Chapter 10

Summary and future perspectives
INTRODUCTION

Amyloidosis is the name of a group of diseases, all characterized by deposition of protein fibrils with a beta-pleated sheet structure. This structure is held responsible for the affinity of amyloid for Congo red dye and the resistance to proteolysis. Amyloid deposition may be localized (restricted to one tissue or organ) or systemic (deposition throughout the body). Three types of systemic amyloidosis are important to be recognized by the clinician: AA, AL, and ATTR amyloidosis. AA amyloidosis is caused by deposition of serum amyloid A protein (SAA), an acute-phase protein which concentration is chronically increased in blood of patients with severe longstanding inflammation. AL amyloidosis is caused by deposition of a lambda or kappa immunoglobulin free light chain that is produced by a monoclonal plasma cell dyscrasia. ATTR amyloidosis is caused by deposition of mutated transthyretin (TTR) that is the result of an inherited point mutation or by deposition of wild type transthyretin in very old age. Signs and symptoms vary considerably among the three types and the choice of treatment differs completely [1, 2].

Amyloid is a histological diagnosis and its presence can be detected in a Congo red-stained tissue specimen. The Congo red-stained amyloid deposits show apple-green birefringence when viewed in polarized light. Aspiration of subcutaneous abdominal fat tissue is a very simple and useful technique to obtain tissue that can be used for the detection of amyloid [1].

SAA, kappa or lambda light chains, and TTR are so-called precursor proteins. Such a precursor protein is characteristic for a particular type of amyloid and immunohistochemistry (using antibodies directed against the different precursor proteins) is used routinely to detect the type of amyloid involved. Beside the characteristic precursor protein, all amyloid deposits invariably contain other constituents, such as glycosaminoglycans (GAGs) and serum amyloid P component (SAP). SAP is a pentraxin that binds in a calcium-dependent way to all amyloid deposits [2]. After labeling with $^{123}$iodine, $^{123}$I- SAP scintigraphy has been used for detection and evaluation of systemic amyloidosis [3].

This thesis has been focused on two diagnostic techniques in the field of amyloidosis: Part I. A fully monoclonal antibody-based ELISA for the quantification of amyloid A protein in fat tissue. Chapters 2-5 describe diagnostic studies using the amyloid A protein ELISA for fat tissue analysis of patients with systemic amyloidosis. Part II. Radiolabeled serum amyloid P component ($^{123}$I-SAP) for
assessing organ involvement and “amyloid load”. Chapters 6-9 describe diagnostic scintigraphic and retention studies using $^{123}$I-SAP.

The remainder of the current chapter contains two sections: the first section (summary) introduces the different chapters and describes their most important results and conclusions, the second section (future perspectives) discusses some of the clinical implications and future developments.

SUMMARY

After a short introduction, this thesis starts in chapter 2 with the description of the development of a new, reproducible, and fully monoclonal ELISA for the quantification of serum amyloid A protein (SAA). The lowest detection limit of the assay was 8 μg/L allowing its use in biological fluids and in in-vitro studies. The 95% upper limit of the basal reference interval for serum of healthy controls was 4.3 mg/L. SAA and CRP concentrations differed among diseases and SAA concentrations were relatively higher than CRP especially in patients treated with glucocorticoids.

In chapter 3 the new ELISA was used for the quantification of amyloid A protein in guanidine extracts of aspirated subcutaneous abdominal fat tissue. The concentrations in 24 patients with arthritis and AA amyloidosis were higher than in controls without arthritis, controls with uncomplicated rheumatoid arthritis, and controls with other types of systemic amyloidosis. The upper reference limit of controls without AA amyloidosis appeared to be 11.6 ng/mg fat tissue. Patients with extensive deposits, as reflected by semi-quantitatively assessed fat smears stained with Congo red, had higher amyloid A protein concentrations than patients with minute deposits.

