A quantitative method for detecting deposits of amyloid A protein in aspired fat tissue of patients with arthritis

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Abstract

Objective—To describe a new, quantitative, and reproducible method for detecting deposits of amyloid A protein in aspired fat tissue and to compare it with smears stained with Congo red.

Methods—After extraction of at least 30 mg of abdominal fat tissue in guanidine, the amyloid A protein concentration was measured by a monoclonal antibody-based sandwich ELISA.

Results—The concentrations in 24 patients with arthritis and AA amyloidosis (median 236, range 1.1–8530 ng/mg tissue) were higher (p<0.001) than in non-arthritic controls, uncomplicated rheumatoid arthritis, and other types of systemic amyloidosis (median 1.1, range 1.1–11.6 ng/mg tissue). Patients with extensive deposits, according to Congo red staining, had higher concentrations than patients with minute deposits.

Conclusion—This is a new, quantitative, and reproducible method for detecting deposits of amyloid A protein in aspired fat tissue of patients with arthritis, even when minute deposits are present as detected in smears stained with Congo red.

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About 3% to 10% of the patients with rheumatoid arthritis develop AA amyloidosis in the course of their disease.1,2 Deposits of amyloid A protein are found in many tissues and in the walls of blood vessels throughout the whole body. These amyloid A protein deposits are derived from serum amyloid A protein (SAA), an acute phase reactant, which is produced by the liver during inflammation. If accumulation of amyloid A protein deposition cannot be stopped or delayed, serious renal and gastrointestinal problems will arise, and most patients will die within two to four years.3,4 However, if the underlying inflammation is adequately suppressed, further loss of renal function and increase of proteinuria can be stopped and survival will improve.5–7 Therefore, AA amyloidosis should be diagnosed as early as possible.

Amyloid is diagnosed by the typical green birefringence in polarised light of a tissue biopsy specimen stained with the Congo red dye. Subsequent immunohistochemistry with anti-AA antibodies establishes the amyloid A nature of the amyloid involved.8 However, minute deposits of amyloid are hard to detect in Congo red stained biopsy specimens, and can be overlooked easily.8 A quantitative biochemical measurement of the amyloid A protein concentration in tissue might therefore improve the diagnostic yield of a biopsy in an independent way. An additional advantage will be the characterisation of the amyloid as the amyloid A protein type. Amyloid A protein has already been quantified in small samples (10–60 mg) of mouse and human necropsy tissues of the spleen.9 However, the clinician who treats patients with arthritis needs a procedure that is easy to perform routinely during life, which can be repeated at intervals, and with results that are reproducible and can be obtained within days. In patients, abdominal subcutaneous fat tissue is very appropriate for this purpose: this tissue is easy to obtain by aspiration, and smears are frequently found to be positive for amyloid by staining these with Congo red.10

The aim of this study was to develop a new, clinically usable, quantitative, and reproducible method for detecting deposits of amyloid A protein in aspired fat tissue of patients with arthritis. The new method was tested in patients with arthritis and AA amyloidosis, in non-arthritic controls, in patients with rheumatoid arthritis without AA amyloidosis, and in patients with other types of systemic amyloidosis. In all patients and controls, the new method was compared with the standard method for detecting amyloid deposits in smears of fat tissue stained with Congo red.

Methods

STUDY DESIGN

We describe a monoclonal antibody-based sandwich enzyme linked immunosorbent assay (ELISA) for measuring the amyloid A protein concentration in extracts of human abdominal fat tissue. This ELISA for quantifying the amyloid A protein concentration was tested in various groups of patients and the results were compared with the semi-quantitative scores of fat tissue smears stained with Congo red. The influence of the concentration of serum amyloid A protein in simultaneously obtained blood samples was investigated as well as the effect of the duration of the AA amyloidosis, the duration of the arthritis, and the age of the patients. Immunoblotting of the fat extracts was used to determine the size of the amyloid A protein fragments involved.

