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### Research Article

A transthyretin variant with a methionine for valine substitution at position 30 [TTR(Met30)] is found in Portuguese patients with familial amyloidotic polyneuropathy (FAP). Effective, rapid, small- and semimicro-scale (immunoblotting) procedures were developed to determine whether or not TTR(Met30) is present in the plasma of an individual subject. The immunoblotting procedure employs only 0.10 ml of serum and can serve as a reliable procedure for the screening of large numbers of persons for the presence of TTR(Met30). In family studies of seven FAP kindreds, TTR(Met30) was found in 21 out of 41 asymptomatic FAP offspring, and its presence was not related to either age or sex. Thus, the mutant TTR segregated in accordance with the known autosomal dominant mode of inheritance of FAP. Total plasma TTR levels were not reduced in asymptomatic FAP offspring who were carriers of TTR(Met30), and no difference was observed between carriers and noncarriers of the mutant TTR. The ratios of the variant to normal TTR in plasma were estimated in asymptomatic FAP offspring and were similar to those found in FAP patients. In contrast, TTR(Met30) was relatively enriched in cerebrospinal fluid samples from two FAP patients. The significance of this finding is not known, but might relate to the preferential deposition of amyloid in the nervous system in FAP. A limited study was conducted involving simultaneous [...]

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# Biochemical Marker in Familial Amyloidotic Polyneuropathy, Portuguese Type

## Family Studies on the Transthyretin (Prealbumin)-Methionine-30 Variant

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### Abstract

A transthyretin variant with a methionine for valine substitution at position 30 [TTR(Met<sup>30</sup>)] is found in Portuguese patients with familial amyloidotic polyneuropathy (FAP). Effective, rapid, small- and semimicro-scale (immunoblotting) procedures were developed to determine whether or not TTR(Met<sup>30</sup>) is present in the plasma of an individual subject. The immunoblotting procedure employs only 0.10 ml of serum and can serve as a reliable procedure for the screening of large numbers of persons for the presence of TTR(Met<sup>30</sup>). In family studies of seven FAP kindreds, TTR(Met<sup>30</sup>) was found in 21 out of 41 asymptomatic FAP offspring, and its presence was not related to either age or sex. Thus, the mutant TTR segregated in accordance with the known autosomal dominant mode of inheritance of FAP. Total plasma TTR levels were not reduced in asymptomatic FAP offspring who were carriers of TTR(Met<sup>30</sup>), and no difference was observed between carriers and noncarriers of the mutant TTR. The ratios of the variant to normal TTR in plasma were estimated in asymptomatic FAP offspring and were similar to those found in FAP patients. In contrast, TTR(Met<sup>30</sup>) was relatively enriched in cerebrospinal fluid samples from two FAP patients. The significance of this finding is not known, but might relate to the preferential deposition of amyloid in the nervous system in FAP. A limited study was conducted involving simultaneous analysis of both stored (collected in 1975) and fresh serum from 20 FAP offspring, all of whom had been asymptomatic in 1975. In every subject, the results obtained with the stored and the fresh serum samples were in agreement. Six of these subjects developed clinical FAP since 1975; TTR(Met<sup>30</sup>) was present in each of these subjects. These several studies strongly suggest that the presence of TTR(Met<sup>30</sup>) in plasma constitutes a predictive biochemical marker of FAP in the preclinical phase of the disease.

### Introduction

Human plasma transthyretin (TTR)<sup>1</sup> has been related to several forms of amyloidosis and particularly to the systemic heredo-

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1. *Abbreviations used in this paper:* AF<sub>p</sub>, amyloid fibril protein from Portuguese patients with FAP; CNBr, cyanogen bromide; CSF, cerebrospinal fluid; FAP, familial amyloidotic polyneuropathy; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; RBP, retinol-binding protein; T4, tryptic peptide 4 from normal TTR; T4\*, aberrant T4 from TTR(Met<sup>30</sup>); TTR, transthyretin; TTR(Met<sup>30</sup>), the variant TTR found in FAP.

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familial amyloidoses of autosomal dominant inheritance. Following the initial report by Costa et al. (1) that a protein component of the amyloid fibrils that accumulate in tissues of Portuguese patients with familial amyloidotic polyneuropathy (FAP) is closely related to TTR, we described the presence of a variant TTR in these patients (2, 3). This variant differs from normal plasma TTR in having a methionine for valine substitution at position 30. We found that this abnormal TTR circulates in relatively low concentrations in the plasma of FAP patients, and selectively deposits in tissues as amyloid fibrils. The same variant TTR [TTR(Met<sup>30</sup>)] has also been demonstrated in FAP patients of Swedish ancestry (4, 5), and in Japanese patients (6, 7). TTR variants having substitutions other than the one found at position 30 have also been reported in an FAP patient of Jewish origin (8, 9).

Detailed studies of the properties of the purified amyloid protein (AF<sub>p</sub>) from Portuguese patients with FAP showed that the protein resembled plasma TTR in forming a stable tetrameric structure, and in its binding affinities for both thyroxine and retinol-binding protein (RBP) (3). Although purified plasma TTR from FAP patients showed no differences from normal TTR with regard to a wide range of physical-chemical properties (10), it was shown (3) that the variant TTR(Met<sup>30</sup>) was present in plasma in these patients, together with larger amounts of normal TTR.

