Chapter 5

Diagnostic performance of amyloid A protein quantification in fat tissue of patients with clinical AA amyloidosis

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INSTITUTIONAL AFFILIATIONS

AMYLOID 2007: 14:133-40
ABSTRACT

Objective. Amyloid A protein quantification in fat tissue is a new immunochemical method for detecting AA amyloidosis, a rare but serious disease. The objective was to assess diagnostic performance in clinical AA amyloidosis.

Methods. Abdominal subcutaneous fat tissue of patients with AA amyloidosis was studied at the start of an international clinical trial with eprodisate (NC-503; 1,3-propanedisulfonate; Kiacta™), an anti-amyloid compound. All patients had renal findings, i.e. proteinuria (≥1 g/day) or reduced creatinine clearance (20-60 mL/min). Controls were patients with other types of amyloidosis and arthritic patients without amyloidosis. Amyloid A protein was quantified by ELISA using monoclonal anti-human serum amyloid A antibodies. Congo red-stained slides were scored by light microscopy in a semi-quantitative way (0 to 4+).

Results. Ample fat tissue (>50 mg) was available for analysis in 154 of 183 patients with AA amyloidosis and in 354 controls. The sensitivity of amyloid A protein quantification for detection of AA amyloidosis (>11.6 ng/mg fat tissue) was 84% (95% CI: 77%-89%) and specificity 99% (95% CI: 98%-100%). Amyloid A protein quantification and semi-quantitative Congo red scoring were concordant. Men had lower amyloid A protein values than women (P<0.0001) and patients with familial Mediterranean fever had lower values than patients with arthritis (P<0.001) or other inflammatory diseases (P<0.01).

Conclusions. Amyloid A protein quantification in fat tissue is a sensitive and specific method for detection of clinical AA amyloidosis. Advantages are independence from staining quality and observer experience, direct confirmation of amyloid AA type, and potential for quantitative monitoring of tissue amyloid over time.
INTRODUCTION

AA amyloidosis is a progressive and often fatal systemic condition occurring in some patients with chronic inflammatory diseases [1]. Because of its long latency period, it frequently remains undiagnosed unless significant organ damage has occurred. Median survival after diagnosis is 2-10 years [2-4]. Renal involvement, reflected by proteinuria or renal insufficiency, is the most frequent and prominent feature of clinical AA amyloidosis [2, 3]. Amyloid deposition is detected by tissue biopsy and histology. Amyloid deposits stain with Congo red and show apple-green birefringence under polarized light [1]. Confirmation of AA type is achieved by immunohistochemistry, using specific antibodies to AA protein [5, 6].

The kidney is the most obvious site to biopsy for detection of amyloid in patients with renal abnormalities [2, 7]. However, kidney biopsy is an invasive procedure and potentially hazardous complications, such as perirenal bleeding, occur in about 0.7% [8]. Aspiration of abdominal subcutaneous fat is easy to perform, has a low risk of complications, and can be used to detect tissue deposition of amyloid [9, 10]. Specimens of abdominal fat tissue reacting positively after Congo red staining confirm the presence of amyloid in 52-88% of patients [11, 12]. Familial Mediterranean fever (FMF), however, may differ in this respect showing negative Congo red staining of abdominal fat tissue in patients with AA amyloidosis [13].

Quantification of amyloid A protein concentration in fat tissue by enzyme-linked immunosorbent assay (ELISA) is a recently developed, different way of detecting AA amyloid deposition [14]. This immunochemical method has potential advantages compared to the Congo red stain, such as independence from quality of staining procedure and observer experience, direct confirmation of the AA type of amyloid, the possibility to become highly automated, and quantitative monitoring of tissue amyloid at predetermined points in time during the course of the disease. Recommendation for routine clinical application requires previous assessment of its sensitivity and specificity. An international study on efficacy and safety of a new drug in patients with clinical AA amyloidosis provided an opportunity for a substudy in which sensitivity of the method was assessed in this well-defined and large group of patients. The aim was to study the diagnostic performance of amyloid A protein quantification in fat tissue of patients with well-defined clinical AA
amyloidosis as well as in fat tissue of patients without AA amyloidosis, but having closely related other diseases.