PATIENTS

The new method was tested from January 1997 on February 1998 in 24 patients with arthritis and biopsy confirmed AA amyloidosis (AA
Detecting deposits of amyloid A protein in aspirated fat tissue of patients with arthritis

The causes of the arthritis were rheumatoid arthritis in 17 patients, ankylosing spondylitis in three patients, juvenile chronic polyarthritis in two patients, psoriatic arthritis in one patient, and SAPHO (an acronym derived from synovitis, acne, pustulosis, hyperostosis, and osteitis) in another patient. Three groups served as controls: 22 members of the hospital staff and patients without arthritis (Control group), 25 patients with active rheumatoid arthritis for many years without clinical signs of amyloidosis (RA group), and 25 patients with other types of systemic amyloidosis (19 with the AL type and six with familial amyloidosis of the ATTR type) and a variable order and semi-quantitatively: (0), no apple-green birefringence detectable; (1+), minute deposits; (2+), moderate deposits; (3+), extensive deposits. Subsequently, the three slides of each individual patient were scored simultaneously to obtain one final score for the individual patient. When the two blinded investigators scored a patient differently, the three slides were discussed to obtain a final score for the patient.

MICROSCOPY

At least four visible fragments of fat tissue were put on each of three glass slides and crushed into a single cell layer by pressing a second slide perpendicularly to the first. The three smears were dried in the air at room temperature, fixed with acetone, and stained with alkaline Congo red dye according to Puchter. The affinity of tissue for Congo red was analysed by the apple-green birefringence in polarised light using the Olympus BX 50 microscope, 100 Watt. Two independent and blinded investigators (JB and BPCH) scored all slides in a random order and semi-quantitatively: (0), no apple-green birefringence detectable; (1+), minute deposits; (2+), moderate deposits; (3+) extensive deposits. Subsequently, the three slides of each individual patient were scored simultaneously to obtain one final score for the individual patient. When the two blinded investigators scored a patient differently, the three slides were discussed to obtain a final score for the patient.

PREPARATION AND EXTRACTION OF THE FAT TISSUE ASPIRATES

The fat tissue was collected from the syringe and separated from the sodium citrate fluid, weighed (wet weight), and washed three times for 10 minutes with 1 ml of phosphate buffered saline (PBS) to remove possible remnants of blood. The washed fat tissue was extracted in 1 ml of a solution of 0.1 M TRIS HCl, pH 8.0, and 6 M guanidine hydrochloride and this suspension was shaken continuously at room temperature overnight. The suspension was centrifuged at 10 000 × g for 10 minutes and the supernatant fat tissue extract was collected. The total protein concentrations in the fat tissue extracts were measured with the Pyrogallol red method (Instruchemie, Hilversum, the Netherlands) and expressed in albumin equivalents. The pellets were stained with

### Table 1 Patients' characteristics, serum acute phase proteins, and fat tissue measurements

<table>
<thead>
<tr>
<th>Control</th>
<th>RA</th>
<th>AA</th>
<th>AL/ATTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>22</td>
<td>25</td>
<td>24</td>
</tr>
<tr>
<td>Men/women</td>
<td>11/11</td>
<td>9/16</td>
<td>5/19</td>
</tr>
<tr>
<td>Age (y)</td>
<td>43 (24–70)†</td>
<td>69 (42–82)</td>
<td>63 (9–88)</td>
</tr>
<tr>
<td>Duration of arthritis (y)</td>
<td>NA</td>
<td>20 (2–57)</td>
<td>22 (9–41)</td>
</tr>
<tr>
<td>Duration of amyloidosis (months)</td>
<td>NA</td>
<td>NA</td>
<td>9 (0–139)</td>
</tr>
<tr>
<td>Serum acute phase proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAA concentration (mg/l)</td>
<td>0.4 (0.4–146)‡</td>
<td>9.3 (3.1–295)</td>
<td>14 (0.4–229)</td>
</tr>
<tr>
<td>CRP concentration (mg/l)</td>
<td>2 (1–198)*</td>
<td>15 (2–210)</td>
<td>17.5 (1–84)</td>
</tr>
<tr>
<td>Amyloid A (µg/mg fat tissue)</td>
<td>1.1 (1.1–4.2)‡</td>
<td>1.1 (1.1–4.2)‡</td>
<td>236 (1.1–850)</td>
</tr>
<tr>
<td>Amyloid A (µg/mg protein)</td>
<td>0.11 (0.01–0.88)‡</td>
<td>0.11 (0.01–0.88)‡</td>
<td>31.5 (0.11–651)</td>
</tr>
<tr>
<td>Total wet weight (mg)</td>
<td>87 (31–278)</td>
<td>88 (32–361)</td>
<td>107 (30–415)</td>
</tr>
</tbody>
</table>

