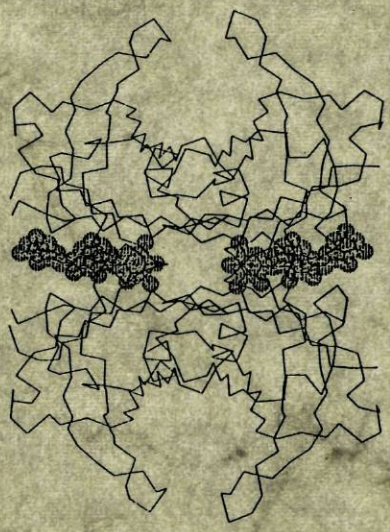
MARIA DO ROSÁRIO RODRIGUES DE ALMEIDA

CARACTERIZAÇÃO GENÉTICA E BIOQUÍMICA DE VARIANTES MOLECULARES DE TRANSTIRRETINA



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Dissertação de candidatura ao grau de Doutor em Ciências Biomédicas, especialidade de Bioquímica, apresentada no Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto.

Orientador - Professora Doutora Maria João Gameiro de Mascarenhas Saraiva (Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal).

PORTO

1995

Ao Pedro e ao Rui,

naturalmente.

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No cumprimento do Decreto-Lei 388/70, esclarece-se serem da nossa responsabilidade a execução das experiências que estiveram na base dos resultados apresentados (excepto quando referido em contrário) assim como a sua interpretação e discussão.

ABREVIATURAS

-ASO- allele specific oligonucleotides, oligonucleotidos especificos de alelo.

-DNA- desoxiribonucleic acid, ácido desoxirribonucleico.

-FAC- familial amyloidotuc cardiomyopathy, cardiomiopatia amiloidótica familiar.

-FAP- familial amyloidothic polyneuropathy, polineuropatia amiloidótica familiar.

-HA- hereditary amyloidosis, amiloidose hereditária.

-HPLC- high performance liquid chromatography, cromatografia líquida de alta resolução.

-HIEF- hybrid isoelectric electrophoresis focusing, electroforese de focagem isoeléctrica híbrida.

-IEF- isoelectric electrophoresis focusing, electroforese de focagem isoeléctrica.

-PAF- polineuropatia amiloidótica familiar, familial amyloidothic polyneuropathy.

-PCR- polymerase chain reaction, reacção de polimerização em cadeia.

-RBP- retinol binding protein, proteina de ligação ao retinol.

-RFLP- restriction fragment lenght polymorphism, fragmento de restrição de tamanho polimórfico.

-SSA- Senile systemic amyloidosis, amiloidose sistémica senil.

-T_4- tiroxina.

-TBG- thyroxine binding globulin, globulina de ligação à tiroxina.

-TTR- transtirretina.

ÍNDICE

1.RESUMO 11

2.INTRODUÇÃO 14

2.1.Estrutura e expressão do gene da TTR

2.1.1.O gene da TTR 15

2.1.2.Expressão do gene da TTR 16

2.1.3.Regiões reguladoras da expressão do gene da TTR 18

2.2.Estrutura e fisiologia da TTR 19

2.2.1.Estrutura da TTR 19

2.2.2.Funções fisiológicas da TTR 23

2.2.2.1.Importância da TTR no metabolismo da vitamina A 23

2.2.2.2.Interacção TTR-RBP 25

2.2.2.3.Importância da TTR no transporte de hormonas da tiróide 26

2.2.2.4.Ligação da T_4 à TTR 27

2.2.2.5.Interacção da TTR com o receptor 29

2.2.3.Outras funções fisiológicas 30

2.2.3.1.Interacção com o ácido retinóico 30

2.2.3.2.Ligação à noradrenalina 30

2.2.3.3.Inibição da produção de interleuquina-I em monócitos e células endoteliais 30

2.2.3.4.Ligação a pterinas 31

2.2.3.5.Ligação a globinas e metais 31

2.2.3.6.Função tímica 32

2.2.3.7.Interacção da TTR com a proteína ß-amilóide 32

2.2.4.Interacção da TTR com algumas drogas e agentes farmacológicos 32

2.2.4.1.Compostos fenólicos 32

2.2.4.2.Compostos bifenílicos 33

2.2.4.3.Compostos tiromiméticos - Milrinona e 3,3'-diiodotironina 33

2.2.4.4.Flavonoides 35

2.2.4.5.Compostos polihalogenados bifenílicos (PHBs) 36

2.3.Variantes moleculares de TTR 37

2.3.1.Variantes amiloidogénicas 40

2.3.1.1.A TTR Met 30 e a Polineuropatia Amiloidótica Familiar (PAF) 41

2.3.1.2.Recorrência da mutação que origina a TTR Met 30 42

2.3.2.Variantes não amiloidogénicas 45

2.3.3.Variantes com alteração da afinidade de ligação à tiroxina 45

2.3.3.1.Variantes com elevada afinidade de ligação 45

2.3.3.2.Variantes com baixa afinidade de ligação 46

2.3.4.Variantes de TTR com alteração de ligação ao RBP 48

2.4.Amiloidogénese 49

2.4.1.Componentes dos depósitos de amilóide 49

2.4.2.TTR e amiloidogénese 50

2.5.Nota final 52

3.PROJECTO DE INVESTIGAÇÃO: OBJECTIVOS, RESULTADOS E DISCUSSÃO 53

3.1.Objectivos 54

3.2.Resultados e discussão 55

4.TRABALHO EXPERIMENTAL 61

4.1.Identification of five different transthyretin (TTR) variants associated with familial amyloidotic polyneuropathy (FAP): TTR Leu 64 and TTR Tyr 77 and first report of TTR Ala 49, TTR Gln 89 and TTR Ala 71 62

4.2.Identification of transthyretin variants associated with cardiac amyloidosis: TTR Ile 122 and first report of TTR Thr 45 andTTR Leu 68 81

4.3.Identification of non-amyloidogenic transthyretin variants in heterozygotic and compound heterozygotic individuals: TTR THR 109 and TTR Met 119 and first report of TTR Asn 90 and TTR Arg 102 95

4.4.Haplotype analysis of common transthyretin mutations 116

4.5.Thyroxine binding in transthyretin compound heterozygotic individuals:

the presence of TTR Met 119 increases T_4 binding affinity 128

4.6.Thyroxine binding to natural and recombinant TTR variants 145

5.CONCLUSÕES, IMPACTO E PERSPECTIVAS 158

6.BIBLIOGRAFIA 163

1. RESUMO

A transtirretina (TTR) é uma proteína transportadora de hormonas da tiróide e de retinol (vitamina A), através da formação do complexo RBP -TTR (RBP-retinol binding protein). Para além da sua função biológica, a TTR tem sido associada a diversas amiloidoses hereditárias que podem originar neuropatias, cardiomiopatias e/ou deposição de amilóide no vítreo sendo variável o grau de envolvimento dos tecidos alvo.

A esta heterogeneidade clínica corresponde também uma heterogeneidade molecular, sendo conhecido um grande número de variantes de TTR. Estas variantes são todas resultantes de mutações pontuais no gene de TTR e diferem da TTR normal por substituição de um resíduo de aminoácido. Não se conhecem no entanto, as razões pelas quais a substituição de um aminoácido induz a precipitação da proteína sob a forma de fibras de amilóide. Assim, propusemo-nos contribuir para o conhecimento dessas razões através da identificação de novas variantes de TTR e de estudos funcionais dessas variantes, nomeadamente estudos de ligação à tiroxina (T_4).

As variantes foram identificadas por análises de proteína e de DNA a que se seguiu a implementação de métodos de diagnóstico molecular através de análise de RFLPs e hibridização com sondas específicas para o alelo mutante. Estes métodos de diagnóstico são extremamente importantes para o diagnóstico diferencial das amiloidoses associadas à TTR e para detecção de portadores assintomáticos.

Identificamos 12 variantes moleculares de TTR incluindo variantes neuropáticas, cardiomiopáticas e variantes não amiloidogénicas. Destas, sete foram descritas por nós pela primeira vez, nomeadamente TTR Thr 45, Ala 49, Leu 68, Ala 71, Gln 89, Asn 90 e Arg 102. Caracterizamos também indivíduos heterozigotos compostos. Em dois dos casos analisados uma das mutações é amiloidogénica e outra não amiloidogénica (Met 30-Met 119 e Met 30-Asn 90) enquanto que num outro caso as duas mutações envolvidas não são amiloidogénicas (Asn 90-Met 119).

A variante amiloidogénica de TTR mais frequente é, sem dúvida, a TTR Met 30. Embora o maior foco da mutação se localize em Portugal, a TTR Met 30 tem sido encontrada em muitos outros locais distribuidos pelo mundo. As relações históricas

encontradas entre esses locais e Portugal levantaram a questão da existência, ou não, de um fundador único para a origem da mutação. Estudos anteriores, efectuados por um grupo japonês, demonstraram a existência de vários haplotipos associados à mutação no Japão, indicando a recorrência desta mutação, facto também apoiado por a mutação ocorrer num dinucleótido CpG. Para averiguar se o mesmo acontecia na Europa, e em particular em Portugal, fizemos estudos similares de determinação do haplotipo associado à TTR Met 30 em várias famílias Portuguesas, Suecas, Italianas, Espanholas, uma família Grega, uma Inglesa e uma Turca. Verificamos que na Europa a mutação que origina a TTR Met 30 teve mais do que uma origem, pois encontramos dois haplotipos diferentes associados à mutação. Porém, em Portugal, todas as famílias Met 30 estudadas apresentaram o mesmo haplotipo associado à mutação, haplotipo I. Assim, sugerimos que em Portugal a mutação tenha tido uma origem única o que justificaria a distribuição da mutação a partir de um foco bem definido no Norte do país. Neste estudo tentamos também relacionar o haplotipo com a variabilidade de expressão clínica, nomeadamente com a idade de início da doença, tendo para isso incluído famílias com início típico e com ínicio tardio. A existência de um único haplotipo associado à mutação não permitiu estabelecer qualquer relação entre o haplotipo e a idade de ínicio da doença, o mesmo acontecendo com as famílias Suecas que são tipicamente tardias.

A ampla distribuição das mutações que identificamos e das outras mutações já conhecidas no monómero de TTR não permitiu identificar regiões específicas na molécula para a localização preferencial das variantes amiloidogénicas ou não amiloidogénicas, não sendo possível estabelecer uma correlação estrutural e fenotípica.

A heterogeneidade clínica e molecular associada a estas amiloidoses, assim como a ausência de mutação no gene de TTR na amiloidose sistémica senil (SSA), verificada por outros autores e confirmada por nós, sugere o envolvimento de outros factores na formação e deposição de amilóide, nomeadamente de factores tecidulares específicos.

Não excluimos, no entanto, a possibilidade de estas substituições de aminoácido induzirem alterações estruturais que possam estar relacionadas com a amiloidogenicidade das proteínas mutantes. No sentido de verificar alterações estruturais das variantes de TTR fizemos estudos funcionais das proteínas, nomeadamente estudos de ligação de T_4,

em que comparamos o comportamento de variantes naturais com o de variantes produzidas sinteticamente num sistema de expressão bacteriano. Estes estudos permitiram-nos concluir que as substituições de aminoácidos nas variantes de TTR podem:

- alterar directamente a ligação de T_4 à TTR como acontece com as variantes cuja substituição de aminoácido ocorre no canal de ligação de T_4, nomeadamente TTR Thr 109 e Met 119;

- alterar indirectamente a ligação de T_4 devido a alterações estruturais induzidas no canal de ligação ou noutra região da molécula, por substituição de aminoácido num local diferente, como se sabe acontecer com a TTR Met 30 e como sugerimos para a TTR Pro 55.

- alterar indirectamente a ligação de T_4 alterando as estruturas terciária e quaternária da proteína, como sugerimos relativamente à TTR Asn 90 por não apresentar a cooperatividade negativa de ligação de T_4, característica da TTR normal;

- não alterar a ligação de T_4 como verificamos para algumas variantes de TTR nomeadamente TTR Ser 6, Ala 49, Arg 102.

Estes resultados permitiram-nos concluir que as variantes moleculares de TTR, amiloidogénicas ou não, podem induzir modificações estruturais e consequentemente alterar a função da proteína. Por outro lado, estes resultados indicam que as variantes amiloidogénicas não induzem necessáriamente alterações estruturais que afectem a ligação de T_4 como se verificou para as variantes amiloidogénicas com afinidade normal para a T_4. Assim, a existência de uma modificação estrutural comum a todas as variantes amiloidogénicas parece-nos pouco provável. Alternativamente, sugerimos que as alterações conformacionais de cada variante podem afectar de modo diferente os processos envolvidos na formação de amilóide.

Estes resultados terão, no futuro, impacto nos estudos de difracção de raios-X com vista à definição da estrutura das proteínas mutantes.

2. INTRODUÇÃO

2. INTRODUÇÃO

Após a descrição da primeira variante de TTR associada à polineuropatia amiloidótica familiar (PAF) em 1984 por Saraiva *et al.*, tem vindo a aumentar continuamente o número de variantes de TTR identificadas em associação com as amyloidoses hereditárias.

A diversidade de expressão clínica destas variantes de TTR tem demonstrado a complexidade da amiloidogenese, sugerindo que este é um processo multifactorial. Pensa-se que, para este processo, podem contribuir alterações conformacionais da TTR, interacções com outras moléculas e/ou proteólise. A importância deste tema tem despertado o interesse de vários grupos sendo muito extenso o número de trabalhos publicados. Assim, no sentido de fundamentar o trabalho de investigação que apresentamos, fizemos uma revisão sumária dos aspectos que consideramos principais. Referimos brevemente a estrutura e expressão do gene da TTR e abordamos a estrutura da proteína especialmente sob o ponto de vista funcional, nomeadamente, ligação de T_4 e compostos análogos. Apresentamos ainda alguns modelos de formação de amilóide, salientando a possível importância das alterações conformacionais.

2.1. ESTRUTURA E EXPRESSÃO DO GENE DA TTR

2.1.1. O gene da TTR

A TTR é codificada por um gene de cópia única localizado no braço longo do cromossoma 18, mais precisamente na região 18q11.2-q12.1 (Whitehead *et al.*, 1984). A estrutura do gene foi determinada após a sua clonagem (Mita *et al.*, 1984; Tsuzuki *et al.*, 1985; Sasaki *et al.*, 1985). O gene abrange cerca de 7.0 Kb e é constituído por 4 exons e 3 introns. O exon 1 codifica para um péptido sinal de 20 aminoácidos e 3 aminoácidos da molécula de TTR madura; o exon 2 codifica para 44 aminoácidos, do resíduo 4 a 47; o exon 3 codifica para 45 aminoácidos, do resíduo 48 a 92 e o exon 4 codifica para 35

aminoácidos, do resíduo 93 a 127. Os introns, designados por A, B e C , têm comprimentos de 934 bp, 2090 bp e 3308 bp, respectivamente. Nos limites de cada intron encontram-se as sequências consensus GT e AG dos locais de excisão («*splicing*»). Nos introns B e C existem duas seqências *Alu* com polaridades opostas, que se pensa poderem formar uma estrutura em ansa («*hairpin*») no mRNA percursor.

No que se refere às sequências promotoras e aos elementos reguladores, foram identificadas várias sequências consensus entre 30 a 101 bp a montante do local de ínicio de transcrição, nomeadamente uma sequência TATA (-30 a -24 bp) seguida de uma região rica em G+C de cerca de 20 bp e uma sequência CAT (-101 a -96). Na região 3' não traduzida, 123 bp a jusante da região codificante, localiza-se um sinal de poliadenilação (AATAAA). Na região 5' a montante do ínicio da transcrição foram detectadas 2 sequências homólogas a um elemento sensível às hormonas glucocorticoides. Estas são sequências conservadas, através das quais as hormonas glucocorticoides induzem os genes da metalotionina (Sasaki *et al.*, 1985).

Tsuzuki *et al.* (1985) referiram ainda a presença de duas «*open reading frames*» com as respectivas sequências reguladoras, uma no primeiro intron e outra no terceiro. Embora não se conheça o significado destas «*open reading frames*», foi sugerido que codifiquem para proteínas reguladoras da expressão do gene (Tsuzuki *et al.*, 1985).

2.1.2. Expressão do gene da TTR

A TTR plasmática é sintetizada principalmente no fígado e secretada para o plasma onde a sua concentração é cerca de 20-40 mg/dl (Gitlin & Gitlin, 1975). No entanto, a concentração de TTR no líquido céfalo-raquidiano é cerca de 12 vezes mais elevada do que o que seria de esperar para uma proteína plasmática por transporte através da barreira hemato-encefálica. Esta elevada concentração de TTR fazia supor a existência de um transporte preferencial de TTR para o líquido céfalo-raquidiano e/ou a síntese de TTR no sistema nervoso (Weisner e Roethig, 1983). Estudos imunocitoquímicos demonstraram uma reactividade positiva para a TTR no retículo endoplasmático e também no complexo

de Golgi nos plexos coroideus indicando síntese *de novo* de TTR (Aleshire *et al.*, 1982). Estudos posteriores de expressão de TTR no rato e no homem por análise de Northern, hibridização «*in situ*» e tradução «*in vitro*» confirmaram a síntese de TTR nos plexos coroideus e demonstraram ser este o único local de síntese de TTR no sistema nervoso (Soprano *et al.*, 1985; Herbert *et al.*, 1986). Estes estudos demonstraram ainda expressão do gene de TTR em muito pequena quantidade (1-2%) no estômago, coração, músculo esquelético e baço (Soprano *et al.*,1985). Jacobsson (1989) demonstraram também síntese de TTR no pâncreas humano e de rato.

O olho é também um local de síntese de TTR nos mamíferos (Martone *et al.*, 1988), sendo o epitélio pigmentado da retina o único local ocular de síntese de TTR (Dwork *et al.*, 1990). Recentemente, verificou-se existir mais um local de síntese de TTR no sistema nervoso central, a glândula pineal, uma estrutura periventricular fotossensitiva (Martone et al., 1993). No entanto, os níveis de síntese de TTR na glândula pineal são mais baixos do que no epitélio pigmentado da retina. A detecção simultânea de síntese de RBP nesta glândula sugere um mecanismo de transporte de retinol, semelhante a outros tecidos envolvendo TTR e RBP.

Curiosamente, o epitélio pigmentado da retina é em termos funcionais e de desenvolvimento, homólogo aos plexos coroideus e tal como estes encontra-se isolado do plasma, tendo sido sugerido que os mecanismos de regulação da expressão de TTR nestes locais sejam diferentes dos mecanismos de regulação no fígado (Martone *et al.*, 1988).

A síntese de TTR nos plexos coroideus parece ser particularmente importante devido à alta actividade transcricional já referida e também porque, em termos de desenvolvimento, é o local onde se inicia a expressão da TTR, por volta da 8ª semana de gestação (Jacobsson, 1989). Por outro lado, ontogenicamente a expressão de TTR parece ter ocorrido primeiro nos plexos coroideus, nos anfíbios e répteis, e só mais tarde no fígado, quando aparecem os mamíferos placentários (Schreiber *et al.*, 1993).

2.1.3. Regiões reguladoras da expressão do gene da TTR

O gene da TTR humana tem grande homologia com o gene da TTR de rato, cerca de 80 % na sequência codificante, e uma homologia ainda maior , cerca de 91%, quando se compara a sequência de aminoácidos (Costa *et al.,* 1986). Esta homologia permite estabelecer um paralelismo entre as estruturas dos dois genes. Assim, as regiões reguladoras do gene de TTR de rato foram estudadas em células humanas de hepatoma (HepG2) (Costa *et al.,* 1986; Costa *et al.,* 1989) e as sequências encontradas foram comparadas com a sequência do gene humano (Sakaki *et al.,* 1989) (Figura 1).

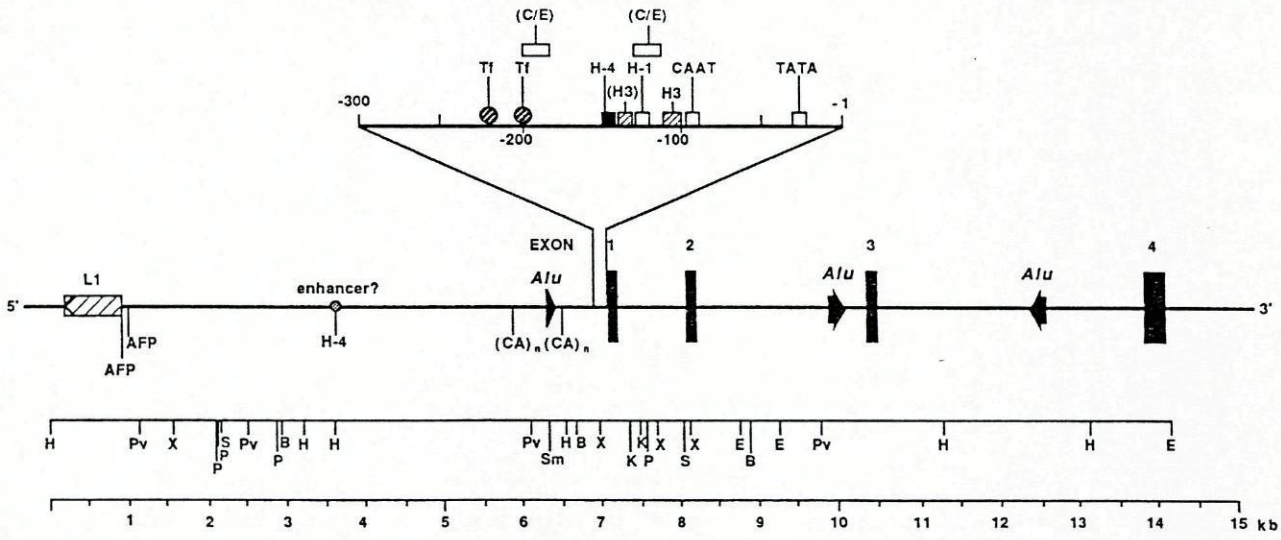


Fig. 1 - Representação esquemática do gene humano de TTR e localização das regiões reguladoras. Os exons, rectangulos a cheio, estão numerados de 1a 4. Alu, L1, (CA)n - referem-se às respectivas sequências; TATA - TATA box; CAAT - CAAT box; H1, C/E, H3, H4 indicam os locais de ligação dos factores nucleares HNF-1, C/EBP, HNF-3 e HNF-4. Tf é um motivo comum ao Tf-LF-1, Tf-LF-2, LF-A1; enhancer- região para homologia com um enhancer tecido-específico do gene de rato. AFP-local de ligação do factor AFP-1. (Sakaki *et al.,* 1989)

No gene de rato existem duas regiões reguladoras, uma sequência promotora localizada entre -50 a -190 nucleótidos e uma região enhancer localizada a cerca de 2 kb do local de ínicio da transcrição (Costa *et al.,* 1986). A região promotora do gene de rato, localizada entre os nucleótidos -90 a -150, é altamente conservada e contém os sítios de ligação para factores nucleares enhancers da transcrição, nomeadamente HNF-1, C/EBP, HNF-3 e HNF-4 (Costa *et al.,* 1989). No gene humano, os locais de ligação para os

factores HNF-3 e HNF-4 são sequências bastante conservadas, o mesmo não acontecendo com as sequências para C/EBP (Sakaki *et al.*, 1989). Estes factores estão presentes no fígado adulto e fetal e nas células do saco vitelino. Contudo, os factores de transcrição parecem não actuar como um grupo pois a ausência de um, como por exemplo o C/EBP, ausente nas células do saco vitelino, não afecta a transcrição do gene.

A região homóloga à sequência enhancer encontra-se mais afastada do local de ínicio de transcrição, aproximadamente a 3.4- 3.6 kb. Esta sequência tem homologia com o local de ligação do factor HNF-4 e parece ser particularmente importante para a expressão da TTR nos plexos coroideus. O gene humano de TTR tem ainda outra sequência reguladora localizada a cerca de 6 kb do ínicio da transcrição. Esta sequência é homóloga aos sítios de ligação do factor AFP1 (hepatoma nuclear protein). No gene humano existem ainda sequências potenciais de ligação para outros factores nucleares hepato-específicos, nomeadamente Tf-LF1, TF-LF 2 e LF-A1 sendo o motivo comum TGG/A A/CCC/T (Sakaki *et al.*, 1989). Estes estudos demonstraram que a expressão hepato-específica depende de elementos *cis* mas também de factores *trans* e que a expressão da TTR nos plexos coroideus deve envolver outras sequências reguladoras e outros factores estimuladores da transcrição (Costa *et al.*, 1990).

2.2. ESTRUTURA E FISIOLOGIA DA TTR

2.2.1. Estrutura da TTR

A TTR é uma proteína tetramérica constituída por quatro subunidades idênticas. Cada subunidade tem 127 resíduos de aminoácidos cuja sequência foi determinada por Kanda *et al.* (1974) (Figura 2). O peso molecular do tetrâmero é de 54,980 KDa, sendo o peso de cada subunidade de 13,745 KDa, tal como calculado pela sua composição em aminoácidos.

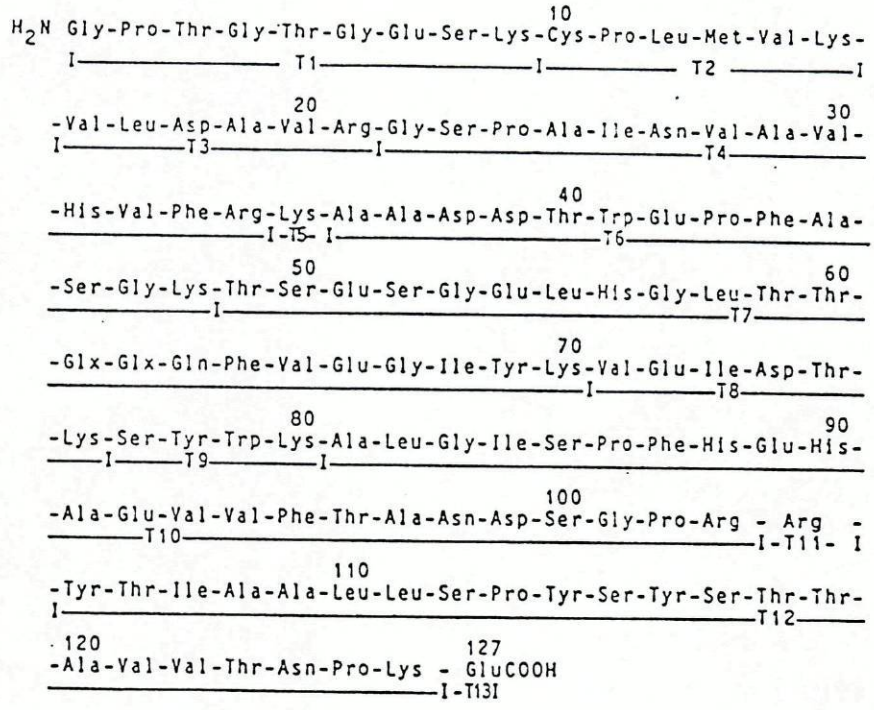


Fig. 2 - Sequência de aminoácidos do monómero de TTR (Kanda *et al.*, 1974). São indicados os péptidos trípticos.

A estrutura tridimensional da TTR, determinada por cristalografia e difracção de raios-X, foi estabelecida por Blake *et al.* (Blake & Swan, 1971; Blake & Oatley, 1977; Blake *et al.*, 1978). Considerando a estrutura secundária da proteína, o elemento principal é a estrutura em folha pregueada ß que engloba cerca de 45% dos aminoácidos organizados em 8 cadeias ß, designadas de A a H (Figura 3).

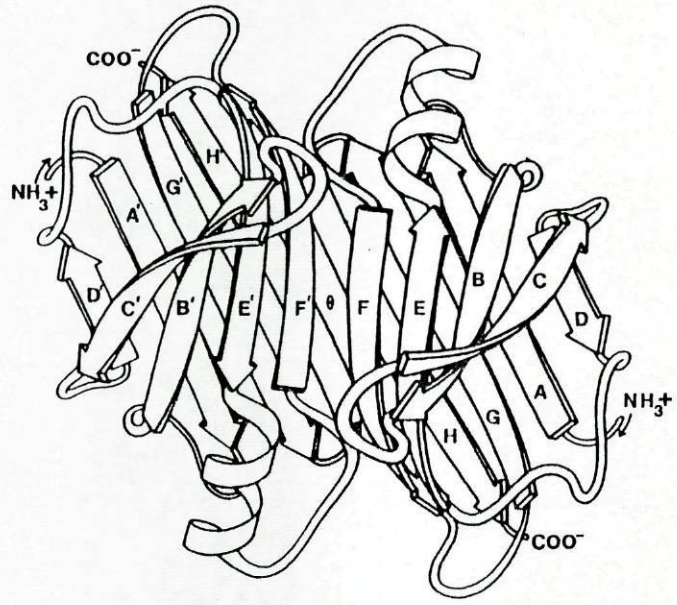


Fig. 3 - Representação esquemática do dímero de TTR (de la Paz *et al.*, 1992).

As cadeias ß são compostas por 6 a 9 resíduos de aminoácidos, exceptuando a cadeia D que é composta apenas por três aminoácidos. As cadeias estão organizadas em duas folhas ß que compreendem as cadeias DAGH e CBEF, de cada monómero. Todas as interacções entre cadeias são antiparalelas, com excepção das que se estabelecem entre as cadeias A e G. Os resíduos 75 a 83 formam um elemento em α-hélice que se localiza na extremidade final da cadeia E. Os restantes resíduos de aminoácidos constituem os 7 *loops* entre as cadeias ß e ainda sequências N- e C-terminal, com 10 e 5 resíduos, respectivamente. Os *loops* que unem as cadeias ß têm um comprimento variável.

A estrutura de cada monómero é essencialmente determinada pelas interacções entre as duas folhas ß, DAGH e CBEF. Entre estas folhas ß projectam-se as cadeias laterais dos seus resíduos de aminoácidos, principalmente aminoácidos alifáticos hidrofóbicos: Ala, Val, Leu e Ile. Esta estrutura parece ser estabilizada por sete resíduos aromáticos: 4 fenilalaninas e 3 tirosinas. Em cada monómero há seis resíduos

completamente internos capazes de formarem ligações de hidrogénio: Thr 49, Tyr 69, Thr 75, His 88, Tyr 116 e Thr 118. Estes resíduos participam numa série de ligações de hidrogénio envolvendo quatro moléculas de água que estabilizam os grupos polares interiores. Os resíduos de tirosina também têm um papel importante na estabilização da estrutura terciária.

Os monómeros de TTR associam-se em dímeros por interacções entre as cadeias F e H de cada monómero. Os contactos extensos, através de ligações de hidrogénio, entre as cadeias F e H de cada monómero fazem com que se considere o dímero, e não o monómero, como a unidade estrutural básica da proteína. Designando as cadeias de um monómero por A-H e as do outro por A'-H' o arranjo das cadeias no dímero é DAGHH'G'A'D' e CBEFF'E'B'C' estendendo as duas folhas ß de 4 cadeias no monómero para duas folhas ß de 8 cadeias no dímero. As interacções entre cada folha envolvem seis ligações de hidrogénio entre átomos da cadeia principal ou entre cadeias laterais. As interacções entre átomos da cadeia principal são essencialmente entre os grupos NH ou CO dos resíduos Glu 89, Val 94, His 90 e Glu 92 de um monómero e os equivalentes no outro monómero. As interacções entre cadeias laterais envolvem, básicamente, os grupos hidroxilo de resíduos de serina (Ser 115) e treonina (Thr 119). Existem ainda interacções hidrofóbicas na interface dos dois monómeros que envolvem resíduos localizados entre as duas folhas ß.

Embora os dímeros se juntem para formar o tetrâmero com as folhas de 8 cadeias DAGHH'G'A'D' opostas face-a-face, os contactos entre os dímeros envolvem apenas resíduos dos *loops* das extremidades das folhas nomeadamente *loops* GH (resíduos 112-115) e AB (resíduos 17-23). Cada *loop* AB interactua com as cadeias H e H' do dímero oposto. Estas interacções podem ser hidrofílicas e hidrófóbicas contrariamente às que se estabelecem entre monómeros e que são predominantemente hidrofílicas.

A estrutura da TTR define duas regiões à superfície da molécula que são complementares em forma e tamanho à cadeia dupla de DNA. Não sendo conhecida nenhuma interacção da TTR com o DNA não se sabe qual é o significado desta complementaridade estrutural. No entanto, foi sugerida uma homologia estrutural entre a

TTR e o receptor nuclear das hormonas da tiróide que pressupunha uma proteína ancestral comum.

Globalmente, esta estrutura quaternária confere à proteína as seguintes características:

- um arranjo compacto que se reflecte numa grande estabilidade da molécula;

- a existência de um canal que atravessa a molécula e é limitado por 16 cadeias ß;

- duas depressões semicilindricas à superfície da molécula complementares em forma e tamanho à estrutura do DNA (Blake *et al.*, 1981) (Figura 4).

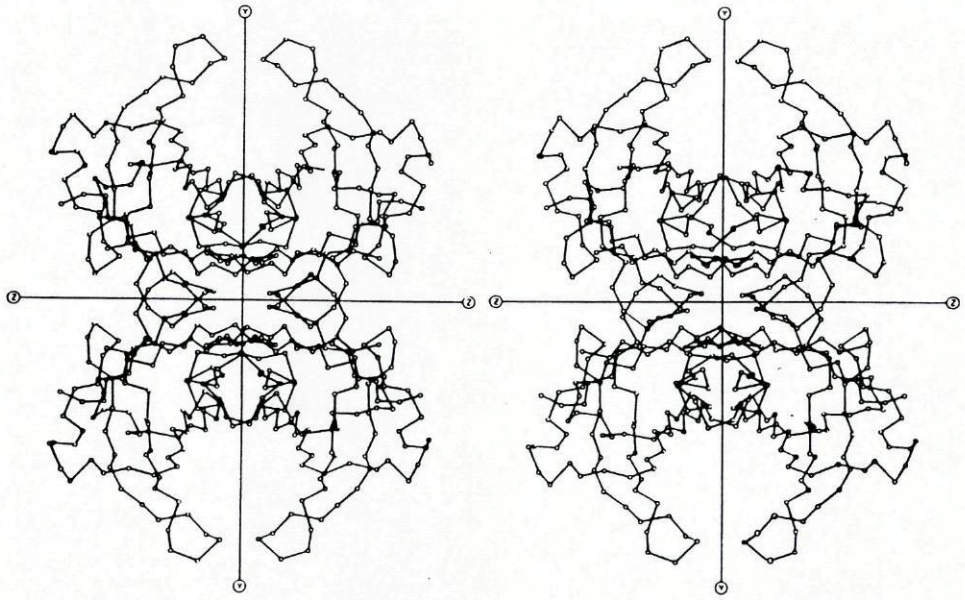


Fig. 4 - Desenhos estereoscópicos da molécula de TTR, olhando ao longo do eixo dos Y. O canal de ligação está localizado paralelamente ao eixo dos Z.

2.2.2. Funções fisiológicas da TTR

2.2.2.1. Importância da TTR no metabolismo da vitamina A

A TTR desempenha um papel fundamental na distribuição de retinol (alcool da vitamina A) aos tecidos através da formação de um complexo proteico com a proteína de transporte do retinol (retinol binding protein-RBP) (Kanai *et al.*, 1968). O retinol e outros

compostos com actividade de vitamina A são necessários para a visão, reprodução e manutenção dos epitélios diferenciados.

Os retinoides , provenientes da dieta, são absorvidos no intestino e transportados pelas quilomicras até ao fígado onde são armazenados. No fígado dois tipos de células são importantes para o metabolismo e armazenamento de retinoides: as células parenquimatosas e as células estreladas. As células parenquimatosas absorvem os retinoides das quilomicras. Os retinoides são então mobilizados das células parenquimatosas para as células estreladas, onde são armazenados. Em função das necessidades do organismo, os retinoides são novamente transferidos para as células parenquimatosas onde formam complexos com o RBP para serem secretados (Soprano & Blaner, 1994). No plasma o holo-RBP forma um complexo com a TTR (Smith & Goodman, 1979). A formação deste complexo TTR-RBP , de peso molecular de cerca de 70,000 KDa, protege o RBP, uma molécula de peso molecular 21,000 KDa, da filtração glomerular e catabolismo renal (Raz *et al.*,1970). No plasma o complexo TTR-RBP-retinol transporta o retinol até às células alvo. O mecanismo de absorção do retinol pelas células não está totalmente esclarecido sendo conhecidas duas hipóteses para esse mecanismo: uma que propõe que a entrada do retinol é mediada por receptores de RBP na membrana celular e outra que considera não ser necessária a intervenção de receptores.

Sivaprasadarao & Findlay (1988) demonstraram a existência de receptores de RBP em placentas humanas e sugerem que o RBP liberta o retinol nas células alvo por interacção com esses receptores na membrana citoplasmática. O retinol é retido pelas células, e o RBP volta à circulação sob a forma de apo-RBP, com baixa afinidade para a TTR, sendo filtrado no rim (Goodman, 1987). Pensa-se que a TTR possa ter um papel regulador na distribuição de retinol pelos tecidos controlando os níveis de RBP livre e holo-RBP (Sivaprasadarao & Findlay, 1988).

Estudos em ratinhos desprovidos de TTR (Episkopou *et al.*, 1993) mostraram que estes apresentavam valores de retinol no plasma abaixo dos níveis de detecção e níveis de RBP muito baixos (cerca de 3% dos valores normais) confirmando a extrema importância da TTR no transporte de RBP. Contudo, estes ratinhos não apresentavam

quaisquer sinais de deficiência de retinol sugerindo a existência de um mecanismo alternativo na distribuição de retinol pelos tecidos.

2.2.2.2. Interacção TTR-RBP

A molécula de TTR possui quatro locais de ligação para o RBP (van Jaarsveld *et al.*, 1973; Kopelman *et al.*, 1976) embora em condições fisiológicas se encontre uma razão molar TTR:RBP de 1:1 (Goodman, 1987). Estudos recentes da estrutura do complexo TTR-RBP demonstram que uma molécula de TTR pode ligar duas moléculas de RBP, impedindo estéricamente a ligação de outras duas moléculas de RBP ao mesmo tetrâmero (Monaco *et al.*,in press).

