largely in patients of Irish ancestry, but 16% of the cohort were Scottish and p.V50M was found in a heterogeneous group of patients: 55% were of Greek-Cypriot / Greek ancestry, 29% were British; the remaining cases were from Portugal (5%), Sweden (4%), Ireland (3%) and the Middle East (4%). The median age at referral in each group was: 76 years (range 47-93); 66
years (40-81) and 45 years (21-86) respectively, although the patients with p.V50M who were of Greek-Cypriot and Greek ancestry were significantly younger than the British (median 35 years (range 21-70) vs 68 years (42-86) P<0.0001).

Forty-two rare or novel \textit{TTR} variants were found in 133 cases (17\%, 64\% were male), demographics are shown in Table 1. Rare variants included: p.S97Y in n=16, p.G67E in n=8, p.G67V n=7, p.V40I n=7, p.F53V n=5, p.I127V n=5, p.E109Q n=7, p.I127F n=4, p.G73E/A n=3, p.V91A n=3, p.S97F n=3, p.A101V n=3 and p.H110D n=3. Five previously unreported variants were identified: p.D58V in three unrelated individuals, two Ghanaian and one Polish; p.E74L in one Belgian and one Scottish/British subject; p.E74Q in two unrelated patients from Romania, one of whom had an extensive family history of early death due rapidly progressive neurological symptoms; p.A101V in a Polish and Russian patients and p.H110D in a large Irish kindred. Interestingly the p.E74L variant is complex resulting from an adenine and guanine nucleotides deletion and insertion of two thymine bases into their position (c.220_221 delGAinsTT). The clinical features of the novel variants were consistent with FAP, and the p.D58V substitution also caused vitreous opacities.

Sixteen patients were homozygotes: 13 for p.V142I (10 were Afro-Caribbean, two Nigerian and one Ghanaian); and single individuals for p.V50M, p.T80A and p.D58V (of Greek, Irish and Ghanaian ancestry respectively). Homozygosity for the p.V142I variant was associated with earlier onset cardiac dysfunction in 12 patients. One p.V142I \textit{TTR} gene carrier was asymptomatic and had family history of amyloidosis from the maternal side. Family history of cardiac death was evident in 4 of the 12 symptomatic cases. The patient homozygous for the \textit{TTR} p.V50M variant presented at the age of 20 years with sensory and autonomic neuropathy. His parents were asymptomatic carriers and his paternal grandmother developed symptoms of peripheral neuropathy at the age of 50 years. The patient homozygous for the \textit{TTR} p.T80A variant presented at the age of 39 years with cardiac symptoms and autonomic
dysfunction. Subsequently he developed symptoms of peripheral neuropathy. The patient’s mother was diagnosed with cardiac amyloidosis at age 69 years and her two sisters died of cardiac amyloidosis in their 6th decade. The patient homozygous for the TTR p.D58V variant presented at the age of 40 years, predominantly with a restrictive cardiomyopathy, but also significant peripheral and autonomic neuropathy. She had strong family history of sudden cardiac death; both parents died at the ages of 54 and 64 years, and two of her siblings died in their 3rd and 4th decade.

The genotype-phenotype correlation of the TTR variants identified in our centre is shown in Figure 1B. As expected, cardiac symptoms were the presenting clinical features in patients with the p.V142I variant; whereas p.T80A was associated with variable degrees of cardiac and neurological involvement. The clinical symptoms caused by the p.V50M substitution varied, depending on late vs. early onset disease: individuals with early onset from Greece/Cyprus had significant neuropathy, whereas the late onset British patient presented with dominant cardiac phenotype. Patients with other, less common variants presented with both neurological and cardiac symptoms and some TTR variants caused vitreous and / or leptomeningeal amyloid fibril deposition including: p.V50M, p.R54G, p.A56P, p.F53V, p.I104S, p.S72P and p.L32P.

Of the 695 patients with ATTRm who were assessed and followed up at the NAC, 293 (42%) have died: 164 (55%) diagnosed with p.V142I; 81 (56%) with p.T80A; 13 (19%) with p.V50M and 35 (52%) with other TTR variants. Median age at death in each group was 78 years (range 63-91), 71 years (60-83), 74 years (37-82) and 65 years (33-87), respectively, with a median survival from diagnosis of 33 months (range 1-240); 64 months (2-146); >10 years (11-220) and 56 months (1-213), respectively (Fig 2).

Interestingly, not all TTR pathogenic variants carriers had amyloid of ATTR type: 5 patients (all of Afro-Caribbean ancestry) with biopsy proven AL amyloidosis (four of lambda and one
of kappa sub type) were also found to carry the p.V142I variant. Four of these patients died of cardiac amyloidosis between the ages of 46 and 55 years. The known protective TTR substitution p.T139M was found in 11 subjects. None of these patients had been diagnosed with ATTR amyloidosis; in eight their amyloid was AL lambda, one AL kappa, and in two there was no evidence of amyloid.