PATIENTS AND METHODS

Study design and patients
The sensitivity of amyloid A protein quantification in fat tissue was studied in patients with clinical AA amyloidosis. The patients were participants enrolled between July 2001 and January 2003 in an international, randomized, placebo-controlled phase II/III clinical trial with eprodisate (NC-503; 1,3-propanedisulfonate; Kiacta™), a potential anti-amyloid compound. The trial was registered with ClinicalTrials.gov (Identifier #NCT00035334). Trial eligibility required that patients have AA amyloidosis proven by a biopsy positively stained with Congo red in combination with positive anti-AA amyloid immunohistochemistry. In addition all patients were required to have renal findings, i.e. proteinuria of $\geq$1 g/day or reduced creatinine clearance between 20 and 60 mL/min.

Specificity was studied in controls without AA amyloidosis, particularly those with closely related other diseases, who had been seen in our hospital in Groningen between October 1996 and April 2005. Controls comprised different groups: healthy controls and patients with diseases unrelated to amyloidosis, and controls with closely related diseases, such as chronic arthritis without any sign of amyloidosis, typical localized amyloidosis, systemic AL (light chain-derived) amyloidosis, and systemic hereditary ATTR (transthyretin-derived) amyloidosis. Different cut-off values of amyloid A protein concentration in fat tissue were used to calculate sensitivity and specificity and to establish the baseline amount of this protein in fat tissue of controls. In all controls concurrent serum amyloid A protein (SAA) concentration in blood was measured to study its relation with amyloid A protein concentration in fat tissue. The local Ethics Committees approved the study and all patients and controls gave informed consent according to the Declaration of Helsinki.

Fat aspiration, Congo red staining, and scoring of staining intensity
Abdominal fat tissue was aspirated as previously described [14]. Skin and subcutaneous tissue were anaesthetized with lidocaine and subsequently a 16
gauge needle connected to a 10 mL-syringe were used to aspirate at least 50 mg of fat tissue. Fat tissue was kept at room temperature, transported, and analyzed in a single center within seven days after aspiration. About 20-30 mg of fat tissue was required to make three fat smears per individual as described [14]. Smears were dried in air at room temperature, fixed with acetone, and stained with alkaline Congo red dye according to Puchtler et al. [15]. Affinity of tissue for Congo red was analyzed by apple-green birefringence in polarized light using an Olympus BX 50 microscope, 100 Watt, equipped with a polarization filter. Congo red-stained slides were scored semi-quantitatively and blinded for clinical data by two independent observers (BPH and JB). Grading was similar to that described before [16] with minor modification: 0 (negative), 1+ (minute, <1% of surface area), 2+ (little, between 1% and 10%), 3+ (moderate, between 10% and 60%), and 4+ (abundant, >60%). Examples are shown in Figure 5.1. Scores of three smears per patient were used to achieve an overall score. When the overall score of a patient differed between the two observers, all three smears were reviewed and discussed to obtain consensus.

**Amyloid A protein quantification**

Amyloid A protein was quantified as described [14]. Remaining fat tissue (at least 20 mg) was collected, weighed, and washed three times for 10 minutes with phosphate-buffered saline to remove remnants of blood. Washed fat tissue was extracted in a solution of 6 M guanidine hydrochloride and 0.1 M Tris-HCl, pH 8.0, mixed thoroughly, and shaken overnight. The suspension was centrifuged at 10,000 x g for 10 minutes and the supernatant fat tissue extract was collected. Microtiter plates were coated with the IgG fraction of the SAA-reactive mouse monoclonal capture antibody Reu.86.5 (Hycult Biotechnology, Uden, The Netherlands). The plates were washed, followed by incubation of the samples. The plates were washed again, followed by incubation with the IgG fraction of the SAA1-reactive mouse monoclonal detection antibody Reu.86.1 (Hycult Biotechnology) coupled to horseradish peroxidase. After washing, the plates were incubated with the chromogen 3’3’5’5’tetramethylbenzidin (TMB, Carl Roth, Karlsruhe, Germany) dissolved in acetate buffer until the reaction was stopped by adding H$_2$SO$_4$. The absorption at 450-575 nm was read in an Emax microplate reader and amyloid A protein concentrations were calculated by SOFTmax$^\text{®}$ PRO software (Molecular Devices, Sunnyvale, USA) according to a standard curve of
purified SAA. The intra-assay and interassay coefficients of variation were both less than 10% and the lower limit of detection of the amyloid A protein in fat extract was 1.6 ng/mL extraction fluid. Amyloid A protein reference range of patients without AA amyloidosis was <11.6 ng/mg fat tissue [14, 16]. Blood levels of SAA

**Figure 5.1.** Examples of Congo red (CR)-scored fat smears in normal light (red-stained deposits) and polarized light (green birefringence), bar length 200 μm. A. Grade 0 (negative). B. Grade 1+ (minute, <1% of surface area), the arrow points to Congo red-positive material. C. Grade 2+ (little, between 1% and 10%). D. Grade 3+ (moderate, between 10% and 60%). E. Grade 4+ (abundant, >60%).
were measured using ELISA (basal control values <4.2 mg/L) as described [14, 17] using the World Health Organization reference standard [18].