A sequência de aminoácidos do RBP foi determinada por Rask *et al.* (1979), e a sua estrutura tridimensional definida por análise de cristais da holoproteína (Cowan *et al.*, 1990; Zanotti *et al.*, 1993). A molécula de RBP tem um núcleo em folha ß constituída por 8 cadeias ß antiparalelas e ainda uma α-hélice. O retinol liga-se ao RBP ficando o anel hidrofóbico protegido no interior da molécula com a cadeia de isopreno e o grupo OH estendendo-se até à superfície da molécula. Os resíduos do RBP que interactuam com a TTR situam-se próximo do local de ligação do retinol, sendo a presença deste essencial para a estabilidade do complexo. A ligação do RBP à TTR, definida recentemente (Monaco *et al.*, in press), parece indicar que as duas moléculas de RBP interactuam com o mesmo dímero de TTR mas cada uma delas estabelece contactos com um dos outros dois monómeros, bloqueando os restantes locais de ligação no tetrâmero.

Apenas cerca de 8% dos resíduos de RBP estão envolvidos em interacções com a TTR. Entre os resíduos em contacto no complexo, sobressaem as isoleucinas 84 de duas cadeias diferentes de TTR, que participam em interacções com cada uma das moléculas de RBP. Por outro lado, o grupo carbonilo do resíduo de Gly 83 da TTR forma uma ligação de hidrogénio com o OH do retinol e interactua com o resíduo Leu 35 do RBP. Esta interacção, mais uma vez, dá apoio à observação de que o retinol estabiliza a ligação do RBP à TTR. Embora o modelo molecular do complexo TTR-RBP descrito por Aqvist & Tapia (1991) previsse muitas das interacções agora descritas, não incluía os resíduos 34-

37, em particular a Leu 35, que representam o fragmento onde ocorrem as maiores alterações durante a passagem da forma de holo a apo-proteína e são talvez responsáveis pela menor afinidade do apo-RBP para a TTR (Monaco *et al.*, 1994).

2.2.2.3. Importância da TTR no transporte de hormonas da tiróide

As hormonas tiroideias (T_4 e T_3) são essenciais para a regulação do metabolismo basal, anabólico e catabólico, consumo de oxigénio e também no controle do crescimento celular e diferenciação. A principal hormona da tiróide em circulação é a tiroxina (T_4), embora esta para ser biologicamente activa, tenha de ser deiodinada a T_3. Cerca de 95% das hormonas da tiróide em circulação encontram-se ligadas a um grupo de proteínas plasmáticas das quais se devem dissociar antes de atingir o seu local de acção, dentro das células. No entanto, só a fracção livre das hormonas é metabolicamente activa ao nível dos tecidos. O papel principal das proteínas de transporte das hormonas da tiróide parece ser o armazenamento extratiroidal de T_3 e T_4 e a capacidade de tamponamento que permite às hormonas serem libertadas quando são precisas, e por outro lado, proteger as células da entrada de excesso de hormonas. A possibilidade destas proteínas terem um papel na entrada das hormonas para os tecidos por interacção com receptores na superfície celular é alvo de investigação e discussão. As proteínas de transporte de hormonas da tiróide no homem são a proteína de ligação à tiroxina (TBG - thyroxine binding globulin), a TTR e a albumina. Outras proteínas, menos importantes, capazes de ligar hormonas da tiróide são as lipoproteínas e imunoglobulinas. Estas proteínas diferem nas suas concentrações no plasma e na sua afinidade para a ligação à tiroxina (T_4). Assim, a TBG, sendo a proteína que existe em menor concentração no plasma, é a que transporta mais T_4 (cerca de 70%) devido à sua alta afinidade para a T_4 (Ka $1x10^{10}$ M^{-1}). A TTR tem uma afinidade intermédia (Ka=$7x10^7$ M^{-1}) quando comparada com os outros transportadores, sendo a albumina a que tem menor afinidade (Ka= $7x10^5$ M^{-1}) (Robbins & Bartalena, 1986). Cada uma destas proteínas liga menos avidamente a T_3 do que a T_4, ao contrário do que acontece com o receptor nuclear, que liga mais fortemente a T_3. A TBG é, normalmente, o principal determinante do nível de T_4 total no soro mas a TTR parece ter um papel

particularmente importante no transporte de hormonas da tiróide no sistema nervoso central. A elevada taxa de síntese de TTR nas células epiteliais dos plexos coroideus e a elevada concentração de TTR no líquido céfalo-raquidiano fazem prever um papel importante da TTR no sistema nervoso central (Dickson *et al.*, 1987). Esta função da TTR é pouco conhecida; porém Dickson *et al.* (1987) propuseram que a T_4 entrasse nas células epiteliais dos plexos coroideus e se ligasse à TTR sintetizada *in situ*. O complexo TTR-T_4 poderia então ser secretado para o líquido céfalo-raquidiano possibilitando a distribuição de T_4 às diferentes regiões do cérebro (Schreiber *et al.*, 1990). Esta função da TTR pode ser particularmente importante durante o desenvolvimento e pode estar relacionada com a fase precoce do desenvolvimento a partir da qual a TTR começa a ser sintetizada nos plexos coroideus.

A importância da TTR no transporte de T_4 para o cérebro é posta em causa pelos recentes estudos de metabolismo das hormonas da tiróide em ratinhos desprovidos de TTR (Palha *et al.*, 1994a). O fenótipo normal destes ratinhos indica que a TTR não é indispensável para o transporte de T_4 para o cérebro.

2.2.2.4. Ligação da T_4 à TTR

A TTR tem dois locais de ligação para a T_4, estruturalmente idênticos e, localizados num canal interior que atravessa a molécula de TTR (Blake & Oatley, 1977). Estes locais de ligação têm afinidades para a T_4 significativamente diferentes, respectivamente, 1.05×10^8 M^{-1} e 9.55×10^5 M^{-1} .A diferente afinidade de cada um destes locais para a T_4 implica existência de cooperatividade negativa na ligação da T_4. Assim, fisiologicamente liga-se apenas uma molécula de T_4 por molécula de TTR. A T_3 também se liga a estes locais mas com menor afinidade.

O canal interno da TTR é formado pela associação dos dois dímeros do tetrâmero de TTR não havendo interacções entre as folhas-ß de cada dímero. Os dímeros interactuam apenas através dos resíduos dos *loops* GH e AB na extremidade das folhas ß. No centro do canal, os resíduos Leu 110, Ser 115 e Ser 117 originam uma constrição que separa os dois locais de ligação de T_4. Os locais de ligação têm uma organização química que permite a

definição de regiões com diferentes polaridades complementares às características químicas da hormona. Assim, a partir do centro do canal localiza-se: (i) uma região hidrofílica formada pelos grupos hidroxilo da Ser 112, Ser 115, Ser 117 e Thr 119; (ii) uma região hidrofóbica formada pelos grupos metilo da Leu 17, Ala 108, Leu 110 e Val 121; (iii) uma região carregada, à entrada do canal, formada pelas cadeias laterais da Lys 15 e Glu 54. Existem ainda moléculas de H_2O localizadas no centro do canal de ligação que formam uma rede de ligações de hidrogénio entre os resíduos de Ser 115, Ser 117 e Thr 119.

Estas regiões formam seis concavidades (ou bolsos) capazes de ligar os átomos de iodo da molécula de T_4 (De La Paz *et al.*, 1992) (Figura 5).

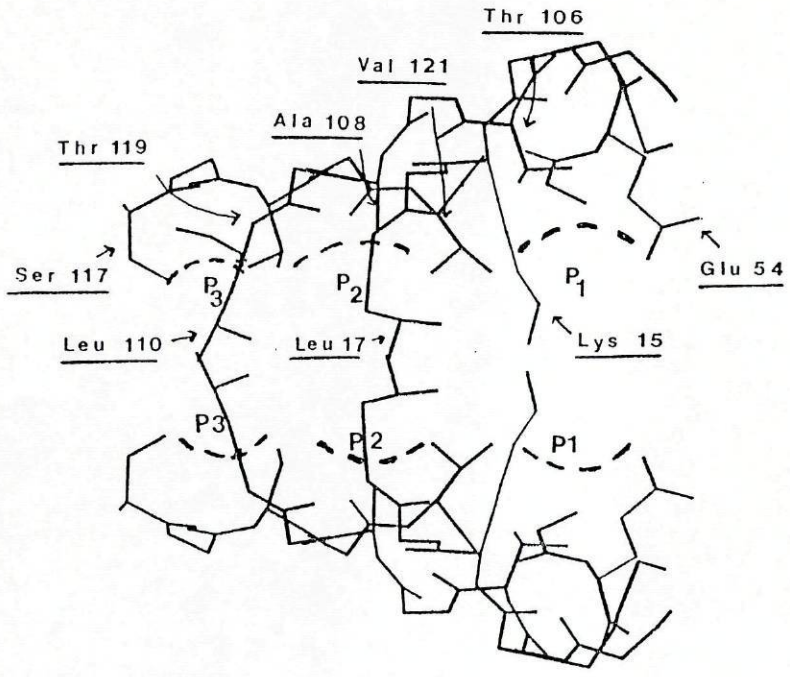


Fig. 5 - Diagrama do local de ligação da T_4 na TTR mostrando as cadeias laterais que se projectam no canal de ligação. A entrada do canal fica à direita. As linhas tracejadas representam os pares de bolsos, P1, P2 e P3, onde se podem ligar os átomos de iodo (de La Paz *et al.*, 1992).

A conformação da hormona é limitada pela geometria do local de ligação; assim, os dois aneis benzénicos da T_4 orientam-se perpendicularmente um ao outro e estabelecem contactos hidrofóbicos com as cadeias laterais da proteína. O grupo OH da hormona

estabelece ligações hidrofóbicas com a Ser 117 e Thr 119. Os grupos α-amino e α-carboxilo da hormona interactuam com os resíduos de Lys 15 e Glu 54 localizados à entrada do canal. Os iodos 3 e 5 da T_4 ligam-se ao par de bolsos P1 e P1' estabelecendo interacções entre grupos metilo e metileno da Lys 15, Leu 17, Thr 106, Ala 108 e Val 121. O iodo 3', próximo do anel de tirosina liga-se ao bolso P2 definido pelas cadeias de Ala 108, Ala 109 e Leu 110 e ainda à Lys 15 e Leu 17. O iodo 5' liga-se ao bolso P3 formado pelas cadeias Ala 108, Ala 109 e Leu 110 e ainda à Ser 117 e Thr 109. As duas concavidades mais próximas da entrada do canal são ocupadas por moléculas de H_2O que se ligam por pontes de hidrogénio à Thr 119 e à Ser 117.

Os bolsos P3 e P2, mais próximos do centro do canal, permitem interacções mais extensas entre os átomos de iodo e a TTR devido às contribuições dos resíduos hidrofílicos de Ser e Thr e de resíduos da cadeia peptídica principal. Por outro lado, a geometria do canal, semelhante a um cone com o vértice no centro, também favorece essas interacções mais extensas devido à menor distância entre a molécula de TTR e os átomos de iodo nos bolsos P2 e P3.

2.2.2.5. Interacção da TTR com o receptor

Estudos em células humanas de hepatoma, HepG2, sugeriram que a captação de TTR nessas células é mediada por receptores, propondo ainda a intervenção destes receptores na internalização das hormonas da tiróide (Divino & Schussler, 1990a). Contudo este receptor nunca foi isolado.

Também foi demonstrada a endocitose de TTR em oócitos de galinha por um mecanismo mediado por um receptor para a TTR (Vieira et al., 1995). Experiências de ligand -blotting e cross-linking mostraram interacção da TTR e do complexo TTR-RBP com uma proteína da membrana de 115 KDa. No entanto, são necessários mais estudos desta proteína para confirmar a sua função.

2.2.3. Outras funções fisiológicas

2.2.3.1. Interacção com o ácido retinoico

O ácido *all-trans* retinoico é um inibidor potente da ligação da T_4 à membrana do eritrócito e também é capaz de competir com a T_4 para a ligação à TTR o que sugere semelhanças entre os locais de ligação de iodotironinas do receptor da membrana do eritrócito e da TTR. Não sendo conhecida nenhuma proteína semelhante ao RBP para ligação do ácido retinoico, é possível que a TTR funcione também como transportador do ácido retinoico.

In vivo, os retinoides podem influenciar a homeostase das hormonas da tiróide. Porém, este efeito não parece ser devido a competição de ligação à TTR entre a T_4 e o ácido retinoico pois a TTR não é o principal transportador de hormonas da tiróide no plasma. Pensa-se que este efeito dos retinoides na homeostasia das hormonas da tiroide seja devido à homologia entre receptores nucleares do ácido retinoico e da T_3. Estes receptores podem ligar-se ao mesmo tipo de sequências no DNA sendo possíveis interacções cruzadas entre estes receptores. (Smith *et al.*, 1994; Cheng *et al.*, 1994).

2.2.3.2. Ligação à noradrenalina

De Vera *et al.* (1988) demonstraram que a TTR é uma das proteínas plasmáticas capazes de ligar noradrenalina embora não se saiba qual o significado dessa ligação. Contudo, Boomsma *et al.* (1991) verificaram que não é a noradrenalina mas os seus produtos de oxidação que se ligam à TTR e a outras proteínas plasmáticas.

2.2.3.3. Inibição da produção de interleuquina-I em monócitos e células endoteliais

A interleuquina-I é uma citoquina com potente actividade imunorreguladora e inflamatória que é libertada pelas células fagocíticas mononucleares, células endoteliais e

outras células em resposta à estimulação imunitária. A TTR interfere com a secreção da IL-I inibindo a pro-IL-1ß convertase (proteína que corta o péptido sinal da IL-I) ou qualquer outro componente da via de exportação. Por outro lado, a transcrição de mRNA de TTR hepática é regulada negativamente durante a fase aguda de resposta à inflamação. Assim, durante a inflamação, baixa a transcrição de TTR e aumenta a produção de interleuquina-I. Este efeito imunomodulador da TTR não ocorre nos plexos coroideus onde a síntese de mRNA de TTR não é regulada do mesmo modo (Borish *et al.*, 1992).

2.2.3.4. Ligação a pterinas

A TTR no plasma encontra-se ligada a compostos amarelos. Na TTR de galinha o principal composto amarelo é um carotenoide, a luteína. Estudos recentes referem que o composto amarelo da TTR humana tem propriedades idênticas a uma pterina reduzida ou a um folato (Ernstrom *et al.*, 1995). Uma das possíveis implicações funcionais baseia-se no facto de a tetrahidrobiopterina ser apontada como um cofactor na deiodinação da T_4 embora não seja requerido para a 5' - deiodinase celular. Por outro lado, também foi sugerido que a TTR possa ser importante como transportador de folatos para o cérebro após absorção dos folatos pelos seus receptores nas células dos plexos coroideus.

2.2.3.5. Ligação a globinas e metais

Verificou-se que a globina é um constituinte menor dos depósitos de amilóide e que a TTR é capaz de se ligar à hemina e hemoglobina sugerindo que esta interacção seja devida ao ferro do grupo heme pois é sensível ao tratamento com EDTA (Martone & Herbert, 1993). Por outro lado, a formação de precipitados congofílicos de TTR induzida pela incubação com uma mistura determinada de metais e a solubilização desses precipitados pelo EDTA confirma a interacção da TTR com metais (Herbert & Martone, 1993).

Estas interacções com globinas e metais sugerem que, a hemólise intravascular ou a interacção de TTR com metaloproteínas, possam contribuir de algum modo para a formação de amilóide.

2.2.3.6. Função tímica

A persistência de actividade tímica em ratinhos timectomizados foi atribuída a um decapéptido corresponente aos 10 resíduos de aminoácido N-terminais da molécula de TTR (Burton *et al.*, 1978; 1987) tendo-se verificado posteriormente que as propriedades imunopotenciadoras da TTR são comuns a outros murinos (Burton *et al.*, 1985). Porém, o significado biológico desta actividade imunológica da TTR não é ainda conhecido.

2.2.3.7. Interacção da TTR com a proteína ß-amilóide

Estudos recentes demonstraram que a TTR forma complexos com o péptido ß-amilóide tendo sido sugerido que servirá para sequestrar o péptido impedindo-o de participar na formação de fibras de amilóide em doentes de Alzheimer (Schwarzman *et al.*, 1994). A importância destes resultados de experiências *in vitro* só poderá ser confirmada pela sua verificação *in vivo.*

2.2.4. Interacção da TTR com algumas drogas e agentes farmacológicos

2.2.4.1. Compostos fenólicos

Estudos da influência de algumas drogas no metabolismo das hormonas da tiróide demonstraram que algumas dessas drogas interferem especificamente com a ligação da tiroxina à TTR, como por exemplo o 2,4-dinitrofenol e o salicilato (Wolff *et al.*, 1961). No entanto, outros derivados do àcido benzoico, como os ácidos gentísico e resorcilico, são inibidores mais potentes da ligação da T_4 à TTR do que o salicilato (Woeber *et al.*, 1964). Ao contrário do salicilato, estes compostos não apresentam efeitos calorigénicos (não induzem desacoplamento da fosforilação oxidativa) tendo por isso sido utilizados em

estudos, *in vivo* e *in vitro*, da influência da TTR no metabolismo periférico da T_4 no homem (Woeber *et al.*, 1964).

2.2.4.2. Compostos bifenílicos

Outras drogas que foram usadas em estudos de competição de ligação de T_4 à TTR e à TBG são compostos bifenílicos derivados do ácido antranílico, como por exemplo os ácidos flufenâmico, meclofenâmico, e mefenâmico (Munro *et al.*, 1989). Estes compostos são particularmente potentes na inibição da ligação da T_4 à TTR. Também o diflunisal, diclofenac e fenclofenac inibem a ligação de T_4 à TTR, embora sejam competidores menos potentes que os anteriores.

Em geral estas drogas não provocam alterações significativas dos níveis de T_3 e T_4 no soro por interactuarem especificamente com a TTR. Como já foi referido, no soro o principal transportador de T_4 é a TBG que transporta cerca de 70% da T_4 circulante. Isto corresponde a uma ocupação de apenas 1/3 da capacidade da TBG para ligar T_4. Assim, os competidores que actuam apenas com um local de ligação na TTR não têm efeito nos níveis de T_4 no soro porque a quantidade de T_4 deslocada da TTR é pequena e pode ligar-se à TBG. No entanto, em condições em que a TTR seja o principal transportador de T_4, como na deficiência de TBG ou ocupação da TBG mais elevada do que o normal, estas drogas podem induzir alterações dos níveis de T_4 no soro (Woeber *et al.*, 1964).

2.2.4.3. Compostos tiromiméticos - Milrinona e 3,3'-diiodotironina

A milrinona é um agente inotrópico cardíaco, isto é, aumenta a contractilidade do miocárdio e a velocidade do coração devido à estimulação da Ca^{2+}-ATPase associada à miosina. Este efeito da milrinona é semelhante aos efeitos das hormonas da tiróide no coração. Estruturalmente a milrinona apresenta semelhanças com a T_4, sendo constituída por uma estrutura bipiridinica, não iodinada, como se pode ver na figura 6.

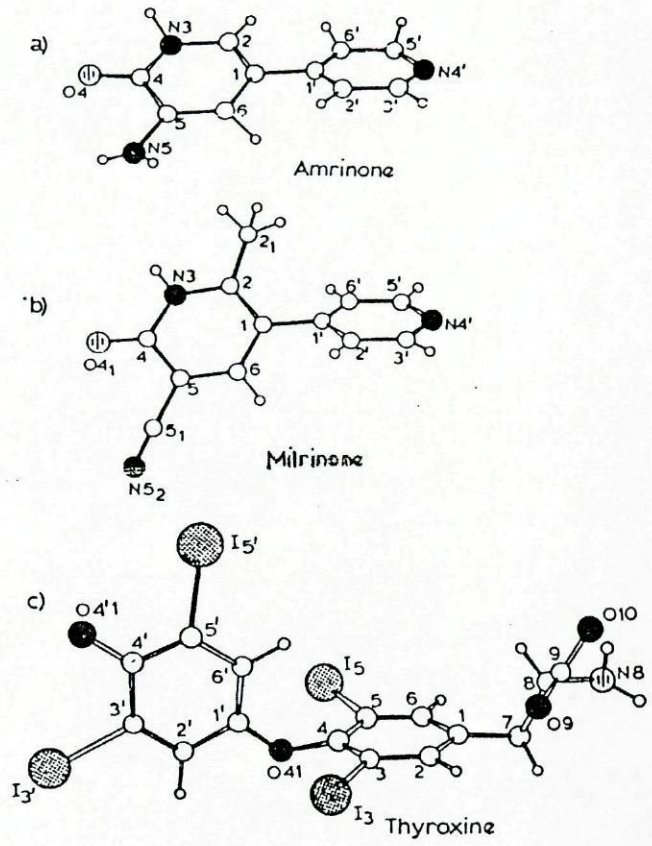


Fig. 6 - Conformação molecular e esquema de numeração da a) amrinona, b) milrinona e c) tiroxina.

No sentido de compreender os mecanismos de reconhecimento molecular para os diferentes metabolitos das hormonas da tiróide assim como dos seus competidores Wojtczak *et al.* (Wojtczak et al. 1992; 1993; Ciszak *et al.*, 1992) utilizaram modelos computacionais para determinar a estrutura dos complexos formados entre esses compostos e a TTR. Um desses compostos é a 3,3'-diiodotironina (3,3'-T_2), um metabolito das hormonas da tiróide (Fig. 6), e outro é a milrinona. Por análise de modelos computacionais de ligação da milrinona à TTR verificou-se que, a melhor sobreposição das estruturas da T_4 e da milrinona se obtinha quando o anel de piridina substituído era sobreposto no anel fenólico da hormona com o grupo ciano do anel de piridina a ocupar o mesmo espaço que um dos iodos fenólicos; deste modo o grupo 2-metil é capaz de encaixar num bolso do canal que em geral não é ocupado pela hormona (Davis *et al.*, 1987). Estudos de competição da milrinona com a T_4 para ligação à TTR mostraram

existir dois locais de ligação para a milrinona com diferentes afinidades sendo a constante de afinidade da milrinona para a TTR cerca de 10 vezes menor que a da T_4. A determinação da estrutura do cristal do complexo TTR-milrinona confirma os modelos computacionais (Wojtczac *et al.*, 1993). O anel de piridona está orientado ao longo do eixo do canal e os grupos 4-ceto e 5-ciano correspondem às interacções do 4'OH e 3'I da 3,3'-T_2 demonstrando que a menor afinidade da milrinona para a TTR deve-se à falta de interacções iónicas à entrada do canal de ligação que na T_4 se estabelecem com a cadeia alanil. A mais forte ligação da milrinona quando comparada com a 3,3'-T_2 deve estar relacionada com as suas interacções hidrofóbicas mais fortes com o local de ligação e com a diferente orientação no canal. A amrinona é um análogo da milrinona mas com uma afinidade muito menor do que a milrinona para a TTR.

2.2.4.4. Flavonoides

Os flavonoides são substâncias naturais amplamente distribuídas nas plantas e que são potentes inibidores de uma das deiodinases da T_4 - T_4-5'-deiodinase (Kohrle *et al.*, 1989). Este efeito inibidor dos flavonoides está relacionado com a semelhança de estrutura e conformação destes compostos com as iodotironinas. Estes compostos interactuam com a TTR deslocando a T_4 ligada. Estudos da relação estrutura-actividade destes compostos levaram a que se desenvolvesse um flavonoide sintético com alto poder de inibição da ligação da T_4 à TTR, o EMD 21388 - 3-metil-4',6-diidroxi-3',5'-dibromoflavona (Kohrle *et al.*, 1989). Este flavonoide tem sido muito utilizado para estudos do metabolismo e fisiologia das hormonas da tiroide, nomeadamente no rato onde a TTR é o principal transportador de hormonas da tiroide (Kohrle *et al.*,1989; Lueprasitsakul *et al.*, 1990, Mendel *et al.*, 1992). Os resultados destes estudos são contra a hipótese de a TTR ter um papel fundamental na transferência da T_4 do plasma para os tecidos por interacção com um possível receptor celular (Divino & Schussler, 1990a; 1990b).

2.2.4.5. Compostos polihalogenados bifenílicos (PHBs)

É conhecido que muitos compostos químicos poluentes do ambiente provocam alterações no metabolismo das hormonas da tiróide, em particular os hidrocarbonetos aromáticos clorados, como a 2,3,7,8-tetracloro-p-dioxina (TCDD), bifenis policlorados (PCBs), bifenis polibrominados, dibenzo-p-dioxinas (PCDDs), dibenzofuranos (PCDFs) hexaclorobenzeno e DDT (McKinney *et al.*, 1985; Lans *et al.*, 1993). Na figura 7 apresentamos as estruturas de alguns destes compostos.

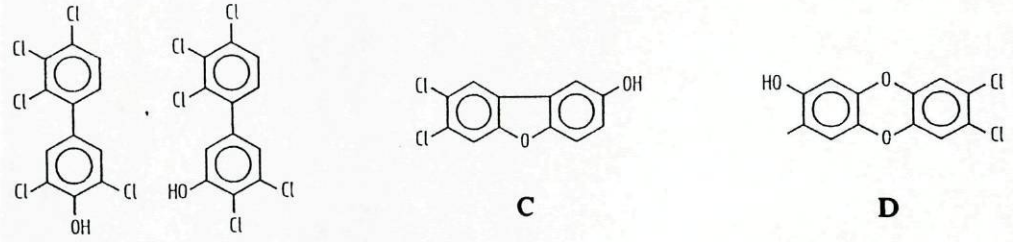


Fig. 7 - Exemplos de estruturas hidroxiladas de PCBs (A e B), PCDD (C) e PCDF (D).

Os derivados hidroxilados destes compostos têm uma estrutura semelhante à T_4 e interactuam específicamente com a TTR não interferindo com a ligação à TBG (Lans *et al.*, 1994). Em termos de estrutura, os requisitos fundamentais para a ligação à T_4 são a presença do grupo hidroxilo em posição *meta* ou *para* e a presença de um ou mais átomos de cloro no anel aromático. Quanto maior for o grau de substituição por cloros maior é a potência inibidora como acontece com o pentaclorofeno (PCP). Estes compostos também interactuam com o receptor nuclear de tiroxina.

Estes compostos podem induzir alterações neurotóxicas por interferirem no metabolismo da T_4 no sistema nervoso central. Estudos em ratos aos quais foi administrado hexaclorobifenil ou pentaclorobifenil revelaram uma deficiente captação de T_4 no cérebro (Raaij *et al.*, 1994). Esta deficiência de T_4 no líquido céfalo-raquidiano pode ter consequências graves no desenvolvimento do cérebro ou no funcionamento normal do

sistema nervoso central nos adultos. Porém, ainda não se conhecem os mecanismos de transporte de T_4 ou de PCP para o líquido céfalo-raquidiano e se a TTR pode estar envolvida ou não nesse processo (Raaij *et al.*, 1994).

A toxicidade destes compostos pode, por outro lado, estar relacionada com o facto de a sua interacção com a TTR impedir a formação do complexo TTR-RBP resultando na excreção acelerada do retinol ligado ao RBP via catabolismo renal (Brouwer *et al.*, 1988).

2.3. VARIANTES MOLECULARES DE TTR

Em 1984 foi descrita pela primeira vez uma variante de TTR, TTR Met 30, que estava associada a uma forma de amiloidose hereditária em famílias Portuguesas, em particular à polineuropatia amiloidótica familiar (PAF) (Saraiva *et al.*, 1984). A partir dessa altura, muitas outras variantes foram descritas, sendo hoje conhecidas 43 variantes de TTR associadas a amiloidoses hereditárias (Tabela I). As amiloidoses hereditárias relacionadas com a TTR podem apresentar expressões clínicas muito diversas, mas, em geral, classificam-se de acordo com a característica clínica predominante que pode ser neuropatia, cardiomiopatia ou deposição de amilóide no vítreo.

Tabela I - Variantes amiloidogénicas de TTR

Posição	Resíduo normal	Resíduo mutante	Característica clínica predominante	Referência
10	C	R	Neurop	Uemichi *et al.* (1992)
18	D	R	Neurop	Pepys *(comun. pessoal)*
24	P	S	Cardio	Araki *et al.* (1994)
30	V	M	Neurop	Saraiva *et al.* (1984)
30	V	A	Neurop	Jones *et al.*(1992)
30	V	L	Neurop	Nakazato *et al.* (1992)
30	V	G	Neurop	Herbert *et al.* (1994)
33	F	I	Neurop	Nakazato *et al.* (1984b)
33	F	L	Neurop	Ii *et al.* (I991)
36	A	P	Neurop	Jones *et al.* (1991)
42	E	G	Neurop	Ueno *et al.* (1990)
45	A	T	Cardio	Saraiva *et al.* (1992)
45	A	D	Cardio	Jacobson *et al.* (1994)
47	G	R	Neurop	Murakami *et al.* (1992)
47	G	A	Neurop	Ferlini *et al.* (1994)
47	G	V	Neurop-CT	Booth *et al.* (1994)
49	T	A	Neurop	Almeida *et al.* (1992)
50	S	R	Neurop	Ueno *et al.* (1990)
50	S	I	Neurop	Saeki *et al.* (1992)
50	S	I	Cardio	Nishi *et al.* (1992)
52	S	P	Neurop	Booth *et al* .(1994)
54	E	G	Neurop	Booth *et al.* (1994)
55	L	P	Neurop	Jacobson *et al.* (1992b)
58	L	H	Neurop-CT	Nichols *et al.* (1989)
58	L	R	Neurop-CT	Saeki *et al.* (1991)
59	T	K	Cardio	Booth *et al.* (1995)
60	T	A	Neurop	Wallace et al. (1986)
61	E	K	Neurop	Shiomi *et al.* (1993)
64	F	L	Neurop	Ii *et al.* (1991)
68	I	L	Cardio	Almeida *et al.* (1991)
69	Y	H	Vítreo	Zeldenrust *et al.* (1994)
70	K	N	Neurop-CT	Izumoto *et al.* (1992)
71	V	A	Neurop	Almeida *et al* .(1993)
77	S	Y	Neurop	Wallace *et al.* (1988b)
84	I	S	Neurop-CT	Dwulet *et al.* (1986)
84	I	N	Vítreo	Skinner *et al.* (1992)
89	E	Q	Neurop	Almeida *et al.* (1992)
97	A	G	Neurop	Yasuda *et al.* (1994)
107	I	V	Neurop	Jacobson *et al.* (1994a)
111	L	M	Cardio	Nordlie *et al.* (1988)
114	Y	C	Neurop	Ueno *et al.* (1990a)
114	Y	H	Neurop-CT	Murakami *et al.* (1994)
122	V	I	Cardio	Saraiva *et al.* (1990)

Neurop- neuropatia amiloidótica

Neurop-CT- neuropatia amiloidótica com síndrome do tunel cárpico

Cardio- cardiomiopatia amiloidótica

Vítreo- amilóide no vítreo

Embora em número muito menor, conhecem-se também variantes de TTR não amiloidogénicas, que apresentamos na tabela II. A alta frequência de algumas destas variantes justifica a detecção de indivíduos portadores de duas mutações em alelos diferentes, heterozigotos compostos, que também incluímos na tabela II.

Foram ainda descritos dois indivíduos portadores de duas mutações no mesmo alelo, sendo o alelo mutante Ser 6-Ile 33 ou Asn 90-Gly 42, também apresentados na tabela II.

Tabela II - Variantes não-amiloidogénicas e heterozigotos compostos

Alelo 1	Alelo 2	Frequência *	Referência
Ser 6	**N**	33/558	Jacobson *et al.* 1995
Ser 6	**Met 30**	nd	Saraiva*(comun.pessoal)*
Ser 6	**Met 119**	nd	Saraiva*(comun.pessoal)*
Ser 6 - Ile 33	**N**	nd	Jacobson *et al.* (1994b)
Ser 6	**Asp 45**	nd	Jacobson *et al.* (1994)
Ser 6	**Gly 54**	nd	Booth *et al.* (1994)
His 74	**N**	1/8,000	Uemichi *et al.* (1994)
Asn 90	**N**	16/12,400	Saraiva *et al.* (1991)
Asn 90	**Met 30**	nd	Saraiva *et al.* (1991)
Asn 90	**Met 119**	nd	Alves *et al.* (1993)
Asn 90 - Gly 42	**N**	nd	Skare *et al.* (1994)
Ag 102	**N**	1/8,000	Almeida *et al.* (1991a)
Thr 109	**N**	1/10,000	Alves *et al.* (1993)
Thr 109	**Met 119**	nd	Izumoto *et al.* (1993)
Val 109		nd	Izumoto *et al.* (1993)
Met 119	**N**	35/10,000	Alves *et al.* (1993)
Met 119	**Met 30**	nd	Alves *et al.* (1993)

*Referente à frequência do alelo mutante
N - normal
nd - não determinada

Os casos de homozigotia para estas mutações são muito raros mas conhecem-se indivíduos homozigotos para a TTR Met 30 em famílias Suecas (Holmgren *et al.*, 1988), numa família Turca (Skare *et al.*, 1990), numa família Japonesa (Ikeda *et al.*, 1992) e numa família Portuguesa. Também foram descritos indivíduos homozigóticos para TTR His 58

(Jacobson *et al.*, 1993) e TTR As 90 (Saraiva *et al.*, 1991). Curiosamente, a maior parte dos portadores de TTR Ile 122 são homozigotos (Jacobson *et al.*, 1990).

Com o objectivo de estudar a estrutura, função e amiloidogenecidade de variantes de TTR homotetraméricas foram criados sistemas de expressão do gene mutante em *E. coli* (Furuya *et al.*, 1989; 1991; Murrell *et al.*, 1992; McCutchen *et al.*, 1993a).

2.3.1. Variantes amiloidogénicas

Como já referimos, a maior parte das variantes moleculares de TTR conhecidas são variantes amiloidogénicas, e em particular variantes neuropáticas. Nestes casos, como a designação indica, a deposição de amilóide ocorre preferencialmente nos nervos periféricos, podendo existir algum envolvimento cardíaco, ocorrência de síndrome do tunel cárpico e/ou deposição de amilóide no vítreo. A mais frequente das variantes amiloidogénicas é a TTR Met 30, que referiremos mais adiante.

A TTR Pro 55 é uma variante amiloidogénica que se encontra associada a uma forma particularmente agressiva de amiloidose. Esta variante foi descrita numa família Americana de ascendencia Holandesa e Alemã (Jacobson *et al.*, 1992b) e numa família Tailandesa (Yamamoto *et al.*, 1994). A idade de ínicio dos sintomas da doença, embora variável, é mais precoce do que noutros casos, podendo ocorrer por volta dos 15 a 20 anos. A evolução da doença é mais rápida, conduzindo à morte em menos de 10 anos. Tal como acontece com outras variantes, para além da polineuropatia grave os portadores apresentam deposição de amilóide no vítreo e cardiomiopatia. No sentido de esclarecer as causas da amiloidogenicidade desta variante, têm sido realizados alguns estudos de estabilidade desta proteína utilizando para isso a proteína produzida sinteticamente num sistema de expressão bacteriano (McCutchen *et al.*, 1993a). Estes estudos serão referidos adiante.

Das variantes cardiomiopáticas salientamos a TTR Ile 122 que foi descrita exclusivamente na população negra, na qual ocorre com alta frequência (2,2% no estado heterozigótico) (Jacobson *et al.*, 1991; Jacobson, 1992). Pensa-se que a amiloidose

cardíaca associada à TTR Ile 122 possa ser uma causa relativamente frequente de colapso cardíaco neste grupo étnico.

A TTR Met 111 é também uma variante cardiomiopática que referimos por ser a mais bem estudada em termos familiares. Esta mutação foi descrita numa família Dinamarquesa (Nordlie *et al.*, 1988) tendo sido analisados 36 indivíduos (Ranlov *et al.*, 1992) demonstrando claramente o caracter familiar da doença.

A TTR Ile 50 é um exemplo da diversidade de expressão clínica das variantes de TTR. A TTR Ile 50 foi associada a polineuropatia numa família Japonesa (Saeki *et al.*, 1992) e associada a cardiomiopatia numa outra família, também Japonesa (Nishi *et al.*, 1992).

O síndrome do túnel cárpico é comum em várias formas de amiloidose associadas à TTR. Porém, a TTR His 114 é uma variante cuja característica principal é o síndrome do túnel cárpico, não apresentando polineuropatia (Murakami *et al.*, 1994).

Do mesmo modo, a deposição de amilóide no vítreo está frequentemente relacionada com variantes de TTR sendo a característica predominante associada à TTR His 69 (Zeldenrust *et al.*, 1994) e TTR Asn 84 (Skinner *et al.*, 1992), .

2.3.1.1. A TTR Met 30 e a Polineuropatia Amiloidótica Familiar (PAF)

A polineuropatia amiloidótica familiar (PAF) foi descrita pela primeira vez por Corino de Andrade em 1952 (Andrade, 1952). A doença, como o próprio nome indica, é caracterizada pela deposição sistémica de amilóide, com especial envolvimento dos nervos periféricos. Tem início, em geral, na terceira década de vida com progressão até à morte em 10 a 15 anos. Costa *et al.* (1978) verificaram que os depósitos de amilóide nos indivíduos com PAF eram constituídos, principalmente, por TTR. Saraiva *et al.* (1984) caracterizaram a TTR dos depósitos de amilóide tendo verificado a existência de uma variante de TTR em que um resíduo de valina na posição 30 da cadeia polipeptídica era substituído por um de metionina. A mutação que origina esta substituição foi identificada

por Sasaki *et al.* (1984) após clonagem do gene. A comparação do gene mutante com o gene normal demonstrou não existir qualquer diferença entre eles a não ser a substituição de uma guanina por adenina no codão da valina 30, localizado no exon 2 do gene (Maeda *et al.*, 1986). Análises de genótipo e fenótipo de indivíduos PAF eram coincidentes demonstrando a associação da TTR Met 30 a este tipo de polineuropatia (Saraiva *et al.*, 1985).

Estudos de expressão do gene de TTR em indivíduos PAF e normais mostraram que os níveis de expressão dos genes normal e mutante são semelhantes (Maeda *et al.*, 1986). O nível plasmático de TTR total é menor nos doentes PAF (Skinner *et al.*, 1985), e o nível de TTR Met 30 é menor do que o de TTR normal (Saraiva *et al.*, 1984). Estes resultados quando comparados com os níveis de expressão indicam que esta diferença é devida à deposição selectiva de TTR Met 30 e não a diferenças nos níveis de síntese de TTR (Mita *et al.*, 1986b).