We now report the development of relatively simple small-scale and semimicro-scale procedures to determine whether or not TTR(Met<sup>30</sup>) is present in the plasma of any particular individual person. Using these procedures, the presence or absence of TTR(Met<sup>30</sup>) was determined in the members of several FAP kindreds, and particularly in a moderately large number of asymptomatic children of FAP patients. In some instances, the ratios of TTR(Met<sup>30</sup>) to normal TTR were estimated. In addition, studies were conducted on TTR(Met<sup>30</sup>) in cerebrospinal fluid (CSF) samples from two FAP patients, and on TTR in stored frozen serum samples collected in 1975 from then asymptomatic FAP offspring. The results reported here show that the presence of the variant TTR(Met<sup>30</sup>) can be used both for the diagnosis of the Portuguese variety of FAP, and for the identification of at-risk offspring in the preclinical phase of the disease.

### Methods

*Samples; subjects studied.* Serum samples (range of volumes 10–20 ml) were collected from the members of several FAP kindreds. Six of these kindreds each consisted of an FAP patient and his or her asymptomatic offspring; the latter comprised a total of 21 children, with ages ranging from 6 to 23 yr. None of these children of FAP parents had clinical evidence of FAP (11) or positive nerve or skin biopsies. In a seventh FAP kindred studied in more extensive detail, serum was collected from offspring of both affected and nonaffected members. This latter study comprised a total of 28 serum samples from individuals with ages ranging from 8 to 51 yr, and belonging to three different generations. A limited number of samples was also collected from FAP patients and some of their children from two other kindreds.

Other serum samples studied included those from individuals (both FAP patients and normal individuals belonging to FAP families), from whom serum samples (0.5–2 ml), collected in 1975 and stored frozen at  $-20^{\circ}\text{C}$  ever since, were available. A total of 20 such frozen and stored serum samples were studied. In addition, serum was obtained fresh from each of these same 20 individuals so that the results obtained with samples collected in 1984 could be compared with those obtained with sera collected in 1975.

CSF (10–15 ml) from two FAP patients and one normal subject was collected by lumbar puncture; sera from these two FAP patients were obtained concurrently.

**Isolation of TTR.** TTR was isolated from the individual serum samples (usual volume used: 10–15 ml) by two different small-scale and rapid procedures. In the study involving the six small FAP kindreds, a two-step procedure was employed that consisted of: (a) chromatography on Cibacron Blue F3-GA (Bio-Rad Affi-gel Blue, Bio-Rad Laboratories, Richmond, CA) with a linear salt gradient in 30 mM phosphate buffer, pH 7.0 (12); and (b) affinity chromatography on human RBP linked to Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as described for the isolation of the  $\text{AF}_p$  protein (3).

Other purified TTR samples (including those from the large FAP kindred) were obtained by a different two-step procedure involving: (a) ion-exchange chromatography on DEAE-cellulose employing a “batch” technique and using the same conditions as those previously used in the isolation of TTR from a large volume of FAP serum (10); and then (b) chromatography on Cibacron Blue agarose gel as described above. In the first step of this procedure, the serum was mixed with an equal volume of DEAE-cellulose (Whatman Inc., Clifton, NJ) that had been equilibrated with 0.05 M phosphate buffer, pH 7.6, containing 0.073 M NaCl; the unbound material was filtered through a Buchner funnel and the resin was washed with the same buffer until no protein could be detected in the eluate. The TTR (and other bound material) was then eluted with the same phosphate buffer containing 0.6 M NaCl.

Both procedures resulted in the isolation of TTR that was estimated to be  $\sim 90\%$  pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The recoveries obtained were of the order of 16% in the case of the RBP affinity column procedure and  $\sim 35\%$  with the second procedure.

Chromatography on Cibacron Blue agarose gel was also employed in the isolation of TTR from CSF samples. This procedure resulted in a TTR preparation that contained two minor contaminants (as revealed by SDS-PAGE analysis) that did not interfere with the structural studies carried out subsequently.

**Identification of TTR(Met<sup>30</sup>) using isolated TTR.** In order to determine the presence or absence of TTR(Met<sup>30</sup>), the TTR samples from individual persons, isolated as described above, were subjected to cyanogen bromide cleavage, followed by combined high performance liquid chromatography (HPLC) and SDS-PAGE analysis. The methods employed were similar to those reported previously for the structural studies of the amyloid protein  $\text{AF}_p$  (3). Thus, the TTR samples (500  $\mu\text{g}$  each) were digested with cyanogen bromide (CNBr) for 24 h and the resulting peptides were separated by HPLC on a  $\mu\text{Bondapak C}_{18}$  column (0.4  $\times$  30 cm) (Waters Associates, Millipore Corp., Milford, MA) with a linear gradient of 10–60% of acetonitrile in 0.05% trifluoroacetic acid. The large peptides eluting towards the end of the gradient (between 35 and 45 min) were collected and analyzed by SDS-PAGE, in 15% Laemmli gels (13), using a silver staining technique for detection (14) of protein/peptide bands.