Of note, in the same period of time, we have seen a dramatic increase in diagnosis of ATTRwt with 929 new cases to date (Fig 3a). This cohort accounts for 10% of all amyloid diagnosis at our centre and for 59% of ATTR patients (Fig 3b and c). As expected, 94% of our ATTRwt patients were Caucasian males, with a median age at disease diagnosis of 78 years (range 51-95). Of them, 305 (33%) have died; the median age at death was 81 years (range 63-96) with a median survival from diagnosis of 60 months (range 1-249).

Discussion

Hereditary ATTR amyloidosis is the commonest form of familial amyloidosis. TTR gene variants have been reported to be associated with variable amyloid organ involvement, but the neurological and cardiac symptoms, often leading to progressive and disabling peripheral and autonomic neuropathy or severe cardiomyopathy are the most commonly reported clinical features (Connors, et al., 2003; Rapezzi, et al., 2013). With the exception of a few TTR variants, including p.V142I, p.V50M and p.T80A, the majority are identified in isolated individuals or kindreds. ATTRm amyloidosis typically presents between the third and seventh decade of life. Untreated disease results in inexorably progressive symptoms with devastating effect on quality of life and reduced survival at 5 to 15 years from the onset of symptoms. ATTRm amyloid deposits within the vitreous causing blindness and the leptomeninges with CNS deficits are also recognised (Connors, et al., 2003; Holmgren, et al., 2005; Long, et al., 2012; Meng, et al., 2015; Uemichi, et al., 1999)
Here, we describe the outcome of TTR gene analysis performed in our Centre since it was implemented into our routine diagnostic work-up for patients with suspected ATTR amyloidosis. Our study has the largest cohort of patients with suspected systemic amyloidosis to have undergone TTR gene sequencing. ATTRm was identified in 17% of cases, while ATTRwt accounted for 20% of diagnoses. The TTR p.V142I variant was the commonest finding and nearly 50% of these had Afro-Caribbean ancestry, our ethnic distribution is in line with the demographics of the South East of the United Kingdom. It is important to highlight that presence of a mutation cannot be relied to confirm the amyloid type as p.V142I was incidentally found in five patients (all of Afro-Caribbean ancestry) who were definitively diagnosed with AL amyloidosis, four of five cases presented with isolated cardiac amyloidosis.

The second most common TTR variant (25%) was p.T80A found predominantly in kindreds of Irish ancestry, consistent with the known founder effect (Reilly, et al., 1995) in whom the median age at diagnosis was 66 years and cardiac symptoms were the principal manifestations, but 80% of cases had some degree of peripheral and autonomic neuropathy. Consistent with a previous report (Sattianayagam, et al., 2012), only 35% of cases had a positive family history of amyloidosis. The TTR p.V50M was found in 16% of all ATTRm cases. There was a significant difference between the age at onset for patients of Greek-Cypriot / Greek ancestry when compared to British subjects (median 35 years (range 21-70) vs 68 years (42-86)) p<0.0001, and also marked difference in the clinical features, with the former suffering predominantly with neurological symptoms while the later had cardiac dominated disease. Forty two other rare variants were found, of which five were novel. The anatomical location of ATTR fibril deposition associated with the novel mutations was consistent with other known variants in that the nerves and the heart were primarily affected.
In the three best recognised TTR substitutions (p.V142I, p.T80A and p.V50M) there was evidence of a gene dosage effect with homozygous patients presenting at a younger age than heterozygotes.

Survival from diagnosis varied, depending on the type of TTR variant. Patients with the p.V50M had over 10 years survival, which was most likely due to successful liver transplantation (LT) and younger age at presentation. LT has been the only curative treatment in this disorder and is considered to be particularly beneficial for early-onset TTR p.V50M patients, while those with other variants were reported to have paradoxical acceleration of transthyretin amyloid deposition in the heart following LT (Stangou and Hawkins, 2004; Stangou, et al., 2005). The recent advances in treatment for ATTR amyloidosis including drugs blocking translation of the TTR protein (Adams, et al., 2017; Adams, et al., 2016; Maurer, et al., 2018) may transform treatment outcomes particularly for older patients with rarer variants for whom the only treatment has been symptomatic.

Although classically regarded as an autosomal dominant condition, family history was rare in our ATTRm cohort, most likely reflecting late disease onset and incomplete penetrance (Hellman, et al., 2008; Plante-Bordeneuve, et al., 2003). Sporadic presentation is well recognised in other forms of hereditary amyloidosis, in particular in the fibrinogen A α-chain amyloidosis (MIM# 105200) caused by the p.E545V variant in the FGA gene (MIM# 134820); (NCBI Reference Sequence NM_000508.3) (Gillmore, et al., 2010; Rowczenio, et al., 2017).