Com o objectivo de estudar *in vivo* a expressão do gene de TTR e a deposição de amilóide, foram criados ratinhos transgénicos através da microinjecção do gene humano de TTR em embriões de ratinhos (Sasaki *et al.*, 1986; Wakasugi *et al.*, 1987; Yamamura *et al.*, 1987). Nestes ratinhos o gene é expresso em vários tecidos incluindo fígado e plexos coroideus. Embora se tenham detectado depósitos de amilóide na mucosa do intestino, glomérulos renais e outros tecidos, em nenhum caso há deposição de amilóide nos nervos periféricos. Pensa-se que isso possa estar relacionado com os baixos níveis de expressão do gene humano nestes animais.

2.3.1.2. Recorrência da mutação que origina a TTR Met 30

A PAF associada à TTR Met 30 tem uma grande incidência no Norte de Portugal, localizando-se o maior foco na Póvoa de Varzim e regiões circundantes, nomeadamente em Vila do Conde, Esposende e Barcelos. A PAF existe ainda noutras regiões litorais, como por exemplo, Matosinhos, Porto, Gaia, Figueira da Foz e em focos menores no interior do país, por exemplo, na Serra da Estrela.

A TTR Met 30 é a variante amiloidogénica mais amplamente distribuída pelo mundo, ocorrendo em focos menores na Suécia, Japão, Espanha, Itália e Brasil. Foram ainda identificadas famílias portadoras de TTR Met 30 de origem Francesa, Inglesa, Grega, Turca e Suiça. As relações históricas existentes entre Portugal e os países onde se encontram os principais focos da doença, estabelecidas pelos navegadores portugueses, pareciam indicar que a mutação tivesse uma origem única - hipótese do fundador único (one founder hypothesis). Por outro lado, a mutação que origina a TTR Met 30 ocorre num dinucleótido CpG, sequências com alta frequência de mutação (Cooper & Youssoufian, 1988), apontando para a possibilidade de a mutação ter ocorrido várias vezes independentemente - hipótese das múltiplas origens.

Estudos da estrutura do gene de TTR em indivíduos Japoneses normais e portadores da mutação Met 30 mostraram que existiam sete substituições intrónicas em ambos os casos (Yoshioka *et al.*, 1986; 1989). A análise destas substituições em vários indivíduos normais e portadores da mutação demonstrou que nenhuma delas estava ligada à PAF, sendo polimorfismos existentes na população em geral. O estudo da transmissão destes polimorfismos em famílias PAF permitiu verificar que eram transmitidos em bloco, definindo-se diferentes haplotipos. Estudos semelhantes em portadores da mutação Met 30 Norte Americanos (li *et al.*, 1993) revelaram a associação da mutação com 4 haplotipos diferentes, sendo dois iguais aos definidos pelos Japoneses e dois diferentes (Figura 8).

HAPLOTIPO	POLIMORFISMOS DO GENE DA TTR					
	1218	2422	2537	5198	5610	5708
I	G	C	A	C	G	T
II	T	G	G	C	G	T
III	T	G	G	A	C	G
IV	G	C	A	A	G	T
V	G	G	G	A	G	T

Fig.8 - Haplotipos no gene de TTR. Adaptado de Yoshioka *et al.* (1986) e li *et al.* (1993).

A conversão de um haplotipo noutro requer uma ou duas recombinações em introns do gene o que é altamente improvável devido à proximidade das substituições polimórficas. Assim, a existência de mais de um haplotipo associado à mutação implica que a mutação tenha tido várias origens.

A análise da sequência da região codificante do gene de TTR demonstrou existirem mais dinucleótidos CpG, que, quando mutados, devido a metilação da citosina em posição 5', poderiam originar diferentes substituições de aminoácido, tal como se apresenta na Figura 9.

Base change	Position																	
	3	5	6	21	29	30	98	99	100	101	103	104	108	109	119	121	122
Normal	Thr	Pro	Gly	Arg	Ala	Val	Asn	Gly	Ser	Gly	Arg	Arg	Ala	Ala	Thr	Val	Val
CpG - TpG	Met			Stop							Cys	Cys			Met		
CpG - CpA*			Ser	Glu		Met		Ser		Ser	His	His		Thr			Ile

* devidas a transições C-T em CpG na cadeia não codificante

Fig. 9 - Possíveis alterações de aminoácido causadas por transições C-T em dinucleótidos CpG. Adaptado de Yoshioka *et al.* (1989).

Algumas destas substituições já foram detectadas, como é o caso da TTR Ser 6, TTR Thr 109, TTR Met 119 e TTR Ile 122, e tal como era esperado são mutações que ocorrem com alta frequência, em particular a TTR Ser 6 (Jacobson *et al.*, 1995), TTR Met 119 (Alves *et al.*, 1993) e TTR Ile 122 (Jacobson *et al.*, 1991).

Destas variantes só a TTR Met 119 foi estudada quanto ao haplotipo associado à mutação. Porém, os indivíduos Americanos portadores da TTR Met 119 apresentaram todos o mesmo haplotipo associado à mutação, haplotipo III, indicando uma origem única para a mutação nesses indivíduos (li et al., 1992).

Num estudo recente de haplotipos associados à TTR His 58, TTR Ala 60 e TTR Tyr 77 (Zhao *et al.*, 1994) foi demonstrado que a mutação que origina a TTR Tyr 77 deve ter tido pelo menos duas origens independentes.

2.3.2. Variantes não amiloidogénicas

As variantes de TTR não amiloidogénicas foram apresentadas na tabela II. Destas variantes algumas não apresentam qualquer característica clínica associada como é o caso da TTR Ser 6, His 74, Asn 90 e Arg 102. As variantes TTR Thr 109, Val 109 e Met 119 encontram-se associadas a situações de hipertiroxinemia eutiroide.

A mais frequente destas variantes é a TTR Ser 6 (Jacobson et al., 1995) tendo-se verificado também uma alta frequência para a TTR Met 119 e a TTR Asn 90 na população Portuguesa (Alves *et al.*, 1993).

2.3.3. Variantes com alteração da afinidade de ligação à tiroxina

2.3.3.1. Variantes com afinidade elevada

As variantes que apresentam uma afinidade elevada para a ligação de T_4 são variantes cujo aminoácido substituído se localiza no canal de ligação da T_4, nomeadamente a TTR Thr 109 (Moses *et al.*, 1990) e Met 119 (Curtis *et al.*, 1994).

A **TTR Thr 109** é uma variante associada a hipertiroxinemia eutiroide. Os portadores desta mutação apresentam níveis elevados de T_4 no soro devido a uma elevada ligação de T_4 à TTR. Moses et al. (1990) verificaram que a TTR destes indivíduos tinha uma afinidade para a T_4 cerca de três vezes superior à afinidade da TTR normal. Estudos da afinidade da variante heterozigótica isolada do soro, e homozigótica sinteticamente produzida em bactérias, confirmaram o aumento de afinidade e demonstraram afinidades semelhantes para as duas formas (Rosen *et al.*, 1993; 1994). A determinação da estrutura da TTR Thr 109 recombinante por cristalografia (Steinrauf *et al.*, 1993) mostrou que a substituição do resíduo de alanina por treonina provoca um afastamento das folhas ß da molécula e consequentemente, um alargamento do local de ligação da T_4. Estas alterações aumentam a distância entre o oxigénio do carbonilo da treonina e um dos iodos da T_4 podendo estabilizar essa ligação e aumentar a afinidade para a T_4. No entanto, estudos de Wojtczak *et al.* (1992) indicam que o resíduo 109 não se projecta no interior do canal, que é

o local onde se estabelecem os pontos de contacto com a T_4. Assim, com o objectivo de esclarecer as interacções da T_4 no canal de ligação, Rosen *et al.* (1994) levaram a cabo estudos de ligação desta variante com análogos de tiroxina. O aumento de afinidade de ligação não foi igual para todos os compostos testados, nomeadamente os ácidos tri- e tetraiodoacético e o flavonoide sintéctico EMD21388. Estas diferenças podem ser devidas à possibilidade de os ligandos terem diferentes orientações no canal de ligação ou a pequenas alterações ao nível da Lys 15 e da Ser 117, dois dos resíduos directamente envolvidos nos contactos com a T_4. A co-cristalização da TTR Thr 109 com a T_4 e, por outro lado, a comparação do comportamento desta variante com outras produzidas por mutagénese dirigida são abordagens possíveis para o conhecimento mais detalhado das interacções entre a TTR e a T_4.

A **TTR Met 119** foi inicialmente descrita como uma variante não patogénica (Harrison *et al.*, 1991). Posteriormente, Scrimshaw *et al.* (1992) verificaram que portadores de TTR Met 119 de uma família Neozelandesa apresentavam níveis elevados de T_4 ligada à TTR. Também Alves *et al.* (1993) referiram niveís elevados de T4 em dois portadores de TTR Met 119 de uma família Portuguesa. Curtis *et al.* (1994) estudaram a ligação de T_4 à TTR semipurificada desses indivíduos tendo verificado um aumento da afinidade de ligação. Pensa-se que a substituição do resíduo de treonina por metionina possibilita uma interacção mais extensa da T_4 com outros resíduos de aminoácido localizados no canal de ligação de T_4, provocando um aumento da afinidade. Dado que estes estudos foram efectuados em indivíduos heterozigóticos resta confirmar este aumento de afinidade na proteína homotetramérica recombinante ou de indivíduos homozigóticos.

2.3.3.2. Variantes com baixa afinidade de ligação

Muitas das variantes amiloidogénicas apresentam baixa afinidade de ligação de T_4 mas não são conhecidas as alterações estruturais que originam essas diferenças. No caso da TTR Met 30, a determinação da sua estrutura por cristalografia fornece algumas evidências para essas alterações estruturais.

Sabe-se que a **TTR Met 30** de indivíduos heterozigotos tem menor afinidade de ligação de T_4 do que a TTR normal (Refetoff *et al.*, 1986) mas a TTR Met 30 de indivíduos homozigóticos apresenta uma afinidade de ligação de TTR muito menor, tendo mesmo sido considerada virtualmente nula (Rosen *et al.*, 1993).

Estudos cristalográficos da TTR Met 30 homotetramérica mostram algumas alterações que podem ser responsáveis pela baixa afinidade da TTR Met 30 para a ligação de T_4. No entanto, estes estudos revelam algumas diferenças entre si. Terry *et al.* (1993) verificaram que as alterações associadas à substituição da valina 30 por metionina afectam principalmente os resíduos 10-14 e 56-58. Estes resíduos fazem parte das cadeias ß A e D da folha interior, que limita o canal de ligação de T_4 e localizam-se próximo da cadeia lateral do resíduo 30 (situado na cadeia B da folha exterior). Assim, as alterações a nível do resíduo 14 poderiam ser transmitidas ao resíduo de Lys 15 que é um dos resíduos envolvidos na ligação de T_4. O mesmo seria possível em relação ao resíduo de Glu 54 que é também um dos resíduos envolvidos na ligação directa da T_4 e que se localiza à entrada do canal. Segundo estes autores, estas modificações podem ser tão pequenas que não são detectáveis devido à resolução da técnica usada. Contudo, Hamilton *et al.* (1993) explicam a menor afinidade da TTR Met 30 para a T_4 como sendo devida à alteração da forma do canal de ligação. No seu modelo cristalográfico verificaram existir um afastamento das duas folhas ß para acomodação da cadeia lateral, mais volumosa, da metionina. Este desvio das folhas ß torna o canal mais elíptico e diminui afinidade de ligação de T_4. Uma análise mais precisa destas interacções de T_4-TTR Met 30 implica a determinação da estrutura cristalográfica do complexo.

Além da TTR Met 30, outras variantes estudadas (investigadas) apresentam baixa afinidade de ligação de T_4 nomeadamente a TTR Tyr 77, TTR His 58 e a TTR Ser 84 de indivíduos heterozigotos e TTR Ile 122 de indivíduos homozigotos (Murrell *et al.*, 1992; Rosen *et al.*, 1993).

As variantes estudadas que não apresentam alteração de ligação à T_4 são a TTR Ser 6 e a TTR Ala 60 (Refetoff *et al.*,1986; Murrell *et al.*, 1992; Rosen *et al.*, 1993).

2.3.4. Variantes de TTR com alteração de ligação ao RBP

As interacções entre a TTR e o RBP têm sido menos exploradas, sob ponto de vista estrutural, do que as interacções TTR-T_4 e só recentemente foi determinada a estrutura do complexo TTR-RBP (Monaco *et al., in press*). Na realidade, só duas variantes de transtirretina foram estudadas relativamente à sua afinidade de ligação de RBP, a TTR Met 30 e a TTR Ser 84.

A ligação de RBP à TTR foi estudada por polarização de fluorescência tendo sido demonstrado que a TTR Met 30 de indivíduos heterozigotos tem afinidade e capacidade de ligação de RBP semelhantes à TTR normal (Saraiva *et al.*, 1983).

Níveis particularmente baixos de RBP foram encontrados em indivíduos portadores de TTR Ser 84 (Benson & Dwulet, 1983). Estudos da interacção RBP-TTR mostraram que a TTR Ser 84 recombinante tem uma afinidade de ligação muito baixa, ou práticamente nula, para ligar RBP (Berni *et al.*, 1994). A recente determinação da estrutura do complexo TTR-RBP revela que os resíduos de Ile 84 participam directamente na interacção da TTR com o RBP (Monaco *et al., in press*) justificando a baixa afinidade de ligação de RBP à TTR quando este resíduo é substituído por um de serina, TTR Ser 84.

Nos indivíduos portadores de TTR Ser 84, heterozigóticos, a redução da concentração de RBP é apenas de cerca de 3 vezes em relação à concentração normal devido à presença de monómero não mutado. Porém, estudos em ratinhos deficientes em TTR demonstraram uma redução de cerca de 30 vezes da concentração de RBP no plasma salientando a importância da formação do complexo TTR-RBP (Episkopou *et al.*, 1993).

2.4. AMILOIDOGÉNESE

2.4.1. Componentes dos depósitos de amilóide

As amiloidoses são doenças caracterizadas pela deposição extracelular de amilóide, uma substância fibrilar proteica que pode ser identificada por coloração com vermelho de Congo. A deposição de amilóide pode ocorrer em vários tecidos ou orgãos conduzindo a processos degenerativos.

Diferentes tipos de amiloidose estão relacionados com diferentes proteínas percursoras da substância amilóide. Na Tabela III apresentam-se os diferentes tipos de amiloidose e respectivas proteínas percursoras.

TABELA III - Amiloidoses e respectivas proteínas percursoras

Clinical syndrome	Precursor protein	Fibril component
Alzheimer's Disease	Amyloid Precursor Protein	β-Protein 1–42, 1–43
Primary Systemic Amyloidosis	Immunoglobulin Light Chain	Intact light chain or fragments thereof
Secondary Systemic Amyloidosis	Serum Amyloid A	Amyloid A (76 residue fragment)
Senile Systemic Amyloidosis	Transthyretin	Transthyretin or fragments thereof
Familial Amyloid Polyneuropathy (I)	Transthyretin	Over 40 transthyretin variants
Hereditary Cerebral Amyloid Angiopathy	Cystatin C	Cystatin C minus 10 residues
Hemodialysis Related Amyloidosis	β_2-microglobulin	β_2-microglobulin
Familial Amyloid Polyneuropathy (III)	Apolipoprotein A-1	Fragments of apolipoprotein A-1
Finnish Hereditary Systemic Amyloidosis	Gelsolin	71 amino acid fragment of gelsolin
Type II Diabetes	Islet of Amyloid Polypeptide-IAPP	Fragment of IAPP
Medullary Carcinoma of the Thyroid	Calcitonin	Fragments of calcitonin
Spongiform Encephalopathies	Prion	Prion or fragments thereof
Atrial Amyloidosis	Atrial Natriuretic Factor-ANF	ANF

Para além das fibras, os depósitos de amilóide são constituídos por componentes extrafibrilares, nomeadamente uma proteína designada por componente P e carbohidratos na forma de glicosaminoglicanos (GAGs) e proteoglicanos (Pgs) (Husby *et al.*, 1994). A

apolipoproteína E é também um componente comum a todos os tipos de amilóide tendo sido sugerido que juntamente com os outros componentes não fibrilares desempenhe o papel de chaperone molecular patológico (Wisniewski and Frangione, 1992). Este conceito de chaperone molecular patológico pressupõe uma interacção destes chaperones com a proteína precursora das fibras induzindo a formação da estrutura ß característica da substância amilóide.

2.4.2. TTR e amiloidogénese

As amiloidoses associadas à TTR podem envolver variantes moleculares de TTR originando neuropatias e cardiomiopatias ou envolver TTR normal no caso da amiloidose sistémica senil (SSA). A SSA é uma forma de amiloidose associada à TTR, diagnosticada em cerca de 25% das pessoas com mais de 80 anos. Neste tipo de amiloidose a amilóide deposita-se preferencialmente no coração, embora ocorra também deposição de amilóide noutros orgãos. Os depósitos de amilóide são compostos por TTR normal ou fragmentos de TTR normal originados por clivagem nas posições 46, 49, 52 (Cornwell III *et al.,* 1988). Estes fragmentos foram também detectados em depósitos amilóide do coração de indíviduos portadores de TTR Met 30, TTR Ile 33, TTR Met 111 e TTR Ile 122. Se a proteólise induzir a formação de fibras de amilóide este poderá ser um mecanismo comum às amiloidoses hereditárias e à SSA (Saraiva & Costa, 1990a).

Outro dos modelos propostos para a polimerização da TTR sob a forma de fibras baseia-se na análise da estrutura da TTR Met 30 (Terry *et al.,* 1993). Globalmente, a estrutura da TTR Met 30 não é muito diferente da TTR normal. Contudo, a substituição do resíduo de valina por metionina induz alterações estruturais nas cadeias A e D provocando também uma maior exposição do resíduo de Cys 10 que na proteína normal se encontra protegido num «bolso» da molécula. No tetrâmero de TTR os 4 grupos -SH localizam-se aos pares em extremidades opostas da molécula não sendo possíveis ligações dissulfureto intramoleculares. Foi então proposto que cada tetrâmero poderia estabelecer ligações dissulfureto com outros tetrâmeros, formando uma estrutura linear com as características das fibras de amilóide. Com base neste modelo, a exposição do grupo -SH da Cys 10,

devido à substituição de valina por metionina, facilita e aumenta a rapidez de um processo que ocorre muito mais lentamente na SSA. Estudos de amilóide do vítreo de indivíduos homozigóticos e heterozigóticos Met 30 mostram evidência para a existência de ligações dissulfureto na TTR das fibras de amilóide (Thylén et al., 1993). Porém, McCutchen & Kelly (1993b) afirmam que as ligações dissulfureto intermoleculares não são necessárias para a formação de amilóide *in vitro* e sugerem que o mesmo acontece *in vivo,* embora não excluam a sua ocorrência. Estas afirmações baseiam-se em experiências de formação de amilóide *in vitro* de uma variante de TTR em que o resíduo de cisteína 10 é substituído por um de alanina. O comportamento desta variante em meio ácido é semelhante ao da TTR normal, ocorrendo formação de fibras de amilóide ao mesmo pH. Por outro lado, foi descrita uma variante amiloidogénica com substituição da cisteína 10 por arginina (Uemichi et al., 1993) confirmando que a formação de fibras de amilóide *in vivo* não implica a existência de pontes dissulfureto entre as moléculas de TTR.

McCutchen *et al.* (1993a) propõem que a formação de fibras de amilóide *in vitro* depende da polimerização de um intermediário monomérico parcialmente desnaturado. A formação deste intermediário *in vitro* poderá ocorrer por desnaturação parcial em condições ácidas que corresponderiam *in vivo* a um envolvimento lisossomal. O envolvimento lisossomal na formação de amilóide a partir de outros percursores já foi proposto anteriormente (Golde et al., 1992). Comparando a estabilidade da TTR Pro 55, TTR Met 30 e da TTR normal em meio ácido Kelly & Lansbury (1994) estabeleceram uma correlação entre o aumento de pH a que se dá a transição de tetrâmero a monómero com o aumento da patogenicidade. Propõem ainda que no intermediário monomérico a região correspondente às cadeias C e D forme um loop único, ficando mais exposta. Este modelo explica a maior amiloidogenicidade da TTR Pro 55 pois este resíduo localiza-se na cadeia D. Segundo este modelo as mutações amiloidogénicas da TTR podem destabilizar o tetrâmero e estabilizar o intermediário estrutural em meio ácido ou apenas afectar a via de desnaturação. Estes autores sugerem ainda o envolvimento de outros factores no processo de desnaturação ou formação de amilóide nomeadamente interacção com componentes da matriz extracelular ou com chaperones moleculares.

Qualquer destes modelos necessita do esclarecimento de muitos aspectos fundamentais para a sua confirmação.

2.5. NOTA FINAL

Na secção anterior foram apresentadas algumas propostas de mecanismos para a formação de amilóide associada à TTR. Embora cada uma destas hipóteses reflita um aspecto predominante, quer seja o envolvimento de pontes dissulfureto ou a estabilidade da proteína, cada vez mais o processo de formação de amilóide revela-se multifactorial (Saraiva & Costa, 1992). Para a formação de amilóide podem contribuir alterações conformacionais da TTR, proteólise da TTR ou interacção da TTR com outros factores. Dada a importância das alterações conformacionais em todos os aspectos da amiloidogénese, orientamos o nosso trabalho no sentido de poder contribuir para a determinação dessas alterações associadas a substituições de aminoácidos.

Para isso caracterizamos variantes amiloidogénicas e não amiloidogénicas e realizamos estudos funcionais dessas variantes. Alterações da função da proteína, neste caso a ligação à tiroxina, poderão reflectir alterações estruturais da proteína.

3. PROJECTO DE INVESTIGAÇÃO: OBJECTIVOS, RESULTADOS E DISCUSSÃO

3. Projecto de investigação: objectivos, resultados e discussão

3.1. Objectivos

Uma das questões fundamentais que se coloca no estudo das amiloidoses hereditárias associadas à TTR é a de definir as razões pelas quais a substituição de um aminoácido na cadeia polipeptídica da TTR induz a sua precipitação sob a forma de fibras de amiloide. Assim, o nosso objectivo foi o de contribuir para o conhecimento dessas razões identificando e caracterizando, sob o ponto de vista genético e funcional, diferentes variantes de TTR. Quando iniciamos este trabalho eram conhecidas apenas cerca de oito variantes moleculares de TTR, todas associadas a neuropatias e/ou cardiomiopatias amiloidóticas familiares. Por isso, o nosso trabalho foi orientado no sentido de caracterizar molecularmente outras variantes de TTR tendo como objectivos imediatos:

- A identificação de novas variantes moleculares de TTR associadas a amiloidoses hereditárias envolvendo neuropatia e/ou cardiomiopatia.
- A identificação de variantes moleculares de TTR não amiloidogénicas.
- A implementação de métodos de diagnóstico das variantes identificadas com vista à detecção de portadores assintomáticos, e aconselhamento genético.
- A verificação de fundador único ou de múltiplas origens das mutações de TTR que ocorrem com maior frequência, pelo estudo de haplotipos.
- A verificação da existência de associação do haplotipo com a expressão clínica, e em particular, no caso da TTR Met 30, com a idade de início da doença.

A caracterização funcional destas variantes no sentido de prever possíveis alterações estruturais das proteínas mutantes. Para tal realizamos:

- Estudos funcionais de variantes naturais de TTR nomeadamente, estudos de ligação de tiroxina.

- Estudos de ligação de tiroxina a variantes de TTR produzidas sinteticamente num sistema de expressão bacteriano.

3.2. Resultados e discussão

Os resultados e discussão das experiências realizadas encontram-se em detalhe nos capítulos seguintes. Neste capítulo vamos resumir os resultados obtidos e apresentar os principais pontos de discussão.

1. Identificação de variantes moleculares de TTR associadas a polineuropatia amiloidótica familiar - caracterizaram-se cinco variantes de TTR em indivíduos com polineuropatia amiloidótica familiar: TTR Ala 49, TTR Leu 64, TTR Ala 71, TTR Tyr 77 e TTR Gln 89. Destas cinco variantes, três foram descritas por nós, pela primeira vez, nomeadamente a TTR Ala 49, TTR Gln 89 e TTR Ala 71. A TTR Ala 49 e a TTR Gln 89 foram detectadas por focagem isoeléctrica em soros de famílias italianas com PAF ao que se seguiu o isolamento e análise dos péptidos resultantes da digestão da proteína com tripsina em cromatografia líquida de alta pressão (HPLC). Os péptidos não comuns à TTR normal foram sequenciados. No caso de não serem detectáveis diferenças no mapa de péptidos trípticos sequenciaram-se os exons do gene de TTR após amplificação por PCR, como foi o caso da TTR Gln 89. A TTR Ala 71 também foi identificada por sequenciação do gene de TTR. As restantes variantes neuropáticas detectadas foram TTR Leu 64 e TTR Tyr 77. A TTR Leu 64 foi detectada por sequenciação de DNA amplificado. A TTR Tyr 77 foi detectada por focagem isoeléctrica e identificada por análise de RFLPs. Em qualquer dos casos estabeleceu-se sempre um método de diagnóstico da mutação para possibilitar a detecção de portadores. Os métodos de diagnóstico estabelecidos basearam-se no estudo de DNA amplificado por análise de RFLPs e/ou hibridização com sondas específicas para o alelo mutante após amplificação do DNA por PCR. Verificou-se que as mutações identificadas ocorrem em pontos distintos da estrutura 3D da molécula não sendo possível estabelecer uma correlação estrutural e fenotípica.Por outro lado, verificou-se que, tal como para outras variantes de TTR, a heterogeneidade clínica não estava associada apenas à heterogeneidade molecular mas que também ocorria para uma mesma variante de TTR, nomeadamente no que se refere à idade de início da doença. Este facto sugere a possibilidade de envolvimento de factores específicos tecidulares no processo de formação de fibras de amilóide e na sua deposição (Trabalho nº 1).

2. Identificação de variantes moleculares de TTR associadas a amiloidoses cardiomiopáticas: embora na maior parte das polineuropatias amiloidóticas se verifique também cardiomiopatia, o envolvimento desta é muito variável. Porém, algumas variantes de TTR originam cardiomiopatia sem polineuropatia como acontece com a TTR Met 111 e TTR Ile 122. Assim, investigamos a presença de variantes de TTR em indivíduos com cardiomiopatias amiloidóticas familiares tendo sido identificadas por nós duas novas variantes de TTR, nomeadamente TTR Thr 45 e TTR Leu 68 e uma variante já descrita, TTR Ile 122.

Em qualquer dos casos começamos por analisar a proteína isolada do plasma destes indivíduos. A análise do mapa de péptidos trípticos revelou as respectivas substituições Ala - Thr 45, Ile - Leu 68 e Val - Ile 122 que foram confirmadas por sequenciação de DNA nos dois primeiros casos e por análise de RFLPs no terceiro. A transmissão familiar da mutação foi confirmada por análise de RFLPs e/ou hibridização com sondas específicas para o alelo mutante de outros indivíduos na família, excepto no caso do indivíduo portador de TTR Ile 122.

A heterozigotia do portador de TTR Ile 122 contrariou a necessidade de homozigotia para a manifestação clínica da doença, tal como tinha sido descrito anteriormente na literatura. Dado que esta variante foi inicialmente descrita num caso de amiloidose sistémica senil (SSA), pesquisamos por RFLPs esta mutação em 3 casos de SSA. Verificamos por sequenciação dos 3 exons do gene de TTR que o gene da TTR na SSA é normal, confirmando por um lado a TTR Ile 122 não está associada à SSA, e por outro que na SSA o gene de TTR não é mutado.

Neste trabalho sugere-se a intervenção de factores tecidulares que condicionam a deposição de amilóide em particular no coração. Não excluimos, contudo, a contribuição de uma alteração estrutural e/ou proteolítica uma vez que se detecta a presença de fragmentos de TTR em fibras de amilóide na amiloidose sistémica senil (Trabalho nº 2).

3. Identificação de variantes moleculares de TTR não amiloidogénicas: estudos de rastreio de mutações de TTR na população normal por focagem isoeléctrica levaram à detecção de duas novas variantes de TTR por nós caracterizadas - TTR Asn 90 e TTR Arg

102. Nestes estudos foram também identificadas a TTR Thr 109 e a TTR Met 119, anteriormente descritas. A identificação da substituição de aminoácido baseou-se na análise de mapas de péptidos e sequenciação de aminoácidos de péptidos específicos. As mutações que originam estas substituições foram confirmadas por análises de DNA, nomeadamente sequenciação e análise de RFLPs, e estudos familiares.

Neste trabalho sugere-se que, embora estas variantes não pareçam ser amiloidogénicas, possam ocorrer modificações estruturais na molécula que alterem a sua função, como acontece com a TTR Thr 109 associada a hipertiroxinemia em indivíduos eutiroides (Trabalho nº 3).

Ainda em estudos anteriores de rastreio de mutações de TTR detectaram-se indivíduos portadores de duas mutações em alelos diferentes, designados por isso como heterozigóticos compostos. Sequenciação e análises de RFLPs de DNA amplificado permitiram a identificação de indivíduos portadores de TTR Met 30 - TTR Asn 90, TTR Met 30 - TTR Met 119 e TTR Asn 90 - TTR Met 119. Embora seja impossível prever as alterações induzidas pela presença de duas mutações, os compostos heterozigóticos portadores de mutações não patogénicas (TTR Asn 90- TTR Met 119) parecem ser indivíduos saudáveis. São, no entanto, necessários estudos a longo prazo para avaliar a sua associação com sindromas clínicos. Por outro lado, os indíviduos portadores de TTR Met 30 e de outra variante não patogénica, TTR Asn 90 ou TTR Met 119, não apresentam uma forma mais severa da doença. Pelo contrário, o portador de TTR Met 30 - TTR Met 119 apresenta uma evolução mais benigna da doença . Assim a presença de uma mutação não patogénica poderá ter algum efeito protector (Trabalho nº 3).

4. Estudo da origem da mutação que originou a TTR Met 30: Dada a frequência em Portugal da mutação que origina a TTR Met 30 e as relações ancestrais com outros países onde a TTR Met 30 também ocorre com alta frequência, tentamos averiguar a possibilidade desta mutação ter tido uma única origem, em Portugal, ou de a mutação ter ocorrido aleatóriamente noutros pontos do globo. Para isso baseamo-nos no trabalho publicado por Yoshioka *et al.* (1989) sobre o estudo da distribuição de haplotipos em indivíduos portadores da mutação TTR Met 30. No nosso estudo incluímos principalmente famílias

Met 30 portuguesas; também pesquisamos famílias de outros países europeus nomeadamente Suécia, Itália, Espanha, Inglaterra e Turquia. Verificamos que todos os portadores de TTR Met 30 Portugueses possuiam o mesmo haplotipo - haplotipo I - associado à mutação Met 30. O mesmo acontecendo com os portadores Suecos. Porém, nas famílias Italianas analisadas identificaram-se dois haplotipos diferentes associados à TTR Met 30 (haplotipo I e haplotipo III). Do mesmo modo o haplotipo III foi encontrado associado à TTR Met 30 numa família Inglesa e numa família Turca. Assim, sugerimos que a mutação que originou a TTR Met 30 deve ter ocorrido mais do que uma vez na Europa o que está de acordo com a hipótese das múltiplas origens da mutação apresentada por Yoshioka *et al.* (1989). No entanto, e ao contrário do que acontece no Japão, a mutação em Portugal parece ter uma origem única; o que está de acordo com a distribuição da mutação a partir de um foco principal localizado no litoral norte de Portugal. A hipótese das origens múltiplas é também apoiada pelo facto de a mutação que origina a TTR Met 30 ocorrer num dinucleótido CpG, local com alta susceptibilidade de mutagenese. Neste estudo tentamos também relacionar a variabilidade de expressão clínica, nomeadamente a idade de início da doença, com o haplotipo. No entanto não foi possível estabelecer qualquer relação: as famílias portuguesas estudadas, incluindo indivíduos com início tardio e com início típico, apresentaram todas o mesmo haplotipo associado à mutação. De igual modo, as famílias suecas estudadas, que se sabe terem um início típicamente tardio apresentaram o mesmo haplotipo das famílias portuguesas (Trabalho nº 4).

5. Haplotipos associados às mutações de TTR mais frequentes em Portugal:

Estudaram-se os haplotipos associados às variantes TTR Met 119, TTR Asn 90 e TTR Ser 6. Verificou-se que as mutações TTR Met 119 e TTR Asn 90 estão associadas ao haplotipo III; o haplotipo III foi anteriormente descrito em associação com a TTR Met 119 em indivíduos Americanos. A TTR Ser 6, mutação que ocorre com maior frequência na população, mostrou-se associada ao haplotipo I. No entanto, dado o número reduzido de indivíduos estudados, não se pode excluir que outros haplotipos estejam associados a estas mutações. Nomeadamente seria de esperar mais do que um haplotipo para mutações em dinucleótidos CpG, tal como é o caso das TTR Ser 6 e TTR Met 119 (Trabalho nº 4)

6. Ligação da tiroxina à TTR de indíviduos heterozigóticos compostos portadores das mutações mais frequentes em Portugal: neste trabalho tentamos estudar a influência de uma segunda mutação na proteína sobre a sua função de ligação de tiroxina. Para isso estudamos a ligação de tiroxina a TTR isolada de plasma de indíviduos heterozigóticos compostos, nomeadamente portadores de TTR Ser 6 - TTR Met 30, TTR Ser 6 - TTR Met 119, TTR Met 30 - TTR Asn 90, TTR Met 30 - TTR Met 119 e TTR Asn 90 - TTR Met 119. Neste estudo também incluímos proteínas isoladas de indivíduos heterozigóticos para cada uma das variantes consideradas ou seja, TTR Ser 6, TTR Met 30, TTR Asn 90, TTR Met 119 e TTR normal. Quanto à afinidade de ligação de T_4 a cada uma destas variantes verificamos que a TTR Ser 6 tem uma afinidade de ligação semelhante à TTR normal e que a TTR Met 119 tem uma afinidade mais elevada que a TTR normal. Quanto à TTR Met 30 confirmamos a afinidade reduzida já descrita na literatura. Em relação à TTR de heterozigóticos compostos verificamos que a presença de TTR Met 119 está relacionada com o aumento da afinidade de ligação à T_4 e que este efeito é independente do outro monómero presente ser normal, TTR Ser 6 ou TTR Met 30.

A TTR Asn 90 apresenta uma afinidade de ligação de T_4 menor que a TTR normal e parece não apresentar cooperatividade negativa na ligação à T_4.

Este comportamento diferente da TTR Asn 90 é também observado em compostos heterozigóticos. Sugerimos que nesta variante a substituição de um resíduo de histidina por um de asparagina provoca alterações nos contactos entre monómeros já que este resíduo está envolvido nessas interacções levando à perda da cooperatividade negativa da ligação (Trabalho nº 5).

7. Ligação de tiroxina a outras variantes de TTR: testamos a ligação de T_4 a outras variantes de TTR . Nestes ensaios utilizamos soros totais e/ou TTR isolada do soro. Na maior parte dos casos não se verificaram alterações significativas da afinidade para a tiroxina excepto nos portadores de TTR Pro 55 , TTR Met 111 e de TTR Thr 109. Assim, verificou-se um aumento considerável na afinidade de ligação à TTR Thr 109 confirmando os valores da literatura; contráriamente, no caso de portadores de Pro 55 e Met 111, verificou-se uma diminuição na afinidade de ligação. Dado que os indivíduos estudados

eram heterozigóticos para a mutação, uma análise mais precisa destes resultados exigia a comparação com os resultados da ligação às correspondentes mutantes sintéticas homotetraméricas que no caso da TTR Met 111 e da TTR Pro 55 existiam no laboratório. Os resultados obtidos no estudo de ligação competitiva de T_4 à TTR Met 111 recombinante sugerem uma alteração na cooperatividade negativa de ligação de T_4 a esta variante que apresenta uma diminuição da afinidade para a T_4. Relativamente à TTR Pro 55, verificamos que a proteína recombinante (homotetramérica) aparentemente não liga T_4, tal como acontece com a TTR Met 30. Este facto poderá dever-se a alterações na estrutura quaternária, consequentes de alterações estruturais das cadeias C e D do monómero de TTR (Trabalho nº 6)

Embora outros autores tenham sugerido que todas as variantes amiloidogénicas de TTR possam apresentar uma alteração comum na estrutura quaternária que predispõe à fibrilogénese, os resultados obtidos por nós no estudo da ligação de T_4 levam-nos a pensar que tal não sucede e que só estudos de cristalografia e difracção de raios-X desta e de outras variantes amiloidogénicas e não amiloidogénicas poderão sustentar essa hipótese. Os nossos resultados no estudo de ligação a diferentes variantes de TTR serão importantes na definição das mudanças conformacionais associadas às diversas mutações.

4. TRABALHO EXPERIMENTAL

4.1. IDENTIFICATION OF FIVE DIFFERENT TRANSTHYRETIN (TTR) VARIANTS ASSOCIATED WITH FAMILIAL AMYLOIDOTIC POLYNEUROPATHY (FAP): TTR Leu 64 and TTR Tyr 77 and first report of TTR Ala 49, TTR Gln 89 and TTR Ala 71

ABSTRACT

We report the molecular characterization of different transthyretin (TTR) variants associated with familial amyloidotic polyneuropathy (FAP). Three of these variants are newly described, namely TTR Ala 49, TTR Gln 89 and TTR Ala 71. The first two TTR variants were detected by isoelectric focusing (IEF) in two Italian families with hereditary amyloidosis (HA), one being neutral and the other basic. Both families presented neuropathy and cardiomyopathy but they differed in other clinical features. By protein and DNA analysis the neutral variant was found to have a substitution of an alanine for a threonine residue at position 49 (TTR Ala 49) of the polypeptide chain. The basic variant had a glutamine residue replacing glutamate at position 89 (TTR Gln 89).

The third new variant characterized by DNA sequencing was TTR Ala 71, found in a Spanish individual with FAP .

Two other families presented TTR variants that have been previously reported. One of these families, with Italian ancestors, had TTR Leu 64 associated with FAP. In the other family, an American family of various European origins, the variant associated with FAP was TTR Tyr 77.