Data are median and (range). RA = rheumatoid arthritis without AA amyloidosis, AA = arthritis with AA amyloidosis, AL/ATTR = AL amyloidosis and ATTR amyloidosis. CRP = C reactive protein, SAA = serum amyloid A protein. NA denotes not applicable.

*p<0.05, †p<0.01, and ‡p<0.001 versus the AA group.
Congo red and extracted again to assess the efficiency of the extraction procedure.

**QUANTIFICATION OF THE AMYLOID A CONCENTRATION OF FAT TISSUE EXTRACTS**

The concentration of amyloid A protein was measured in the fat tissue extracts by a monoclonal antibody-based sandwich ELISA as described previously for serum samples. Human SAA was purified from the HDL3 fraction of acute phase serum. The purified apo-SAA was linked to helix pomatia haemocyanin and injected into Balb/c mice to produce monoclonal antihuman–SAA antibodies. One monoclonal antibody Reu.86.2 (ICN Biomedicals, Zoetermeer, the Netherlands) displays a high affinity for tissue deposits of amyloid A protein in immunohistochemistry. Two other monoclonal antibodies Reu.86.5 and Reu.86.1 demonstrate high affinity for purified SAA and for purified amyloid A protein both in ELISA and in immunoblotting techniques. Monoclonal antibody Reu.86.5 reacts with all acute phase subtypes of SAA, whereas Reu.86.1 reacts only with SAA1, the major SAA subtype. Reu.86.5 and Reu.86.1 were used in the sandwich ELISA.

Microtitre plates (HB, FB, Corning Costar, Badhoevedorp, the Netherlands) were coated at room temperature on an EASIA shaker (500 rpm) with the IgG fraction of the pan-reactive capture antibody Reu.86.5 (diluted to 0.5 µg/well in 0.01 M PBS, pH 7.4) during one hour. Then the plates were washed with the washing solution of 0.025 M TRIS-HCl, pH 8.0, 0.15 M NaCl, and 0.05% Tween-20. This was followed by incubation of the samples in serial dilutions, starting with 1:20. Incubations were done in duplicate at room temperature on the EASIA shaker in 0.01 M PBS, pH 7.4, 1% BSA, and 0.05% Tween-20 in a final volume of 0.1 ml/well during one hour. The plates were washed, followed by incubation with the IgG fraction of the SAA1 reactive detection antibody Reu.86.1 coupled to horseradish peroxidase (diluted to 0.2 µg/well in 0.01 M PBS, pH 7.4). One mg of the chromogen 3′3′5′5′ tetramethylbenzidin (TMB, Carl Roth, Karlsruhe, Germany) was dissolved in 11 ml 0.1 M acetate buffer, pH 6.0, and 0.004% H2O2. After washing, the plates were incubated with the chromogen solution at room temperature until the reaction was stopped after 20 minutes by the addition of 1 M H2SO4. The absorption at 450–575 nm was read in an Emax microplate reader and the concentrations were calculated by SOFTmax PRO software (Molecular Devices, Sunnyvale, USA). Reconstitution of an exact quantity of purified apo-SAA into normal plasma provided the ultimate standard of the assay. Recently the international standard for SAA protein (0.15 IU per ampoule, WHO code 92/680) has been published, and we recalculated the standard of our assay according to this WHO standard. No effects were seen of sample storage, repeatedly freezing and thawing, or the addition of increasing concentrations of guanidine hydrochloride in the assay (up to 0.75 M). The intra-assay and interassay coefficients of variation were both less than 10%; the lower limit of detection of the amyloid A protein was 1.6 ng/ml (or 1.6 µIU/ml).

