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Serum transthyretin levels in senile systemic amyloidosis: effects of age, gender and ethnicity

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Abstract

Serum transthyretin (TTR) levels are reduced in familial amyloidotic polyneuropathy (FAP). A single study of patients with senile systemic amyloidosis (SSA) in Sweden found that those individuals also had a significantly lower mean serum TTR concentration than age- and gender-matched controls. To determine if the same phenomenon prevailed in an ethnically more heterogeneous population, we compared the serum TTR levels, as determined by ELISA, in 45 documented SSA patients with congestive heart failure, 20 AL patients with congestive heart failure and population controls. Serum TTR concentrations in the controls were influenced in a statistically significant manner by age, gender and ethnicity. Although it is unlikely that such differences are clinically relevant, they must be considered when assessing the meaning of serum TTR concentrations in any clinically defined population. The serum concentrations in patients with SSA did not differ from age, gender and ethnically matched controls or from a group of AL patients with significant clinical cardiac involvement. We also compared TTR concentrations in 12 African-Americans carrying the TTR V122I allele with those in 826 African-Americans who were homozygous wild type at the TTR locus. The TTR V122I carriers had significantly lower serum TTR concentrations than appropriate controls even though the majority of such individuals had not reached the age of clinical or anatomic risk, i.e. over 60. Thus, as in carriers of other TTR mutations the serum TTR level is lower than normal, despite having a much later appearance of clinical disease.

Keywords

Transthyretin; senile systemic amyloidosis; TTR V122I; serum transthyretin

Introduction

Senile systemic amyloidosis (SSA) originally described as senile cardiac amyloidosis (SCA) is characterised by homogeneous, eosinophilic, congophilic deposits in the cardiac atria and ventricles [1]. Later studies showed that the subjects frequently had similar deposits in other

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organs, particularly the lungs and gut, resulting in its designation as SSA rather than SCA [2,3]. Some authors felt that it represented a pathologic entity, common at autopsy in the elderly with only a small proportion of subjects actually showing clinical evidence of associated disease in life [4,5]. However, several careful retrospective analyses with clinical correlation showed that many of the subjects with cardiac amyloidosis had significant functional compromise including congestive heart failure and arrhythmias, particularly atrial fibrillation without evidence of other significant cardiac pathology [6–12]. One report indicated that the disorder was the proximal cause of death in half of the affected individuals [13].

It was clear in the early studies that isolated atrial deposits did not seem to have the clinical impact of ventricular amyloid deposition [4,5]. We now know that the amyloid precursors for the two forms of deposition are different. The atrial deposits are derived from atrial natriuretic factor (ANF) and appear to be more common in individuals with longstanding congestive heart failure, perhaps, as a result of chronic over-production of ANF in response to the cardiac pathology [14–16]. The ventricular fibrils, like those seen in familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC) are derived from the serum protein transthyretin (TTR). In contrast with the familial disorders, in which the TTR is mutant, in SSA the deposits are composed of wild type (TTR) and/or its fragments [17–19]. The precise definition of the disease became less clear with the description of a subset of mutations in the TTR gene resulting in amino acid substitutions with the clinical phenotype of late onset cardiac amyloidosis, i.e. after age 60 [20–23]. It has been suggested that wild type TTR amyloid deposition does not occur until age 80, whereas the mutations cause significant cardiac pathology earlier [24]. However, recent experience in our laboratory and others suggests that many individuals with wild type cardiac TTR amyloidosis manifest their disease as early as the seventh decade (Lachmann H, Hawkins PH, personal communication). In the patients described in the present report, there were as many individuals diagnosed before age 70 as after age 80 with the majority of cases diagnosed in their 70s.

In population studies, it has been shown that the mean serum TTR concentration increases until adolescence, is stable until the sixth decade then decreases with increasing age [25]. Because of its sensitivity to both nutritional status and inflammation, serum TTR is a useful clinical indicator of acute systemic disease, particularly under-nutrition and infections as seen post-surgically and in the developing world. It has been utilised to evaluate patients at risk in those settings [26]. The physiology underlying the phenomenon is rooted in the hepatic regulation of transcription of the TTR gene by transcription factors (HNF3α, HNF 6) sensitive to the cytokines IL1, IL6 and TNF, hence its inclusion, with albumin, as a negative acute phase reactant [25].

In 1985, it was reported that the mean level of serum TTR was significantly lower relative to age-matched controls in 13 Swedish patients over age 80 with congestive heart failure related to the presence of wild type TTR cardiac amyloidosis [27,28]. The observation has neither been confirmed in the literature nor has the issue been addressed in other populations. In the current study, we explore the question in a larger sample set with much greater ethnic diversity than that in the original report.