**Identification of TTR(Met<sup>30</sup>) by immunoblotting.** For the detection of TTR(Met<sup>30</sup>) in much smaller aliquots (0.1–0.2 ml) of serum, a sensitive immunoblotting technique was developed. This semi-micro-method consisted of the following steps: (a) 100  $\mu\text{l}$  of serum was diluted (1/10, vol/vol) with 30 mM phosphate buffer, pH 7.0, and applied to a small Cibacron Blue agarose column (1 ml vol). The column was eluted with the same phosphate buffer until no more protein appeared in the eluate (zero absorbance at 280 nm); the eluate was then dialyzed against distilled  $\text{H}_2\text{O}$  and lyophilized. (b) The resulting preparation was digested with CNBr in 70% formic acid for 24 h, dialyzed against distilled  $\text{H}_2\text{O}$ , and

lyophilized. The CNBr digest was applied to 15% SDS gels, and electrophoresis was carried out according to the method of Dreyfuss et al. (15). The peptide fragments were then transferred onto nitrocellulose paper (0.2  $\mu\text{m}$ ) (Schleicher and Schuell, Keene, NH), according to the method of Towbin et al. (16). The blots were incubated with the IgG fraction purified from an anti-TTR antiserum and the bands were visualized by autoradiography using  $^{125}\text{I}$ -Protein A (New England Nuclear, Boston, MA) (17). The IgG fraction was purified by affinity chromatography on Protein A-Sepharose (Pharmacia Fine Chemicals).

**Ratio of TTR(Met<sup>30</sup>) to normal TTR in biological samples.** The ratio of the variant TTR(Met<sup>30</sup>) to normal TTR was estimated both in serum and in CSF samples by tryptic peptide mapping of the TTR isolated from the respective biological samples, using HPLC techniques as described previously (3). In brief, after reduction and alkylation with iodoacetic acid, the TTR samples were digested with trypsin (*N*-tosyl-L-phenylalanine chloromethyl ketone-treated from Worthington Biochemical Corp., Freehold, NJ) for 90 min with an enzyme-to-substrate ratio of 1:100; the reaction was stopped with an equal volume of 1 N HCl, followed by lyophilization. The tryptic peptides were separated on a  $\mu\text{Bondapak C}_{18}$  column (Waters Associates) using a phosphoric acid-acetonitrile system. Tryptic peptides T4\* and T4 (residues 22–34) (see reference 3) were identified by parallel analyses of standard samples of digests of  $\text{AF}_p$  and normal TTR, respectively. Peptide T4\* is the tryptic peptide 4 (residues 22–34) derived from the variant TTR(Met<sup>30</sup>); peptide T4 is tryptic peptide 4 from normal TTR. The relative quantities of these two peptides were calculated by integration of the areas of the corresponding peaks.

**Other methods.** Serum TTR and RBP concentrations were measured by single radial immunodiffusion using immunodiffusion plates purchased from Calbiochem-Behring Corp., La Jolla, CA. The rabbit anti-human TTR antiserum used in the immunoblots was prepared in this laboratory (10).

## Results

**Detection of TTR(Met<sup>30</sup>) in asymptomatic FAP children.** In order to address the question of whether TTR(Met<sup>30</sup>) circulates in the plasma of asymptomatic FAP children, we isolated TTR from individual small serum samples of 21 offspring of FAP patients. The presence of the variant TTR was determined by the detection of “abnormal” peptides (as compared with those obtained with normal TTR) by both HPLC and SDS-PAGE analysis, as we have described previously (3). Thus, upon CNBr treatment of amyloid protein  $\text{AF}_p$  and TTR from the plasma of FAP patients, two extra peptide fragments are produced (as compared with normal TTR): (a) an intermediate peptide, residues 14–30, which is readily observed on HPLC analysis of the CNBr digest; and (b) a larger fragment, residues 31–127, which can be visualized as a single peptide band of apparent molecular weight of  $\sim 12,000$  on SDS-PAGE analysis. We have never detected either of these fragments upon analysis of CNBr digests of normal TTR. In contrast, we have found both of these “abnormal” fragments in CNBr digests of TTR from every patient with clinical FAP whom we have studied (a total of 18 patients so far).

In our first study involving six small FAP kindreds, TTR(Met<sup>30</sup>) was detected in the plasma of slightly more than half of the asymptomatic offspring of FAP patients. The results are summarized in Table I. The variant TTR was found in 12 of the 21 offspring. Within this group of offspring there was no relationship of the presence (or absence) of the variant TTR with either age or sex. Thus, within the age range of offspring covered in this initial study (6–23 yr), the variant TTR was detected as early as 7 yr. Moreover, the 12 positive offspring included five males and seven females. The negative offspring (normal TTR only) comprised five males and four females.