**SERUM ACUTE PHASE PROTEINS**

Blood concentrations of SAA were measured by ELISA as described above. Basal control values of healthy controls are below 4.2 mg/l. Blood concentrations of C reactive protein (CRP) were measured by ELISA. No effects were seen of sample storage or the addition of increasing concentrations of guanidine hydrochloride in the assay (up to 0.75 M). Basal control values of healthy controls are below 2.1 mg/l.

**IMMUNOBLOTTING OF FAT TISSUE EXTRACTS**

Fat tissue extracts of the patients and controls were applied to a mini-gel format (Mini-Protean II, Biorad, Venendaal, the Netherlands) of a 15% sodium dodecyl sulphate poly acrylamide gel (SDS-PAGE), according to the method of Laemmli. The proteins were separated by this SDS-PAGE technique and electroblotted onto a nitro-cellulose sheet. Remaining binding sites were blocked with 4% milk powder dissolved in 0.01 M PBS, then incubated with the pan-reactive antibody Reu.86.5, followed by incubation with rabbit antimouse immunoglobulin coupled to horseradish peroxidase (Dakopatts, Copenhagen, Denmark), and finally visualised with the chemiluminescence technique (SuperSignal Substrate, Pierce, Rockford, IL, USA). Rainbow molecular weight markers were used in the range from 2.35 to 46 kDa (Amersham, Buckinghamshire, UK).

**STATISTICAL METHODS**

The k statistic was used to measure the interobserver agreement of the two blinded investigators who scored the Congo red stained slides. Statistical analysis was performed by using the statistical package GraphPad Prism, version 2.01 (GraphPad Software Inc, San Diego, CA, USA). Non-parametric tests were used all the time. The Mann-Whitney or Student t test for correlation was used in patients with AA amyloidosis to detect correlations between the amyloid A protein concentration and fat tissue measurements between the patients with AA amyloidosis and the other three groups. These tests were also used for differences of concentrations of amyloid A protein in fat tissue between patients with AA amyloidosis having more or less amyloid in the fat smears stained with Congo red. The Spearman test for correlation was used in patients with AA amyloidosis to detect correlations between the amyloid A protein concentration and variables such as age, disease duration, and acute phase proteins. In all tests two tailed p values < 0.05 were considered significant.

**Results**

The aspiration of fat was a gentle and almost painless procedure, without any adverse effects
Detecting deposits of amyloid A protein in aspirated fat tissue of patients with arthritis

The amyloid A protein concentration (ng/mg tissue) of aspirated fat tissue of 22 controls, 25 patients with rheumatoid arthritis without AA amyloidosis (RA), 24 patients with arthritis and AA amyloidosis (AA), and 25 patients with AL or ATTR amyloidosis (AL/ATTR). Horizontal lines indicate median values. The limit of detection is 1.1 ng/mg tissue. The asterisk (*) indicates the difference (p < 0.001) between the patients with arthritis and AA amyloidosis and each of the three other groups.

The two investigators did not detect any amyloid in the Congo red stained smears, the median amyloid A protein concentration increased parallel to this score, as shown in figure 2. Deposition of amyloid was absent (0) in two, minute (1+) in eight, moderate (2+) in six, and extensive (3+) in eight patients. The concentration of amyloid A protein was higher (p < 0.05) in the patients with extensive (3+) deposits than in the patients with minute (1+) deposits (median 1240 v 110 ng/mg). When amyloid was present, its presence was not always detected in all three glass slides of a patient. Amyloid was overlooked four times in one of the three glass slides, twice by each observer, namely in three patients with minute (1+) deposition and in one patient with moderate (2+) deposition.