Material and methods

Populations

Forty-five patients with SSA and a history of congestive heart failure were chosen as study subjects. All patients had biopsy-proven amyloidosis, confirmed by positive Congo red staining of a heart or fat tissue specimen. All patients were evaluated for a plasma cell dyscrasia by serum and urine immunofixation electrophoresis and by bone marrow biopsy with immunohistochemical examination to confirm the absence of a monoclonal population of plasma cells. Cardiac involvement was defined as a cardiac biopsy showing amyloid deposits, and/or a history of congestive heart failure with myocardial wall thickening on echocardiogram without a history of significant hypertension, valvular or coronary disease and a non-cardiac biopsy showing amyloid deposition. Heart failure was defined as jugular venous distention with or without peripheral edema and/or the presence of radiographic features of congestive heart failure. In the absence of a monoclonal serum or urine light chain and the absence of a monoclonal plasma cell population in the bone marrow, subjects underwent screening for ATTR amyloidosis by isoelectric focusing of serum and/or TTR gene mutation testing by direct DNA sequencing [29]. If both a clonal light chain and a variant TTR were excluded and the patient presented with amyloid cardiomyopathy at an advanced age, the diagnosis of SSA was made [30].

As a comparison group, 20 patients with AL amyloidosis involving the heart who had been evaluated during the same time period were selected from a larger group of AL subjects. Because all patients with SSA had congestive heart failure, we selected patients with AL amyloidosis who also had heart failure. The patients with AL amyloidosis had biopsy-proven amyloidosis and all had a plasma cell dyscrasia with clonal plasma cells in the bone marrow and/or a monoclonal gammopathy detected by immunofixation electrophoresis of serum or urine proteins. These patients gave informed consent for the data and sample collection/analysis with the approval of the Institutional Review Board at Boston University School of Medicine in accordance with the Declaration of Helsinki.

Frozen sera from 828 Caucasians and 826 self-identified African-Americans who initiated an appointment in the Kaiser Health Appraisal Clinic as participants in the Kaiser-Permanente of San Diego Health Maintenance Organisation served as population controls. All the donors gave informed consent allowing the determination of serum markers related to the development of liver disease [31]. Only a portion (586) of the African-Americans consented to genetic analysis and only 12 of the 16 TTR V122I positives had sera available for TTR quantitation.

Samples

Aliquots of frozen plasma were thawed to perform the TTR ELISA. DNA had been extracted from peripheral blood buffy coat preparations by standard techniques and was maintained frozen at −20°C [31]. Sera were stored at −20°C until used.

Methods

TTR ELISA:Immulon 4 HBX 96-well plates (Dynex Technologies Cat 3855 or Fisher 14-245-153) were coated with 1:1000 rabbit anti-human prealbumin, DakoCytomation). The wells were treated with blocking buffer (15 g non-fat) milk carnation in Tris HCl buffer (final pH 7.5) for 60 min at 37°C and then washed with Tris buffer. Standards were prepared with recombinant wild type human TTR diluted in blocking buffer. Sera were diluted 1:500 in blocking buffer. The standards and samples (100 μl) were added to each well in triplicate, covered and incubated for 75 min at 37°C. Plates were washed with 1 × Tris buffer using a SkanWasher 300. Conjugated antibody (Goat anti-human prealbumin alkaline phosphatase, EY Laboratories, San Mateo, CA, 1 mg/ml, AA-2112-1, 1-800-821-0044) was diluted 1:1000 and 100 μl was added to each well and incubated at 37°C for 75 min. The plate was washed with 1 × Tris buffer. NPP was added and the plate placed in the dark on rocker for 15 min and then read at 405 nm, using ELISA SoftmaxPro software and the signal converted to μg/ml.