INTRODUCTION

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant disease characterized by the systemic deposition of amyloid with particular involvement of the peripheral nerves. In FAP, the amyloid deposits are mainly composed of a mutated transthyretin (TTR). Different TTR variants are associated with different forms of amyloidosis and the organs involved in the amyloid deposition are variable. In most cases, there is amyloid deposition in the peripheral nerves causing FAP (Saraiva, 1991); in others, the amyloid deposits preferably in the heart (familial amyloidotic cardiomyopathy - FAC) and still in other cases, into the vitreous body (Saraiva *et al.,* 1993). The distinction between these forms of hereditary amyloidosis (HA) is difficult due to the involvement of multiple organs in the disease.

The most common TTR variant associated with FAP has a substitution of methionine for valine at position 30 of the polypeptide chain, TTR Met 30 (MIM 176300.0001), which has been found in different populations (Saraiva *et al.,* 1988b). Therefore samples from individuals with FAP are first investigated for the presence of the TTR Met 30 mutation. If the Met 30 mutation is not present, the analysis proceeds to search for the presence of other TTR mutations. Isoelectric focusing has been used as a screening method of TTR variants in the sera of individuals from families with HA (Altland *et al.,* 1987). By this method two Italian families from Sicily were found to have two non-Met 30 TTR variants (Saraiva *et al.,* 1988a). One of them was a neutral variant whereas the other one was basic. In both families there was a late onset of both neuropathy and cardiomyopathy.

We report the biochemical and genetic analyses of these families which started with the study of the primary structure of the protein by tryptic peptide mapping and aminoacid sequencing, and was followed by the definition of the genetic defect by DNA sequencing, allele specific oligonucleotide (ASO) hybridization and/or restriction fragment lenght polymorphisms (RFLP) analysis.

We also present the characterization of a new TTR variant found in a Spanish individual with FAP with amyloid present in a nerve biopsy. Since several Spanish families have already been reported as carriers of the Met 30 mutation this mutation, was first excluded. Analysis of the DNA sequence showed a mutation in the codon for aminoacid residue 71 of the TTR monomer, originating a substitution of alanine for valine - TTR Ala 71.

Studies on other FAP families revealed different TTR variants associated with the disease; one American family with Italian ancestors presented TTR Leu 64 and in another American family with heterogeneous European origin, TTR Tyr 77 was diagnosed.

MATERIAL AND METHODS

Individuals studied:

Kindred I: An Italian kindred from Sicily with hereditary amyloidosis confirmed by skin and/or nerve biopsy (Salvi *et al.,* 1990). Three patients have been observed with a long follow-up (more than four years). The disease started in the 5th decade of life with appearance of vitreous opacities followed, several years later, by polyneuropathy and cardiomyopathy (Salvi *et al.,* 1991). We studied eleven individuals from this kindred, including two patients and their asymptomatic offspring.

Kindred II: An Italian kindred from Sicily with hereditary amyloidosis. Three patients presented carpal tunnel syndrome as an initial clinical manifestation of the disease. Many years later it was followed by polyneuropathy and cardiomyopathy responsible, in one patient, for intractable heart failure and death (Salvi *et al.,* 1990). We analysed 12 individuals belonging to this kindred, three patients and nine asymptomatic subjects.

Kindred III: The propositus was a 32 years old man from a Spanish family with a history of hereditary amyloidosis, who presented a sensory motor neuropathy of the lower limbs, loss of weight and severe constipation. Amyloid was demonstrated in a nerve biopsy. Immunoblotting of transthyretin isolated from the serum showed that TTR Met 30 was not present (Saraiva *et al.,* 1985). We studied, also, 3 brothers of the propositus.

Kindred IV: The propositus was a 67 year old man of Italian origin, living in California, who began to suffer from slowly progressive sensory and motor neuropathy and cardiac arrythmias after age sixty. Two of his brothers also had a progressive neuropathy with fatal outcome; in one of them, amyloid deposits were detected in biopsies of bowel and urinary bladder. Blood for DNA analyses was available from the propositus, one symptomatic brother and their asymptomatic offspring.

Kindred V: The propositus was a 48 year old woman belonging to an American family of European origin with amyloidosis history. The father and an uncle of the propositus had an onset of the disease in their early sixties, with loss of sensation in lower and upper limbs, gastrointestinal disturbances with diarrhea, heart problems that led to the use of a pace maker, developed pulmonary disease and died from pneumonia. We studied the propositus and four asymptomatic individuals of this family: one sister, two cousins and an uncle.

Isoelectric focusing: TTR variants were screened by a hybrid isoelectric focusing (IEF) method (Altland and Banzhoff, 1986; Altland *et al.,* 1987). The gels consisted of 4-7 immobilized pH gradient with an immobiline buffering power of 1 meqv/pH unit per litre and a separation distance of 14 cm. The central part, between 4 and 10 cm, was flattened to 0.05 pH units/cm. These gels were poured by the use of computer controlled burettes in the laboratory of Dr. Klaus Altland, in Germany. The dried gels were rehydrated 2-3 hours with a solution 8M urea, 0.5% Pharmalites, 10% Dextran. The gels were pre-focused at constant voltage, aproximately 300V and cooled to 10ºC. Focusing was run at 4000V for 6 hours or overnight. The gels were stained by Coomassie Blue or silver staining.

TTR isolation from plasma: TTR was isolated from plasma by procedures described previously (Saraiva *et al.,* 1985), including ion-exchange and affinity chromatography on Blue-Sepharose.

Peptide mapping and sequence analysis: Comparative tryptic peptide mapping of plasma TTR from the patient and of normal TTR was performed by high performance liquid chromatography (HPLC). Approximately one miligram of each TTR sample was digested with trypsin, and the resulting peptides were separated by HPLC on a reverse-phase C8 column with a gradient of 0 to 30% of acetonitrile in 0.1% trifluoroacetic acid, for 85 minutes (Saraiva *et al.,* 1990b). Some of the peptides were collected and subjected to sequence analysis on an Applied Biosystems model 470A gas-phase sequencer equipped with a 120A

PTH analyzer. The analyses were performed in the Protein Core Laboratory at Columbia University, by Dr. MA. Gawinowicz.

DNA isolation: DNA was isolated from leucocytes by phenol extraction after cell lysis with proteinase K and SDS, as previously described by Kan & Dozy (1978).

In some cases we extracted DNA from fixed tissues in paraffin blocks (Wright *et al.,* 1990). We used several sections of 5-10 µm thick and extracted the paraffin with xylene and ethanol. Then, the tissue was digested with proteinase K and the lysate was used for DNA amplification.

DNA amplification: Exons 2, 3 and 4 of the TTR gene were amplified by the polymerase chain reaction (PCR) using the method of Saiki *et al.* (1985) with the appropriate flanking primers for the region of interest. The primers used for the amplification of exon 2 were: 2A (5' GTTAACTTCTCACGTGTCTT 3') and 2B (5' AAGTCCTGTGGGAGGGTTCT 3') with cycles of 1' 92°C, 2' 50°C and 3' 72°C. For the exon 3 the primers used were 3A (5' TCCTCCATGCGTAACTTAAT 3') and 3B (5' ACTGTGCATTTCCTGGAATG 3') with cycles of 1' 92°C, 2' 55°C and 3' at 72°C. Alternatively, we used another pair of primers for TTR exon 3 amplification: 23A - 5' ACTTGGTGGGGGTGTATTAC 3' and 23B - 5' GGAAAGGGAACCTTTGGTCA 3' with PCR cycles of 1 min at 92 °C, 1 min at 50°C and 1 min at 72°C. Primers for the amplification of exon 4 were: 4A (5' CACGTTTTTCGG GCTCTGGTCG 3') and 4B (5' GAAATCCCATCCCTCGTCCTTC 3') and the amplification cycles were 1' at 92°C, 1' at 60°C and 1' at 72°C (Almeida *et al.,* 1990).

DNA sequencing: Exons 2, 3 and 4 were symetrically amplified with the appropriate pair of primers, as described before (section 4.1).Then, we performed an asymetric PCR with 1 µl of the symetric PCR product and 50 pmol of one of the primers for each exon (McCabe, 1990). The asymetric product was then precipitated with ethanol and ressuspended in 10 µl of TE (Tris 0.1 M, EDTA 1mM pH 8) buffer.

In some instances, an avidin capture method was used to isolate single strand DNA. For this purpose, each exon of the TTR gene was amplified asymetrically with 5 pmol of biotinylated primer A and 100 pmol of primer B. The amplified DNA was linked to avidin-agarose (Mitchell *et al.*, 1989) . The slurry was applied to a Sephadex G-50 column and the amplified strand (anti-sense) was eluted with NaOH 0.2 M; after neutralization with ammonium acetate 5M, the single strand DNA was precipitated with ethanol and diluted with 20 µl of TE buffer.

Seven µl of the single strand DNA was sequenced with 10 pmol of primer A using Sequenase version 2.0 (USB) with α-^{32}P-dATP. The sequencing gel was exposed to X-ray film for 24 h.

Allele specific oligonucleotide (ASO) hybridization: one third (about 20 µl) of the amplification mixture was denatured with NaOH, neutralized and dotted onto a Zeta probe membrane (Bio-Rad). The ASO probes employed for kindred I were: normal (Thr 49) - 5'TATAGGAAAACCAGTGAGT3' and mutant (Ala 49) - 5'ACTCACTGGCTTTCCTATA3'. The probes were labeled with γ-^{32}P-ATP by the T_4 kinase reaction for 30 min at 37°C. Hybridization with ASO probes was carried out at 30°C, followed by washes under stringent conditions at 55°C (Almeida *et al.*, 1990). The blots were then exposed to X-ray film for 2-4 h. The ASO probes for the basic variant were: normal (Glu 89) - 5' CCATTCCATGAGCATGCAG 3' and mutant (Gln 89) - 5' CCATTCCATCAGCATGCAG 3'. The conditions of hybridization were the same as described for kindred I except that the washing temperature was 53°C.

RFLP analysis: One fourth of the symmetrically amplified TTR exons was digested with the appropriate enzymes according to the instructions of the manufacturer. The digested DNA was analysed by electrophoresis in a 4% Nusieve (FMC) agarose gel stained with ethidium bromide.

RESULTS

Kindreds I and II (Italian kindreds)

We studied two different Italian families with HA, both from Sicily. Sera from the FAP patients and their asymptomatic offspring had been first screened for the presence of TTR Met 30 by immunoblotting and it It was found that the individuals were not carriers of the TTR Met 30 mutation; Therefore, to check for other mutations, we performed IEF analysis on their sera as shown in Figure 1.

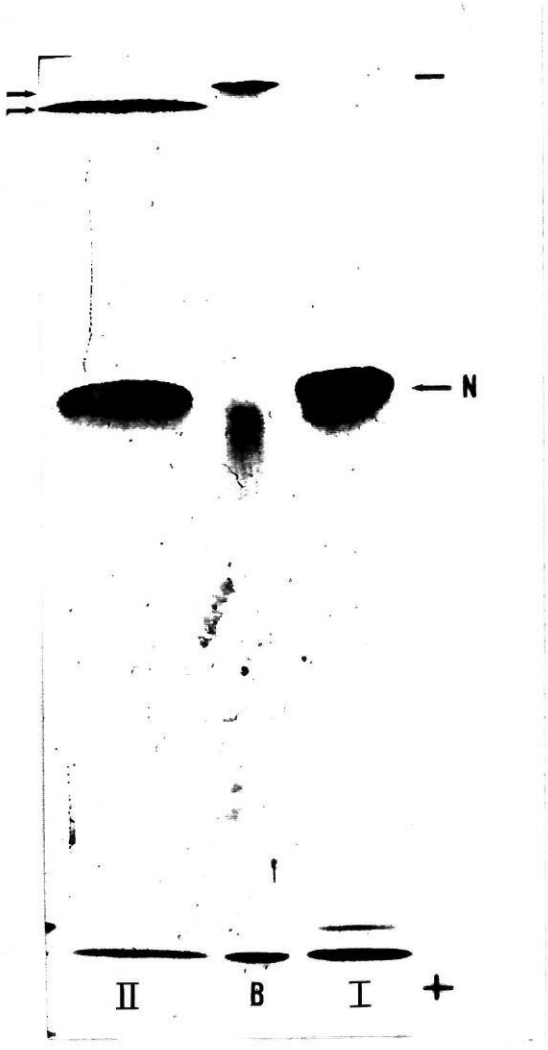


Fig. 1 - IEF under denaturing conditions of TTR variants from Italian kindreds. The middle lane contains a non-pathogenic TTR basic variant, present in the German population, where a central normal band (diffuse here, due to ageing of the sample), an anodic oxidation product of TTR and a cathodic basic TTR monomer are detected. The left-hand lane shows the basic TTR variant (kindred II). The right-hand lane of the gel shows the pattern observed in kindred I.

For comparative purposes, a sample from an individual with a TTR variant previously described in the German population (Altland and Banzhoff, 1986) was applied to the gel. For this sample, represented in the midle lane, three bands were observed: (i) a normal TTR monomer band, labelled N, (ii) an anodic band, which is an oxidation product of the TTR subunit, and (iii) a cathodic band representing the basic TTR variant monomer, not present in the normal serum.

The IEF pattern obtained for the propositus from kindred II is shown on the left hand side. An extra protein band was observed in the cathodic part of the gel, representing a basic variant TTR monomer. In the case of the propositus from kindred I, no evidence for a charged TTR variant monomer was obtained. However, as shown on the right hand side of the gel, a splitting of the normal TTR monomer band, and an additional oxidation product on the anodic part of the gel, were observed. The splitting of the monomer band suggested that a neutral substitution might be present in kindred I.

TTR was isolated from the serum of the individual presenting the neutral variant and analysed by comparative tryptic peptide mapping (Figure 2).

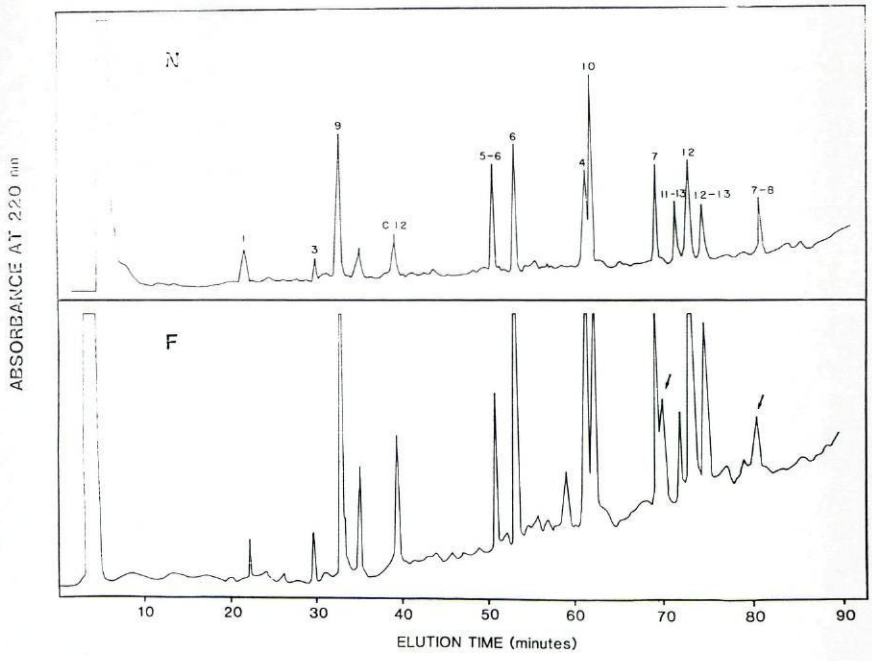


Fig. 2 - Tryptic peptide map of isolated TTR from a normal individual (N) and from a carrier of the neutral TTR variant (F). The abnormal peptides are labeled with an arrow.

The analysis of the peptide map revealed the presence of an abnormal peptide, labeled 7*, eluting in a position close to the normal peptide 7 (residues 49-70). Normally, peptide 7 also elutes at the end of the gradient as an uncleaved product containing peptide 8 (peptide 7-8); however the mutant peptide 7-8 (also labeled) is not separated from the normal counterpart under the gradient conditions used. Amino acid sequencing of peptide 7* showed the substitution of an alanine for threonine at position 49 of the polypeptide chain. This substitution could be explained by a G for A change in the codon of threonine (Thr (ACC)- Ala (GCC)), which was confirmed by DNA sequence analysis. In order to study other members of the family, we synthesized ASO for the mutation to perform allele specific hybridization since this mutation does not create or abolish any restriction site for endonucleases.

From the eleven individuals analysed by ASO hybridization we found six carriers of the mutation (Figure 3): two FAP patients and four asymptomatic carriers. All the carriers were heterozygous for the mutation since their DNA hybridized with both the normal and the mutant probe.

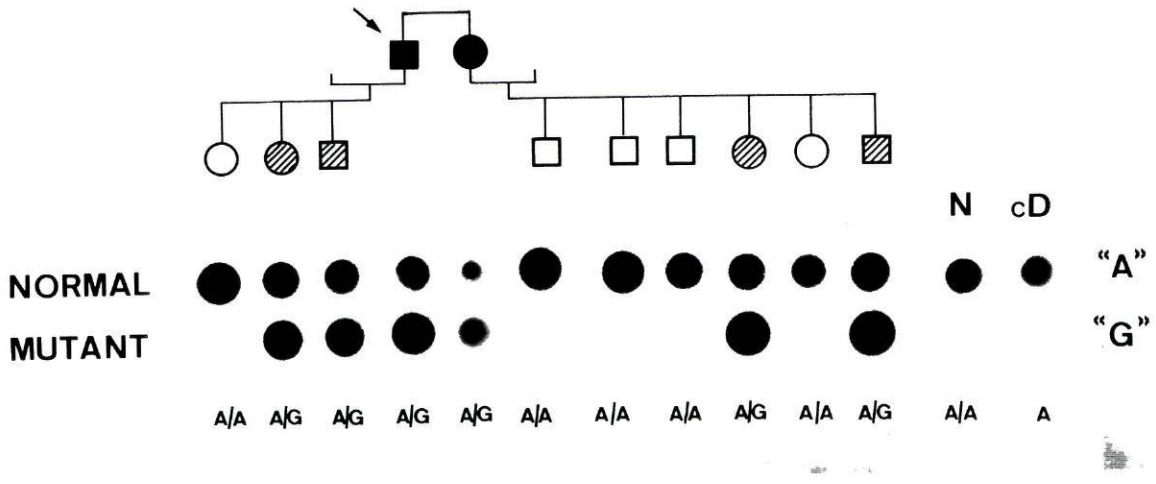


Fig. 3 -Genotype analysis of the family with the neutral TTR variant (kindred I) - TTR Ala 49 - by ASO hybridization. A normal (N) and a cDNA (cD) were used as controls.
●, ■ patients; ⊘, ▨ asymptomatic carriers; ○, □ non-carriers;

Concerning the other family with the basic TTR variant (kindred II), we isolated TTR from the serum of the propositus and analysed it by comparative tryptic peptide mapping.

The peptide map obtained for the basic TTR variant was similar to that of the normal protein. Since this was not conclusive we proceeded with the DNA analysis of the propositus. We sequenced PCR amplified exons of the TTR gene and found a mutation in exon 3. This mutation was a single base change of a cytosine for a guanine giving a substitution of glutamine (CAG) for glutamate (GAG) at position 89 of the TTR polypeptide chain (Figure 4).

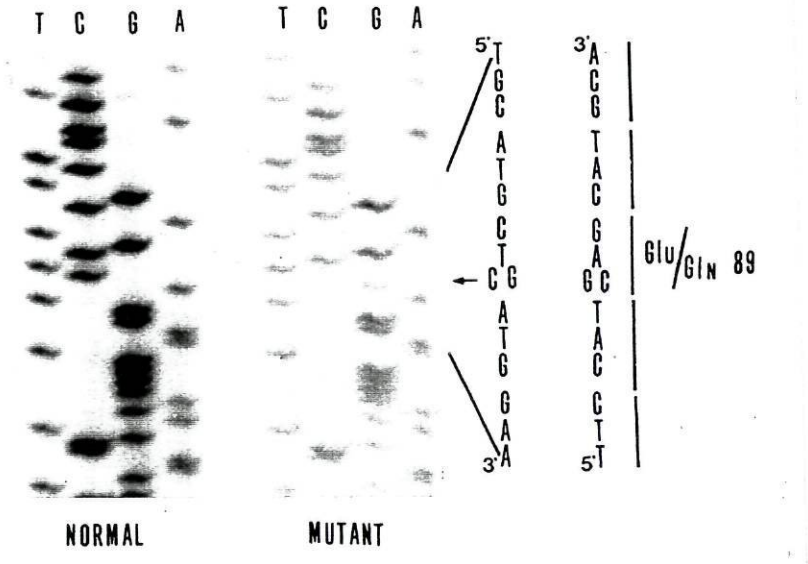


Fig. 4 - Sequencing analysis of PCR amplified exon 3 of TTR gene from kindred II. Comparison with normal DNA sequence. The mutant sequence shows both guanine and cytosine at the first base of codon 89, resulting in a substitution of glutamine for glutamate.

In order to confirm the substitution at the protein level we sequenced peptide 10 (aminoacids 81-103) and found a Gln for Glu substitution at position 89. This result is in agreement with the basic behaviour of the mutant protein since there is a substitution of a basic aminoacid residue for an acidic one. The base substitution corresponding to this mutation abolishes a restriction site for endonuclease NlaIII; however the fragments originated could not be separated by agarose gel electrophoresis given their size proximity -

6 bases. Thus, to investigate the presence of this TTR variant (TTR Gln 89) in the members of the family we performed hybridization with allele specific probes. We found 3 carriers of the mutation and 8 non carriers as shown in Figure 5.

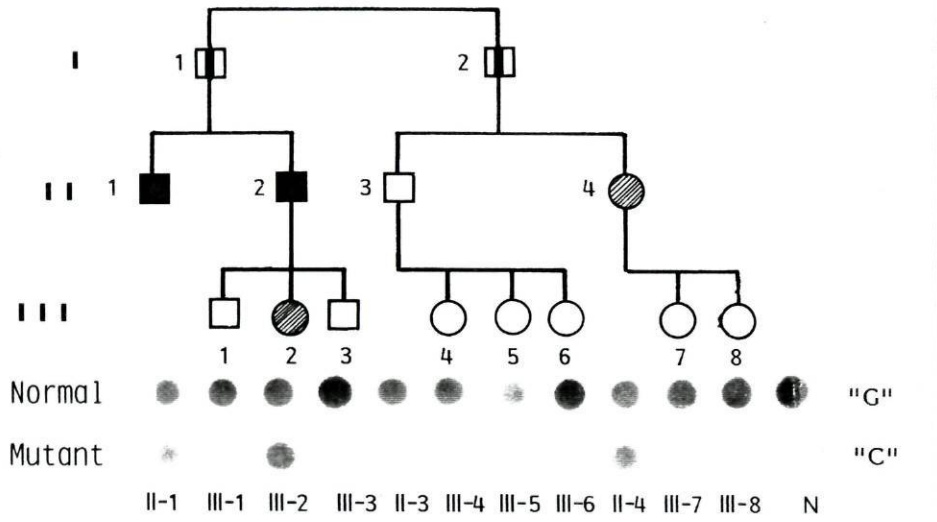


Fig. 5 - Genotype analysis of the family with the basic TTR variant (kindred II) - TTR Gln 89 - by ASO hybridization.
▯▯ , individuals described as affected by the disease. The other symbols are the same as in Fig. 3.

Kindred III (Spanish kindred)

The presence of amyloid in a nerve biopsy of the propositus and the exclusion of the presence of TTR Met 30 in the patient's serum led us to suspect that the amyloidosis in the propositus should be related to a different TTR variant.

In order to identify this hypothetical TTR variant we sequenced amplified DNA extracted from a paraffin liver biopsy. Sequence analysis showed a cytosine for thymine substitution in the second base of the codon for amino acid residue 71 of the polypeptide chain (Figure 6A). This mutation corresponds to a substitution of alanine (GCG-mutated residue) for valine (GTG-normal allele) originating TTR Ala 71.

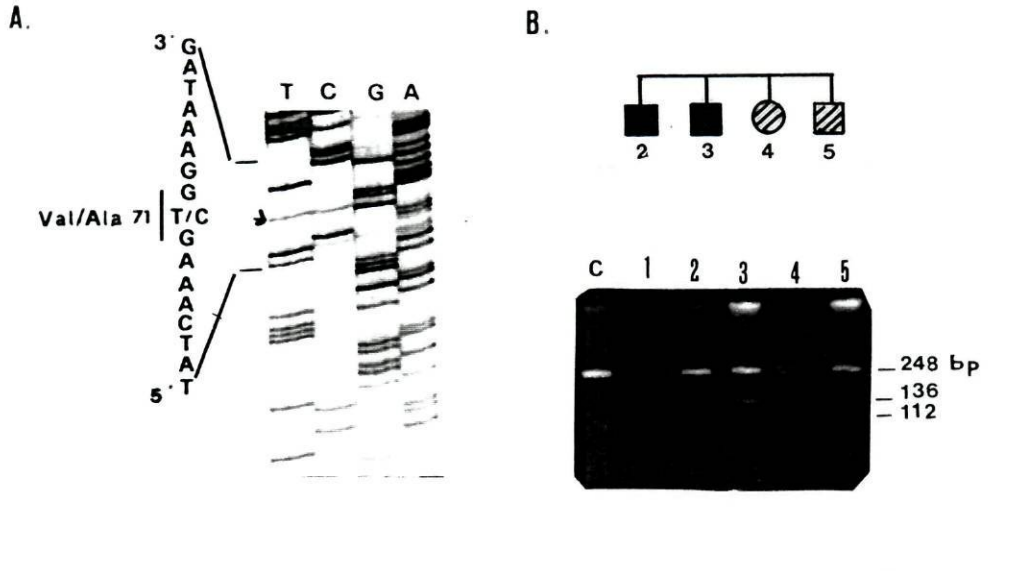


Fig. 6 - Analysis of the amplified exon 3 of the TTR gene. **A.** DNA sequencing. **B.** Digestion with Aci I. C- control DNA; 1 - DNA from a paraffin liver biopsy and 2- DNA from leucocytes of the propositus; 3,4 and 5- DNA from 3 siblings of the propositus.

To make possible a simple and rapid diagnosis of other individuals at risk, we searched for an alteration of a restriction site originated by this mutation and found that one restriction site was created for endonuclease Aci I 5'...GCGG...3'. Digestion of the amplified exon 3 of the TTR gene with Aci I (NEB) originates 2 fragments of 112 and 136 bp, when the mutation is present. The fragment of 248 bp corresponds to the undigested amplified DNA. Thus, we analysed DNA from the propositus and 3 brothers by RFLP analysis and found that the propositus and all the siblings were carriers of the mutation (Figure 6B). One of these siblings had also developed clinical symptoms of the disease.

Kindred IV (American kindred of Italian origin)

Since the propositus of this kindred had amyloidosis confirmed by biopsy and developed a sensory and motor neuropathy, we searched for a TTR variant that might be associated with the disease. Sequence of exon 3 of the TTR gene showed a substitution of

cytosine for thymine in the first base of codon 64 corresponding to a substitution of leucine for phenylalanine (Figure 7).

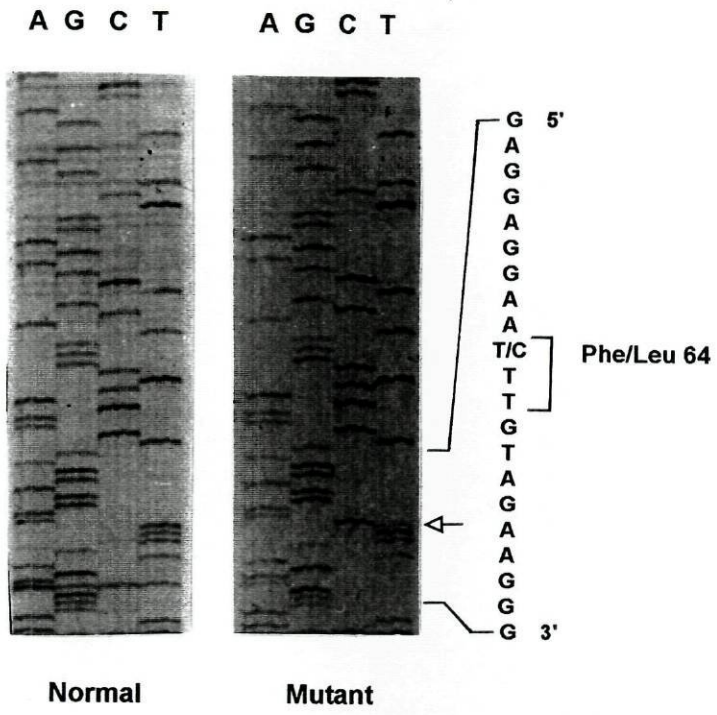


Fig. 7 - Sequencing analysis of amplified exon 3 of the TTR gene from a control and a carrier of a TTR variant (kindred IV). The first base of codon 64 of the TTR polypeptide chain presents both cytosine and thymine corresponding to a substitution of leucine for phenylalanine.

This substitution abolished a restriction site for the enzyme Apo I (NEB), which allowed the diagnosis of nine other members of the kindred. The result of the RFLP analysis is shown in Figure 8. Sample 1 is a control DNA, samples 2, 3 and 4 correspond to the amplified DNA of the propositus, a symptomatic brother and one asymptomatic nephew, respectively. The DNA fragment of 348 bp corresponds to the amplified fragment of exon 3 and fragments of 221 and 127 bp are the resulting fragments after digestion of the normal sequence with Apo I. As can be seen in the ilustration, and contrary to sample 1, samples 2 , 3 and 4 were not completely digested, indicating that these individuals were carriers of

the mutation. The other exons were screened for the presence of other mutations by SSCP analysis, which detects most of the known mutations, and none were found (Torres *et al.*, in press).

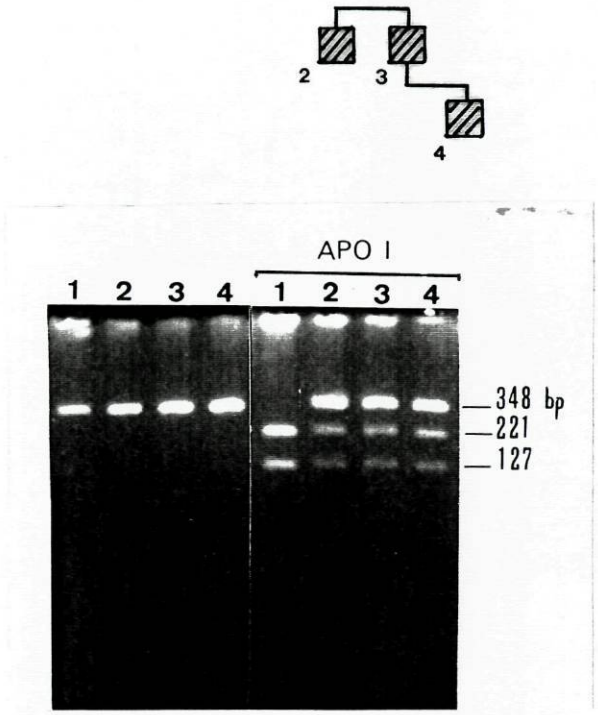


Fig. 8 - RFLP analysis of amplified exon 3 of the TTR gene. Samples digested with endonuclease Apo I are indicated. Sample 1 is a control and samples 2, 3 and 4 correspond to carriers of the mutation.

Kindred V (American family of European origin)

Sera from the propositus of this kindred was first studied by IEF and the pattern observed was similar to that found for TTR Tyr 77 (previously described) (Wallace *et al.*, 1988b) (Altland K. personal communication). Since this mutation creates a restriction site for endonuclease Ssp I (BRL) we digested amplified DNA from exon 3 (247 bp) with this enzyme and analysed it by electrophoresis in a Nusieve agarose gel (Fig. 9). We concluded that the propositus and her sister were carriers of TTR Tyr 77 since only their amplified

DNA could be digested with Ssp I giving origin to the expected fragments of 130 and 117 bp each.

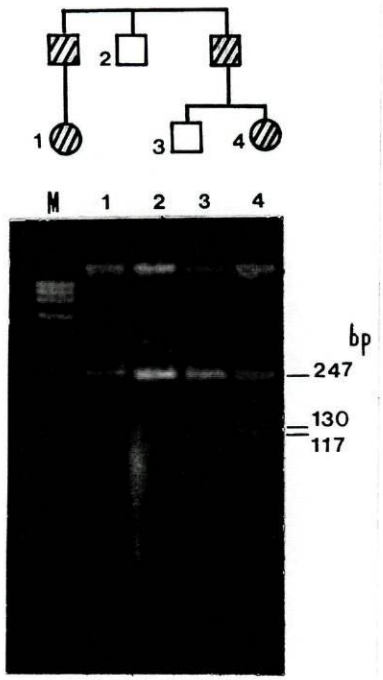


Fig. 9 - RFLP analysis of amplified DNA from exon 3 of individuals from kindred V. The carriers of the TTR Tyr 77 present two extra bands of 130 and 117 bp corresponding to the fragments originated by digestion with Ssp I.

DISCUSSION

Most of the TTR related amyloidoses give rise to neuropathy as the main clinical feature. However, cardiomyopathy, amyloid deposition in the vitreous, carpal tunnel syndrome and other clinical manifestations occur, in most, cases with a variable degree of relevance.

Two of the new TTR variants presented here, TTR Ala 49 and TTR Gln 89, add to the list of variants associated with amyloid neuropathy and cardiomyopathy. Though in this

group of variants, neuropathy and cardiomyopathy coexist, they are predominantly neuropathic making them neuropathic forms of HA. TTR Ala 49 is also associated with amyloid deposition in the vitreous, resembling the clinical picture of TTR Pro 36 (Jones *et al.*, 1991) and TTR Ser 84 (Wallace *et al.*, 1988a). TTR Pro 55 (Jacobson *et al.*, 1992b) is also associated with neuropathy, cardiomyopathy and amyloid deposition in the vitreous but has an earlier onset (in the second decade of life) and a particularly aggressive and rapid progression. Two other TTR variants with predominant amyloid deposition in the vitreous, are TTR His 69 (Zeldenrust *et al.*, 1994) and TTR Asn 84 (Skinner *et al.*, 1992), but in these cases no significant neuropathy and/or cardiomyopathy are observed.

The TTR Gln 89 mutation was associated with neuropathy, cardiomyopathy and carpal tunnel syndrome similarly to TTR His 58 (Nichols *et al.*, 1989), TTR Arg 58 (Saeki *et al.*, 1991), TTR Ala 60 (Wallace *et al.*, 1986), TTR Asp 70 (Izumoto *et al.*, 1992) and TTR His 114 (Ueno *et al.*, 1990).

If we localize the neuropathic mutations described in this work in the molecule, we find that they lye apart, as can be seen in Figure 10.

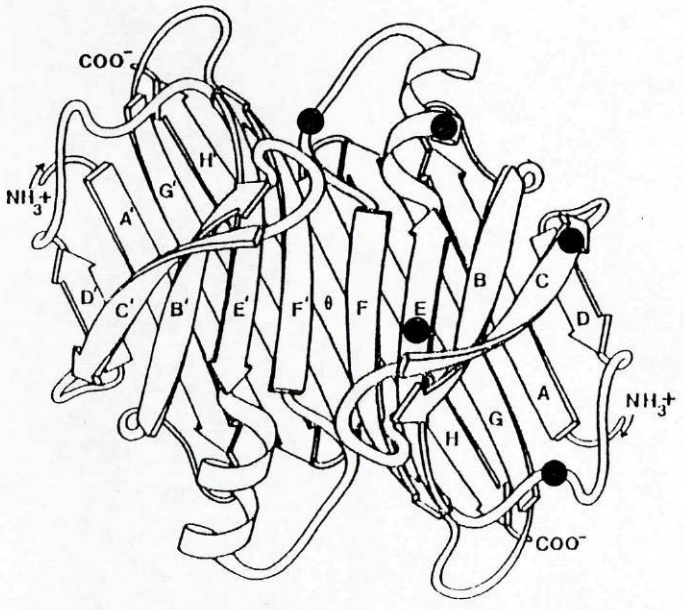


Fig. 10 - Schematic representation of the TTR dimer with the localization of the aminoacid substitutions of the neuropathic TTR variants described in this work.

Two of the mutations are located in ß strands (TTR Ala 49 and TTR Ala 71), one is located in the α-hélice (TTR Tyr 77) and the other two are in different *loops* of the TTR monomer (TTR Leu 64 and TTR Gln 89). Thus, we corroborate that TTR neuropathic mutations are not located in a specific region of the molecule. Whether they induce a common conformation is still a matter to be further investigated. This aspect will be discussed in later sections (sections 5 and 6).

The lack of clinical and genetic and/or structural correlation observed in the neuropathic forms of HA is further evident on the heterogeneity of clinical expression in different kindreds with the very same mutation. For instance, though TTR Leu 64 was first described in a family from Italy, with carpal tunnel syndrome (Ii *et al.,* 1991), in the case we analyzed no carpal tunnel syndrome was observed. Similarly, TTR Ala 49 has recently been reported in a French family, associated with neuropathy, cardiomyopathy and, contrary to what we reported, also with carpal tunnel syndrome (Benson II *et al.,* 1993). However, the most striking case seems to be that of TTR Ile 50 (Saeki *et al.,* 1992; Nishi *et al.,* 1992) which was desbribed in two kindreds from different islands in Japan. In one case the TTR Ile 50 is associated with polyneuropathy and in the other case only with cardiomyopathy. The high clinical heterogeneity for a same genetic variant indicates that other factors, either genetic or environmental, are involved.

The genotypic and phenotypic diversity of the TTR amyloidoses imply precise and sensitive diagnostic methods for the identification of the TTR mutation associated with a particular clinical syndrome. On the other hand, the similarity of some syndromes requires a differential genetic diagnosis. That was the case of TTR Ala 71, that is clinicaly similar to TTR Met 30. TTR Ala 71 was the first and unique non-Met 30 FAP kindred reported in Spain and it is possible that it occurs in other kindreds. The same holds true for FAP in Portugal. Only a precise genetic diagnosis of Portuguese FAP kindreds will exclude the sole occurrence of TTR Met 30 associated with FAP in Portugal.