In chapter 4, fat tissue specimens of 112 Egyptian patients with longstanding rheumatoid arthritis were screened for the presence of amyloid by Congo red-stained smears. Eight patients appeared to have amyloid deposits in their fat tissue, resulting in a prevalence of amyloid in Egyptian patients with longstanding RA of about 7%. Quantification of the concentration of amyloid A protein in fat by ELISA (using the upper reference level that had been established in Dutch controls) identified six of the eight patients with AA amyloidosis, whereas none of the remaining 104 patients had increased concentrations. Contrary to the
expectations, proteinuria was not a discriminating feature in the eight patients, whereas long disease duration, constipation, bronchopulmonary symptoms, and a moderate to low number of red blood cells might help to identify the arthritis patients with amyloid in this population.

In chapter 5, the diagnostic performance of the ELISA for quantification of amyloid A protein in fat tissue was studied in 154 of 183 patients with clinical AA amyloidosis and in 354 controls. Amyloid A protein quantification in fat tissue proved to be a sensitive (84%) and specific (99%) detection method. Advantages are its independence from staining quality and observer experience, the direct confirmation of amyloid AA type, and its potential for quantitative monitoring of tissue amyloid over time. Amyloid A protein quantification and semi-quantitative Congo red grading were concordant. Men had lower amyloid A protein values than women and patients with Familial Mediterranean Fever (FMF) had lower values than patients with arthritis or other inflammatory diseases. These remarkable differences between both sexes and between inflammatory diseases cannot be explained easily. The capacity of fat cells to produce SAA as well as the mechanisms that produce and deposit amyloid A fibrils in fat tissue may be influenced directly by sex hormones and cytokines. This seems to be an interesting area for future fundamental research.

In chapter 6, serum amyloid P component (SAP) was isolated on a column of pyruvate-rich agarose from the blood of healthy donors. The method turned out to be effective and the purity of isolated SAP was high (>99%), so the isolated SAP was suitable for use in scintigraphy studies of patients with systemic amyloidosis.

In chapter 7, kinetic turnover studies of $^{123}$I-SAP were performed in 49 patients with systemic amyloidosis of the AA and AL types. Systemic amyloidosis was characterized by accelerated initial clearance of $^{123}$SAP from plasma and increased interstitial exchange rate and extravascular retention. These findings reflect reversible binding of radiolabeled SAP to amyloid deposits and provide clinically useful information for diagnosis, monitoring of the effect of therapy and for the assessment of the prognosis in patients with systemic amyloidosis.

In chapter 8, the diagnostic accuracy and additional information of $^{123}$I-SAP scintigraphy was studied in 167 patients with systemic amyloidosis. Diagnostic sensitivity of SAP scintigraphy for systemic AA, AL, and ATTR amyloidosis was 90%, 90%, and 48% respectively, and specificity was 93%. The distribution of amyloid was less diverse in AA than in AL type. Myocardial uptake
was not visualized in any patient. Splenic amyloid was very frequent (80%) in AA and AL type, but rarely detected clinically (14%). Abnormal tracer uptake in the liver and kidneys correlated with disturbed liver function and proteinuria, respectively. Bone marrow uptake was specific for AL (21%), and was more frequent in AL kappa than AL lambda. Localized amyloid deposits were not imaged. It is concluded that SAP scintigraphy is diagnostic of amyloid in most patients with AA and AL type but to a lesser extent in patients with hereditary ATTR type, relating to differences in distribution and amyloid load among these disorders. It usually reveals more widespread organ involvement than is identified clinically, and certain patterns of distribution of SAP uptake are characteristic of particular fibril types.

In chapter 9, the diagnostic performance and prognostic value were studied of EVR$_{24}$, a simple parameter describing extravascular $^{123}$I-SAP retention after 24 hours. In this study the same patients with systemic amyloidosis were used as in chapter 8. The EVR$_{24}$ appeared to have no additional value to $^{123}$I-SAP scintigraphy in the detection of systemic amyloidosis. In AL amyloidosis the EVR$_{24}$ was strongly associated with organ involvement (the number of organs and the severity of involvement of liver and kidney) and with prognosis and appears to be a suitable indicator of the amyloid load in the body.

**FUTURE PERSPECTIVES**

The ultimate goal of these studies was to improve the tools for diagnosis and characterization of systemic amyloidosis and to obtain a better understanding of the amyloid load in the body of patients with systemic amyloidosis.