Table I. Distribution of TTR(Met<sup>30</sup>) in Asymptomatic Offspring of FAP Patients\*

Kindred	FAP propositus	Asymptomatic offspring	
		TTR(Met <sup>30</sup> ) carrier	Noncarrier
A	32M	8F, 10M, 14F	6F, 12M
B	42F	19F	23F
C	45M	21M, 22M	14M, 16M, 23M
D	41M	15F, 15M‡	
E	42F	17F, 19F, 21M	6F, 13M
F	35F	7F	9F

\* In this table the numbers represent the age of the subjects studied; sex is indicated by F (female) and M (male).

‡ Dizygotic twins.

This initial study demonstrated that the frequency and distribution of the variant TTR in the FAP offspring were consistent with the autosomal dominant manner of inheritance typical of the Portuguese form of FAP (18). This apparent autosomal dominant form of inheritance was observed clinically in the full pedigrees of all of the families included in this study (data not shown).

This distribution was also evident on analysis of plasma TTR of offspring of affected members of a seventh large kindred with FAP. The results of the analyses carried out on this kindred are shown in Fig. 1. In this more extensive study, which included three generations of individuals with no clinical evidence of FAP, TTR(Met<sup>30</sup>) was detected in 9 out of 20 asymptomatic offspring of FAP patients. Moreover, the variant TTR was never found in offspring of nonaffected family members.

We measured the serum levels of TTR and of RBP of the offspring who were apparent carriers of the gene for TTR(Met<sup>30</sup>), and compared these levels with the corresponding levels of TTR and RBP of their apparently normal siblings. The results are

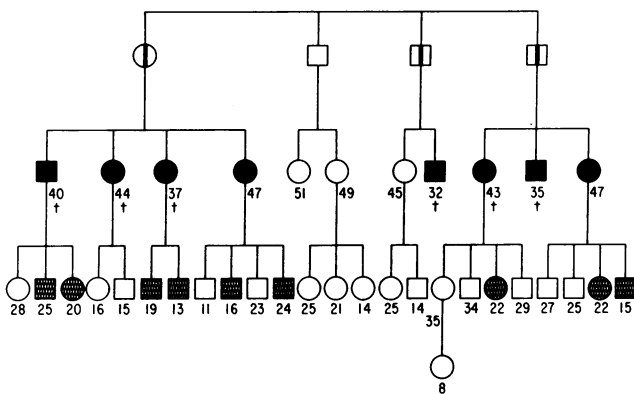


Figure 1. Distribution of TTR(Met<sup>30</sup>) in an FAP kindred. Symbols: □, ■, male; ○, ●, female; ■, ●, FAP patients both by clinical evidence and positive nerve biopsy; □, ○, healthy persons found to be noncarriers of TTR(Met<sup>30</sup>); ■, ●, asymptomatic carriers of the variant TTR. □, ○, not observed, dead, described as having the disease. The numbers indicate the ages of the offspring at the time the sera were collected, or at the time of death. The 28 subjects whose sera were analyzed in this study were all asymptomatic individuals: the healthy older members of the kindred (ages 51, 49, and 45), the 24 offspring of the FAP patients and these healthy subjects, and finally the 8-yr-old girl.

shown in Table II. TTR and RBP levels were normal in the asymptomatic FAP offspring who were carriers of the TTR(Met<sup>30</sup>) gene. The levels of TTR and of RBP in the TTR(Met<sup>30</sup>) carrier asymptomatic offspring did not differ significantly from the corresponding levels observed in their normal siblings.

Relative levels of TTR(Met<sup>30</sup>) in asymptomatic FAP offspring. The reduced levels of TTR(Met<sup>30</sup>) relative to normal TTR usually found in FAP patients (3) prompted us to investigate the relative levels of TTR(Met<sup>30</sup>) compared with normal TTR present in the plasma of asymptomatic FAP offspring who were carriers of the TTR(Met<sup>30</sup>) gene. For this study we used comparative tryptic peptide mapping, and considered the ratio of peptides T4\* to T4 to represent the ratio of the levels of TTR(Met<sup>30</sup>) to normal TTR present in the original plasma. We have found (reference 3 and unpublished observations) that peptide T4\* is always present in tryptic peptide maps of TTR from plasma of FAP patients and is always absent in parallel maps of normal TTR. In contrast, in AF<sub>p</sub> tryptic maps the normal peptide T4 is not detected and peptide T4\* is present in relatively large amounts.

Fig. 2 C shows a portion of a tryptic peptide map of an asymptomatic carrier of TTR(Met<sup>30</sup>). The ratio of the amount of peptide T4\* to peptide T4 (~0.55 in this particular subject) gives us an estimate of the relative amounts of TTR(Met<sup>30</sup>) and normal TTR present in plasma. Parallel tryptic maps of the amyloid protein AF<sub>p</sub> and of TTR from an FAP offspring who was not a carrier of TTR(Met<sup>30</sup>) are also shown. We measured this ratio in six additional asymptomatic carriers of TTR(Met<sup>30</sup>), and observed that the values varied a good deal from individual to individual. The results are presented in Table III. No correlation was found between the relative levels of the abnormal TTR and either age or total TTR serum levels. The ratios shown in Table III (mean [±SD] 0.38±0.16) were not different from the ratios we have found in FAP patients when assayed individually. Thus, in a study of three individual FAP patients, the mean (±SD) ratio of peptide T4\* to peptide T4 was 0.47±0.13. Hence, the percentage of the total plasma TTR represented by the variant TTR(Met<sup>30</sup>) was, on the average, 27% (±9%) for asymptomatic FAP offspring; for FAP patients, in this particular study, it was 32% (±6%).