Although protein analysis by HPLC tryptic peptide mapping and amino acid sequencing of the abnormal peptide is important for mutation identification, and should be used to identify post-translational modifications, it can present drawbacks. For instance, protein analysis did not allowed the identification of TTR Gln 89.

Since the TTR gene is a single copy gene with 4 exons of about 200bp each, sequencing of PCR amplified DNA is a more sensitive and simple method to identify mutations in the TTR gene. However more simple and reliable methods of diagnosis are needed for asymptomatic carrier detection and genetic counseling purposes, such as RFLP analysis.

RFLP analysis of PCR amplified DNA is easily used when the mutation creates or abolishes a restriction site for an endonuclease. That was the case of TTR Ala 71, TTR Leu 64 and TTR Tyr 77. RFLP analysis can substitute other methods as new enzymes are available. For instance, we have used a recent enzyme (Apo I) to identify carriers of TTR Leu 64 whereas in the past, this mutation was screened by an allele specific probe. ASO hybridization was used by us when there was no altered restriction site, as was the case of TTR Ala 49 and TTR Gln 89. However, it is possible to construct specific primers, that when included in the PCR amplification, create a restriction site. This method is termed PCR induced mutation restriction analysis (PCR-IMRA) or restriction site generation by PCR (RG-PCR). This last method has been used by other authors for the detection of TTR Ala 49 and TTR Gln 89 (Ferlini *et al.,* 1992; Benson II *et al.,* 1993).

In conclusion, the definition of the mutated residue is not sufficient to characterize or predict the clinical expression of a variant. It seems also important to assess the function of these modified proteins, namely T_4 and RBP binding, and to establish the structure of these variants by X-ray cristalography for comparison purposes. As more TTR variants are characterized, we will get a better insight into the factors or processes involved in the pathogenesis underlying FAP.

4.2. IDENTIFICATION OF TRANSTHYRETIN VARIANTS ASSOCIATED WITH CARDIAC AMYLOIDOSIS: TTR Ile 122 and first report of TTR Thr 45 and TTR Leu 68

ABSTRACT

Some cases of TTR related familial amyloidotic cardiomyopathy (FAC) were investigated for the presence of a mutated TTR. In one of these cases a new TTR mutation (TTR Thr 45) was identified in an individual of Italian origin. After protein characterization the amino acid substitution was confirmed by DNA sequencing and the mutation was screened in the family by ASO hybridization.

Another case of TTR related cardiac amyloidosis described in a German individual was studied. Electrophoretic analysis of plasma TTR showed the presence of an electrically neutral variant. Protein analysis by comparative peptide mapping and amino acid sequencing combined with DNA sequencing revealed the presence of a new TTR mutation: a substitution of leucine for isoleucine at position 68 - TTR Leu 68.

TTR Ile 122 has been reported associated with senile systemic amyloidosis (SSA), which is characterized by massive heart amyloid deposition and occurs late in life, in individuals homozygous for the mutation. In this work, the heterozygous condition for TTR Ile 122, previously reported by protein analysis in an individual of black origin, was confirmed by DNA sequencing and RFLP analysis of PCR amplified DNA.

The Ile 122 mutation was absent in five other cases of SSA, suggesting that TTR Ile 122 is not associated with SSA.

INTRODUCTION

Cardiac amyloidosis is characterised by the deposition of amyloid fibrils in the heart. In the hereditary forms of cardiac amyloidosis the protein subunit in amyloid is normal and/or mutated TTR. All the known TTR variants arise from single amino acid substitutions due to single point mutations in the coding region of the TTR gene. Most of these variants are related with neuropathies and show variable degrees of cardiac involvement. However, there are TTR variants associated with cardiac amyloidosis without neuropathy. Such is the case of TTR Met 111 described in a Danish kindred presenting amyloid cardiomyopathy (Nordlie *et al.,* 1988). Systemic senile amyloidosis (SSA) is a

condition occuring in about 25% of individuals over the age of 80 and presents massive heart infiltration of amyloid (Cornwell III *et al.,* 1981). TTR Ile 122 has been referred to as being associated with SSA in homozygous individuals for this mutation (Gorevic *et al.,* 1989) leading to the hypothesis that clinical the expression only occur in homozygous individuals (Jacobson *et al.,* 1990). However, study of TTR isolated from a black individual showed that this mutation was present both in circulation and in the amyloid deposits along with normal TTR, questioning whether SSA is associated with homozygoty for Ile 122. Furthermore, the amyloid deposits are mainly composed of normal TTR, or more precisely, fragments of normal TTR (Westermark *et al.,* 1990).

In this work we present the study of several cases of cardiac amyloidosis related with TTR to further elucidate the ethiology of these diseases.

We studied an individual with a history of familial cardiac amyloidosis in which the onset of the disease occured at about age 50. Protein analyses of isolated serum TTR revealed the substitution of a residue of threonine for alanine at position 45 in the TTR monomer. These results were confirmed by amplified DNA sequencing.

We also investigated a form of TTR related cardiac amyloidosis that was previously detected in a German individual (Hess *et al.,* 1990). Electrophoretic analysis (Altland *et al.,* 1987) of his plasma TTR revealed the presence of a neutral TTR variant. We have characterized this TTR variant by protein and DNA analysis and found a substitution of leucine for isoleucine at position 68 of the TTR monomer.

We also confirmed, by RFLP analysis, the heterozygous condition for TTR Ile 122 in an individual with late-onset of amyloid cardiomyopathy previously reported by Saraiva *et al.* (1990b) and searched for the presence of TTR Ile 122 in three individuals with SSA.

MATERIAL AND METHODS

Case 1:

The subject, a 58-year-old male Irish and Italian descent, first presented an enlarged heart at age 50 years. At age 53 years he showed persistent diarrhea and genitourinary disturbances. Since age 54 years, progressive symptoms of heart failure with dyspnea and

pedal edema have been evident. He has shown no ocular symptoms or peripheral neuropathy. Biopsies of his skin, rectal fat, and bladder showed the presence of amyloid. The amyloid deposits in the skin biopsy stained positively with anti-TTR antibodies. His mother, from Naples, was reported to have died of amyloidosis, and one sister has pedal edema. The mother's sister (the patient's aunt) died of amyloid heart disease, and autopsy reports described massive cardiac involvement of amyloid and cardiomegaly.

Case 2:

A German individual of 63 years old was reported to have cardiomyopathy and signs of polyneuropathy. Amyloid material from a cardiac biopsy stained positively with TTR antibodies. Analysis of his plasma TTR by double one-dimensional electrophoresis in polyacrylamide gels followed by isoelectric focusing were performed by Dr. Klaus Altland in Germany. This electrically neutral variant was also identified in the serum of the patient's son. Peripheral blood was collected from the propositus for TTR and DNA isolation.

Case 3:

The subject was a 64 year-old black man from Barbados who died of progressive heart failure at age 64 with echocardiographic signs of dilated cardiomyopathy. At age 47 he presented enlarged heart and hypertension. Congestive heart failure with edema of the legs and dyspnoea was present at age 62, after which refractory heart failure with atrial fibrillation developed until death. Family history is mostly unavailable; the only daughter was reported to have an enlarged heart. The post-mortem histological examination showed massive cardiac amyloid deposition; the only other amyloid deposit was in the artery of the adrenal gland (Saraiva *et al.*, 1990b).

SSA cases:

We studied three subjects in their sixties, from the Mayo Clinic, with no family history of amyloidosis. They presented cardiomyopathy and TTR amyloid had been confirmed by heart biopsies. Blood was available for DNA analyses.

Protein analysis: TTR isolation from plasma and comparative tryptic peptide mapping were performed as described in the previous section (section 4.1).

DNA analyses: DNA isolation, PCR amplification of exons from the TTR gene and sequencing were performed as described in section 4.1.

ASO hybridization was carried out with primers designed to differentiate the normal from the mutated sequence for TTR Ala 45: a «normal» one, a nonedecamer with the normal antisense sequence (5'ACCCAGAGGCAAATGGCTC3'), and another one differing from the previous one by a single base change of A for G (5'GAGCCATTTACCTCTGGGT3') and referred to as «mutant» probe; in this last case we choose to use the sense sequence. For the stringent washes we used (6x SSC, 0.1% SDS) and the temperatures were 60°C for normal and 58°C for the mutant probe.

For the RFLP analyses one fourth of the amplification product was digested with the appropriate enzyme following the instructions of the manufacturer. The digested samples were analysed by Nusieve agarose gel electrophoresis and ethidium bromide staining.

RESULTS

In all cases presented, TTR was isolated from the serum of the patient with cardiac amyloidosis and the proteins were characterized by comparative tryptic peptide mapping and amino acid sequence analysis of the abnormal peptide. The amino acid substitutions detected were then confirmed by DNA sequence analysis.

Case 1: Comparative tryptic peptide mapping of TTR from the propositus and of TTR from normal plasma showed 2 abnormal peptides peaks eluting close to the normal peptides 6 and 5+6 (5+6 results from incomplete cleavage at a lysine residue). Thus, the abnormality in the patient's TTR might be related with peptide 6, which encompasses residues 36-48 in the TTR sequence. Amino acid sequence analysis of these abnormal peptides revealed the presence of a threonine residue at position 45 in parallel with the normal residue, alanine.

We concluded that the patient had an abnormal plasma TTR, with a substitution of a threonine for alanine at position 45, along with the normal TTR (Figure 1).

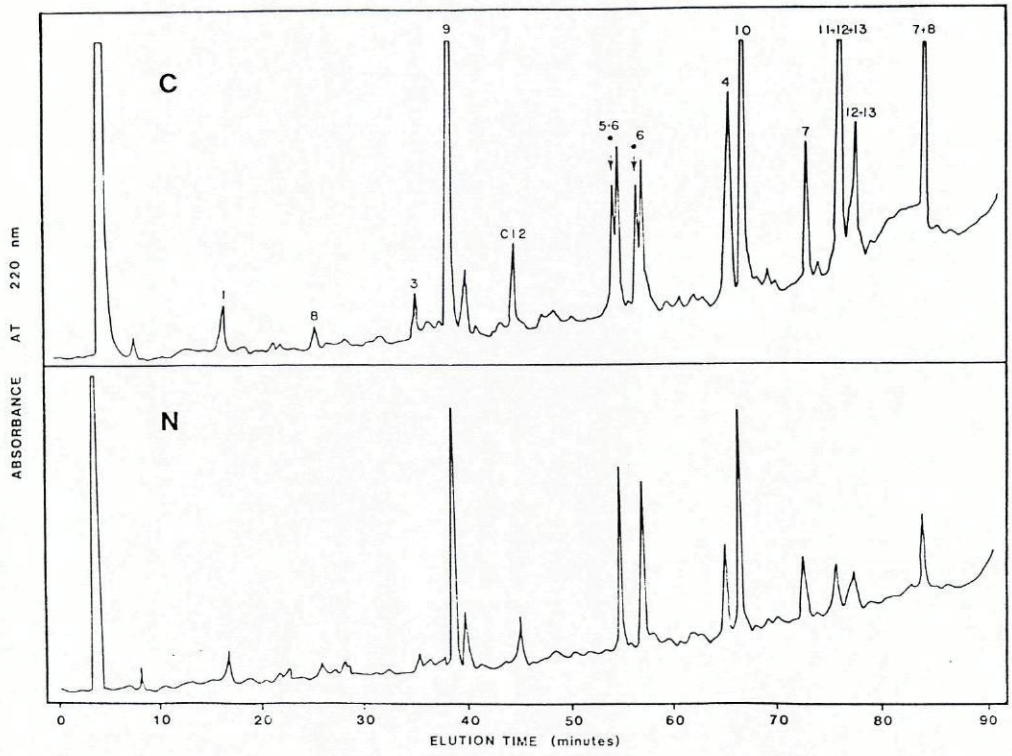


Fig. 1 - Tryptic peptide map of isolated TTR from a control individual (N) and from a carrier with cardiac amyloidosis. The abnormal peptides are labelled with arrows.

This substitution (Ala 45 - Thr) could be explained by a single base change of adenine for guanine in the alanine codon. Ala 45 is coded by GCC, and one of the possible threonine codons is ACC. In order to confirm this substitution and to show that no other change has occurred, we amplified exon 2 of the TTR gene from normal genomic DNA and from genomic DNA from the patient, and sequenced both in parallel. Figure 2 shows the results obtained for the patient DNA, where both C and T were detected as the first base of codon 45 (corresponding to G and A, respectively); normal DNA contained only C (thus G) (not shown); sequencing of the other exons failed to reveal any other change in the DNA sequence. This result confirmed the A for G change in exon 2 of the TTR gene of the patient and also showed the patient to be heterozygous for this mutation.

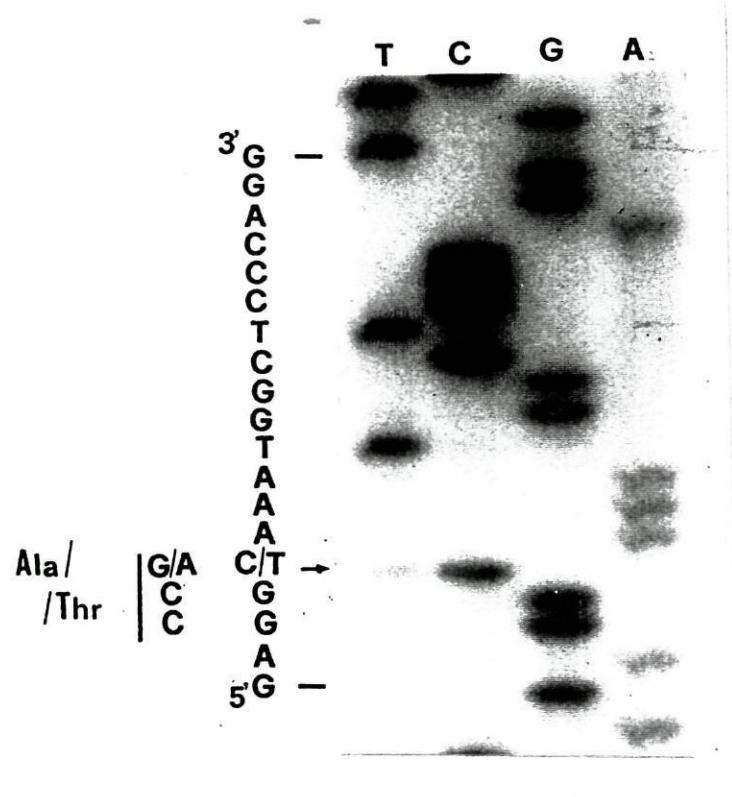


Fig. 2 - DNA sequence analysis of the region spanning the mutation (Ala 45 - Thr) in the TTR gene of the propositus. Both a C and a T (corresponding to a G and an A in the sense strand) were detected.

We next analyzed the DNA from one relative who died with proven cardiac amyloidosis (the propositus aunt). Besides proving the genetic nature of the mutation, our aim was to develop a general method for detection of the mutation. Since the A for G does not modify any known enzyme restriction site in the DNA, we synthesized two ASO probes for ASO hybridization. We amplified exon 2 of the TTR gene from genomic DNA obtained from the patient, from control genomic DNA and from DNA extracted from the heart of the propositus aunt. As shown in Figure 3, all the samples gave a signal with the normal ASO probe. The normal exon 2 (lane N) did not show any signal with the mutant ASO probe. In contrast, DNA from the patient (lane C) as well as DNA from his deceased aunt (lane A), did show a strong signal with the mutant probe. Thus, this experiment gave evidence for the genetic nature of this case and provided a diagnostic tool to be used in future familial cardiac amyloidosis typing.

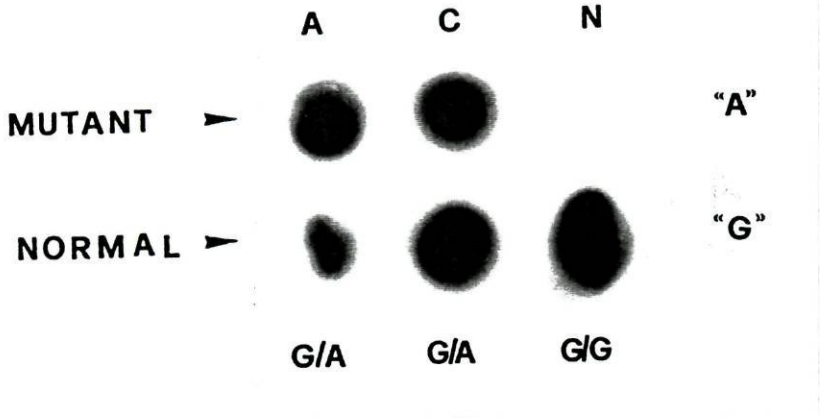


Fig. 3 - DNA analysis by ASO hybridization. PCR-amplified DNA from the propositus (lane C) and from biopsy material from his diceased aunt (lane A), as well as normal DNA (lane N), were hybridized with labeled ASO probes.

Case 2: Comparative tryptic peptide analysis showed the presence of an abnormal peak, peak 7 (labelled 7*) (Figure 4). This peptide corresponds to amino acids 49-70 of the polypeptide chain. Amino acid sequencing of the abnormal peptide 7* revealed the substitution of a leucine residue for isoleucine at position 68 of the TTR monomer.

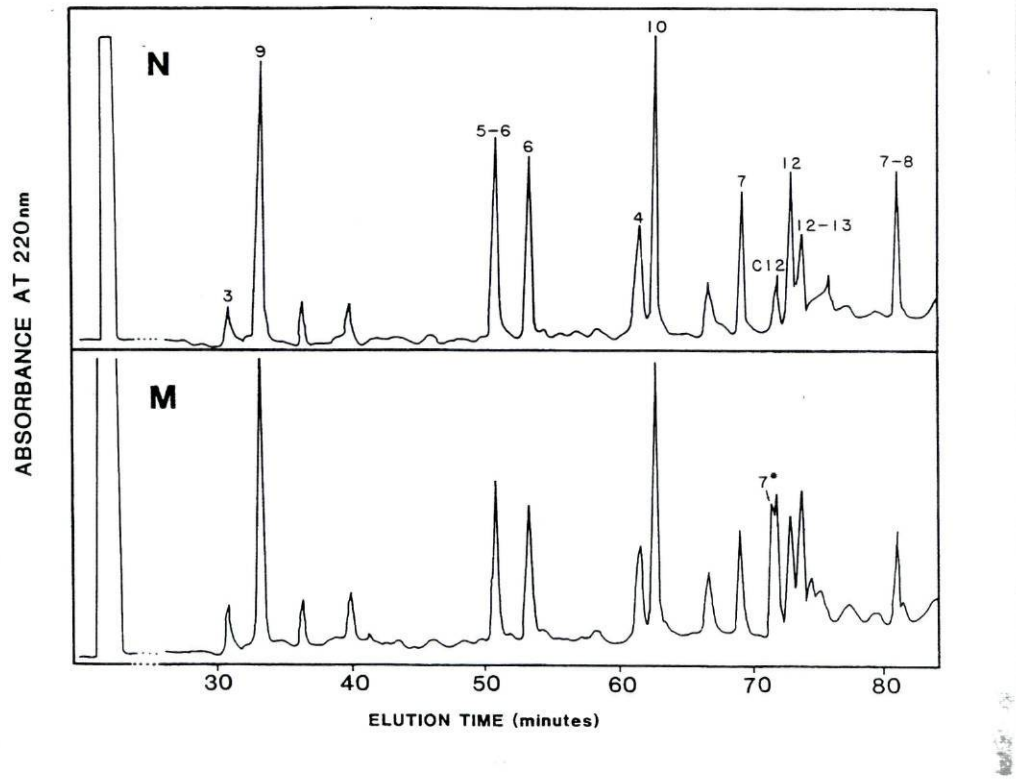


Fig. 4 - Tryptic peptide maps of normal (N) and the mutant (M) TTR. The abnormal peak is labeled with an *.

Assuming that this substitution originated from a single point mutation at the DNA level, it could be explained by a substitution at the first base of codon for Ile; in this case we antecipated to find a C for A or a T for A substitution. In order to confirm this substitution, we performed sequence analysis of PCR amplified TTR exon 3 which encodes amino acids 48 to 92, and found both thymine and adenine in the first base of codon for amino acid residue 68 (Figure 5). This corresponds to the presence of the normal (ATA-Ile) and the variant (TTA-Leu) alleles, indicating that the patient was heterozygous for the mutation. The substitution of leucine for isoleucine at position 68 is in accordance with the electrophoretic observation of an electrically neutral variant.

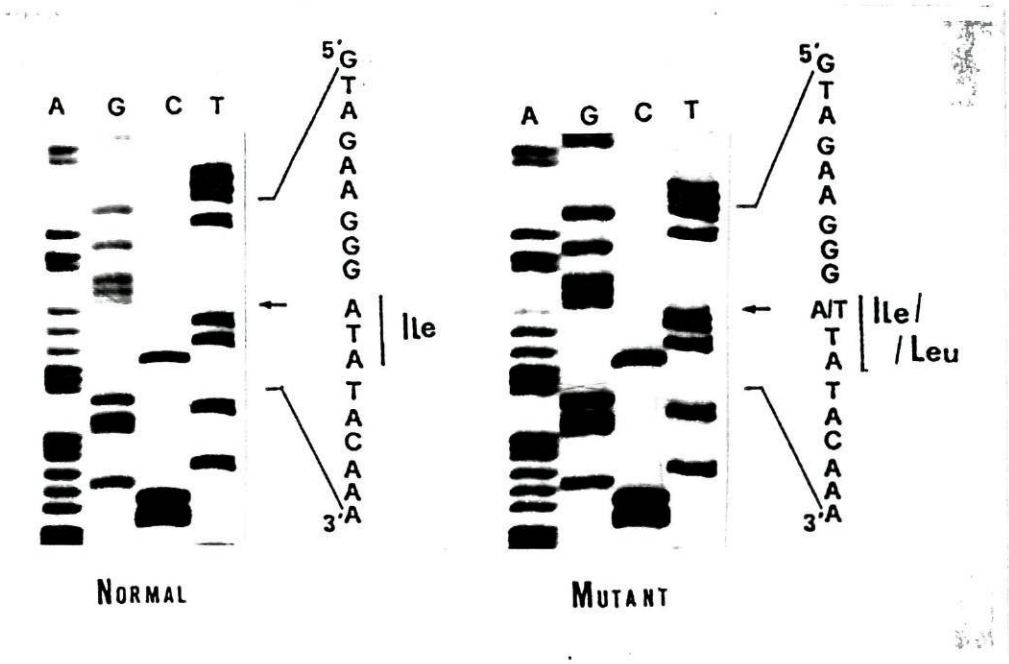


Fig. 5 - DNA sequence analysis of amplified TTR exon 3 from normal and mutant DNA. The mutation is indicated by an arrow.

Case 3: Since a TTR variant, TTR Ile 122, has been previously described in the propositus by protein analysis (comparative peptide mapping and amino acid sequence analysis) (Saraiva *et al.*, 1990b) we sequenced amplified exon 4 of the TTR gene (Figure 6).

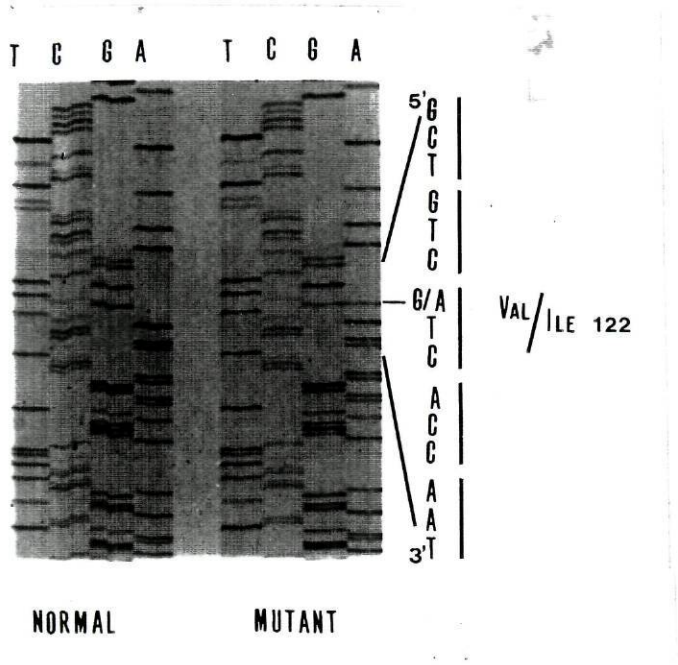


Fig. 6 - DNA sequence analysis of amplified TTR exon 4 from a carrier of TTR Ile 122 and from a control individual (normal).

As expected we found adenine and guanine in the position corresponding to the first base of codon for residue 122 on the TTR monomer. This result corresponds to the occurrence of both the normal and mutant allele, in accordance with what was found by protein analysis. This result further supports the heterozygous condition of the individual for this mutation. Since this mutation destroys a restriction site for endonuclease Mae III, it can be detected by RFLP analysis of PCR amplified DNA from exon 4 (Figure 7).

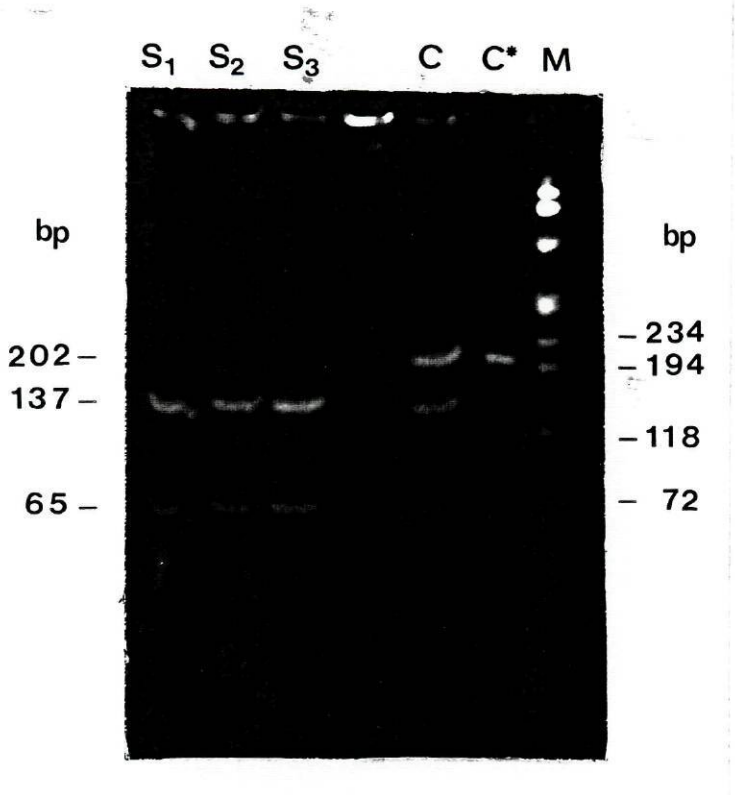


Fig. 7 - RFLP analysis of amplified TTR exon 4. The samples were digested with endonuclease Mae III. C- individual with cardiac amyloidosis; S1, S2, S3 - individuals with SSA. All the samples were digested except the one labelled with an *.

SSA cases: We sequenced amplified TTR exons 2, 3 and 4 of three individuals with systemic senile amyloidosis and we did not find any mutation in the TTR gene. In order to further exclude the occurrence of TTR Ile 122 mutation, we digested amplified TTR exon 4 from these individuals and found that they were not carriers of the TTR Ile 122 mutation since their amplified DNA was completely digested (Figure 7).

DISCUSSION

Cardiac amyloidosis results from massive deposition of amyloid in the heart originating cardiac conduction disturbances, restrictive cardiomyopathy and heart failure. The plasma proteins associated with these amyloid deposits are, frequently, immunoglobulin light chains (AL amyloid) and, with less frequence, serum amyloid A in reactive amyloidosis

(secondary amyloidosis) (Hess *et al.*, 1993). In addition, there are familial forms of cardiac amyloidosis due to deposition of TTR variants (Nordlie *et al.*, 1988).

We presented here the characterization of two new TTR variants associated with cardiac amyloidosis: TTR Thr 45 and TTR Ile 68.

TTR Thr 45 was found in a patient with hereditary amyloidosis, who had died with the autopsy diagnosis of hereditary amyloidosis with massive cardiac involvement. The patient's amyloidosis has been characterized clinically by prominent cardiomyopathy, gastrointestinal alterations, and by the absence of either peripheral neuropathy or vitreous involvement. The lack of peripheral neuropathy distinguishes this case from most forms of familial amyloid neuropathy. TTR Leu 68 was described in a German individual presenting mainly cardiomyopathy with subclinical symptoms of upper limb neuropathy and no signs of vitreous involvement, a common finding in the TTR related amyloidoses. In both cases the onset of the disease occured in the 6^{th} decade of life. These two mutations add to the list of exclusively cardiopathic forms of TTR amyloidoses that includes also TTR Ile 50 (Nishi *et al.*, 1992), TTR Lys 59 (Booth *et al.*, 1995) and TTR Met 111 (Nordlie *et al.*, 1988).

Cardiac amyloidosis may occur very late in life as is the case in the senile forms. The senile forms of cardiac amyloidosis are related with the deposition of atrial natriuretic peptides, found in nearly 95% of the octagenarians or with the deposition of TTR, found in the heart of 25% of individuals over the age of 80 (Cornwell III *et al.*, 1981). This last form of amyloidosis has also been termed senile systemic amyloidosis (SSA). The first protein study in a patient reported to have SSA demonstrated that the isolated amyloid fibrils contained a TTR variant with a substitution of isoleucine for valine at position 122 (Gorevic et al., 1989). However, Westermark *et al.* (1990) reported several other cases of SSA that presented only normal TTR by protein analysis of amyloid fibrils, and no mutation was detected in the TTR cDNA isolated from the liver of an SAA patient (Christmanson *et al.*, 1991). The symptomatology of TTR Ile 122 is very similar to that of SSA . The later onset of SSA is not sufficient to distinguish these two forms of cardiac amyloidosis. The characterization of these forms must thus be based on the presence (FAC) or absence (SSA) of a mutation in TTR.

FAC associated with TTR Ile 122 has been always described in homozygous individuals (Jacobson *et al.,* 1990) and it was thought that the homozygous condition was needed for the clinical expression of the disease. However, Saraiva *et al.* (1990b) reported a case of cardiac amyloidosis in a patient heterozygous for the TTR isoleucine 122 variant. We have confirmed the results by Saraiva *et al.* (1990b) by DNA sequencing and RFLP analysis and showed furthermore that no other mutation was present in the same individual. This finding raises the question of whether there is an absolute need of homozygous TTR Ile 122 for the manifestation of the disease or if the heterozygous state is sufficient for that expression. It is curious that similarly to the TTR Met 30 homozygous carriers (Holmgren *et al.,* 1988a) the TTR Ile 122 homozygous carriers do not present a more severe form of the disease than the heterozygous.

It is very interesting that all the carriers of TTR Ile 122 are black individuals and that this mutated gene has been shown to occur in the Black population with a frequency of 1.1% (Jacobson *et al.,* 1991; Jacobson, 1992a) and yet there are no reports of the amyloid cardiomyopathy being frequent in the Black population.This may be due to misdiagnosis of FAC or low penetrance of the mutation. It is also possible that the clinical expression varies widely and that other factors are needed for the development of cardiac amyloidosis. In opposition to this variant, that seems to occur only in the Black population or at least with a high frequency in this population, TTR Met 30, the most frequent and widespread amyloidogenic TTR variant, was never found in Black individuals, with the exception of a recent reported case (Léger *et al.,* 1994).

The reasons for the predominant deposition of certain mutant TTRs in the heart, which lead to FAC, are not obvious and remain unsolved. Similarly to FAP, it is possible that a particular conformation of TTR induced by cardiomyopathic TTR mutations may lead to fibril formation. Similarly to the neuropathic TTR variants referred to in the previous section, the cardiopathic TTR variants studied in this section are not localized in a specific region of the molecule, as shown in Figure 8.

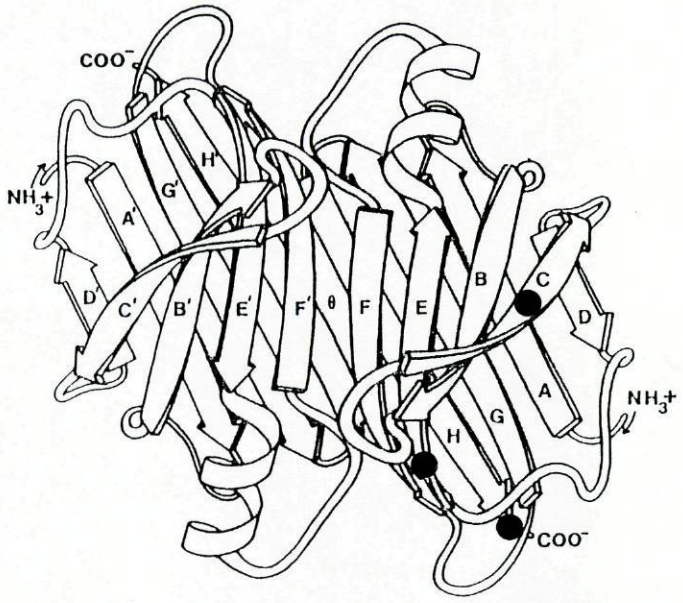


Fig. 8 - Schematic representation of the TTR dimer with the localization of the amino acid substitutions of the cardiopathic TTR variants described in this work.

Whether there are distinct conformations associated with FAP and FAC is not known and should be confirmed by crystallographic and X-rays studies of cardiopathic TTR mutations. On the other hand, in cases like SSA, other factors like proteolysis and tissue contribution might add to amyloid formation (Saraiva, 1991) and, in this regard, the heart is a particular organ for deposition of TTR related amyloid. Characterization of the structural changes ocurring in TTR mutants associated with familial amyloid cardiomyopathies, as well as the factors that influence normal TTR to deposit in SSA, may help in our understanding on the normal and pathological role of TTR in the heart.

4.3. IDENTIFICATION OF NON-AMYLOIDOGENIC TRANSTHYRETIN VARIANTS IN HETEROZYGOTIC AND COMPOUND HETEROZYGOTIC INDIVIDUALS: TTR THR 109 and TTR MET 119 and first report of TTR ASN 90 and TTR ARG 102.

ABSTRACT

Transthyretin (TTR) is a polymorphic protein with many known variants, in most cases associated with different clinical expressions. Screening studies have been carried out in different groups of the Portuguese and German populations to detect more TTR variants. These studies, performed by hybrid isoelectric focusing analysis (HIEF), revealed two new variants, which we have identified as a basic variant - TTR Arg 102 - apparently non-pathogenic, reported in a German kindred, and an acidic variant - TTR Asn 90 - also non-pathogenic, present in both populations. Other non-amyloidogenic TTR variants, that had been previously detected, were also identified, namely TTR Thr 109 and TTR Met 119. The identification of these variants was achieved by DNA sequencing and RFLP analyses of amplified DNA.

These screening studies had revealed also the occurrence of individuals carrying two different TTR variants and, consequently, having no normal monomer. We performed RFLP analysis of PCR amplified DNA to characterize the mutations found in these individuals and their relatives. The segregation of the mutations in the families demonstrated that the carriers inherited one mutated allele from each parent, being compound heterozygotes. The compound heterozygotes analysed were carriers of TTR Met 30 -TTR Asn 90, TTR Met 30 - TTR Met 119 and TTR Asn 90 - TTR Met 119.

INTRODUCTION

Transthyretin (TTR) is a plasma protein carrier of thyroxine and retinol through its association with retinol binding protein (RBP). The TTR molecule is a tetramer composed of 4 identical subunits of 14 400 Da each. The TTR monomer is codified by a single copy gene with four exons and three introns. About 45 different TTR variants have been described, all due to point mutations in exons of the gene. The genetic heterogeneity observed is associated with diverse clinical expression; in particular, most variants are related to different forms of hereditary amyloidosis. In these diseases, TTR deposits extracellularly in tissues as amyloid. The reasons why TTR forms amyloid are not known

and the study of both amyloidogenic and non-amyloidogenic mutants may contribute to the knowledge of structural domains involved in amyloid formation and, at the same time, contribute to the study of structure and function of TTR. The known TTR variants have been identified, in most cases, because they were associated with a disease. Clinically silent variants have been detected in screening studies of the population, namely the Portuguese and German populations. These screening studies were performed using double one-dimensional (D1-D) electrophoresis in polyacrylamide gels followed by isoelectric focusing (Altland *et al.*, 1981, 1987).

We present the characterization of some of the variants detected in the above mentioned screening studies. One is an acidic variant - TTR Asn 90 - found in Portugal and Germany; the other is a basic variant - TTR Arg 102 - detected in a German family. Protein analysis revealed the amino acid substitutions that were subse confirmed at the DNA level by DNA sequencing and RFLP analysis of amplified DNA.

In addition, two other variants that were detected in the screening of the Portuguese population (Alves *et al.*, 1993) and were identified by us by DNA sequencing: TTR Thr 109 and TTR Met 119. These two variants had been previously described in the American population (Moses *et al.*, 1990; Harrison *et al.*, 1991). Further family studies of carriers of these variants were performed by RFLP analysis of amplified DNA.

Although in most cases of hereditary amyloidosis the carriers of TTR variants are heterozygotic individuals that inherited a normal allele from one parent and one mutated allele from the other progenitor, there are some reports of homozygotic individuals, namely for TTR Met 30 (Holmgren *et al.*, 1988a; Skare *et al.*, 1990), TTR His 58 (Jacobson *et al.*, 1992), TTR Asn 90 (Alves *et al.*, 1992) and TTR Ile 122 (Jacobson *et al.*, 1990). However, most interesting is the occurrence of individuals that carry two different TTR variant monomers, namely in the Portuguese population (Alves *et al.*, in preparation). Family studies indicated that these individuals inherited one mutated allele from one parent and the other mutated allele from the other and, therefore, are designated by compound heterozygotes. In this work we performed DNA analysis of three compound heterozygotic individuals from different families and their relatives, in order to characterize the mutations and study their segregation in each family. These individuals were found to be carriers of

TTR Met 30 - TTR Asn 90, TTR Met Asn 90 - TTR Met 119 and TTR Met 30 - TTR Met 119.