Genotyping was performed using a real time polymerase chain reaction assay to identify TTR V122I based on the detection of hybridisation with probes specific for each allele which quenched fluorescence in the absence of hybridisation (Black Hole Quenchers, Biosearch Technologies, Novato, CA). Thus, wild-type specific oligonucleotides did not fluorescence until they bound to the wild-type allele and mutant-specific probes did not fluorescence until they found their complement. Controls were DNAs from individuals previously documented to be homozygous wild type (V122V), homozygous mutant (V122I) and heterozygous. Fluorophore labelled probes were specific for the wild type 5′-HEX–CCACGGCTGTCGTCAC CAATCC–BHQ-3′; and the mutant 5′-FAM–CCA CGGCTGTCATCACCAATCC–BHQ-3′. The primers for the DNA segment being amplified were designated Fok1L 5′-GCTGAGCCCCTACTCC TATTC-3′; Fok1R 5′-CTGGTCCTTCAGGTC CACTG-3′ based on our prior work identifying the polymorphic sequence around the Fok1 site. The HEX and FAM fluors had absorption maxima of 534 nm and quenched in the range of 480–580 nm. All reactions were carried out in 20 μl volumes in a multiplex format containing the two Fok 1 primers and the two labelled primers in an MJ Opticon instrument using cycles of 2′ at 50°C, 5′ at 95°C, 15 s at 95°C, 1′ at 69°C, then read at the wavelengths specific for HEX and FAM. Homozygous wild-type individuals were only positive for HEX whereas the heterozygotes gave both HEX and FAM signals.

Statistics

Descriptive statistics were tabulated using Graphpad Instat 3 Software (GraphPad Software, San Diego, CA). We employed three-way fixed-effects analysis of variance (ANOVA), with dichotomous factors sex (male or female), race (Caucasian or African-American) and age (< 60 years old or ≥60) to compare the serum TTR concentrations and the log transformed serum TTR concentrations, in the Kaiser cohort. The results were virtually identical, and we here report solely the findings with the untransformed data for simplicity. The estimates of serum TTR levels for the various subgroups utilised the ANOVA model incorporating each of the significant factors (sex, race and age). We used van Elteren's stratified Wilcoxon test [32] to compare serum TTR levels between V122I carriers and wild-type TTR carriers in the

Kaiser African-American cohort. Stratification factors were sex and age (< 60 years old or ≥60).

Results

Serum TTR concentrations in senile systemic and AL amyloidosis

All but two of the SSA patients were Caucasian males over age 60 (Table I). One was female and one male was African-American. The male preponderance is characteristic of this disorder [33]. Six of the 20 AL patients were African-American. The sample sizes did not have sufficient power to determine if differences between males and females or Caucasians and African-Americans with AL heart disease were significant. Comparison of the TTR concentrations of the SSA patients with those of the matched Caucasian male control population revealed that the levels were higher in the controls but the difference was not significant. Similarly, although the eight male Caucasian AL patients had lower serum TTR levels than the age, gender and ethnically matched Kaiser group, the difference was not significant. When the SSA patients were compared with the entire group of AL patients, the serum TTR concentrations were neither significantly different nor did they differ significantly when only the Caucasian male patients of each group were used in the comparison.

Serum TTR concentrations in Caucasians and African-Americans

We examined the joint relationship of race, gender and age on serum TTR levels via standard fixed effects three-way analysis of variance. We utilised the Kaiser cohort of 828 Caucasians and 826 African-Americans, with factors sex (male *vs.* female), race (Caucasian *vs.* African-American) and age (< 60 *vs.* 60 and older). As shown in Table II, we found that each of the three factors was significantly related to serum TTR. TTR levels were significantly higher in males when compared with females, Caucasians compared with African-Americans and younger subjects compared with older subjects. There were no significant interactions among any of these three main effects, i.e. age, gender and ethnicity behaved as independent variables. Model estimates of the serum TTR levels for each relevant subgroup are given in Table III.

Role of the amyloidogenic TTR V122I allele on serum TTR concentration

The frequency of amyloidogenic TTR mutations is very low in the U.S. Caucasian population with most cases of amyloidosis being of the senile systemic type with the deposits consisting of the wild type protein [34]. In contrast, large population studies have shown that 3–4% of African-Americans carry the amyloidogenic TTR V122I allele [35,36]. Genotyping of the 526 African-American participants in the Kaiser cohort identified 16 individuals carrying the amyloidogenic allele, 12 of whom had sera available for the determination of serum TTR. Overall, after adjusting for age and sex, the serum TTR concentrations in this group were significantly lower than in those carrying V122V (van Elteren's $Z = 2.408$, $2p = 0.016$). Summary statistics comparing the two allele groups are given in Table IV. When we compared the TTR levels in Caucasians and African-Americans eliminating the TTR V122I carriers, the significant differences between Caucasians and

African-Americans remained, indicating that the lower levels in the allele carriers did not statistically bias the more broadly based comparisons.

Discussion

For many years, it has been known that serum TTR concentrations are relatively low in carriers of autosomal dominant amyloidogenic TTR mutations even before the signs and symptoms of FAP become apparent [37–41]. The levels become even lower with the onset of disease. On the basis of those observations and the single study of Swedish subjects with SSA, we expected the levels in our SSA cohort to be lower than in age, gender and ethnically matched controls.