Studies of TTR(Met<sup>30</sup>) in CSF samples. A preliminary study was carried out to examine the relative levels of the variant TTR compared with normal TTR in CSF from two FAP patients.

Table II. TTR and RBP Serum Levels in FAP Offspring\*

Subjects‡	n§	Age	Sex¶	TTR	RBP
				µg/ml	µg/ml
Carriers of TTR(Met <sup>30</sup> )	27	17.7±6.4 (6-33)	14F; 13M	274±69	41±16
Noncarriers of TTR(Met <sup>30</sup> )	27	19.3±8.3 (6-35)	13F; 14M	294±67	34±11

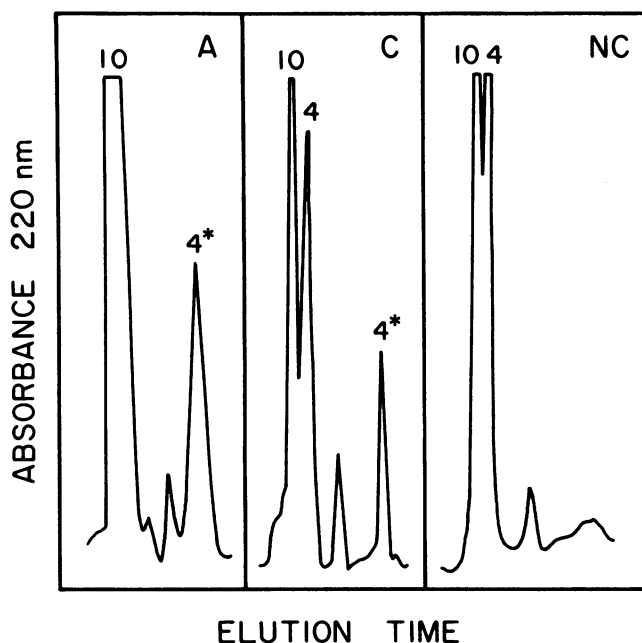
\* Values are mean±1SD.

‡ The study comprised 54 asymptomatic offspring from nine FAP kindreds (seven of these kindreds are represented in Table I and Fig. 1).

§ n, the numbers of subjects in each group.

|| The range of ages of the subjects studied are given in parentheses.

¶ 14F, 14 females; 13M, 13 males.



**Figure 2.** Comparative tryptic peptide mapping of AF<sub>p</sub> protein (A) and of serum TTR from asymptomatic FAP offspring. C and NC are, respectively, TTR samples from a carrier and a noncarrier of TTR(Met<sup>30</sup>). 500 µg of each protein was digested with trypsin for 60 min and the digest was analyzed by HPLC to separate the tryptic peptides on a C<sub>18</sub> µBondapak column. Peptides were detected by the 220-nm absorbance of the effluent at a full scale of 0.1. In this figure, only a portion of each of the observed peptide maps, comprising the regions where peptides 10, 4, and 4\* elute, is shown. The identities of the peaks are indicated by the numbers (10, 4, 4\*) above them.

Using the methodology described above, we conducted tryptic peptide mapping on individual TTR samples isolated from CSF. The ratio of peptide T4\* to peptide T4 was concurrently estimated on TTR isolated from serum samples collected concurrently from the same patients. The ratios of TTR(Met<sup>30</sup>) to normal TTR found in the CSF samples were much higher than those found in the sera of the same individuals. Thus the ratios found for CSF were 1.4 and 1.1, whereas the corresponding ratios for the serum samples were 0.35 and 0.47, respectively. These results suggest that TTR(Met<sup>30</sup>) is present in relatively high amounts in the CSF of FAP patients. Future studies, with CSF from additional patients, will be needed to explore this issue in a definitive way.