MATERIAL AND METHODS

Screening of TTR variants:

Screening studies by IEF had been conducted in Portugal by Dr. Isabel Alves and/or Dr. Klaus Altland in Germany. The mutations associated with the different TTR variants detected were further studied by us.

Case I:

Peripheral blood was available from a woman carrier of a basic TTR variant detected in a screening study of 4,000 sera from midtrimester pregnant women in the Province of Hessen in Germany (Altland *et al.,* 1983). The variant was also found in the serum of the sister and mother of the female propositus and all three (the older was 50 years old) had no detectable signs of FAP.

Case II:

Blood was colected from an individual that had been identified, in a screening of 5,000 Portuguese individuals, as carrier of a TTR variant and also from her offspring. Further clinical examination of this family revealed familial goiter. We studied the propositus and two of her children.

Case III:

A screening study was performed on 1,200 Portuguese serum samples including 700 individuals not related to FAP families and 500 individuals belonging to FAP families. An individual carrier of a TTR acidic variant was detected among the 700 individuals not related to FAP families. In this study, another carrier of the acidic TTR variant seemed to present

only the acidic monomer (i.e., was apparently homozygous for this variant). We investigated also the presence of the mutation in one brother and a daughter of the propositus.

Case IV:

Among the 500 individuals belonging to Met 30 FAP families, from the screening referred in case III, an individual that was simultaneously a TTR Met 30 carrier and a carrier of an acidic TTR variant was detected.

Case V:

As part of a screening to detect additional TTR mutations, sera from 100 affected and non-affected members of Met 30 - related FAP families from the Northern Portugal, routinely diagnosed at Centro de Estudos de Paramiloidose (Porto), were analysed by HIEF and cyanogen bromide (CNBr) cleavage for TTR Met 30. Sequencing of an abnormal CNBr peptide detected in a sample was compatible with the possibility of this variant beeing TTR Met 119, previously described in the United States.

Case VI:

The propositus was an individual detected in the screening study abovementioned above (case V). The propositus that was a carrier of an acidic TTR variant, also presented an abnormal CNBr peptide and was not a carrier of TTR Met 30, as judged by HIEF, being possibly a Met 119 carrier. Whole blood was collected from this individual and his parents.

Case VII:

5,000 samples from the Portuguese population were screened by HIEF in extremely flattened immobilized pH gradients (IPG) allowing the detection of even neutral aminoacid substitutions. As controls, sera samples from carriers of TTR Met 30, TTR Asn 90 and TTR Met 119 were used. In this study, one individual was found to carry two mutations that migrated as TTR Met 30 monomer and TTR Met 119 monomers .

Protein analyses:

TTR was isolated from plasma and analysed by comparative tryptic peptide mapping and amino acid sequence analysis using procedures detailed in section 4.1.

DNA analyses:

DNA isolation from leucocytes, PCR amplification of TTR exons and DNA sequencing were performed as previously described in section 4.1. RFLP analyses of the amplified DNA were performed with the appropriate enzymes for each case, using standard conditions. The digested DNA samples were analysed in a 4% Nusieve agarose gel and stained with ethidium bromide.

RESULTS

Case I:

In the screening for TTR variants, a sample with a basic monomer (about 0.3 pH units cathodic to the normal major TTR zone - pH 5.7) was detected. This result indicated that this variant should contain a gain of one positive or the loss of one negative charge unit.

In order to define the amino acid substitution, we isolated plasma TTR and digested it with trypsin. Comparative tryptic peptide mapping showed the presence of an abnormal peptide, marked * in Figure 1, not present in normal TTR digests. Amino acid sequencing of this peptide revealed a substitution of an amino acid residue of arginine for proline at position 102 of the polypeptide chain.

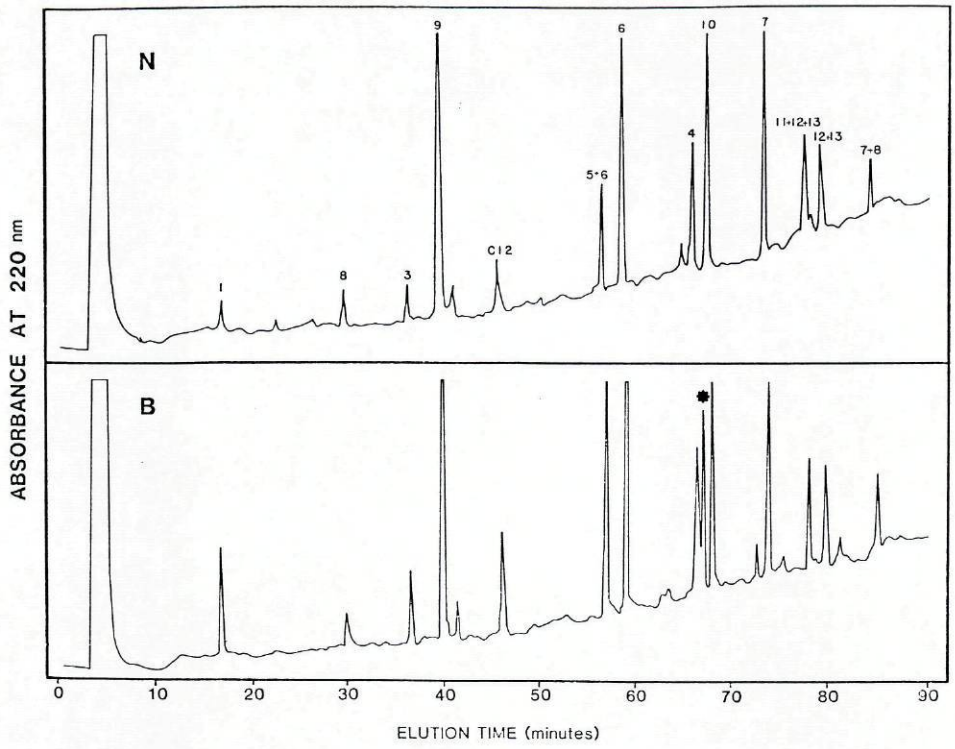


Fig. 1 - Comparative tryptic peptide map of normal (N) and basic TTR (B).

Since this substitution could be explained by a point mutation in the codon for proline 102 (Pro (CCC)- Arg (CGC)), which creates a new restriction site for endonuclease Eag I and abolishes one for Sau 96I, we performed RFLP analyses of amplified TTR exon 4. The cleavage pattern for Eag I and Sau 96I is represented in Figure 2A. As predicted, digestion of DNA from the individual with the basic TTR variant produced two fragments of 77 bp and 125 bp while DNA from a normal individual was not cleaved by Eag I (Figure 2B). Digestion of normal DNA with Sau 96I produced three fragments of 101 bp, 74 bp and 27 bp respectively, whereas digested DNA from the individual with the basic TTR variant presented fragments of 175 bp and 27 bp as expected when the restriction site is abolished. These results confirmed the predicted substitution of Arg for Pro at position 102 of the polypeptide chain.

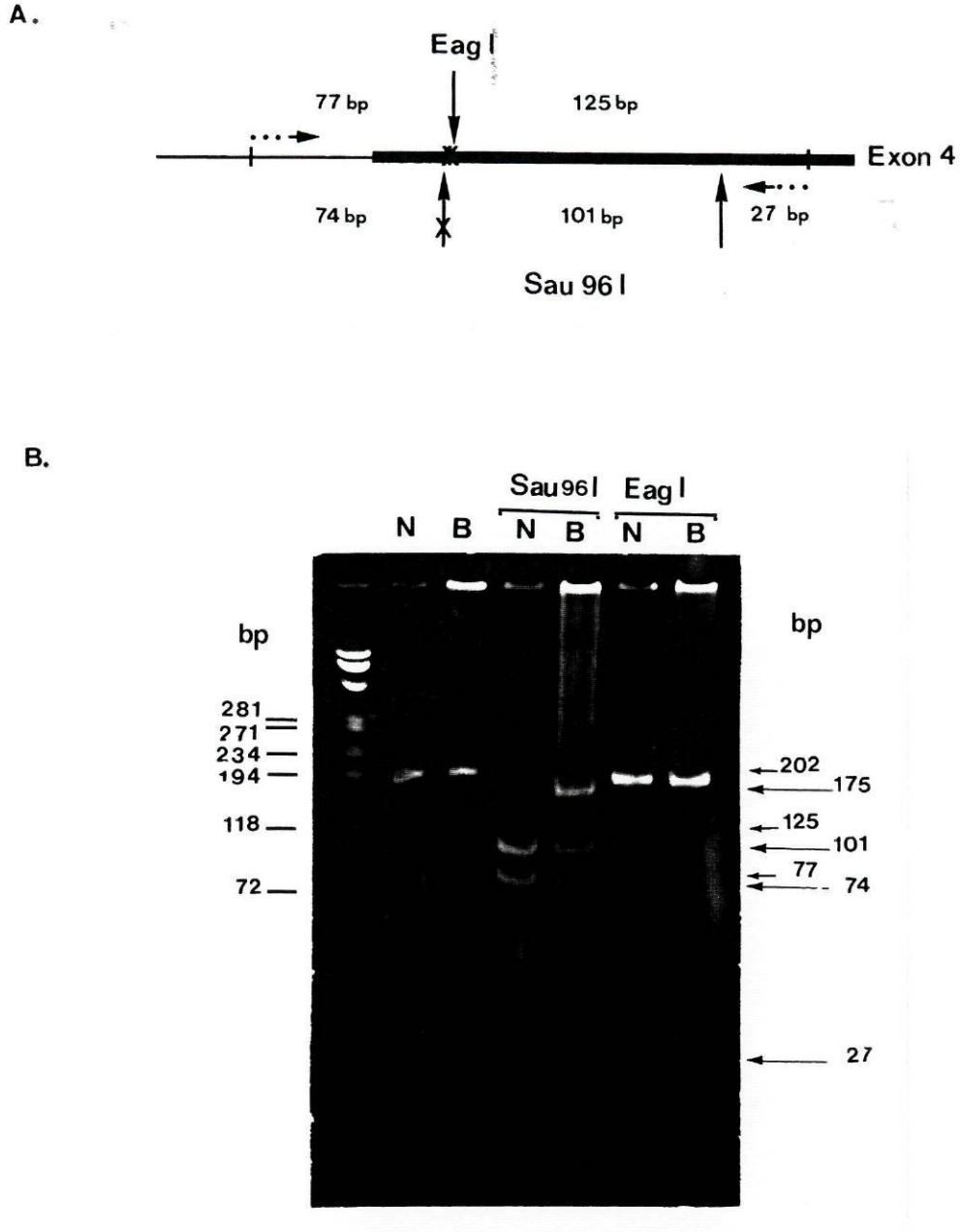


Fig. 2 - A. Schematic representation of amplified TTR exon 4 and sites of cleavage by Eag I or Sau 96I. **B.** RFLP analysis of amplified exon 4 of the TTR gene. N- amplified leucocyte DNA from the normal individual. B- amplified leucocyte DNA from the individual with basic TTR. The digested samples are indicated.

Case II:

We sequenced amplified TTR exons 2, 3 and 4 from a sample that presented a TTR mutation by HIEF analysis. Sequencing of amplified exon 4 presented both G and A at the first base of the codon for residue 109 of the TTR monomer (Figure 3). This result corresponded to a substitution of a residue of threonine for alanine at this position.

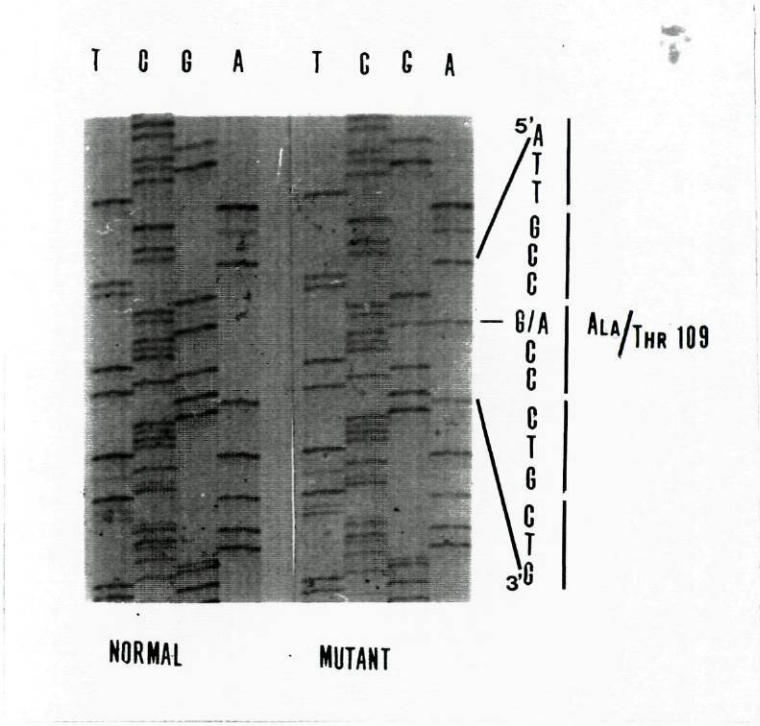


Fig 3 - Sequence analysis of amplified TTR exon 4 from a carrier of TTR Thr 109 (mutant) and from a control (normal).

We have also investigated the presence of the mutation in the descendants of the propositus by RFLP analysis, with Fnu 4HI, known to distinguish this mutation (Moses et al., 1990) (Figure 4), two of them were also carriers of the mutation.

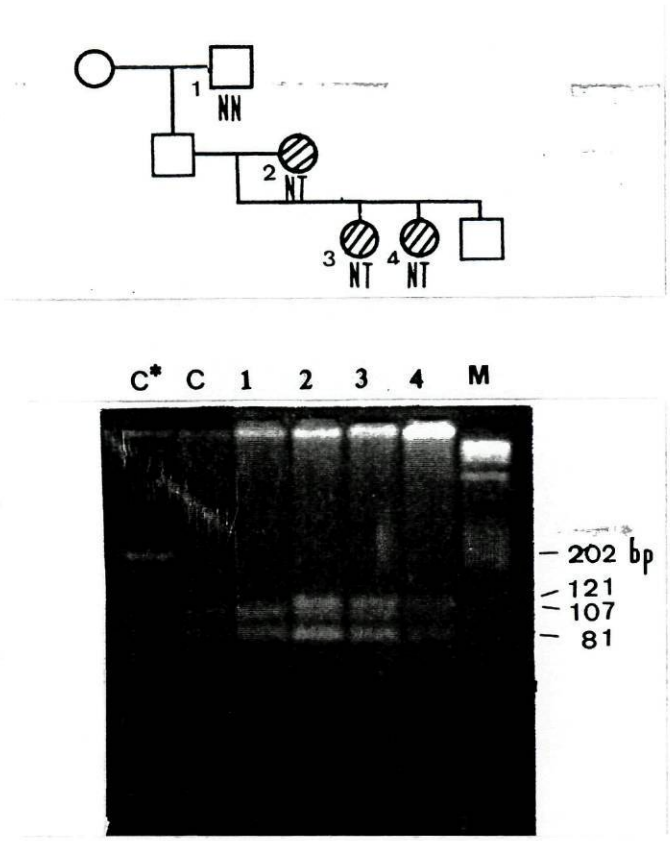


Fig. 4 - RFLP analysis of amplified exon 4 from individuals belonging to a family with TTR Thr 109. The samples were digested with endonuclease FnuHI. C*- control undigested; carriers of the mutation, ⊘, ▨; non-carriers of the mutation ○, □.

Case III:

In the screening for non-pathogenic mutations in the Portuguese population an acidic TTR monomer was detected together with the normal TTR monomer. The variant was indistinguishable, by its pI, from two other acidic TTR variants previously found in two non-related German families (Altland *et al.,* 1987). Structural studies on plasma isolated TTR were next undertaken to elucidate the nature of the change in the pI of the protein. Comparative tryptic peptide mapping showed an abnormal peptide (not shown). Amino acid sequence analysis of this peptide demonstrated that it corresponded to residues 81 to 103 of normal TTR except that histidine (His) at position 90 was replaced by asparagine (Asn). This amino acid substitution was in agreement with the observed shift of pI to a more acidic

level, since the positively charged imidazole group of His was replaced by an electrically neutral Asn residue. The amino acid substitution can be explained by a single base substitution (i.e., A for C) at the first position of the codon for His 90 (CAT). The change creates a new restriction site for restriction enzyme Bsm I and abolishes the restriction site for the enzyme Sph I. Figure 5 shows the DNA patterns, obtained after digestion with either Sph I or Bsm I.

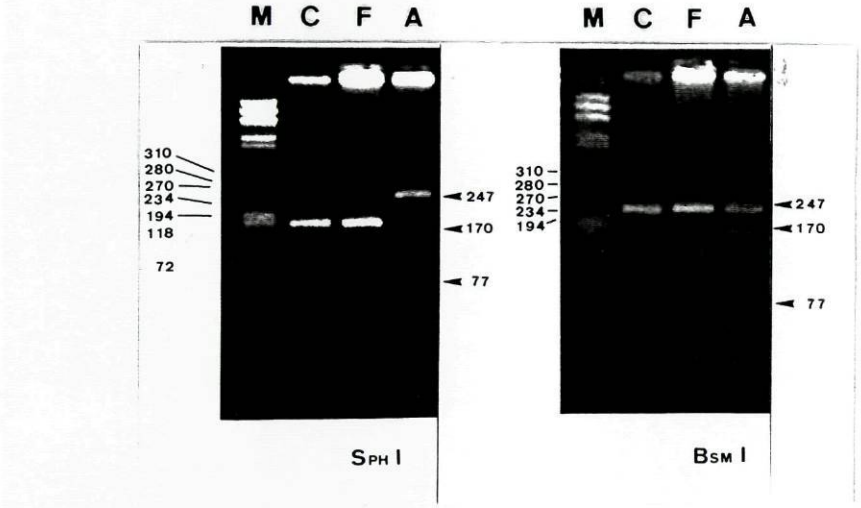


Fig. 5 - RFLP analysis of amplified TTR exon 3 of a heterozygotic carrier of TTR Asn 90. C- control DNA; F-FAP; A - DNA from the carrier of TTR Asn 90; M - molecular weight (MW) markers.

The control amplified DNA was totally digested originating 2 fragments of 170 and 77 bp respectively. The heterozygotic carriers of the acidic variant showed a partial digestion of the amplified DNA, thus presenting one fragment of 247 bp corresponding to the amplified mutant exon 3 and two fragments corresponding to the digested normal exon. Parallel analyses of the same samples by Bsm I confirmed our interpretation of the results. Control DNA was not cleaved by the enzyme, whereas the DNA from the acidic variant was partially cleaved.

We have also analysed DNA from another sample that presented this acidic variant. However, in the HIEF, this sample showed only the acidic monomer and no normal

monomer. DNA from the brother and daughter of the propositus were also tested, by RFLP analysis of the amplified TTR exon 3, for the presence of the mutation originating TTR Asn 90. The results of these analyses are presented in Figure 6.

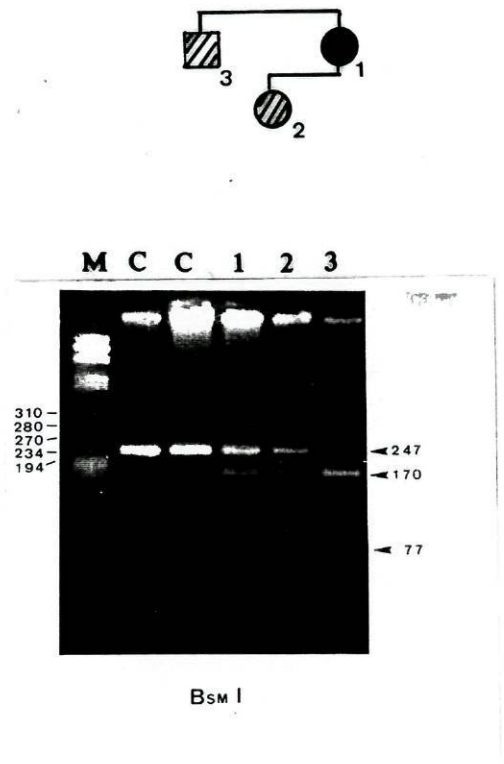


Fig. 6 - RFLP analysis of amplified TTR exon 3. The samples were digested with Bsm I. The samples of the propositus (1), homozygotic carrier of TTR Asn 90, a daughter (2) and a brother (3). Lanes C are control DNAs; M - MW markers.

As expected, the sample of the propositus was completly digested by Bsm I showing that this individual was a homozygous carrier for the Asn 90 mutation. The samples of the daughter and brother were partialy digested with Bsm I demonstrating that these individuals were heterozygous for the mutation. These results were further confirmed by DNA sequencing of the PCR amplified TTR exon 3. Figure 7 shows that the sample from the propositus presented only the mutated base (A) in Asn 90 codon, confirming the homozygosity for this mutation. However, amplified DNA from her brother presented A and C in the same position, while the sample from a control showed only the normal base C.

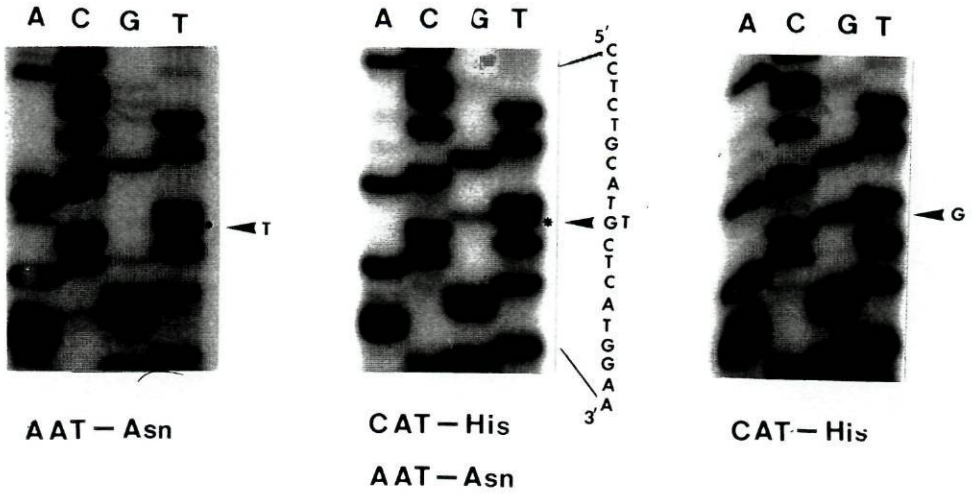


Fig. 7 - Sequence analysis of the coding region around codon for amino acid 90. From left to right: homozygotic carrier of TTR Asn 90, heterozygotic carrier, control DNA.

Case IV:

Several screening studies had been undertaken in order to search for non-pathogenic TTR variants. The first screening in the Portuguese population involved samples from the area of Póvoa de Varzim where FAP is prevalent. 500 of the 1,200 samples analysed belonged to FAP families including TTR Met 30 carriers and non-carriers. In this study, a 28 year-old woman was found to carry a normal TTR monomer and an acidic TTR monomer. This woman was from a TTR Met 30 family, her father had FAP. The analysis, by HIEF, of more relatives demonstrated that one brother presented no normal TTR monomer, carrying a TTR Met 30 monomer and an acidic TTR monomer. Since the mother was not a TTR Met 30 carrier he should have inherited the acidic variant from the mother and the TTR Met 30 variant from the father, being thus a possible compound heterozygote. The acidic variant was characterized by tryptic peptide mapping and amino acid sequencing as having a substitution of asparagine for histidine at position 90. We confirmed these results by DNA analysis of the compound heterozygotic individual. Thus, since the TTR Met 30 mutation is due to a G for A substitution in exon 2 of the TTR gene creating a new restriction site for Nsi I, we amplified exon 2 and digested it with Nsi I and found the DNA fragments expected for the TTR Met 30 mutation (Figure 8A). The mutation originating TTR Asn 90 is located in

exon 3 of the TTR gene and can be detected by digestion with Sph I or Bsm I. In Figure 8B, digestion of the compound heterozygote amplified exons with Sph I was incomplete as expected since the mutation abolishes one restriction site for this enzyme.

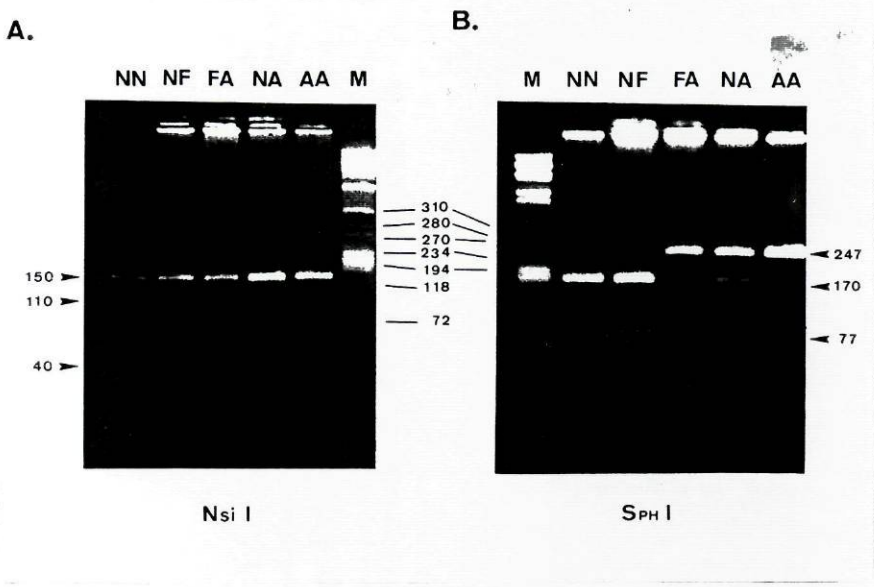


Fig. 8 - A. RFLP analysis of amplified TTR exon 2. The samples were digested with Nsi I. **B.** RFLP analysis of amplified TTR exon 3. The samples were digested with Sph I. NN, NF and AA are control samples of, respectively: normal, heterozygotic Met 30 and homozygotic Asn 90. NA and FA are the samples of the propositus (heterozygotic Asn 90) and her brother, compound heterozygote Met 30-Asn 90. M - MW markers.

Case V:

This case represented a possible TTR Met 119 mutation, previously reported by Harrison *et al.* (1991), that could be confirmed by RFLP analysis using the endonuclease Nco I (BRL). Thus, we averiguated for the presence of the mutation in the propositus and other members of the kindred digesting amplified TTR exon 4 with Nco I. In Figure 9 we present the result obtained for the propositus and two other siblings, one carrier and one non-carrier.

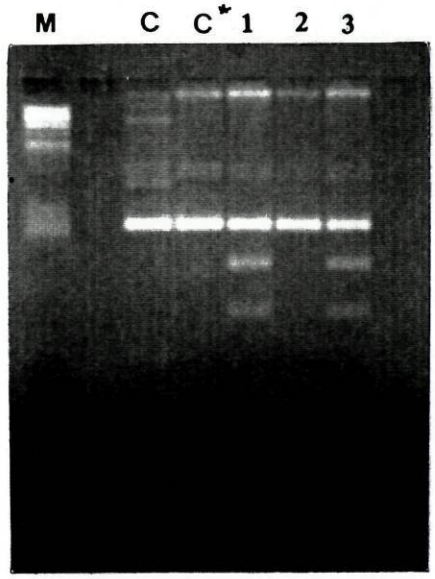


Fig 9 - RFLP analysis of amplified TTR exon 4 of individuals from a kindred with TTR Met 119. C - control DNA; 1,3 - carriers of the mutation; 2- non carrier. All the samples were digested with Nco I except the one labelled with an asterisk.

Case VI:

In this case, where an acidic monomer and a mutation involving a methionine were present, we identified the possible mutations by RFLPs and analysed amplified DNA from exon 2, 3 and 4 of the propositus and from some relatives, in particular the progenitors. Amplified exon 2 was digested with Nsi I to confirm the absence of the Met 30 mutation (Figure 10-A). Exon 2 from the propositus (MA) and his parents (NM and NA) was not cleaved by Nsi I indicating that they were not carriers of the TTR Met 30 mutation. In Figure 10-B we present the digestion of amplified exon 3 with Bsm I. Only the amplified exon 3 from the propositus and his father were digested by Bsm I. This result confirmed the detection of TTR Asn 90 by HIEF in the propositus and showed that he inheritided this mutation from the father. TTR Met 119 mutation creates a new restriction site for Nco I in exon 4. Thus, in Figure 10-C only exon 4 from the propositus and his mother were digested by Nco I, demonstrating that the propositus inherited the Met 119 mutation from his mother, therefore being a TTR Asn 90 - TTR Met 119 compound heterozygote.

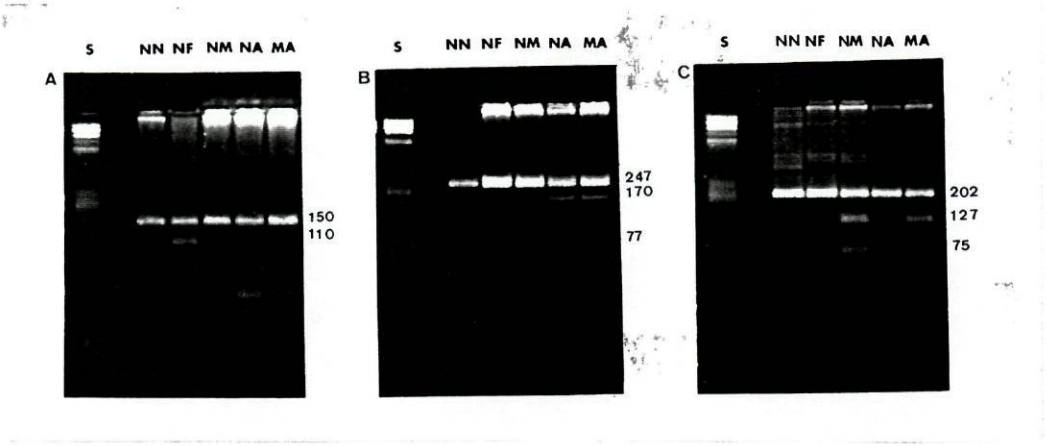


Fig. 10 - RFLP analyses of the kindred with compound heterozygote of TTR Asn 90 - TTR Met 119. From left to right, amplified exon 2 digested with Nsi I(**A.**), amplified exon 3 digested with Bsm I (**B.**) and amplified exon 4 digested with Nco I (**C.**). The symbols mean: NN - control, normal; NF - control, heterozygotic Met 30; NM - (mother of the propositus) heterozygotic Asn 90; NA - (father of the propositus) heterozygotic Asn 90; MA - (propositus) compound heterozygotic Asn 90 - Met 119.

Case VII:

The propositus presented, by HIEF, a pattern consistent with the coexistence of TTR Met 30 and TTR Met 119 monomers. We analysed DNA from this individual and from his relatives to confirm the presence of both mutations. Therefore, TTR exons 2 and 4 were PCR amplified and digested, respectively, with Nsi I and Nco I. The results, presented in Figure 11, confirmed the result of HIEF: the propositus was a compound heterozygote carrying the Met 30 and Met 119 mutations. Since the mother was a heterozygotic carrier of TTR Met 30, the Met 119 mutation should have been inherited from the father in a different allele as inferred by genotypic analysis of the brothers.

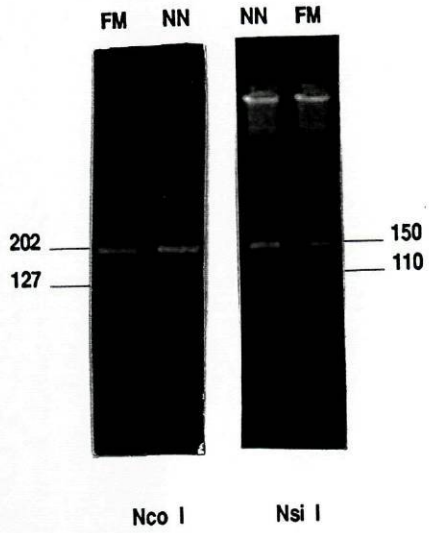


Fig. 11 - RFLP analysis of amplified exons 2 (right) and 4 (left) of a compound heterozygotic individual for TTR Met 30 - TTR Met 119 (FM); NN - control DNA.

DISCUSSION

In this study, we reported the identification of four different non-amyloidogenic TTR variants, namely TTR Asn 90, TTR Arg 102, TTR Thr 109 and TTR Met 119. Although TTR Thr 109 had been previously reported associated with euthyroid hyperthyroxinemia (Moses *et al.,* 1990), the other variants were not associated with any clinical manifestations since the carriers did not present any signs of disease.

TTR Asn 90 has been first reported in an American family of Italian origin with amyloidosis (Skare *et al.,* 1989). However, parallel comparison of the Portuguese and American TTR Asn 90 variants by isoelectric focusing showed different migration for the two proteins (Alves *et al.,* 1992). This puzzling result prompted later the authors to further study that family and cautious analysis of DNA sequencing revealed an additional mutation in the American sample originating a substitution of glycine for glutamate at position 42 (Skare *et al.,* 1994). This result demonstrated the relevance of both protein and DNA analysis in the characterization of a TTR variant.

TTR Asn 90 and TTR Met 119 had been found in the Portuguese population with a high frequency of occurrence, respectively, 0.24% and 0.7% (Alves *et al.,* 1993). TTR Met 119 has also been reported in American individuals of northern- and western-European descent with a frequency of 0.6% (li *et al.,* 1992). It is known that mutations occurring in CpG dinucleotides, originating C-T transitions, have a high frequency of occurrence. Yoshioka *et al.* (1989) found ten CpG dinucleotides in the coding region of the TTR gene and predicted the possible substitutions originated by C-T transitions at these positions. TTR Thr 109, that was found by us and Moses *et al.* (1990), and TTR Met 119 are among those variants. However that is not the case for TTR Asn 90.

TTR Ser 6 is also a TTR variant originated by a mutation at a CpG dinucleotide and seems to be the most frequent TTR variant occurring in the Portuguese and American populations (Jacobson *et al.,* 1995). However this variant did not appear in these screening studies since it is not detected by the technique used. Although HIEF is a powerful technique to analyse charged and non charged proteins, depending on the pH gradient, it does not allow the detection of all the mutations. Thus, other methods have been also used namely to detect DNA mutations like the recently developed single strand conformation polymorphism (SSCPs) analysis (Orita *et al.,* 1991). This technique, based on analysis of PCR amplified DNA, can be used as a mutation exon scanning method allowing the detection of about 90% of the mutations and the concomitant selection of the mutated exon for further analysis. The high sensitivity of this technique allows its use to screen routinely TTR mutations (Torres *et al.,* in press).

The high frequency of the referred mutations in the population raised the possibility that they could also occur in FAP kindreds. Thus, some screening studies were performed in TTR Met 30 kindreds to detect additional TTR variants. In fact, these studies detected individuals carriers of two different TTR mutations. We identified those TTR mutations and studied its segregation in the family and concluded that those individuals were compound heterozygotic carriers of amyloidogenic and non-amyloidogenic TTR variants. Concerning the non-amyloidogenic variants, we characterized an individual carrier of TTR Asn 90 - TTR Met 119. Each of the mutations involved is non-amyloidogenic; however, it is not possible to predict the effects when both mutations occur in the same molecule. So far, this

compound heterozygotic individual seems to be healthy. We also characterized compound heterozygotic individuals carriers of amyloidogenic (TTR Met 30) and non-amyloidogenic TTR mutations, namely TTR Met 30 -TTR Asn 90, TTR Met 30 - TTR Met 119. Although in most cases only one compound heterozygotic individual of each type was identified, in the case of the TTR Met 30 - TTR Met 119 several cases were reported (Alves *et al.*, in preparation) probably due to the high frequency of occurrence of both mutations, per se, in the population studied.

Other cases of compound heterozygotic individuals carriers of non-amyloidogenic variants have been described: one, that has also been detected in our laboratory was a carrier of TTR Ser 6 - TTR Met 119 (Alves *et al.*, in preparation); two other cases were TTR Ser 6-TTR Asp 45 (Jacobson *et al.*, 1994) and TTR Ser 6 - TTR Gly 54 (Booth *et al.*, 1994); yet another, described by Izumoto *et al.* (1992), was a carrier of TTR Val 109 - TTR Met 119 two variants individually related with euthyroid hyperthyroxinemia.

In addition to the compound heterozygotic individuals that carry two different mutations in different alleles, there are cases of double heterozygotes meaning that both mutations occur in the same allele, thus being inherited from one of the parents. That is the case of TTR Gly 42-Asn 90 reported in an Italian family with FAP (Skare *et al.*, 1994) and TTR Ser 6-TTR Ile 33 reported in an Israeli patient «SKO» also with FAP (Jacobson *et al.*, 1994).

Concerning the compound heterozygotes carriers of TTR Met 30 and another non-amyloigenic variant, that is to say, carriers of TTR Met 30 - TTR Asn 90 or TTR Met 30 - TTR Met 119, they do not present a more severe form of the disease when compared with heterozygous Met 30 carriers. In fact, one of the carriers of TTR Met 30- TTR Met 119 presented a more begnin evolution of the disease then expected when compared with heterozygous Met 30 carriers (Coelho *et al.*, 1992). These individuals need a careful and long follow up in order to elucidate the effects of one mutation over the other. It is possible that the non-amyloidogenic TTR monomer/s exert a «protective» effect over the amyloidogenic monomer/s.

Similarly to what we found for the neuropathic and cardiomyopathic TTR mutations, the non-amyloidogenic TTR mutations, although in less number, are not restricted to a specific region in the molecule as shown in Figure 12.

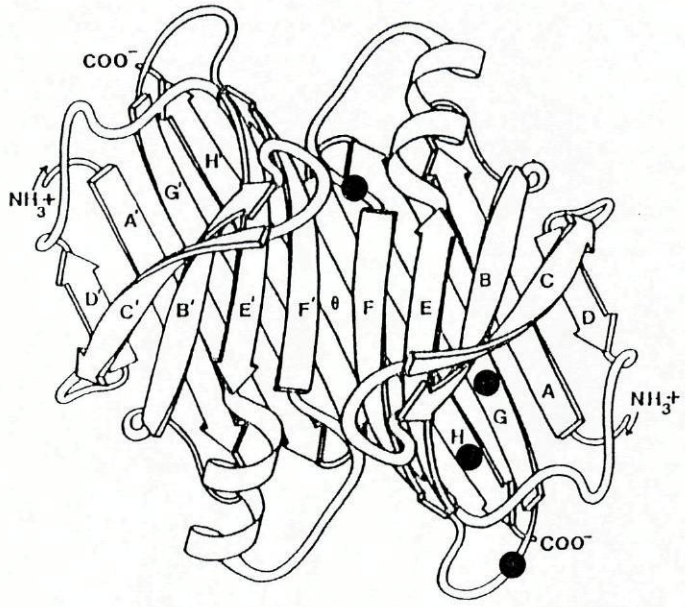


Fig. 12 - Schematic representation of the TTR dimer with the localization of the amino acid substitutions of the non-amyloidogenic TTR variants described in this work.