In this study, the mean serum TTR levels in patients with clinically significant SSA were no different from patients with congestive heart failure caused by another form of systemic amyloidosis, i.e. AL, neither population having lower mean values than age- and gender-matched controls. The finding was somewhat surprising because the phenomenon of ‘cardiac cachexia’ reported in patients with severe chronic congestive heart failure is cytokine-mediated and we expected to find lower serum TTR concentrations in both patient groups [42,43]. Our analyses suggest that the original observation regarding lower serum TTR levels in Swedish patients with congestive heart failure as a result of SSA may have been correct but that the difference between those patients and age-matched Swedish controls may be greater than that recognisable in a larger, genetically more heterogeneous population [27]. It is also curious that in that study the mean TTR level of their 6 FAP patients was higher than in the controls, a finding at variance with the studies in Japanese and Portuguese TTR V30M carriers, V30M carriers in Boston and TTR S84I carriers in Indiana. It is, thus, possible that the regulation of serum TTR concentration may be different in Swedish individuals than in members of other ethnic groups. This possibility should be formally tested experimentally.

As in other familial TTR amyloidoses, the carriers of the TTR V122I amyloidogenic mutation have a lower level of the circulating protein than carriers of two copies of the wild-type protein, despite the fact that this allele in the heterozygous state does not produce tissue deposition until after age 60 [34]. Although the number of V122I carriers was not sufficient to parse the sample by age and gender, comparison with the African-American cohorts above and below age 60 showed the serum TTR concentration in the carriers to be significantly lower than both, a finding similar to that reported for other autosomal dominant TTR mutations with clinical penetrance at a much earlier age [37,40,41].

It is not clear why serum TTR values are reduced in patients with TTR amyloidosis related to mutations but not in individuals with tissue deposits of the wild-type protein. It is possible that the greater proportion of misfolded monomer due to the physical effects of the mutations results in greater endoplasmic reticulum-associated degradation (ERAD) and/or ubiquitin/proteasome-related proteolysis in the hepatocyte. In addition, there is general consensus that inflammatory cytokines reduce the transcription of the TTR gene, hence its behaviour as a negative acute phase reactant. Measurements of circulating cytokine levels in FAP patients have not been published. However, in population studies there is a relationship

between serum TTR and serum IL-6 as well as TTR and plasma HDL levels [44,45]. It is possible that the increased serum IL-6 concentration seen in the elderly could be responsible for the general reduction in serum TTR with aging [46]. Interestingly, in the controls, we noted that in both Caucasian and African-American populations in males, over 60 serum TTR concentrations were higher in males than in females. Perhaps, this contributes to the male preponderance in the occurrence of SSA.

The few studies of TTR production in mutation carriers suggested that, in the liver the transcription rate is no different between the mutant and normal allele [47,48]. Several studies of serum turnover of iodinated normal and mutant TTR have suggested that the mutant proteins are turned over at a faster rate than the wild type molecule in both humans and mice [49,50] but not in the rat [51]. However, these studies were relatively small and performed at a single age, and thus cannot account for the age-associated decay in serum concentration of both wild-type and mutant TTR's or the relationship to tissue deposition. In the human studies, it was not clear whether the more rapid turnover resulted from deposition, catabolism or renal excretion. In the murine studies, tissue deposition was not a measurable factor and the interpretation was that it reflected enhanced catabolism.

Recent studies, although not addressing the problem directly, have indicated that in the serum of V30M patients the circulating monomers are largely wild type, rather than mutant, suggesting that the mutant molecules may be more predisposed to aggregate and be cleared more quickly, a finding consistent with the *in vivo* mouse experiments [52]. However, the questions of clearance and disposition were not posed experimentally in that study.

The observations of mutant TTR deposition in recipients of ''domino'' liver transplants from FAP donors suggest another explanation, i.e. that the liver's capacity to refold or degrade misfolded TTR molecules may become compromised with either age or a lifetime of coping with a significant quantity of misfolded TTR synthesised in the liver [53]. In such a case, perhaps with time, a greater proportion of the misfolded TTR transits the secretory pathway into the circulation and is routed to the tissue deposits with the serum concentration being diminished as a consequence. Such a phenomenon has been hypothesised and modelled as part of an overall view that secretion of misfolded proteins is not governed by some fixed quality standard, but rather by the relative energetics of the interaction of the protein with the secretory and ERAD machinery and those governing the misfolded proteins' tendency to aggregate [54]. Hence, if a misfolded protein has a more favourable thermodynamic/kinetic interaction with the secretory machinery than with either the ERAD or itself it will be secreted rather than aggregate intracellularly or be degraded. However, there are no published data examining the actual rate of TTR secretion *in vivo*. Thus, partially misfolded proteins predisposed to aggregate, may exit the cell and lodge elsewhere. This may be less true for wild-type TTR than with mutant forms nonetheless even the wild-type protein always has a certain fraction of its monomers that is misfolded and at risk for aggregation.