The amyloid A protein concentration of fat tissue of the 17 patients with AA amyloidosis and rheumatoid arthritis (median 358 ng/mg) did not differ significantly from the seven patients with other types of arthritis (median 144 ng/mg). In none of the 24 patients with AA amyloidosis a correlation was found between the amyloid A protein concentration of fat tissue and the age of the patients, the duration of the AA amyloidosis and the arthritis, and the blood concentrations of SAA and CRP. Table 1 shows the blood concentrations of CRP and SAA.
weight markers were chosen from 2.35 to 46 kDa (Amersham, Buckinghamshire, UK).

polyacrylamide gel, immunoblotted, detected with the monoclonal antibody Reu.86.5 raised

amyloidosis (lane 5), and of a patient with AL amyloidosis (lane 7) run on 15%

amyloidosis (lane 4, 6, and 8), of a patient with rheumatoid arthritis without AA

extracts (lane 3–8) of a healthy control (lane 3), of patients with arthritis and AA

Figure 4 SDS-PAGE of non-acute phase serum (lane 1), acute phase serum (lane 2), fat

pellets were lower than 4.2 ng/mg of the origi-

protein concentrations in the re-extracts of these

pellets were stained with Congo red. No amy-

amyloid was observed in the Congo red stained

of the fat tissue with guanidine, the remaining

polyclonal and monoclonal anti-SAA antibodies. The third advantage is its

availability and constant quality of

material can be used directly in the assay instead

of first being lyophilised. The second advantage

is the general availability and constant quality of

the assay by using monoclonal instead of polyclonal antibodies. The third advantage is its

applicability to aspirated fat tissue of live patients instead of necropsy tissues. However, a

possible disadvantage of the assay may be the lack of reactivity of the SAA1 reactive mono-

clonal antibody Reu.86.1 with SAA2. Although other techniques can detect amyloid A deposi-

tion in fat tissue of live patients, such as immunodiffusion,22 immunoblotting,23 and im-

munofluorescence microscopy,24 these techni-

ques provide qualitative instead of quantitative

results. Having quantitative instead of qualita-

tive information concerning amyloid A protein may be important for monitoring disease

progression or the results of treatment. This will

be studied in the near future.

The immunoblotting technique of this study also provided qualitative results that confirm

the presence of amyloid A protein-like material in the fat extracts (see fig 4). Amyloid A protein

has a molecular weight of about 8 kDa, whereas its secretory precursor SAA has a molecular

weight of about 12 kDa. Immunoblotting with polyclonal and monoclonal anti-SAA antibod-

ies showed several bands with molecular
gests that this quantitative measurement may represent an estimation of the amount of amy-

loid present in fat tissue. The highest value of amyloid A protein was 515 µg/mg protein (see

table 1), which would mean that amyloid A protein can constitute up to 51% of all protein

in fat tissue.

Both sensitivity and specificity of the method for detecting AA amyloidosis seem to be

appropriate, although this should be confirmed in a larger group of patients with AA amyloidosi-

s of variable duration and severity, as well as in larger control groups. If the prevalence of

AA amyloidosis is 5% in patients with rheumatoid arthritis, the figures found for sensitivity,

92% (95% confidence intervals 73%, 99%), and for specificity, 100% (97.5% confidence intervals 95%, 100%), will yield a positive pre-

dictive value of 100% and a negative predictive value of 99%. The coefficient of variation

between the amyloid A protein concentration on either side of the umbilicus (within the

patient) was 22%. This within subjects coeffi-

cient of variation shows the good reproducibil-

ity of the method. The interobserver agreement

of the standard Congo red method was good (k

of the slide scores was 0.61) to very good (k

of the final scores was 0.88). However, it appeared

to be useful to score three glass slides of an indi-

vidual patient because in some patients (espe-

cially those with minute deposits of amyloid)

amyloid was overlooked in one of the three glass

slides by one of the two observers.