Eventhough the variants presented here were not associated with amyloidosis in some cases we can predict that they induce functional alterations. For instance, in the case of TTR Thr 109, associated with familial goiter, and TTR Met 119, it is expected that they affect T_4 binding because they are localized in the T_4 binding channel. However, that is quite impredictable for other TTR mutations. The problem becomes even more complex in compound heterozygotes, when considering the coexistence of two different variant monomers and the occurrence of different tetrameric hybrid species. An adequate approach to assess modifications of the TTR structure in these cases seems to be the study of the protein function and the comparison of the protein from compound heterozygotes with heterozygotes of each of the mutations involved. These studies may reflect structural characteristics of the compound protein that affect affinity for T_4 binding, coooperativity of binding interaction betwen TTR subunits and amyloidogenicity.

Thus, functional studies of TTR from these compound heterozygotes are of most importance in order to evaluate the structural and amyloidogenic effects of both mutations. These studies are presented in sections 4.5 and 4.6.

4.4. HAPLOTYPE ANALYSIS OF COMMON TRANSTHYRETIN MUTATIONS

ABSTRACT

The most frequent TTR variant associated with hereditary amyloidosis is TTR Met 30 that has its major focus in Portugal, although it occurs also in many other countries. The distribution of the mutation and the fact that it occurs in a CpG dinucleotide lead us to question the origin of the mutation and the possibility of being originated in Portugal. In order to investigate these questions we studied the distribution of haplotypes associated with the Met 30 mutation in families from different European countries. All the Portuguese families analysed presented the same haplotype associated with the Met 30 mutation (haplotype I). The same haplotype was found for the Swedish and Spanish families studied. However, a distinct haplotype (haplotype III) was found in three families, one Italian, one English and one Turquish. These results suggest that although the Portuguese Met 30 carriers might have one founder, the mutation probably recurred in populations in Europe in a similar manner as reported in Japan. In this study we have also analysed the haplotypes associated with other TTR variants frequent in the Portuguese population.

INTRODUCTION

Several mutations have been identified in the transthyretin (TTR) gene originating different TTR variants (Saraiva, 1995). Most of these variants are present in hereditary amyloidosis with or without polyneuropathy. The most frequent variant associated with familial amyloidotic polyneuropathy (FAP) is TTR Met 30 which has a high prevalence in the Portuguese population but which is also present, though with lower frequency, in other countries namely Sweden (Holmgren *et al.,* 1988), Japan (Tawara *et al.,* 1983; Nakazato *et al.,* 1984a), Italy (Salvi *et al.,* 1990), Spain (Munar-Qués *et al.,* 1990) and Greece (Saraiva *et al.,* 1986).

The origin of the TTR Met 30 mutation has been object of study by Yoshioka *et al.,* (1989) that defined three different haplotypes in the Japanese population. Later, Ii *et al.,* (1993) reported four haplotypes associated with the Met 30 mutation in the American individuals, two of which were newly described. Each haplotype is defined by a set of seven polymorphic substitutions located in the three introns of the TTR gene. The TTR gene, located in chromossome 18, is 7 Kb long and has 4 exons of approximatlly 200 bp each

(Sasaki *et al.*, 1985). The 6 most informative polymorphisms are located at positions 1218 (intron 1), 2422 and 2537 (intron 2), 5198, 5610 and 5708 (intron 3) and the bases at these positions are: haplotype I: G-C-A-C-G-T; haplotype II: T-G-G-C-G-T; haplotype III: T-G-G-A-C-G; haplotype IV: G-C-A-A-G-T; haplotype V: G-G-G-A-G-T. The probability of conversion of an haplotype into another is very low since that would imply the occurence of two recombination events (Yoshioka *et al.*, 1989). Thus, different haplotypes indicate different origins of the Met 30 mutation. In addition, the TTR Met 30 mutation originated by a G to A substitution can be explained by a C to T transition in the anti-sense strand due to the occurrence of cytosine deamination in a CpG dinucleotide, known to be a hot spot mutation site (Cooper *et al.*, 1988).

Other possible mutations occuring in CpG dinucleotides in the TTR gene have been predicted (Yoshioka *et al.*, 1989), and some of them have recently been found such as TTR Ser 6, TTR Thr 109, TTR Met 119 and TTR Ile 122. Some of these are frequent in the population as is the case of TTR Ser 6 (Jacobson *et al.*, 1995) and TTR Met 119 (Alves *et al.*, 1993). TTR Met 119 is also found frequent in American individuals with ancestors of northern- and western-Europe (Harrison *et al.*, 1991; li *et al.*, 1992) and TTR Ile 122 has a high frequency in the black population (Jacobson *et al.*, 1991). TTR Asn 90, although it is not explained by a CpG mutation hot spot, has a high frequency in the Portuguese population (Alves *et al.*, 1993).

In this work we expanded the study of the haplotypes associated with the TTR Met 30 mutation to sixty families, originating not only from Portugal but also from other European countries, in particular Sweden, Italy, Spain, Greece, England and Turkey. We have also tested the haplotype associated with TTR Ser 6, TTR Asn 90 and TTR Met 119 in a few Portuguese kindreds.

MATERIAL AND METHODS

62 individuals (51 Met 30 carriers) belonging to 27 different Portuguese FAP families and 35 individuals (non Met 30 carriers) not related with FAP families, each from a different family were investigated. We studied also 5 individuals (4 Met 30 carriers) from 2 different Spanish FAP families, 19 individuals (14 Met 30 carriers) from 6 FAP families from Italy

including one American family with Italian ancestors, 3 individuals from a Greek FAP family (one Met 30 carrier), twenty seven Swedish TTR Met 30 carriers, each from a different family, 5 individuals (4 Met 30 carriers) from a Turkish family and 3 individuals (2 Met 30 carriers) from an English family.

We have also studied haplotype distribution in 4 families with carriers of two different TTR mutations: 2 families with TTR Met 30 and TTR Met 119 (2 Met 30 carriers, 3 Met 119 carriers and 4 compound heterozygotes Met 30 - Met 119); one family with TTR Ser 6 and TTR Met 119 (4 carriers of Met 119, 1 carrier of Ser 6, and 1 compound heterozygote of Ser 6 - Met 119); one family with TTR Asn 90 and TTR Met 119 (1 carrier of Asn 90, 1 carrier of Met 119 and one compound heterozygote of Asn 90 - Met 119). We analyzed a family with carriers of TTR Asn 90 (3 carriers including one homozygotic individual). We also studied 6 isolated carriers of TTR Ser 6.

Based in the work of Yoshioka *et al.,* (1989) the intronic substitutions defined were analysed by hybridization with allele specific oligonucleotides (ASO) probes and RFLP analysis of PCR amplified DNA. The conditions for the amplifications were similar to the previous description (Yoshioka *et al.,* 1989) except that two new primers were included in order to group the polymorphisms of each intron in an amplified DNA fragment. Accordingly, primers for the analysis of polymorphism 1 (intron 1) were the same as previously described by Yoshioka *et al.,* (1989); for polymorphisms 2 and 3, in intron 2, the primers used were: 5'GAAGAGATGGATCCATGAGG3' and 5'AGAATAGTCCTGTAACCACT3' and for polymorphisms 4, 5 and 6, in intron 3, were: 5'CAAGACATTGCCCCTAGAGT3' and 5'AATCCATGAAGTGAAGAGGC3'. Four of these polymorphisms were detected by ASO hybridization and the other two were detected by RFLP analysis. Hybridization conditions and RFLP analyses were the same as referred to by Yoshioka *et al.,* (1989).

RESULTS

To determine the haplotype associated with the mutation, we studied the segregation of the polymorphisms in several families. In cases with insufficient informative individuals, when the Met 30 carrier was heterozygous for two haplotypes we inferred that the haplotype

associated with the mutation could be one of two. This work was done in colaboration with Prof. Y. Sakaki from the Human Genetics Center in Tokyo; thus, 3 polymorphisms were tested in his laboratory in Japan and the other four by us.

In the Portuguese families analysed, the TTR Met 30 mutation was unequivocally associated with haplotype I, in 24 families and in the other 3 families the Met 30 mutation could be associated with either haplotype I or III; some of these families are presented in Figure 1. We have also determined the haplotypes in 35 control individuals (from non FAP Met 30 families). In those individuals we calculated a frequency of 50% for haplotype I, 47.4% for haplotype III and 2.6 % haplotype II.

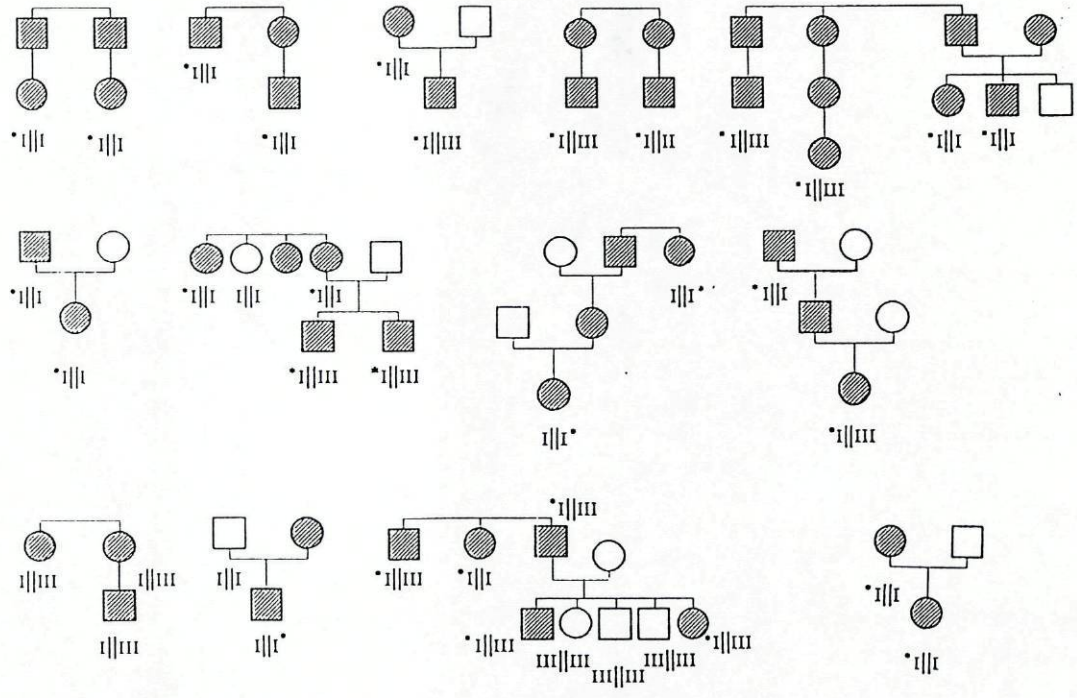


Fig. 1 - Diagram of some of the more informative FAP Met 30 Portuguese families analysed. Study of the segregation of the haplotypes in the families and determination of the haplotype associated with the Met 30 mutation. The symbols ▨,⊘indicate carriers of the Met 30 mutation; symbols □,○indicate non carriers of the Met 30 mutation. The haplotype associated with the Met 30 mutation is marked with an asterisk.

From the 22 Swedish FAP families studied, 16 presented haplotype I associated with the TTR Met 30 mutation and the other 6 could have haplotype I or III. The two Spanish families and the Greek family analysed had haplotype I associated with the Met 30 mutation (Fig. 2). Haplotype I was also the haplotype associated with the Met 30 mutation in five Italian families (Fig. 2).

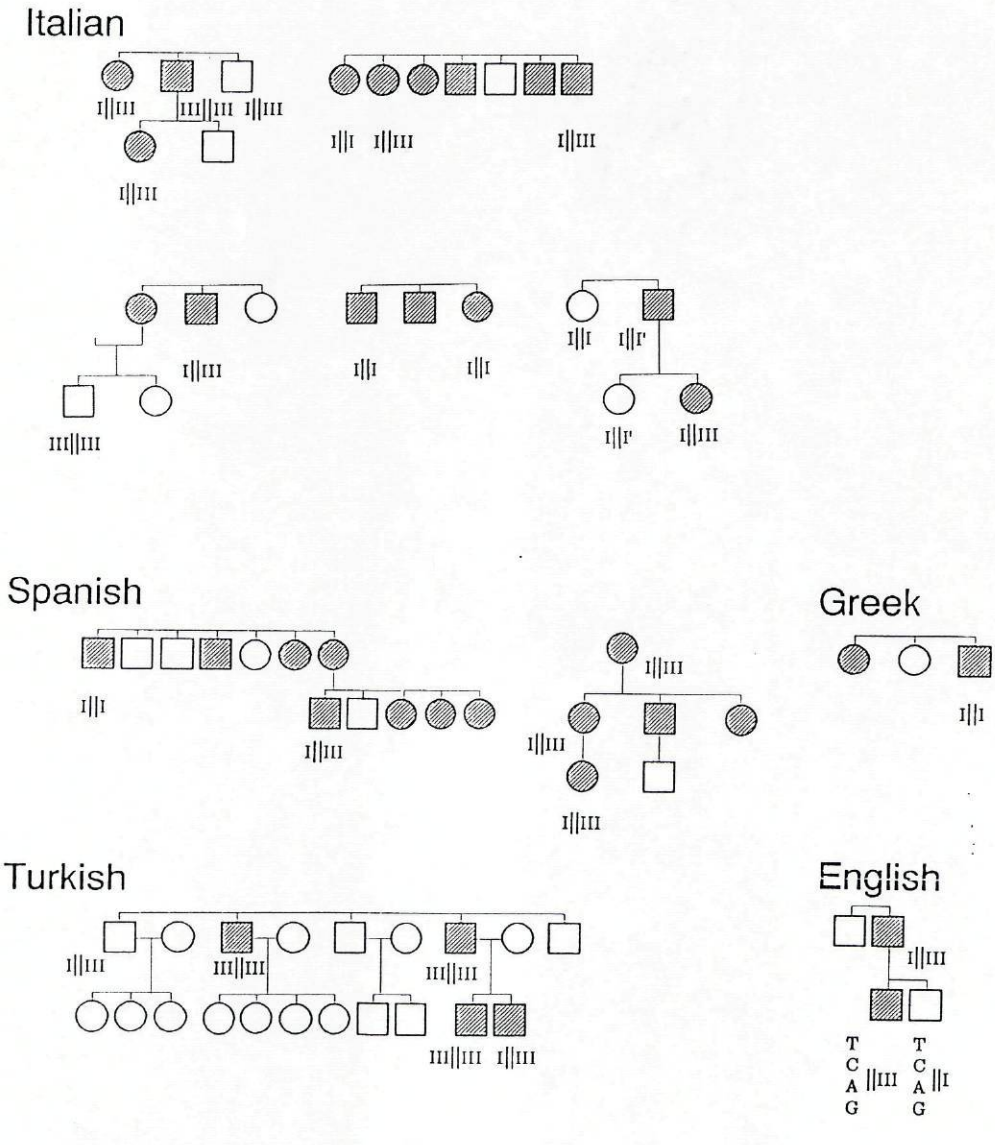


Fig. 2 - Diagram of the non-Portuguese Met 30 families studied and segregation of the haplotypes associated with the mutation. The symbols are the same as in Figure 1.

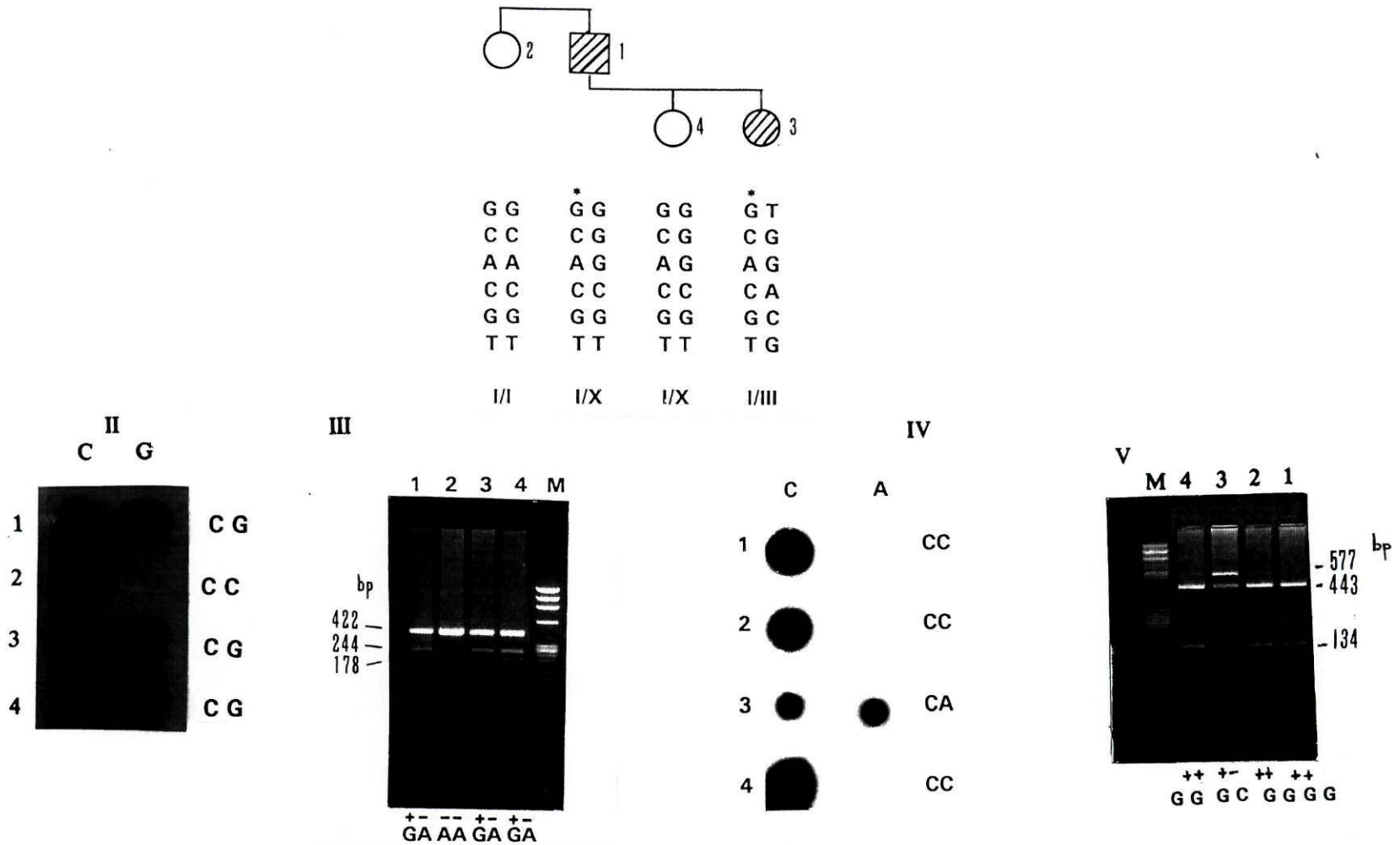


Fig. 3 - Segregation of haplotypes in an Italian kindred. Definition of the new haplotype designated I' and signed with an *. This new haplotype is not associated with the Met 30 mutation. As shown, the haplotype associated with the Met 30 mutation is haplotype I. The symbols are the same as in fig. 1.

In these Italian families we report an haplotype different from the previously five combinations referred to in the introduction. As shown in Figure 3, this new haplotype differs from haplotype II just in the first polymorphism, i.e., instead of a thymine it presents a guanine (it could result from a recombination between haplotype II and III). However, this new haplotype was not associated with the TTR Met 30 mutation.

The Met 30 mutation was associated with haplotype III in one Italian family and also in the Turkish and the English families (Fig. 2). The results for all the Met 30 families analysed are summarized in table I.

TABLE I - Haplotypes associated with TTR Met 30 mutation in FAP families

Origin	Number of families	Number of individuals		Haplotype			Reference (for the source of families)
		Met 30	Non-Met 30	I	III	I/III	
Portugal	27	51	11	24	0	3	Unpublished
Sweden	22	26	1	16	0	6	Unpublished
Italy	6	14	5	5	1	0	Ferlini et al., 1988; Saraiva et al., 1988b
Spain	2	5	0	1	0	0	Munar-Qués et al., 1990
Greece	1	3	0	1	0	0	Saraiva et al., 1986
Turkey	1	4	1	0	1	0	Skare et al., 1990
England	1	2	1	0	1	0	Unpublished

In Figure 4 we show some of these families including those families with compound heterozygotic individuals. In family A, having a compound heterozygotic member carrier of TTR Ser 6 -TTR Met 119, we could associate TTR Ser 6 with haplotype I and TTR Met 119 with haplotype III. TTR Asn 90 was associated with haplotype III in a family with a homozygotic carrier for this mutation (family B). In family C, having a compound heterozygote member of TTR Asn 90 - TTR Met 119, TTR Asn 90 and TTR Met 119 were both associated with haplotype III. TTR Met 119 is also associated with haplotype III in family D. However in family E we could not determine what was the haplotype associated with each of the mutations, TTR Met 119 and TTR Met 30. We also could not define the haplotype associated with TTR Ser 6 in isolated individuals carriers of TTR Ser 6 (not included in these families since we do not have informative relatives). In conclusion, we

found TTR Ser 6 associated with haplotype I, and TTR Asn 90 and TTR Met 119 associated with haplotype III.

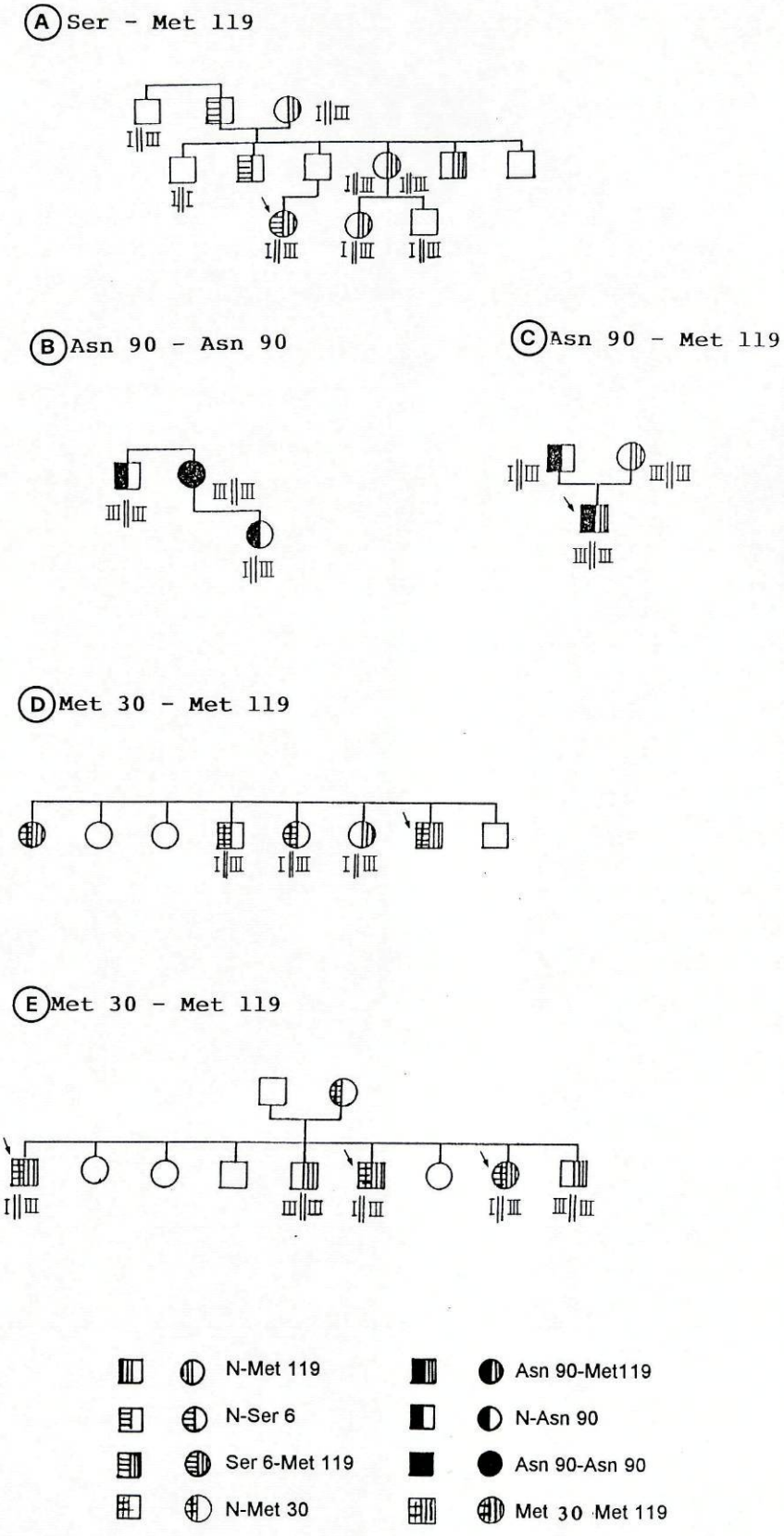


Fig. 4 - Diagram of some of the non-Met 30 families analysed. Segregation of the haplotypes associated with each mutation.

Concerning the families with TTR mutations other than Met 30, the results are summarized in table II.

TABLE II - Haplotypes associated with non-Met 30 TTR variants in Portuguese families

TTR variant	Number of families	Number of individuals		Haplotype			References (for the source of families)
		Carriers	Non-carriers	I	III	I/III	
Ser 6	7	7	0	1	0	6	Unpublished
Asn 90	2	5	1	0	2	0	Saraiva et al., 1991
Met 119	4	14	8	0	3	1	Alves et al., 1993

DISCUSSION

Familial amyloidotic polyneuropathy associated with TTR Met 30 mutation is a widely distributed disease with a major focus in Portugal. The historical connections between the affected populations, namely due to the Portuguese discoveries in the 16th and 17th century, suggested that the mutation occured once and was spread since then to other areas (one founder effect). However, since the Met 30 mutation occurs in a CpG dinucleotide known as a hot spot mutation site (Yoshioka *et al.,* 1989) this would account for the possibility of the mutation occuring several times independently - multiple origin hypothesis. This last hypothesis is supported by the different haplotypes associated with the Met 30 mutation in the various populations studied (Yoshioka *et al.,* 1989; Ii *et al.,* 1992).

In the present work the haplotype study was extended to FAP families from Portugal where the disease has a high prevalence and to FAP Met 30 families from other European countries. The Portuguese Met 30 families studied were from different regions of the country with high incidence of the mutation namely Póvoa de Varzim, Braga, Valença, Espinho, Figueira da Foz, Leiria, Unhais da Serra and Lisboa. All the FAP Met 30 Portuguese families analysed presented only haplotype I associated with the mutation favouring the one founder hypothesis. Haplotype I was also the only haplotype associated with the Met 30 mutation in the Swedish, Spanish and Greek families studied although in the last two cases a small number of families was investigated. However, in the Italian FAP families, the Met 30

mutation was found associated with haplotype I and haplotype III. In addition, at least one Met 30 family from England and one from Turkey had haplotype III associated with the mutation. Thus it seems that, in Europe, the Met 30 mutation could have occured more than once. The comparison of the distribution of the haplotypes in the populations studied also reflects differences in the genetic origins of these populations. For instance two of the haplotypes described in the American population were not found in Europe or in Japan; also the new haplotype (I') that occurs in two Italian families, although not associated with the Met 30 mutation, was not reported before and not among the other kindreds studied, probably because it has a higher frequency in the Italian population. Also, haplotype II found in Japanese Met 30 families has not been associated with the Met 30 mutation in Europe (this study) or in the American individuals studied (Ii *et al.,* 1993).

Concerning the Portuguese FAP Met 30 families it is interesting to note that only haplotype I was found associated with the mutation in spite of the similar frequence of haplotypes I and III in the normal population. Furthermore, previous studies of the incidence of HLA antigens in FAP Met 30 patients revealed a higher incidence for antigens HLA-A2 and HLA-A9 in these patients as compared to the normal population (Martins da Silva *et al.,* 1980). These facts, together with the distribution of the disease in a major area in the North of Portugal, suggest a single origin for the Met 30 mutation in the Portuguese.

As already mentioned, other mutations were predicted by Yoshioka *et al.,* (1989) based in the existing CpG dinucleotides in the TTR gene; similarly to the Met 30 mutation it was reasonable to expect different haplotypes associated with these mutations. Curiously, haplotype III associated with the Met 119 mutation in the Portuguese individuals analysed was the same as reported for the American individuals (Ii *et al.,* 1992). Furthermore, TTR Ser 6 that seems to be the most frequent non-amyloidogenic TTR variant found in the Portuguese and American populations (occuring with a frequency of 12%) (Jacobson *et al.,* 1995) was found associated with haplotype I; however, we expect that upon further extensive studies more than one haplotype will be found associated with this polymorphism.

Concerning TTR Asn 90, this mutation has been also reported in German individuals. Thus, it would be interesting to see if the haplotype associated with the mutation in those individuals was also haplotype III or another haplotype. Another case reported with TTR Asn

90 occured in an Italian family (Skare *et al.,* 1994). In this last case the mutation occurs in the same allele as another mutation - TTR Gly 42. These two mutations are 2 Kb apart; thus the chance of a recombination between these two mutations is very low. Consequently it would be quite improbable that the mutation TTR Gly 42, also occuring in Japan (Ueno *et al.,* 1990), would have the same origin (Skare *et al.,* 1994). The same applies to the Asn 90 mutation. Although it is probable that the Portuguese and the German TTR Asn 90 mutation have the same origin, TTR Asn 90 mutation in that Italian family should have a different origin. A recent study by Zhao *et al.,* (1994) reported haplotype analysis in families with FAP associated with TTR Tyr 77. The study included one French and three American families with FAP associated with Tyr 77. In the French family Tyr 77 was associated with haplotype III and in the American families with haplotype I suggesting that, like other mutations in the TTR gene, the mutation that originates the TTR Tyr 77 in these families had occurred independently.

Since this study confirmed the multiple origins of the Met 30 mutation, similarly to other TTR mutations, it would be interesting to further study the genetic background in the different populations affected with a same mutation. This is particularly important when considering the differences of clinical expression, namely age of onset of the disease, associated with a given variant. No relationship between the haplotype associated with the Met 30 mutation and the age of onset of the disease was observed. Thus, this study included Portuguese families with typical (onset in the third decade) (Coutinho *et al.,* 1980) and late onset (onset in the sixth or seventh decade) (Bastos Lima and Martins da Silva, 1980) all of which had haplotype I associated with the Met 30 mutation. The results were similar for the Swedish FAP Met 30 families that are known to have a characteristic late onset of the disease (Anderson, 1976). Therefore the study of more markers could provide some insights into the genetic factors influencing age of onset of the disease.

4.5.THYROXINE BINDING IN TRANSTHYRETIN COMPOUND HETEROZYGOTIC INDIVIDUALS: THE PRESENCE OF TTR MET 119 INCREASES T_4 BINDING AFFINITY

ABSTRACT

The majority of the known TTR variants are associated with amyloidosis but there are also variants associated with euthyroid hyperthyroxinemia and others are apparently non-pathogenic. Most of the carriers of TTR variants are heterozygotic individuals. However, but screening studies for TTR variants revealed individuals carriers of two different TTR mutations in different alleles - compound heterozygotes.

We characterized these compound heterozygotic individuals and found carriers of two non-amyloidogenic variants, TTR Ser 6 - TTR Met 119 or TTR Asn 90 - TTR Met 119 , and carriers of one amyloidogenic and one non-amyloidogenic variants, TTR Ser 6 - TTR Met 30 or TTR Ser 6 - TTR Met 119 or TTR Met 30 - TTR Met 119.

We compared T_4 binding to TTR from each of these individuals with T_4 binding to TTR from heterozygotic individuals of each of the variants involved and with normal TTR. We also studied T_4 binding to the parallel synthetically produced variants. We found a decreased T_4 binding affinity to TTR Met 30 and an increased T_4 binding affinity to TTR Met 119. We also observed that TTR Met 119 from heterozygotic individuals and compound heterozygotes has a consistent effect of increasing T_4 binding affinity that is independent from the mutation present in the other allele. On the contrary, the Met 30 mutation always decreased the affinity for T_4. Concerning TTR Asn 90, this variant presented a low binding affinity for T_4 but normal binding capacity suggesting that it lacks negative cooperativity. We speculate about possible structural alterations that would give origin to these effects and concluded that further structural studies could help to understand what is the influence of these mutations on amyloid formation.

INTRODUCTION

Transthyretin (TTR) is a thyroxine (T_4) binding protein carrying about 20% of total serum T_4. TTR is also a carrier of retinol binding protein. The TTR molecule is a tetramer of identical subunits with 127 amino acid residues each, codified by a single copy gene. About 50 different point mutations have been described in TTR. Most of them are associated with FAP, a disease characterized by the deposition of TTR as amyloid; others are responsible for euthyroid hyperthyroxinemia and there are still some apparently non-pathogenic

mutations. Screening studies in the Portuguese population revealed a high frequency of occurrence of some of these non-pathogenic variants, in particular TTR Ser 6, TTR Asn 90 and TTR Met 119 (Alves *et al.,* 1993; Jacobson *et al.,* 1995). Concerning the amyloidogenic variants, TTR Met 30 is the most frequent TTR variant and has its major focus in Portugal. These facts, together, probably contributed to the occurrence, in the Portuguese population, of individuals carriers of two different TTR mutations in different alleles - compound heterozygotes.

The effects of the non-amyloidogenic TTR on the amyloidogenicity of TTR Met 30 are not known and structural-functional studies will help to elucidate this aspect. Therefore, we started a series of studies on the T_4-TTR binding properties in different compound heterozygotes, respectively, TTR Ser 6 - TTR Met 30, TTR Ser 6 - TTR Met 119, TTR Met 30 - TTR Asn 90, TTR Met 30 - Met 119 and TTR Asn 90 - TTR Met 119. Some studies have already been performed in TTR from heterozygotic individuals carriers of each of these four variants. TTR Ser 6 was first reported by Fitch *et al.* (1991) and described in association with hyperthyroxinemia; however, recent studies on recombinant TTR Ser 6 have demonstrated that this variant has a normal T_4 binding affinity (Murrell *et al.,* 1992). TTR Met 30, the most studied TTR variant, in the heterozygotic form has low T_4 binding affinity (Refetoff *et al.,* 1986) and the homozygotic TTR Met 30 has almost no affinity for thyroxine (Rosen *et al.,* 1993). To date, there are no T_4 binding studies toTTR Asn 90. TTR Met 119 T_4 binding affinity raised more controversy. This mutation was initially described as benign and its carriers presented no differences on thyroid hormone levels as compared to normals (Harrison *et al.,* 1991). Scrimshaw *et al.* (1991) referred normal levels of free T_4 in carriers of TTR Met 119, but increased T_4 binding to TTR. A study by Alves *et al.* (1993b) reported normal T_4 levels in the serum of TTR Met 119 carriers but an increased T_4 binding potencial as compared to control, attributed to the relatively high levels of TTR in their serum. However, recently Curtis *et al.* (1994) described an increased T_4 binding affinity for transthyretin semi-purified from sera of TTR Met 119 carriers. To further study this question we performed T_4 binding assays in serum and isolated TTR from sera of heterozygotic carriers of TTR Met 119 and from compound heterozygotic individuals carriers of TTR Met 30 - TTR Met 119. In either case TTR may be a hybrid form composed by two different

kinds of TTR monomers. Thus, in order to compare these results with that of homotetrameric TTR we performed the assays also with recombinant proteins synthetically produced in an *E. coli* expression system (Furuya *et al.,* 1991).

MATERIAL AND METHODS

Samples

T_4 binding assays were performed in whole serum and/or serum isolated TTR from: heterozygotic individuals carriers of different TTR mutations namely TTR Ser 6, TTR Met 30, TTR Asn 90 and TTR Met 119; from homozygous individuals for TTR Met 30 and for TTR Asn 90; and from compound heterozygotic individuals carriers of two different TTR mutations, one in each allele, namely TTR Ser 6 -TTR Met 30, TTR Ser 6 -TTR Met 119, TTR Met 30 - TTR Met 119 (Alves *et al.,* in preparation), TTR Met 30 - TTR Asn 90 (Saraiva *et al.,* 1991) and TTR Asn 90 - TTR Met 119 (Alves *et al.,* 1993b).

The same studies were carried out with recombinant proteins synthetically produced in an *E. coli* expression system: normal TTR, TTR Met 30, TTR Asn 90 and TTR Met 119 (Furuya *et al.,* 1991).

TTR isolation

Plasma was dialysed against phosphate buffer 50 mM, 77mM NaCl, pH 7.6. The dialysed plasma was passed through an ion exchange column of DEAE-cellulose.The column was washed with the phosphate buffer and the fraction containing TTR was eluted increasing the ionic strentgh, phosphate buffer 50 mM, 600 mM NaCl. The TTR fraction was dialysed, liophilised and rechromatographed in a Blue-Sepharose column. Then, TTR was isolated by preparative electrophoresis in a native Prosieve agarose (FMC) gel following instructions from the supplier. After electrophoresis, a slice of the gel was stained with Coomassie Blue to localize the TTR band. This band was excised from the gel and electroeluted in a Elutrap (Schleicher & Schuell) in 5mM Tris, 38 mM glycine pH 8.3 buffer for 3h at 200 V or overnight at 50 V (4°C). The protein was further purified by gel filtration in

high performance liquid chromatography (HPLC) using a Ultraspherogel column (Beckman) in buffer 0.1 M KPO_4, 0.1 M Na_2SO_4, 0.05 % NaN_3.

The recombinant proteins were isolated by the same process after osmotic shock of the bacteria (Furuya *et al.*, 1991).