We have found that pre-symptomatic African-American carriers of the amyloidogenic TTR V122I allele had reductions in the serum TTR concentration similar to those seen in subjects with other TTR mutations associated with FAC or FAP. In contrast to those findings, the

serum TTR levels in a large group of U.S. Caucasians with SSA were no lower than that of age- and gender-matched controls. Our data suggest that the lesser tendency of wild type TTR to misfold may allow a greater proportion of the protein synthesised in the liver to be secreted than is the case with the mutant molecules. To the extent that this aspect of hepatic function is diminished by aging the serum concentration of wild type TTR is no different in the presence or absence of tissue deposition.

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Abbreviations

TTR	transthyretin
FAP	familial amyloidotic polyneuropathy
FAC	familial amyloidotic cardiomyopathy
SSA	senile systemic amyloidosis
SCA	senile cardiac amyloidosis
ANOVA	analysis of variance
ERAD	endoplasmic reticulum associated degradation

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Table I

Comparison of serum TTR levels in patients with senile systemic and AL amyloidoses with congestive heart failure with age, gender and ethnically matched controls.

Group	Number	Serum TTR (μg/ml)	Range
SSA Caucasian males > 60	43	248 *	112–610
Caucasian males > 60	128	250 *†	69–606
AL Caucasian males > 60	8	195 †	159–422
All SSA	45	237 ‡	112–610
All AL	20	195 ‡	98–506

* p=0.72,

† p = 0.24,

‡ p = 0.59.

Table II

Analysis of variance of serum TTR levels, with factors sex, race and age.

Source	Sum-of-squares	df	Mean-square	*F*-ratio	*p*
Sex	0.266	1	0.266	30.725	< 0.001
Race	0.072	1	0.072	8.293	0.004
Age	0.059	1	0.059	6.837	0.009
Race*sex	0.004	1	0.004	0.463	0.496
Age*sex	0.030	1	0.030	3.440	0.064
Age*race	0.000	1	0.000	0.011	0.915
Error	14.141	1636	0.009		

Sex (female *vs.* male), race (African-American (814) *vs.* Caucasian (828)) and age (< 60 years old *vs.* 60 or older) are categorical variables in this analysis of variance. The *p*-values correspond to the F-statistics for the main effects (sex, race and age) and the first-order interactions (race*sex, age*sex and age*race).

Table III

Serum TTR levels in Caucasians and African–Americans.

Group	Caucasians (μg/ml)	African-Americans (μg/ml)
Total	262.7 (18–752)	248.1 (90–708)
Males	275.1 (29–752)	263.7 (103–708)
Females	250.2 (18–663)	232.5 (90–674)
Under 60	269.5 (29–52)	254.5 (90–708)
60 and over	255.8 (18–606)	241.8 (101–− 538)
Males under 60	287.5 (29–752)	274.0 (103–708)
Males 60 and over	261.9 (69–606)	254.4 (122–478)
Females under 60	251.7 (103–663)	234.9 (90–674)
Females 60 and over	249.9 (18–453)	228.9 (101–538)

Values are the serum TTR levels for each subgroup as determined from the three-way analysis of variance model of Table II, along with the observed range of serum TTR levels within that subgroup. The analyses included 828 Caucasians and 812 African-Americans.

Table IV

Comparison of mean serum TTR levels in African-Americans homozygous for the wild-type TTR (V122V) gene with those heterozygous for the amyloidogenic (TTRV122I) allele.

Group	Number	Serum TTR (μg/ml)	Range
V122V	814	229*	90–708
Males	400	246	103–708
Females	414	215	90–674
Under 60	578	231	90–708
60 and over	236	238	101–538
Males under 60	274	216	90–674
Males 60 and over	126	242	122–478
Females under 60	305	216	90–674
Females 60 and over	109	210	101–538
V122I	12	171*	80–488
Males	5	146	141–205
Females	7	199	80–408
Under 60	9	146	80–488
60 and over	3	205	118–228

* $p = 0.01$.