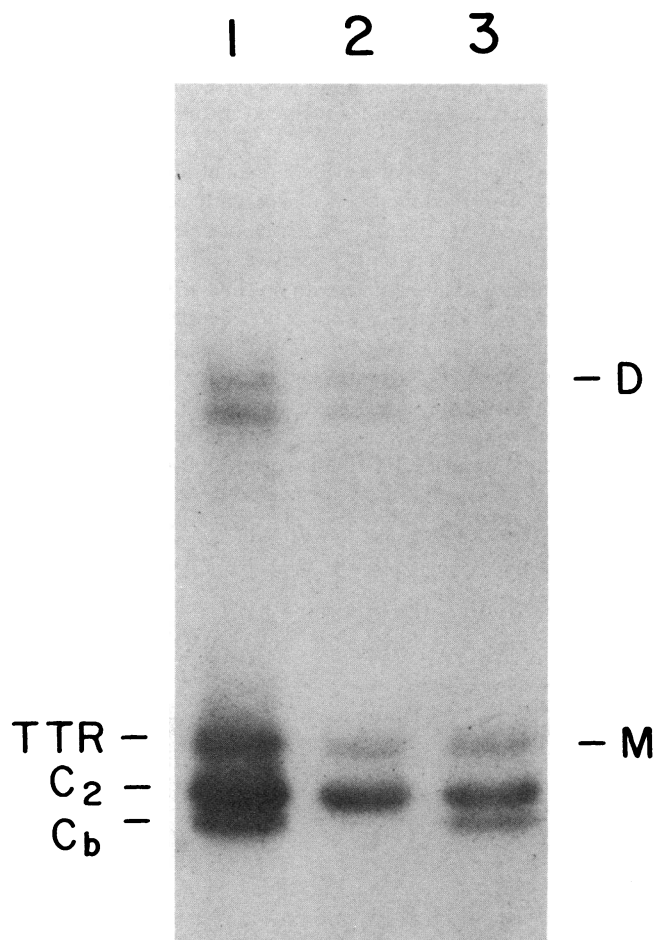
**Table III.** Relative Levels of TTR(Met<sup>30</sup>) and Normal TTR in Asymptomatic FAP Offspring Who Were TTR(Met<sup>30</sup>) Carriers

Age	Peptide ratio: T4*/T4	Serum TTR
yr		µg/ml
14	0.2	184
19	0.2	298
25	0.32	247
24	0.35	353
22	0.4	384
16	0.55	278
20	0.63	247

**Detection of TTR(Met<sup>30</sup>) by immunoblotting.** In order to investigate the presence or absence of TTR(Met<sup>30</sup>) in stored frozen samples, a sensitive immunoblotting microtechnique was developed, as described in the Methods section.

Fig. 3 shows the results obtained by this immunoblotting procedure. The samples shown were derived from serum of a patient with clinical FAP, aged 26 (lane 3), and from her serum collected in 1975 when she was 16 yr old and was in the pre-clinical phase of the disease (lane 1). Lane 2 shows TTR from a control serum sample from an unaffected 45-yr-old member of an FAP family. Someone this age is unlikely to develop the disease. Fig. 3 shows that TTR(Met<sup>30</sup>) is not present in this individual.

To test further the applicability of this immunoblotting technique as a diagnostic and screening procedure, we tested all the serum samples from the seven kindreds studied above (Table I and Fig. 1). For all the samples studied, the data obtained by immunoblotting agreed with the results obtained previously by



**Figure 3.** Detection of TTR(Met<sup>30</sup>) by an immunoblotting procedure. Fresh serum (100 µl) from an FAP patient (lane 3) and serum collected in 1975 from the same person (lane 1) and from an unaffected member of a FAP kindred (lane 2) were subjected to immunoblotting analysis, as described in Methods. In this figure, C<sub>2</sub> represents a normal CNBr fragment (residues 14–127 of TTR) and C<sub>b</sub> represents the “abnormal” smaller CNBr peptide (residues 31–127) derived from TTR(Met<sup>30</sup>) (3). TTR, intact TTR. The marks on the right, labeled M and D, represent the migration positions for the TTR monomer and dimer, respectively.

HPLC and SDS-PAGE analysis of the CNBr-treated isolated protein. Thus, this immunoblotting technique constitutes a reliable and easy semi-microtechnique for both the diagnosis of FAP and the detection of carriers of TTR(Met<sup>30</sup>).

*Retrospective-prospective study.* Definitive proof that the presence of the variant TTR can be used as a preclinical biochemical marker of the disease will require longitudinal studies in families to determine whether offspring of patients with FAP who have the variant TTR(Met<sup>30</sup>) will always develop the clinical disease, whereas offspring without the variant TTR remain healthy. Since this kind of longitudinal prospective study will require many years of future follow up, we have turned to frozen stored sera to try to obtain preliminary data concerning this question. In this limited retrospective-prospective study, the presence or absence of TTR(Met<sup>30</sup>) in both fresh and stored serum (collected in 1975) from the same individual was investigated by the immunoblotting technique illustrated in Fig. 3. The samples tested were obtained both from FAP patients and from asymptomatic younger offspring of FAP patients. In addition, sera collected from a healthy 45-yr-old unaffected member of an FAP family were also analyzed.

The results obtained in this study are summarized in Table IV. Several conclusions can be drawn from these data. First of all, in every one of the 20 subjects, the results obtained with the stored serum sample collected in 1975 agreed with those obtained with the fresh sample collected 9 yr later. Thus, either

Table IV. Detection of TTR(Met<sup>30</sup>) in FAP Patients and Their Asymptomatic Offspring, in Fresh and Stored Old Sera

Subject no.	Current clinical status*	Age in 1984	TTR(Met <sup>30</sup> )†	
			Fresh serum (1984)	Stored serum (1975)
		yr		
1	FAP	25	+	+
2§	FAP	26	+	+
3	FAP	27	+	+
4	FAP	34	+	+
5	FAP	35	+	+
6	FAP	42	+	+
7	Normal	18	-	-
8	Normal	20	+	+
9	Normal	20	+	+
10	Normal	22	-	-
11	Normal	23	-	-
12	Normal	23	+	+
13	Normal	23	+	+
14	Normal	23	+	+
15	Normal	25	-	-
16	Normal	25	+	+
17	Normal	27	-	-
18	Normal	28	-	-
19	Normal	32	-	-
20	Normal	45	-	-

\* None of the current FAP patients had clinical evidence of the disease when the sera were first collected in 1975.