Improvements in diagnostics, in particular the widespread implementation of CMR and ⁹⁹Tc-DPD scintigraphy (Gillmore, et al., 2016) has resulted in a dramatic change in the demographics of amyloidosis in the UK. The number of referred cases with ATTR amyloidosis has increased from less than 2% in 2000 to 23.7 % of new referrals in the last 2 years. The majority of whom were ATTRwt. In total 929 patients have been diagnosed with
ATTRwt amyloidosis, accounting for 59% of all ATTR diagnoses. ATTRwt is predominantly found in elderly Caucasian males (median age at onset 78 years) who almost exclusively presented with restrictive cardiomyopathy, similar to patients with the p.V142I mutation. With increasing longevity and improvement in diagnosis the incidence of ATTRwt amyloidosis may be much more prevalent than previously estimated.

ATTR accounts for a steadily increasing proportion of newly diagnosed amyloidosis. This probably reflects advances in imaging modalities rather than a true change in disease demographics.

Our cohort reflects a mixed ethnicity population with a relatively low frequency of TTR p.V50M compared to reports from other countries (Araki, et al., 1980; Lobato, 2003; Suhr, et al., 2003). We identified 45 different TTR variants, which can be found in the online registry for hereditary amyloidosis at: www.amyloidosismutations.com. The majority were associated with a mixed cardiac and neurological phenotype; 10 were found to cause isolated cardiac amyloidosis and 9 had been associated with isolated neurological symptoms. Early disease onset (median 43 years (range 32-46)) and poor survival was seen with the rare TTR variants: p.L32P, p.F53L, p.G67E and p.V91A which presented as aggressive, rapidly progressing and disabling peripheral and autonomic neuropathy. However, the incidental finding of TTR variants in some cases with AL amyloidosis shows the importance of interpreting genetics in clinical context.

Our findings of ATTRm in 17% of screened patients and the late presentation of TTRv amyloid, with over 70% aged 60 and 80 years old at diagnosis, highlights the importance of genetic testing in routine clinical evaluation of patients with suspected systemic amyloidosis. Previously TTR gene screening was used in the diagnostic work up – to avoid misdiagnosis of AL amyloidosis, which can be treated with chemotherapy, to provide information for family members and to allow highly selected patients to be considered for liver
transplantation. With advances in therapy and the promising results from silencing RNA and antisense oligonucleotides clinical trials we are on the threshold of a new era in the treatment of TTR amyloidosis with the imminent licencing of targeted gene silencing agents and thus an even greater importance for establishing the exact nature of the precursor protein (Adams, et al., 2017; Adams, et al., 2016; Maurer, et al., 2018)

Acknowledgements
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References:


**Figure 1.** A) The types of mutations identified at the NAC between 1992–2017 and the number of cases with each variant. B) The genotype-phenotype correlation associated with the TTR variants identified at the NAC.
Figure 3. New diagnosis of ATTR amyloidosis at the NAC since 2000. A) An increase in diagnosis of ATTRwt. This cohort accounts for 10% of all amyloid diagnosis at our centre and for 59% of ATTR patients (Fig 3B and C).
Table 1. Demographic data of the ATTRm patients identified at the NAC between 1992 and 2017

<table>
<thead>
<tr>
<th>Demographic data</th>
<th>TTR p.V142I</th>
<th>TTR p.T80A</th>
<th>TTR p.V50M</th>
<th>Other rare TTR variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Characteristic Ethnicity</td>
<td>African or Caribbean</td>
<td>Irish</td>
<td>Greek, British, Portuguese, Swedish, Spanish</td>
<td>World-wide (&gt;isolated cases)</td>
</tr>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>72</td>
<td>66</td>
<td>75</td>
<td>64</td>
</tr>
<tr>
<td>Female</td>
<td>28</td>
<td>34</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>Age at referral</td>
<td>Median (range)</td>
<td>76 (47-93)</td>
<td>66 (40-81)</td>
<td>35 (21-70) in Greek</td>
</tr>
<tr>
<td>Age at death</td>
<td>Median (range)</td>
<td>55 (63-91)</td>
<td>56 (60-83)</td>
<td>19 (37-82)</td>
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<td>(%) Died</td>
<td></td>
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<tr>
<td>Median survival from diagnosis (months) (range)</td>
<td></td>
<td>33 (1-240)</td>
<td>64 (2-146)</td>
<td>&gt; 10 years (11-220)</td>
</tr>
<tr>
<td>Predominant Clinical phenotype</td>
<td>Cardiac</td>
<td>Mixed, but significant cardiac involvement</td>
<td>Mixed, but predominant late-onset cardiac in British</td>
<td>Depending on the mutation, but early onset and shorter survival with severe progressive neuropathy</td>
</tr>
<tr>
<td>N (%) of all ATTRm cases</td>
<td>323 (42)</td>
<td>193 (25)</td>
<td>121 (16)</td>
<td>133 (17)</td>
</tr>
</tbody>
</table>