Measurement of the amyloid A protein concentration in fat tissue using ELISA shows high accuracy for diagnosing AA amyloidosis. Therefore this amyloid A ELISA applied to fat tissue is a reliable diagnostic tool that can be used for detection and characterization of AA amyloidosis. Immunochemical quantification of other amyloid constituents such as SAP, TTR, light chains, laminin, entactin, collagen IV, apolipoprotein E, and GAGs may also be considered, although one should keep in mind that the extraction procedure of proteins from amyloid fibrils by guanidine may induce conformational changes of some of the epitopes that will result in decreased antigen recognition by the antibodies used. In case
immunochemical methods will fail to detect and quantify the proteins mentioned above, other specific chemical techniques, such as two-dimensional blotting in combination with mass spectrometry, should be considered [4]. Application of these techniques to fat tissue is at this moment already useful and may be even more promising for the detection of changes of amino acid sequence and composition, specific mutations and variants, and assessing relative amounts of mutations versus wild-type proteins. A first goal of these proteomic studies in the near future should be to detect and diagnose a particular type of amyloid with almost complete certainty and to exclude all other possible types with the same certainty. A second goal may be the unraveling of the role of wild-type protein, subtypes, variants, and mutated proteins, the role of enzymatic cleavage and other modifications of precursor proteins, and the role of other amyloid constituents such as SAP, laminin, entactin, collagen IV, and GAGs in amyloid fibril formation [5, 6].

Fat tissue can easily be obtained for analysis at regular intervals to monitor the actual course of amyloid deposition at tissue level. This monitoring of fat tissue may be useful in AL and ATTR amyloidosis because fat tissue in these diseases is not actively involved in the production of the precursor protein. Fat tissue in AA amyloidosis, however, may behave differently in this respect because of active production of SAA by adipocytes in fat tissue [7-9]. An in vitro monocyte culture system of amyloidogenesis has been developed in the mouse [10, 11]. A fully human cell culture model of amyloidogenesis, however, is not yet available [12]. Fat tissue cultures may provide the means to further elucidate the regulation of SAA synthesis, the processing of SAA into AA amyloid fibrils in a human system, the mechanisms of fibril deposition, amyloid toxicity in tissue, and the role of other amyloid components such as SAP.

$^{123}$I-SAP scintigraphy appears to be a very useful tool for diagnosing systemic AA and AL amyloidosis and in particular for showing involvement of some specific organs, such as liver, spleen, kidneys, adrenal glands, bone marrow, and joints [3]. The pattern of organ involvement in patients with AA amyloidosis as displayed using $^{123}$I-SAP scintigraphy shows remarkable similarity to the well-known mouse model. In that model the spleen is affected first followed by the kidney and other organs such as the liver [13]. $^{123}$I-SAP scintigraphy is currently the best method for detecting liver or spleen involvement, before other clinical signs such as organ enlargement or abnormal laboratory function tests have become apparent. This knowledge, as well as the utility of serum NT-proBNP [14], has been
added in our hospital to the current clinical consensus criteria concerning organ and tissue involvement [15]. The EVR$_{24}$, a simple parameter measuring extravascular tissue retention, appears to be a useful marker of severity of the amyloid load in patients with systemic AL amyloidosis. This marker may also be helpful for assessing the risks of chemotherapy in AL amyloidosis patients with heart involvement.

Until the introduction of $^{123}$I-SAP scintigraphy, regression of amyloidosis was supposed to occur occasionally as documented in biopsy studies of case reports, but convincing proof of regression was lacking. A real breakthrough of $^{123}$I-SAP scintigraphy is that this technique undoubtedly has shown that regression of the amyloid load of the body is indeed possible and should become a realistic goal. However, the amyloid load of the body is still more a theoretical concept than a practically measurable variable. To understand the dynamics of amyloid deposition and the background of therapy, it may be useful to construct a model of the total amyloid load of the body. The “sink” model - as everyone knows from daily life – might be such a way of looking at the amyloid load (Figure 10.1).