Statistical analysis
Statistical analysis was performed by using the statistical package GraphPad Prism, version 4.02 (GraphPad Software Inc., San Diego, CA, USA). Receiver operating characteristic (ROC) curve analysis was used to visualize different possible cut-off points of amyloid A protein values of fat tissue. The unpaired t test was used to detect differences between two groups. One-way ANOVA was used in combination with Bonferroni’s multiple comparison test to detect differences among multiple groups. Pearson test for correlation was used to detect correlations. In case of strongly skewed distribution, data were log transformed to approach normal distribution. Fisher’s exact test was used to calculate differences in 2 x 2 tables and Chi-square test was used for trend of 2 x k tables where appropriate. In all tests two-tailed P values <0.05 were considered significant.

RESULTS

Patients
One hundred and eighty-three patients with clinical AA amyloidosis from 27 sites in 13 countries were enrolled in the study. Twenty-five sites participated in the fat aspirate substudy. Ten fat aspirates (6%) were either missing or not analyzable and in 19 patients the amount of fat tissue (about 20-30 mg) was insufficient to allow for additional quantitative amyloid A protein quantification and was used only for Congo red-stained fat smears. The final substudy group comprised 154 patients from whom ample fat tissue (at least 50 mg) was available to be used for both Congo red staining and amyloid A protein quantification. The AA type of amyloid was confirmed by immunohistochemistry in tissue other than fat tissue in 83% of the 154 patients. Patients were subdivided in three disease categories: 93 with chronic arthritis, 25 with FMF, and 36 with a different chronic inflammatory disease or more than one of such diseases. Table 5.1 shows age and gender of the study group. The 29 patients in whom insufficient fat tissue was available for amyloid A protein quantification did not differ from the 154 of the study group with respect to gender, age, and disease category. However, all 29 patients belonged to 10 sites
(accounting for 82 patients enrolled) whereas sufficient fat tissue was obtained from the remaining 15 sites (accounting for 101 patients).

Table 5.1. Characteristics of 154 patients with AA amyloidosis and 354 controls.

<table>
<thead>
<tr>
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<th>AA amyloidosis</th>
<th>Controls</th>
<th>Amyloidosis</th>
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<tr>
<td></td>
<td>Arthritis</td>
<td>FMF</td>
<td>Other</td>
</tr>
<tr>
<td>Number</td>
<td>93</td>
<td>25</td>
<td>36</td>
</tr>
<tr>
<td>Median age (yr)</td>
<td>57</td>
<td>42</td>
<td>51</td>
</tr>
<tr>
<td>Age range (yr)</td>
<td>(23-77)</td>
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FMF, familial Mediterranean fever; other, other inflammatory diseases; local, localized amyloidosis; AL, systemic AL amyloidosis; ATTR, systemic ATTR amyloidosis.

Three hundred and fifty-four controls without AA amyloidosis were studied: 36 healthy controls, 79 patients with miscellaneous diseases unrelated to amyloidosis, 95 patients with chronic arthritis without amyloidosis, 87 patients with systemic AL amyloidosis, and 30 patients with systemic ATTR amyloidosis (see Table 5.1). Apart from minor bruising in some patients, all biopsies were taken without any complication.

Figure 5.2. Receiver operating characteristic curve of amyloid A protein concentration in fat tissue of 154 patients with clinical AA amyloidosis and 354 controls without AA amyloidosis. Some cut-off values (ng/mg fat tissue) are specifically shown.
Diagnostic performance of amyloid A protein quantification

In Figure 5.2 the ROC curve displays different cut-off values for amyloid A protein concentration in fat tissue. Area under the ROC curve was 0.97 (95% confidence interval (CI): 0.95-0.98). The currently used cut-off level of 11.6 ng/mg fat tissue as assessed in earlier studies [13, 15] was associated with sensitivity of 84% (95% CI: 77%-89%) and specificity of 99% (95% CI: 98%-100%) in the present populations of patients with clinical AA amyloidosis and controls.