† +, present; -, not present.

§ These samples are represented in Fig. 3.

TTR(Met<sup>30</sup>) was present or was absent in both samples from each subject. Secondly, TTR(Met<sup>30</sup>) was present in every one of the six subjects who developed clinical FAP between 1975 and 1984, and had been present in these subjects in the preclinical (1975) phase of the disease. Third, of the 14 currently clinically healthy subjects studied, six have TTR(Met<sup>30</sup>) and eight do not. The six positive subjects currently range in age from 20 to 25, and are hence still too young to be expected to have developed the clinical disease. These six persons require further follow-up to determine whether they will develop clinical disease as they become older.

## Discussion

The major goals of the studies reported here were to determine whether the presence of the variant TTR(Met<sup>30</sup>) in plasma can be used as a predictive biochemical marker of FAP in the preclinical phase of the disease, and to develop effective procedures for the screening of large numbers of persons for the presence of TTR(Met<sup>30</sup>). Information was also sought about the levels of TTR in plasma in FAP offspring, and about the relative levels of the variant TTR in asymptomatic carriers of the TTR(Met<sup>30</sup>) gene.

As reported here, we have developed effective, relatively simple and rapid, small-scale and semimicro-scale (immunoblotting) procedures to determine whether or not TTR(Met<sup>30</sup>) is present in plasma (or in other biological fluids) from individual subjects. These procedures involve the demonstration of aberrant CNBr peptides for the identification of the presence of TTR(Met<sup>30</sup>). The small-scale procedure employed TTR isolated from 10–15 ml of serum by one of two two-step procedures. The even simpler immunoblotting procedure employed only 0.10 ml of serum subjected to a single, rapid chromatographic step before digestion with CNBr. Identical results were obtained with both procedures in all the serum samples studied in the seven kindreds reported in Table I and Fig. 1.

Recent reports have indicated that the variant TTR(Met<sup>30</sup>) circulates in plasma in some asymptomatic offspring of FAP patients of various ethnic origins (19–21). Two kinds of studies, reported here, were conducted to explore the effectiveness and validity of using the presence of the variant TTR as a preclinical marker of the disease. First, family studies were carried out with both affected and nonaffected members of seven FAP kindreds (Table I and Fig. 1). The expected distribution of a biochemical marker among asymptomatic offspring for a disease, such as FAP, that displays an autosomal dominant manner of inheritance (18) is 50%. In the seven kindreds reported here, TTR(Met<sup>30</sup>) was found in 21 out of 41 asymptomatic children of FAP patients. Within this group of FAP offspring there was no relationship of the presence of TTR(Met<sup>30</sup>) with either age or sex. The identification of a 7-yr-old TTR(Met<sup>30</sup>) carrier suggests, in fact, that the variant TTR might possibly circulate since early life. Thus, the segregation of the mutant TTR among asymptomatic FAP offspring was fully in accordance with the known autosomal dominant mode of inheritance of the disease. The results of these family studies hence support the conclusion that the presence of TTR(Met<sup>30</sup>) can serve as an effective preclinical biochemical marker of FAP.

Additional evidence in support of this conclusion was obtained from a second kind of study, namely, the limited retrospective-prospective study reported here. Simultaneous analysis of both stored (9-yr-old) and fresh serum from six current FAP

patients showed that in all of these patients the mutant TTR(Met<sup>30</sup>) had been present in the circulation in the preclinical (1975) phase of the disease. None of the four oldest currently healthy subjects (ages 27–45 in 1984) was a TTR(Met<sup>30</sup>) carrier. Further longitudinal follow-up of the younger currently healthy subjects (ages 18–25) will be needed to determine whether the six TTR(Met<sup>30</sup>) positive subjects develop clinical FAP as they become older, whereas the noncarriers of the mutant TTR remain healthy. With the information available, we anticipate that this will indeed be the case.

We have previously found (10) that total plasma TTR levels are significantly reduced in patients with FAP. In contrast, plasma TTR levels were not reduced in asymptomatic FAP offspring who were carriers of the variant TTR(Met<sup>30</sup>), and no difference was observed between carriers and noncarriers of the mutant TTR (Table II). One possible explanation for these findings might be that selective deposition of the abnormal TTR(Met<sup>30</sup>) in the tissues of FAP patients leads to a reduction in plasma total TTR level. The ratios of abnormal to normal TTR observed in sera from individual asymptomatic FAP offspring (Table III) did not, however, provide support for this hypothesis. Similar, relatively low ratios of TTR(Met<sup>30</sup>) to normal TTR were found in both the asymptomatic FAP offspring and in the FAP patients. Moreover, in the FAP offspring, there was no correlation of this ratio with either age or total TTR levels.