![Figure 10.1. The “sink” model of the total amyloid load of the body in patients with systemic amyloidosis. Tap, precursor producing process; A, amyloid growth; B, amyloid breakdown; C, the amount of fluid in the sink is the amyloid load.](image)

This simple model may illustrate the dynamics of amyloid deposition in the whole body as well as in individual organs. Variable A, amyloid growth, may be thought to
be a product of the increased concentration of the precursor protein in the blood and the deposition rate. Variable B, amyloid breakdown, is caused by a yet completely unknown mechanism and may differ considerably among individual patients and various organs. Variable C, the amyloid load, is reflected by signs and symptoms of the amyloidosis. The current aim of therapy is to reduce C. This should be done in the first place by elimination of A (decrease to zero) or in the second place by decreasing A or increasing B to such an extent that A becomes smaller than B, not only for the body as a whole, but also for the affected individual organs. The additional approach of reducing C should be to treat the signs and symptoms in such a way that the suffering caused by the amyloid load will decrease. A benefit of such a model is that it helps to focus our future research to study the dynamics of underlying mechanisms, such as the precursor production rate, the deposition rate, and the amyloid breakdown rate.

Although $^{123}$I-SAP scintigraphy and EVR$_{24}$ appear to be useful tools in clinical practice, further improvements should be sought for because $^{123}$iodine is expensive and SAP needs to be isolated and purified from the blood of healthy donors. Until now, the use of other isotopes has not been successful in replacing $^{123}$iodine. SAP is a relatively large protein of 127 kD and this high molecular weight may be held responsible for a reduced penetration of the basement membrane of blood vessels in cardiac tissue and for a reduced penetration of the intact blood-brain barrier. Smaller molecules than SAP with similar binding characteristics to amyloid are interesting because they will probably increase the current yield of scintigraphy in systemic amyloidosis, especially for detection of heart involvement. In case such molecules and other ones will be developed, they need to be compared with $^{123}$I-SAP scintigraphy as the current gold standard.

It should be said here that the prospects for many patients with systemic amyloidosis are still grim, although for some of the patients considerable progress has been made: e.g. the introduction of intensive chemotherapy with stem cell rescue in highly selected patients with AL amyloidosis [16], the introduction of liver allograft transplantation in highly selected patients with ATTR amyloidosis [17], and the effect of complete suppression of chronic inflammation in patients with AA amyloidosis [18, 19]. New developments are underway, such as the introduction of lenalidomide in AL amyloidosis [20], the recently started clinical trial with diflunisal in ATTR amyloidosis [21], and the GAG-mimetic drug eprodisate in AA amyloidosis [22]. Also other possible useful drugs should be mentioned here, such as IDOX
Important in this respect is the development of an accurate set of criteria for assessing progression, stable disease and regression of the amyloidosis as well as the underlying disease. Quantification in serum of precursor proteins such as SAA for AA amyloidosis and immunoglobulin free light chains for AL amyloidosis are very helpful for monitoring and assessing the activity of the underlying disease process [18, 19, 26]. A first set of clinical criteria for assessing clinical signs of (AL) amyloidosis has already been established by an International Consensus Committee in Tours in 2004 [15]. However, direct measurement and monitoring of the actual deposition of amyloid needs to be studied too. In this respect analysis of fat tissue at regular intervals in combination with SAP scintigraphy will probably yield important information at the microscopic as well as macroscopic level of amyloid deposition. This direct assessment of amyloid deposition will become increasingly important with further development of methods for therapeutic intervention. Especially in circumstances where the other criteria are not helpful, such as early detection of relapse of amyloidosis in case of only partial response of the underlying disease, direct assessment of amyloid deposition may have a key position.

I would like to finish with three comments. Firstly, I would like to thank the UMCG, the “Dutch Arthritis Association”, and the “Jan Kornelis de Cock Stichting” for the financial support that enabled us to do most of the research presented in this book. Secondly, I want to state that amyloidosis research really is a fascinating and promising area of research. Thirdly, I hope that effective treatment modalities that arrest and control these devastating diseases will be developed in the near future resulting in better prospects not only for selected groups, but for all patients with systemic amyloidosis.

REFERENCES