Because of skewed distribution, amyloid A protein concentration values in fat tissue were log-transformed to obtain normal distribution. Amyloid A protein concentration in fat tissue appeared to be lower in men (mean 63 ng/mg) than in women (mean 315 ng/mg), as shown in Figure 5.3A. The sensitivity of the test was lower in fat samples from men (73%) than women (91%) (P<0.01). No correlation with age was found. In patients with FMF, amyloid A protein concentration in fat (mean 24 ng/mg) was lower than in patients with chronic arthritis (mean 280 ng/mg) or patients with other diseases (mean 151 ng/mg), as shown in Figure 5.3B. The sensitivity was lower in FMF patients (68%) than in patients with chronic arthritis (90%) (P<0.01). The sensitivity in patients with other diseases (77%) did not differ from the sensitivity in patients with FMF and arthritis, respectively.

In the group of patients with chronic arthritis, amyloid A protein concentration in fat was lower in men (mean 131 ng/mg) than in women (mean 394 ng/mg) (P<0.05) and this difference was also seen in the group with other diseases (mean 44 vs. 655 ng/mg for men and women respectively) (P<0.01). In the group of patients with FMF, amyloid A protein concentration in fat did not differ between men (mean 17.7 ng/mg) and women (mean 32 ng/mg) (P=0.41).

Semi-quantitative Congo red score

Congo red-stained slides were positive in 143 of the 154 patients, resulting in sensitivity of the Congo red method of 93% (95% CI: 88%-96%). Sensitivity of Congo red was 95% in women and 90% in men (difference not significant). Sensitivity was lower in FMF (80%) than in chronic arthritis (96%) (P<0.05) and did not differ from the group with other diseases (94%). Median Congo red score was lower for men (2+) than for women (3+) (P<0.0001) and lower for patients with FMF (1+) than for those with chronic arthritis (3+) or other diseases (3+) (P<0.0001).
Figure 5.3. Amyloid A protein concentration in fat tissue of patients with clinical AA amyloidosis. The dotted line marks the upper reference limit of controls (11.6 ng/mg fat). Asterisks (*), (**), and (***), represent P<0.05, P<0.001, and P<0.0001, respectively. The horizontal lines denote the means of the log-transformed values. A. Men (N = 63) and women (N = 91). B. Patients with chronic arthritis (N = 93), patients with FMF (N = 25), and patients with other diseases or more than one disease (N = 36). C. Congo red (CR) stain scores of fat tissue: CR negative (N = 14), CR 1+ (N = 25), CR 2+ (N = 34), CR 3+ (N = 55), and CR 4+ (N = 45).
The semi-quantitative Congo red method and quantitative amyloid A protein measurement were concordant: mean amyloid A protein concentration was 2.1, 10.1, 82, 383, and 1,393 ng/mg fat tissue for negative, 1+, 2+, 3+, and 4+ positive Congo red specimens, respectively (see Figure 5.3C). The median Congo red score for the 19 patients in whom only Congo red-stained specimens were available (3+) did not differ from the median score for the 154 patients (3+) of the study group (P=0.74).

**Figure 5.4.** Amyloid A protein concentration in fat tissue and concurrent serum amyloid A protein (SAA) concentration in blood in 354 controls without AA amyloidosis. The solid line is the linear regression line and the dotted line marks the upper reference limit (11.6 ng/mg fat).

**Amyloid A protein in fat tissue and SAA levels in blood of controls**

Amyloid A protein concentrations in fat tissue of 354 controls were used to assess specificity of different cut-off values, as shown in Figure 5.2. Concurrent SAA concentrations in blood of controls correlated positively (r 0.37, P<0.0001) with amyloid A protein concentrations in fat tissue within the reference range (Figure 5.4). Amyloid A protein concentration in fat tissue of controls did not correlate with age, and no differences were found between the sexes and among the various disease groups.
DISCUSSION

Amyloid A protein quantification in fat tissue (cut-off point 11.6 ng/mg) has a good diagnostic performance with high sensitivity (84%) and high specificity (99%) for detection of clinical AA amyloidosis. Noteworthy is that in this study sensitivity of Congo red-stained tissue is even somewhat higher (93%) than amyloid A protein quantification. Even in FMF, sensitivity of Congo red-stained fat tissue (80%) is much higher than reported [13]. Therefore the Congo red method retains its established place in the diagnosis of amyloidosis. However, to achieve this high sensitivity the Congo red method needs strict quality control of staining procedure, a high-quality microscope, as well as experienced and well-trained observers. The less subjective immunochemical quantification of amyloid A protein bypasses these specific demands for patients suspected to have AA amyloidosis.