The relative amount of TTR(Met<sup>30</sup>) has also been estimated in asymptomatic carriers of other ethnic origins (20–22). Since a specific antibody against the intact abnormal protein is still not available, the methods used for its estimation all rely on the cleavage of the mixture of the two TTRs (normal and abnormal) in plasma, followed by the quantitative analysis of the abnormal fragments by either peptide mapping (as reported here), amino acid sequence analysis (20), or radioimmunoassay (22). These procedures all give approximate values for the level of the mutant TTR in asymptomatic carriers, and the values found by the different procedures may vary. As in the present studies, a relatively low percentage of the variant TTR relative to the normal protein was found in one asymptomatic carrier of Swedish origin (20); higher levels of this TTR variant were, however, reported in Japanese FAP offspring (21). It is possible that the accumulation of the abnormal TTR in the tissues occurs gradually in the course of life, in such a way that it is not deposited in aggregate size, so as to permit its observation as amyloid, until many years have elapsed. It has been suggested that a neuropathic process precedes the formation of amyloid in the Portuguese type of FAP, based on alterations found in the nerve fibers of some asymptomatic children (23). It is also possible that only when sufficient variant TTR has deposited in tissues does sufficient perturbation of TTR metabolism occur to lead to a reduced plasma level. Information is clearly needed about the mechanism of amyloid formation in FAP, as well as about the factors that affect the synthesis and degradation rates of the amyloid-forming protein.

CSF samples from two FAP patients were found to be much more enriched in TTR(Met<sup>30</sup>), relative to normal TTR, than were the serum samples obtained from the same patients. Thus, the ratio of TTR(Met<sup>30</sup>) to normal TTR was approximately threefold higher in CSF than in serum. It is well known that normal CSF is relatively enriched in TTR (24, 25), and TTR has been localized in the choroid plexus in immunohistochemical

studies (26) (Kato, M., D. R. Soprano, K. Kato, and D. S. Goodman, manuscript submitted for publication). Very recent studies in rats have shown that the brain contains significant amounts of TTR mRNA, which is localized in the choroid plexus ([27], and Kato, M., D. R. Soprano, K. Kato, and D. S. Goodman, manuscript submitted for publication.) It was suggested (27) that TTR in the brain and CSF may result from de novo synthesis, and that TTR may play a significant physiological function, as yet unknown, within the nervous system. It is possible that the neuropathy typical of FAP is due in part to a derangement in this putative normal function of TTR, caused by the mutant, structurally abnormal TTR(Met<sup>30</sup>). In any event, the relatively high concentration of TTR(Met<sup>30</sup>) in CSF is certainly in accord with the heavy infiltration of amyloid observed in the nervous system of FAP patients (25), as well as with the prominent neuropathy characteristic of the disease.

The goal of establishing the presence of TTR(Met<sup>30</sup>) as a predictive biochemical marker of FAP has practical implications with regard to the screening of asymptomatic members of FAP kindreds. FAP is highly prevalent in certain areas of Portugal and constitutes a serious national health problem. There are several hundred known families with FAP in Portugal (28), and it has been estimated that there is a much larger number (of the order of 3,000 or more) of asymptomatic persons at risk for FAP. These persons represent a population that could be screened for the presence or absence of TTR(Met<sup>30</sup>), in order to determine which individuals are carriers of the genetic defect that leads to FAP. The screening of such a large population requires a rapid, sensitive, and reliable method for the detection of TTR(Met<sup>30</sup>). The immunoblotting method reported here meets these requirements. In this procedure several samples can be processed simultaneously in a relatively rapid manner. The fact that identical results were obtained with the more elaborate small-scale procedure and with the immunoblotting method shows that the immunoblotting technique is reliable for use as a diagnostic tool. Furthermore, we envisage its use for the detection of TTR(Met<sup>30</sup>) in body fluids other than serum, such as CSF and amniotic fluid, which we have tested. Since the immunoblotting procedure does not require special equipment, it can have a widespread use in epidemiologic studies of FAP in other countries. Such an epidemiologic program is being started in Portugal with the aim of identifying the carriers of the TTR(Met<sup>30</sup>) gene.

Once the TTR(Met<sup>30</sup>) carriers are identified, genetic counselling approaches could be employed to reduce the continued propagation of the FAP genetic defect into the next generation. This would constitute a reasonable public health strategy, in the absence of effective therapeutic approaches to the disease. In addition, identification of TTR(Met<sup>30</sup>) carriers could, as previously pointed out (3), be important in future attempts at prevention or early treatment of this disease.

Another approach for controlling the continued spread of the TTR(Met<sup>30</sup>) gene would focus on the prenatal diagnosis of the presence of this genetic defect. This approach requires the use of recombinant DNA techniques, such as those recently described by Japanese investigators (29). We have also developed these diagnostic techniques, and plan to use them in the future for the prenatal diagnosis of FAP. The use of recombinant DNA techniques will also be important in future studies of the factors that modulate the expression of the variant and normal TTR genes.



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