The new method is not the first published

assay for the quantification of amyloid A protein

in tissue. Amyloid A protein has already been

quantified by inhibition ELISA in small samples

(10–60 mg) of mouse and human necropsy tis-

sues of the spleen.10 However, our new sandwich

ELISA offers at least three important advan-

tages. The first advantage is that the extracted

material can be used directly in the assay instead

of first being lyophilised. The second advantage

is the general availability and constant quality of

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Figure 4  SDS-PAGE of non-acute phase serum (lane 1), acute phase serum (lane 2), fat

elements (lane 3–8) of a healthy control (lane 3), of patients with arthritis and AA

amyloidosis (lane 4, 6, and 8), of a patient with rheumatoid arthritis without AA

amyloidosis (lane 5), and of a patient with AL amyloidosis (lane 7) run on 15%
Detecting deposits of amyloid A protein in aspirated fat tissue of patients with arthritis

weights ranging between 10 and 4 kDa, indicating the presence of amyloid A protein and some of its degradation products. Although monoclonal antibody Reu.86.1 does not recognise SAA2 and its fragments, it recognised the same bands as the pan-reactive monoclonal antibody Reu.86.5 and the polyclonal antibody RA-29. It remains speculative whether the 12–14 kDa band (see lane 4 of fig 4) represents “fresh” or “ongoing” deposition of SAA into amyloid, dimers of smaller amyloid A fragments, or pollution with SAA from accidently obtained peripheral blood. The absence of this band in controls with raised serum SAA concentrations make the latter explanation less likely.

Concentrations of amyloid A protein in fat tissue above the detection limit were found in 18% of the 72 controls (see fig 1). In RA patients without AA amyloidosis, the SAA blood concentrations were higher in patients with measurable amyloid A protein levels in fat tissue than in those without. Although the tissue was washed vigorously, pollution with SAA from accidently obtained peripheral blood (with concentrations about 100 times higher than those of the fat tissue) will be the most likely explanation. Theoretically, however, some amyloid A protein has to be present in fat tissue as a substrate for the generation of amyloid A fibrils. In this respect, the presence of measurable amyloid A protein in fat tissue in patients with high serum SAA concentrations might be related to a normal role in fat tissue of SAA as an HDL3-associated apolipoprotein. If this is the case, the pathophysiological phenomenon of amyloid A deposition in fat tissue might be linked to a derangement of the normal function of SAA.

Amyloid deposition is a dynamic process that can progress, stabilise, and regress. The dynamics of this process has been documented by clinical examination, organ function assessment, echography, and serum amyloid P component (SAP) turnover studies. We hypothesise that quantification of the amyloid A protein concentration of tissue on regular occasions will similarly reflect the accumulation, stabilisation or even regression of the amyloid deposited. Abdominal subcutaneous fat tissue seems to be very suitable for this purpose, because it is easy to obtain by aspiration and it can be obtained repeatedly. The combination of the Congo red stain with the quantitative measurement of amyloid A in fat tissue may thus become the microscopic counterpart of SAP scintigraphy, the best currently available tool to monitor ongoing deposition, stabilisation or regression of amyloid in the whole body. It could be used to monitor the effect of treatment. If a partial response or even complete remission of AA amyloidosis is achievable, we will need some well defined tools to measure it.

In conclusion, this study shows that quantification of the amyloid A protein concentration of abdominal subcutaneous fat aspirates in patients with arthritis is a new, gentle, and reproducible procedure that discriminates between patients with and without AA amyloidosis. An advantage of the new method is that minute deposits of amyloid can be detected more easily than with the standard Congo red method. Whether this method will detect amyloid earlier than the Congo red method has to be studied in a prospective way. It is also an excellent method for differentiating between AA amyloidosis and other types of systemic amyloidosis. Patients with extensive amyloid A deposits in the fat smear have higher concentrations of amyloid A protein than those with minute deposits. Therefore this new method may have the potential for monitoring actual deposition of amyloid A protein in tissue of patients with AA amyloidosis.

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Vesalius 1543: The eleventh plate of the muscles.
Vesalius provides no introductory remarks to this plate. The dissection has been carried a stage further by the reflection, in the case of the torso, of the latissimus dorsi which is seen hanging down from its insertion on the left side of the figure. Serratus posterior superior and inferior are crudely represented, no doubt due in part to the difficulty of expressing such delicate muscles in woodcut.