By measuring the amyloid A protein content in a large number of patients with non-AA types of amyloidosis or with chronic inflammatory diseases without AA amyloidosis, it was possible to determine cut-off values to clearly differentiate patients with AA amyloidosis. Fat tissue appears to play a substantial role in inflammation, recently designated as metabolic syndrome [19], with adipocytes releasing such adipokines as leptin, resistin, adipsin, visfatin, and adiponectin [20], and with modulating factors such as apolipoprotein A-I [21], lipoxin A4 [22], and SAA [23]. In this respect it is interesting to recall the correlation in controls between amyloid A protein concentration in fat tissue and blood levels of SAA. The most likely explanation is contamination of fat tissue by SAA derived from direct contact with blood during fat aspiration. It is, however, conceivable that SAA is locally induced by the same circulating cytokines that stimulate SAA production in the liver [24, 25]. Therefore, knowing that a low concentration of amyloid A protein is always present in fat tissue of patients with AA amyloidosis unrelated to amyloid deposition, the cut-off values with high specificity are very important to avoid false positives.

The clinical value of this method was further supported by analyzing the fat aspiration biopsies of patients with confirmed AA amyloidosis. Lower amyloid A protein concentrations in fat tissue of FMF patients as well as lower sensitivity (68%) and lower median Congo red score (1+) than in the other groups of patients with chronic arthritis (3+) and other diseases (3+) all indicate that deposition in subcutaneous fat tissue in FMF is less prominent than in the other inflammatory
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diseases. It is therefore more difficult to detect AA amyloid in FMF than in chronic arthritis and this might explain lower sensitivity reported in the literature [12]. However, in this study sensitivity of amyloid A protein quantification of fat tissue is 68% and sensitivity of the Congo red method 80%. This means that two-thirds of FMF patients with clinical AA amyloidosis can be detected with this immunochemical method and one-third should be detected in another way, such as through a kidney biopsy. Therefore, we recommend that the first diagnostic step for detection of AA amyloidosis should be analysis of subcutaneous fat tissue, even in FMF patients.

Higher amyloid A protein concentration in fat tissue of women was an unexpected finding and may indicate influence of sex hormones on amyloid deposition in fat tissue. In mice the numbers of fat tissue macrophages are higher in females compared with males, but it is unknown whether the same holds true for humans [26]. The number of macrophages present in fat tissue is directly correlated with adiposity and with adipocyte size in both humans and mice [27, 28]. Fat tissue seems to be directly involved in AA amyloidogenesis and is therefore a suitable target for future research in this field.

In conclusion, amyloid A protein quantification in fat tissue has a good diagnostic performance with high sensitivity and high specificity and without any significant complications. Advantages are independence from staining quality and experience of observers, direct confirmation of amyloid AA type, and the possibility of quantitative monitoring of tissue amyloid over time. Although in men and in patients with FMF sensitivity is somewhat lower than in women and in patients with chronic arthritis, amyloid A protein quantification in fat tissue is a valuable technique currently being utilized in specialized centers for detection of AA amyloidosis. General availability of the technique may make it an appropriate first step for early detection of clinical AA amyloidosis.

ACKNOWLEDGEMENTS

In addition to the authors, the following investigators participated in the Eprodisate For AA Amyloidosis Trial Group study: Gartnavel General Hospital, Glasgow, UK: J.A. Hunter; Le Mans General Hospital, Le Mans, France: X. Puéchal; University Hospital Germans Trias I Pujol, Badalona, Spain: X. Tena Marsa; University
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Hospital Bellvitge Llobregat, Barcelona, Spain: J. M. Valverde Garcia; Mayo Clinic, Rochester, USA: A. Dispenzieri; Hospital Claude Huriez, Lille, France: E. Hachulla; Hospital Clinic, Barcelona, Spain: J. Munoz-Gomez; Bnai Zion Medical Center, Haifa, Israel: I. Rosner; Boston University, Boston, USA: L.M. Dember; Tenon Hospital, Paris, France: G. Grateau; Hospital Clinico San Carlos, Madrid, Spain: J.A. Jover. We thank A. Herrera-Gayol for critically reviewing the manuscript.

The study was supported in part by FDA Orphan Products Development (OPD) grant # FD-R-002007.

D. Garceau and W. Hauck are employees of Neurochem Inc. All other authors declare that they do not have conflicts of interests. There is no financial support or other benefits from commercial sources for the work reported on in the manuscript, nor any other financial interests that any of the authors may have, which could create a potential conflict of interest or the appearance of a conflict of interest with regard to the work.

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