TTR quantification

TTR in whole serum was quantified by rocket immunoelectroforesis (Laurell, 1967) using rabbit anti-human TTR with a titre of 450 mg/l in a concentration of 0.75 µl/cm^2 (DAKO). Isolated TTR variants were quantified by the Bio-Rad micro plate assay.

T_4 binding assays

^{125}I-T_4 purification: Labelled L-^{125}I-Thyroxine (^{125}I-T_4) (1300µCi/µg) (DuPont NEN) was purified using LH-20 Sephadex chromatography (Otten *et al.*, 1984) . The column (1 ml bed volume) was washed with 0.1 M HCl to elute the free iodine, then washed with water and finally the purified ^{125}I-T_4 was eluted with a solution of 0.25% ammonium in methanol. Labelled T_4 was dried under nitrogen and ressuspended in the appropriate buffer.

Competition assays: T_4 binding assays were based in a gel filtration procedure described by Somack *et al.* (1982) with minor modifications (Lans *et al.*, 1993). Briefly, for the isolated proteins, 100 µl of TTR 60 nM in Tris buffer pH 8 (Tris 0.1M, NaCl 0.1M and EDTA 0.001M) were incubated with 100µl of T_4 solutions of variable concentrations ranging from 0-1000 nM and with a constant amount of labelled ^{125}I-T_4 (~50,000cpm). This solution was counted in a gamma spectrometer and incubated at 4°C overnight. Protein bound ^{125}I-T_4 and free ^{125}I-T_4 were separated by gel filtration through a 1ml BioGel P6DG (Bio-Rad) column. The protein bound fraction was eluted, by centrifugation at 1,000 rpm, with Tris buffer in the two first 200 µl fractions, while free T_4 was retained on the BioGel matrix. The first eluates containing the bound T_4 were collected and counted. Free T_4 remaining bound to the Biogel was eluted as described above and radioactivity was counted. T_4 bound was expressed as % of total T_4 added.

The same procedure was applied for the T_4 binding assays in whole serum. However, in this case, after TTR quantification, the serum was diluted to a TTR concentration of 20 nM (approximately 400 times dilution).

TTR dose response assays: the procedure was the same as for the competition assays but using a fixed concentration of T_4 (20 nM) and variable TTR concentrations ranging from 5 to 120 nM.

Each assay was performed in duplicate or triplicate and was repeated at least twice. For analysis of the binding data we used the GraphPAD InPlot computer program (version 3.15, San Diego, CA) and for statistical analysis of grouped values we used Student's test. $P < 0.05$ was considered statiscally significant.

RESULTS

In order to confirm the tetrameric form of the isolated proteins for the binding studies, the last step of protein isolation was a gel filtration chromatography in HPLC. We used albumin, M.W. 64,000, and ribonuclease, M.W. 13,700, as standards. In Figure 1 we present the chromatogram of normal TTR isolated from serum which is superimposed with the chromotogram of the molecular weight standards. The TTR peak eluted in a position corresponding to the tetramer, near the peak of albumin.

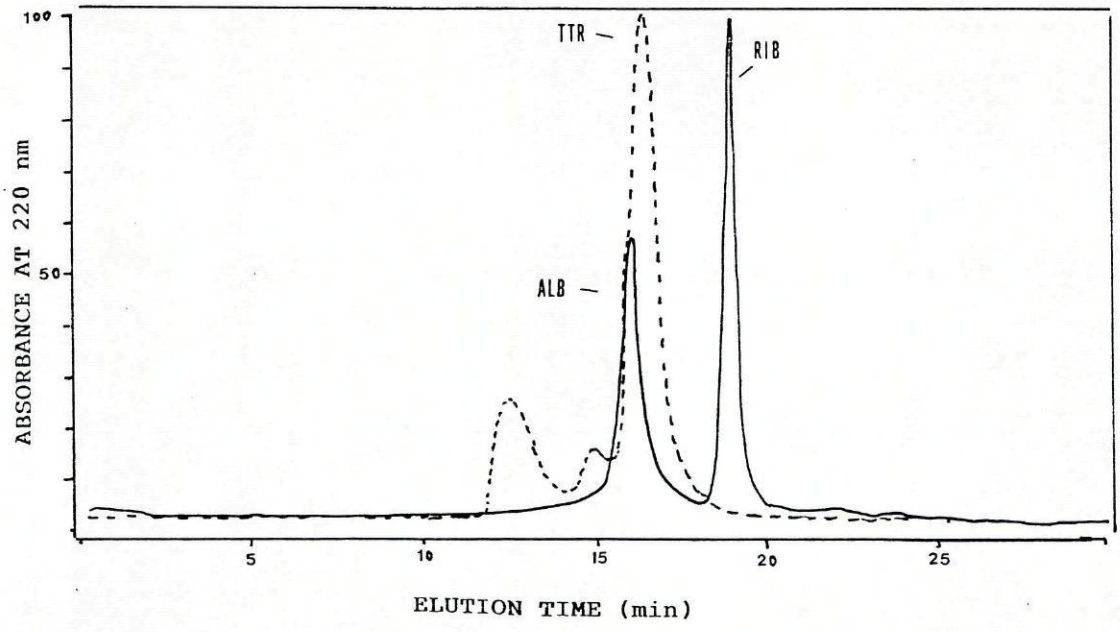


Fig. 1- Chromatogram of the separation of TTR by gel filtration in HPLC (dashed line). Comparison with the chromatogram of albumin (Alb) and ribonuclease (Rib) (full line).

T_4 binding was assayed in whole serum samples from heterozygotic carriers of TTR Met 30 and TTR Met 119 and also on serum from compound heterozygotic individuals carriers of TTR Met 30-TTR Met 119. TTR concentration in serum was determined by rocket immunoelectrophoresis. The results obtained (Table I) showed that, although within the normal range, TTR concentration was elevated in TTR Met 119 carriers sera which is in agreement with previous reports (Alves *et al.,* 1993b).

TABLE I - Serum TTR concentration (mg/dl) determined by rocket imunoelectrophoresis

	N	N/Met 30	N/Met119	Met30/Met119
	31.7	16.1	38	38
	33.3	22.4	39.5	46.5
	27	14.5	34.9	29.4
	26.3	24.7	40.3	29.4
	30.2	27	28.6	34.9
Mean+SD	29.7+3.0	20.9+5.4	36.3+4.8	35.6+7.1
P*		0.02	0.04	0.145

P* - relative to values of N

The results of binding assays are shown in Figure 2; Figure 2A shows the graph for the competitive binding assay and Figure 2B is the Scatchard representation of the data in Figure 2A. For the competition curves we plotted percentage of T_4 bound against total T_4 concentration in the assay. Binding affinities were calculated from the slope of the Scatchard curves obtained when the T_4 bound to free ratio was plotted against bound T_4. Each curve represents the mean values obtained for 5 different serum samples, except for the homozygous TTR Met 30. The affinity constants (Ka) obtained for each variant (Table II) are different, as inferred from the different slopes of the Scatchard analysis.

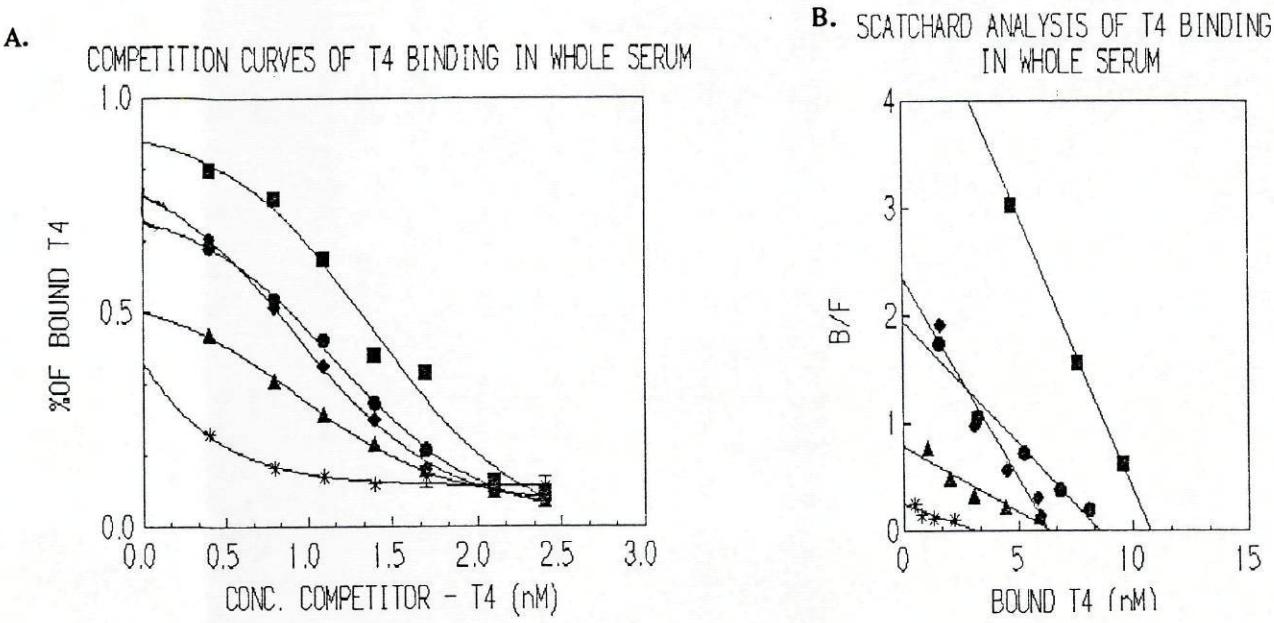


Table II

SERA	Ka x 10^{-9}	Ka rel.
N - N	2.4 ± 0.32	1
N - Met 30	1.58 ± 0.64	0.65
N - Met 119	4.43 ± 1.63	1.85
Met 30 - 119	4.03 ± 0.65	1.68
Met 30 - Met 30	0.77	0.32

Fig. 2 - T_4 binding in whole serum. Panel **A** - competition studies; Panel **B** - Scatchard analysis. ● N-N; ▲ N-Met 30; ■ N-Met 119; ♦ Met 30-Met 119; ✳ Met 30-Met 30.

Concerning TTR Met 30, the heterozygotic carriers presented low T_4-TTR binding affinity and the homozygotic carrier seemed to have no specific T_4 binding which is in accordance with previous reports (Refetoff *et al.*, 1986; Rosen *et al.*, 1993). TTR Met 119 carriers presented an affinity for T_4 binding that is approximately 1.5-2 times higher than the affinity of normal serum TTR, similarly to the results reported by Curtis *et al.* (1994). The results obtained for the T_4 -TTR affinity constant in the compound heterozygote carrier of TTR Met 30-Met 119 indicate that the T_4 affinity constant is slightly (but not statistically significant) lower than the affinity for TTR Met 119. Thus, the presence of Met 119 has an

effect of increasing T_4 binding affinity constant whereas TTR Met 30 has the opposite effect - decreases T_4 binding affinity. These effects were also observed when we studied T_4 binding to TTR in serum of compound heterozygotes carriers of TTR Ser 6 (Figure 3). In this case we analysed one individual carrier of TTR Ser 6-TTR Met 30, one carrier of TTR Ser 6-TTR Met 119 and another heterozygotic carrier of TTR Ser 6. TTR Ser 6 presented a T_4 binding affinity similar to that of normal TTR ; however, TTR Ser 6-Met 119 presented an increased affinity when compared to TTR Ser 6. On the contrary, and as expected, TTR Ser 6-TTR Met 30 had a lower affinity then TTR Ser 6 (Table III).

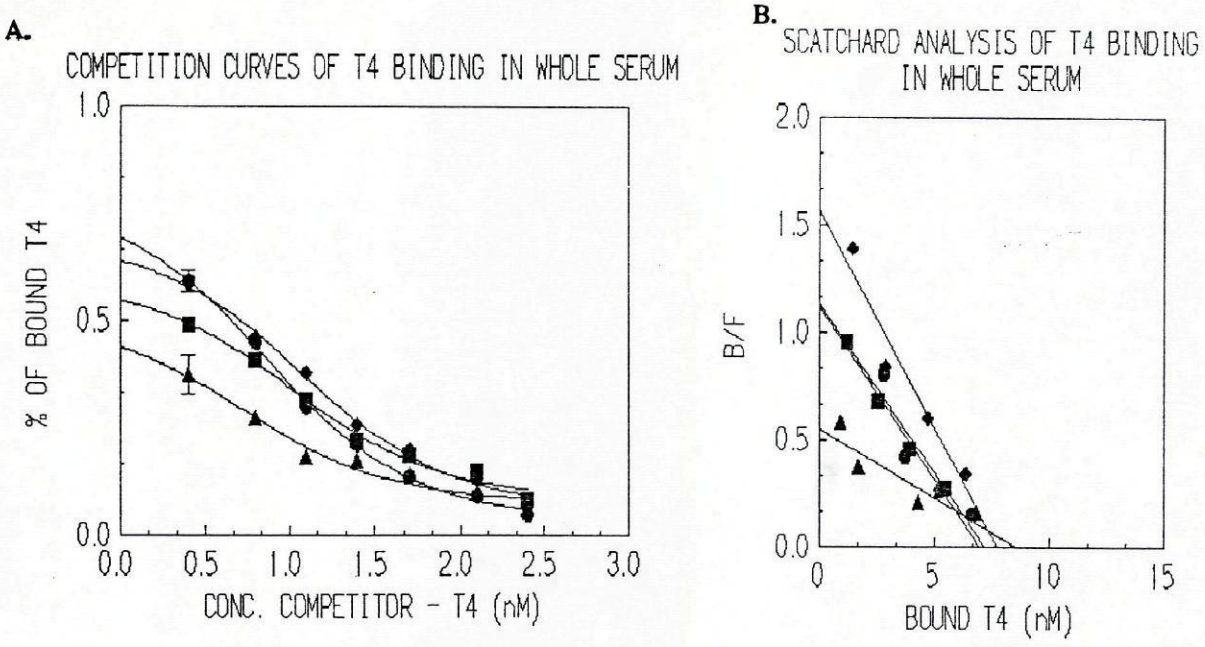


TABLE III

SERA	Ka x 10^{-8}	Ka rel.
N - N	1.56 ± 0.45	1
N - Ser 6	1.6 ± 0.015	1.02
Ser 6 - Met 30	0.65 ± 0.22	0.41
Ser 6 - Met 119	2.02 ± 0.038	1.26

Fig. 3 - T_4 binding in whole serum. Panel **A** - competition studies; Panel **B** - Scatchard analysis. ● N-N; ■ N-Ser 6; ▲ Ser 6-Met 30; ◆ Ser 6-Met 119.

We have also analyzed T_4 binding to TTR in serum from compound heterozygotic individuals carriers of TTR Asn 90 - TTR Met 119, TTR Met 30 - TTR Asn 90 and from heterozygotic and homozygotic carriers of TTR Asn 90 (Figure 4). T_4 binding to TTR in serum from the homozygotic carrier of TTR Asn 90 has a low binding affinity constant (about half) when compared to normal serum. However, T_4-TTR binding in serum from the heterozygotic carrier of TTR Asn 90 presented an affinity constant slightly higher than that obtained for normal serum (Table IV). Respecting the influence of TTR Met 30 and TTR Met 119 in the presence of TTR Asn 90 we found that the compound heterozygotic carriers of TTR Met 30 - TTR Asn 90 presented a T_4 binding affinity similar to the heterozygotic carriers of TTR Asn 90. However, the compound heterozygotic carriers of TTR Asn 90 - TTR Met 119 presented a much higher binding affinity than the heterozygotic carrier of TTR Asn 90. This seems to indicate that TTR Met 30 does not influence T_4 binding affinity of TTR Asn 90 and that, on the contrary, TTR Met 119 increases significantly T_4 binding in the presence of TTR Asn 90.

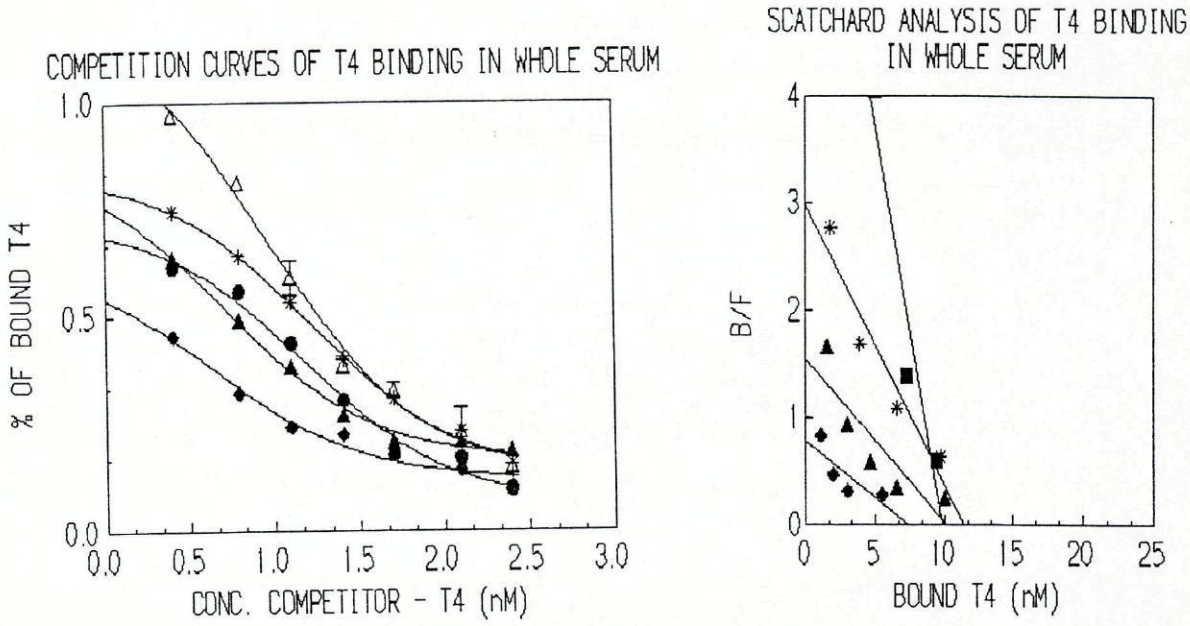


TABLE IV

SERA	Ka x 10^{-8}	Ka rel.
N - N	1.97 ± 0.07	1
N - Asn 90	2.62 ± 0.55	1.33
Met 30 - Asn 90	2.55 ± 0.59	1.29
Asn 90 - Asn 90	1.06 ± 0.56	1.19
Asn 90 - Met 119	7.8 ± 2.4	3.96

Fig. 4 - T_4 binding in whole serum. Panel **A** - competition studies; Panel **B** - Scatchard analysis. ● N-N; ✳ N-Asn 90; ▲ Met 30-Asn 90; ♦ Asn 90-Asn 90; ■ Asn 90-Met 119 30.

The same binding studies were performed on purified proteins from the serum of heterozygotic carriers of TTR Met 30, TTR Met 119 and compound heterozygotes of TTR Met 30 -Met 119 (Figure 5). Although the binding affinity constants (Ka) were slightly lower than the ones obtained in the whole serum, the relative values were similar, i.e., TTR Met 119 variant increases T_4 binding affinity about two fold when compared to normal TTR and this effect is independent of the other TTR monomer present; normal TTR in heterozygotes or TTR Met 30 in compound heterozygotes.

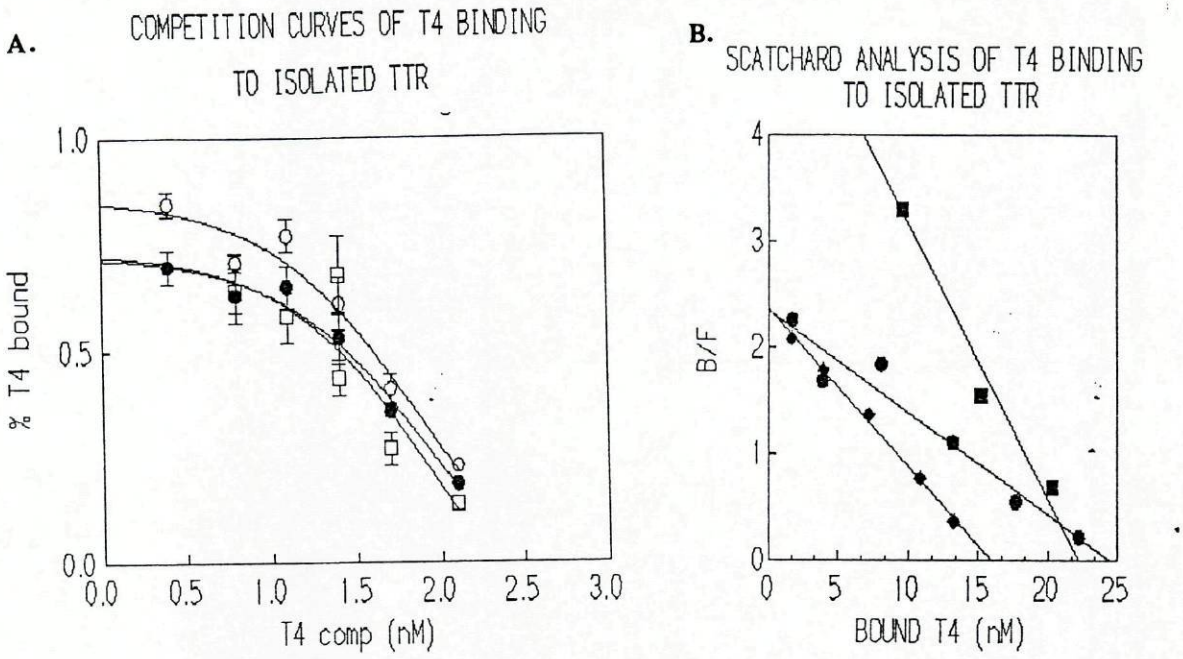
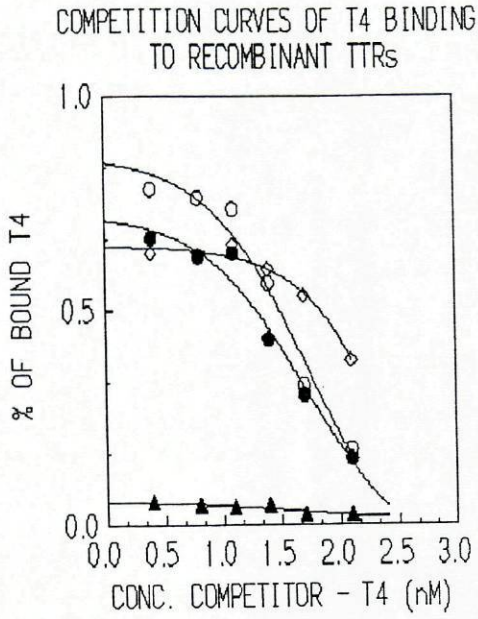


TABLE V

Isolated TTR	Ka x 10^{-8}	Ka rel.
N	1.18 + 0.26	1
Met 119	2.1 + 0.41	1.78
Met119 - 30	2.14 + 0.41	1.81

Fig. 5 - T_4 binding to isolated TTR. Panel **A** - competition studies; Panel **B** - Scatchard analysis. ● N; ■ Met 119; ♦ Met 119-Met 30.

These mutated proteins in the serum or purified from serum are, probably, a complex mixture of heterotetramers composed by different proportions of mutated and normal TTR monomers. In order to evaluate the influence of each mutation, per se, we studied T_4 binding in homotetrameric mutant proteins produced by recombinant bacteria. Thus, we studied T_4 binding in recombinant normal TTR, TTR Met 30, TTR Asn 90 and TTR Met 119, as shown in Figure 6.



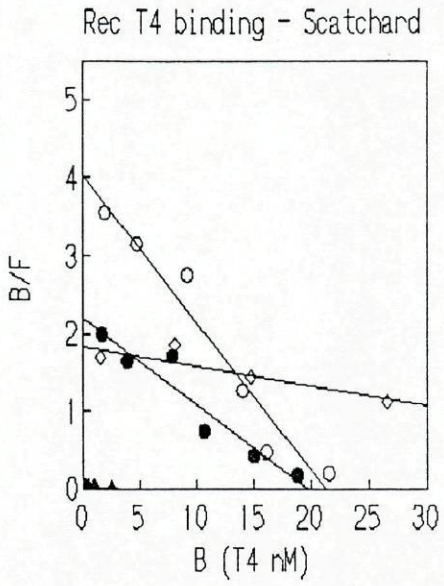


TABLE VI

Recomb. TTR	Ka x 10^{-8}	Ka rel.
Rec N	1.1 ± 0.02	1
Rec Met 30	0.12 ± 0.04	0.11
Rec Asn 90	0.26 ± 0.03	0.24
Rec Met 119	2.3 ± 0.25	2.1

Fig. 6 - T_4 binding to recombinant TTR. Panel **A** - competition studies; Panel **B** - Scatchard analysis. ● Rec N; ▲ Rec Met 30; ◊ Rec Asn 90; O Rec Met 119.

The values obtained for the affinity constants are shown in Table VI. Recombinant TTR Met 30 has virtually no affinity for T_4, similarly to what was found in the protein from serum of the homozygous carrier of TTR Met 30. However, T_4 binding affinity for TTR Met 119 is twice the value of normal TTR. Concerning TTR Asn 90 the results were quite different from those of the other variants; the Scatchard curve, did not suggest the existence of two binding sites with different binding affinities. This fact might represent the loss of negative cooperativity in the Asn 90 variant. In order to further investigate this hypothesis we studied comparatively the binding capacities of N, Met 30 Met 119 and Asn 90 recombinant variants (Figure 7).

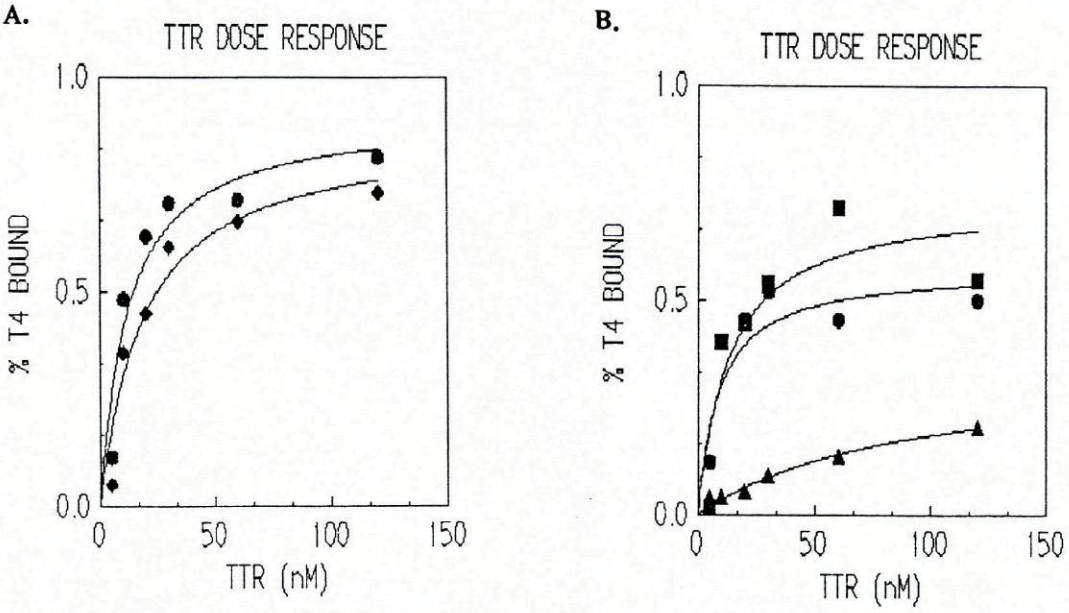


Fig. 7 - TTR dose response curves. Panel **A** - ● Rec normal, ♦ Rec Asn 90; Panel**B** - ● Rec Normal, ▲ Rec 30, ■ Rec Met 119.

Since the binding capacity for Asn 90 is normal, the abnormal behaviour on the competition experiments most likely represent a loss of negative cooperativity. This effect is only evident in the homotetrameric form of Asn 90.

DISCUSSION

In a previous work Alves *et al.* (1993) reported the detection of TTR compound heterozygotes, individuals that have one mutation in each allele of the TTR gene. In order to study the mutual influence of each mutation, we carried out functional studies of TTR from these individuals. Thus, we compared T_4 binding to TTR in heterozygotes, homozygotes and compound heterozygotes of TTR Ser 6, TTR Met 30, TTR Asn 90 and TTR Met 119. In parallel, and for comparative purposes, we tested recombinant homotetrameric mutants. Concerning the natural variants, T_4 binding to TTR was studied using diluted whole serum and serum TTR isolated. Although the absolute values obtained for T_4-TTR binding affinity constants in both cases were different, the relative values were similar; these results further validate the application of this methodology for T_4 binding in diluted serum as previously described by Refetoff *et al.* (1986).

From the results obtained we concluded that the occurrence of TTR Met 119 consistently increases TTR-T_4 binding affinity independently from the other TTR monomer present in the tetramer: normal, Ser 6 or Met 30. These results are in agreement with the data reported by Curtis *et al.* (1994) that employed identical methodology using semi-purified TTR and also found increased T_4 binding affinity for TTR Met 119 carriers. However, Alves *et al.* (1993) tested T_4 binding in whole serum from TTR Met 119 carriers by equilibrium dialysis and step wise saturation of iodothyronine binding sites and found that, when compared with parallel normal serum samples, TTR Met 119 carriers had an increased T_4 binding potencial. This was attributed to an increased concentration of TTR in TTR Met 119 carriers as compared to normals implying that the T_4-TTR affinity found was normal. Also discrepant T_4 binding data was found for TTR Met 30. Homozygotic TTR Met 30 isolated from serum or in whole serum presented a very low T_4 binding affinity and has been reported to bind virtually no T_4 (Rosen *et al.,* 1993). However, isolated recombinant TTR Met 30 showed capacity to bind T_4 in qualitative assays (Furuya *et al.,* 1991). In addition, determination of serum thyroxine levels in TTR-null mutant mice (Episkopou *et al.,* 1993) compared to their human TTR Met 30 transgenic counterparts showed that human TTR Met 30 homotetramers do have the ability to bind T_4 as demonstrated both «in vitro»

and *in vivo* (Palha *et al.,* 1994). We attributed both situations to components in serum that may interact with the distinct TTR variants and might give differences in binding parameters depending on the methodologies used.

Comparing the T_4-TTR affinity constants (Ka) obtained for TTR isolated from TTR Met 119 carriers with the Ka for recombinant homozygotic TTR Met 119 we did not find a relevant difference; on the contrary, T_4 affinity constant for TTR Met 30 in homozygotes was much lower than in heterozygotes (Murrell *et al.,* 1992; Rosen *et al.,* 1993). Neverthless, studies of TTR Thr 109, a TTR variant that also increases T_4-TTR binding affinity showed that the homozygotic recombinant TTR Thr 109 did not present a higher affinity for T_4 than the heterozygotic protein (Rosen *et al.,* 1994). This may suggest that, contrary to what happens with TTR Met 30, the mutations involving Thr 109 and Met 119 do not induce significant structural alterations outside the binding channel and therefore do not interfere with the negative cooperativity of T_4 binding.

According to Blake *et al.* (1977) the methionine for threonine substitution at position 119 would destabilize the thyroxine binding since the threonine 119 and serine 117 establish hydrogen bonds with a water molecule that interacts with the hydroxyl group of T_4 in the binding channel. On the other hand, this substituion of methionine for threonine increases the hydrofobicity in the T_4 binding channel and may contribute to stabilize T_4 binding. Our results are in agreement with the latter hypothesis.

Concerning TTR Asn 90, the different shape of the competition curve and the low slope of the Scatchard representation suggested that this variant, at least the homozygotic native or recombinant, lack negative cooperativity for T_4 binding. Thus, although TTR Asn 90 presented a low binding affinity, the binding capacity was found normal. This may justify the significant increase of T_4 binding to TTR Asn 90 - TTR Met 119 compound heterozygotes. Structurally, residue 90 is involved in interactions between TTR monomers. The main chain C=O of histidine 90, the normal residue, of one monomer forms a hydrogen bond with the main chain N-H of valine 94 in the other monomer via a water molecule (Blake *et al.,* 1978). When histidine 90 is substituted by asparagine 90 this bond may be slightly weaker because the asparagine side chain will tend to displace electrons from C=O

bond (Skare *et al.*, 1990). Destabilization of these interactions probably disrupts the negative cooperativity of T_4 binding to TTR Asn 90.

Further binding studies with T_4 analogues and X-ray analysis of the mutated proteins, in particular recombinant TTR Asn 90 and TTR Met 119, are underway to elucidate the structural alterations induced by these mutations.

4.6. THYROXINE BINDING TO NATURAL AND RECOMBINANT TTR VARIANTS

ABSTRACT

T_4 binding is the best characterized TTR function, concerning structural aspects. Thus, to assess structural changes of TTR we studied T_4 binding to amyloidogenic and non-amyloidogenic TTR variants in whole serum or isolated from serum. We also compared the heterozygotic form of some of these variants with the corresponding homozygotic condition. The TTR variant studied in which an amino acid substitution occurs in the binding site of T_4 (TTR Thr 109) presented an increased T_4 binding affinity. The amyloidogenic variants TTR Met 111 and TTR Pro 55 presented low T_4 binding affinities. We also found amyloidogenic and non-amyloidogenic TTR variants with normal binding affinity. Thus, we could not correlate the amyloidogenicity of the variant with the respective T_4 binding affinities. We speculate about the structural alterations affecting T_4 binding in some of these variants.

INTRODUCTION

Transthyretin is a tetramer of identical subunits with a predominant ß-structure characteristic of the amyloidogenic proteins. The eight ß-chains of each monomer are organized in two four stranded parallel ß-sheets. The four monomers are organized in the tetramer forming a central channel running through the molecule where the two thyroxine (T_4) binding sites are located (Blake *et al.*, 1978). Within each binding site there is an organizaton of the elements that interact with T_4 that define the orientation of T_4 in the binding site. Both binding sites are identical. Thus, the different binding affinities for each binding site can only be justified by the negative cooperativity of T_4 binding to TTR (Blake *et al.*, 1977).

Several TTR variants due to single amino acid substitutions have been described. In most cases these variants are associated with different forms of amyloidosis but there are also non-amyloidogenic TTR variants. Some TTR variants have already been studied concerning T_4 binding and it was found that they can increase, decrease or have no effect on T_4 binding. Most of the amyloidogenic TTR variants tested presented decreased T_4 binding namely heterozygotic or homozygotic TTR Met 30, heterozygotic TTR Ser 84 and

TTR Tyr 77 and homozygotic TTR Ile 122. T_4 binding was not altered in heterozygotic TTR Ala 60 and TTR Ile 122 (Rosen *et al.,* 1993). On the contrary, heterozygotic TTR Thr 109 has an increased T_4 binding affinity (Moses *et al.,* 1990; Rosen *et al.,* 1993). TTR Met 119 referred to in the previous work (section 4.5) presented also an increase of T_4 binding affinity (Scrimshaw *et al.,* 1992; Curtis *et al.,* 1994). These latter two substitutions occur in the T_4 binding channel being predictable that they could interfere with T_4 binding. However, the mechanism whereby single amino acid substitutions may affect the binding affinity for T_4 is not completely understood.

In this work we add to the number of TTR variants studied under the point of view of T_4 binding; we investigated the binding properties of amyloidogenic variants TTR- Ala 49, -Ala 71, -Gln 89, -Met 111 and Pro 55 and also a non-amyloidogenic variant, TTR Arg 102. Some variants already reported by other groups (Rosen *et al.,* 1993) concerning T_4 binding were also included for comparison purposes namely TTR Thr 109, TTR Tyr 77 and TTR Ile 122.

Since in most cases the variant proteins are from heterozygotic individuals, recombinant proteins have been used to analyse T_4 binding to the homotetrameric form of some variants namely recombinant TTR Ile 33, -Ser 84, -Pro 55, -Met 111 and TTR Ile 122.

MATERIAL AND METHODS

Samples

T_4 binding to TTR was assayed in whole serum and/or isolated TTR from carriers of TTR variants and in TTR variants synthetically produced in an *E. coli* expression system.

The whole serum samples assayed were from heterozygous carriers of TTR Ala 49, TTR Pro 55, TTR Ile 68, TTR Ala 71, TTR Gln 89, TTR Arg 102, TTR Thr 109 and TTR Met 111.

TTR was isolated from the sera of heterozygous carriers of TTR Ala 49, TTR Ile 64, TTR Ile 68, TTR Tyr 77, TTR Pro 55, TTR Thr 109.

Homozygous recombinant proteins TTR Ile 33, TTR Ser 84, TTR Ile 122 and TTR Met 111 were synthetically expressed in an *E.coli* expression system that has been developed by

Furuya *et al.* (1991) and TTR Pro 55 was produced in a similar system develloped by McCutchen *et al.* (1993).

Methods:

TTR isolation, quantification and T_4 binding assays were performed using methods described in the previous section (section 4.5).

Isolation of the tetramer of recombinant TTR was performed by HPLC gel filtration using a Ultraspherogel column as described in section 4.5.

RESULTS

1.T_4 binding in whole serum:

We first studied the T_4 binding to TTR in whole serum samples from carriers of some of the TTR variants characterized and presented in the in previous sections (section 4.1,4.2 and 4.3) together with other variants available in the laboratory. The results of the assays performed in whole serum are shown in Figure 1. We present the percentage of T_4 bound (relative to the T_4 added) in the presence of increasing concentrations of T_4 as competitor using whole serum from carriers and the Scatchard representation of the results. To facilitate the visual interpretation of the data we have grouped the samples in different plots. On the top pannels of Figure 1 we present the results obtained for sera from TTR Ala 49, Ala 71 and Gln 89 in comparison with normal sera. On the middle pannels, T_4 binding to sera from TTR Arg 102, Met 111 carriers and normal is represented. Finally on the bottom pannel, we present the results from sera of TTR Pro 55, Ile 68 and Thr 109 carriers and normal serum. Taken together we can group the results into three categories: (I) binding that did not differ from the normal control and in this group we include TTR Ala 49, Ala 71 and Arg 102; (ii) binding with increased affinity: TTR Thr 109 (2 fold increase); (iii) binding with decreased affinity (0.6-0.7) present in the sera of a carrier of TTR Ile 68, Gln 89, TTR Met 111 and TTR Pro 55.

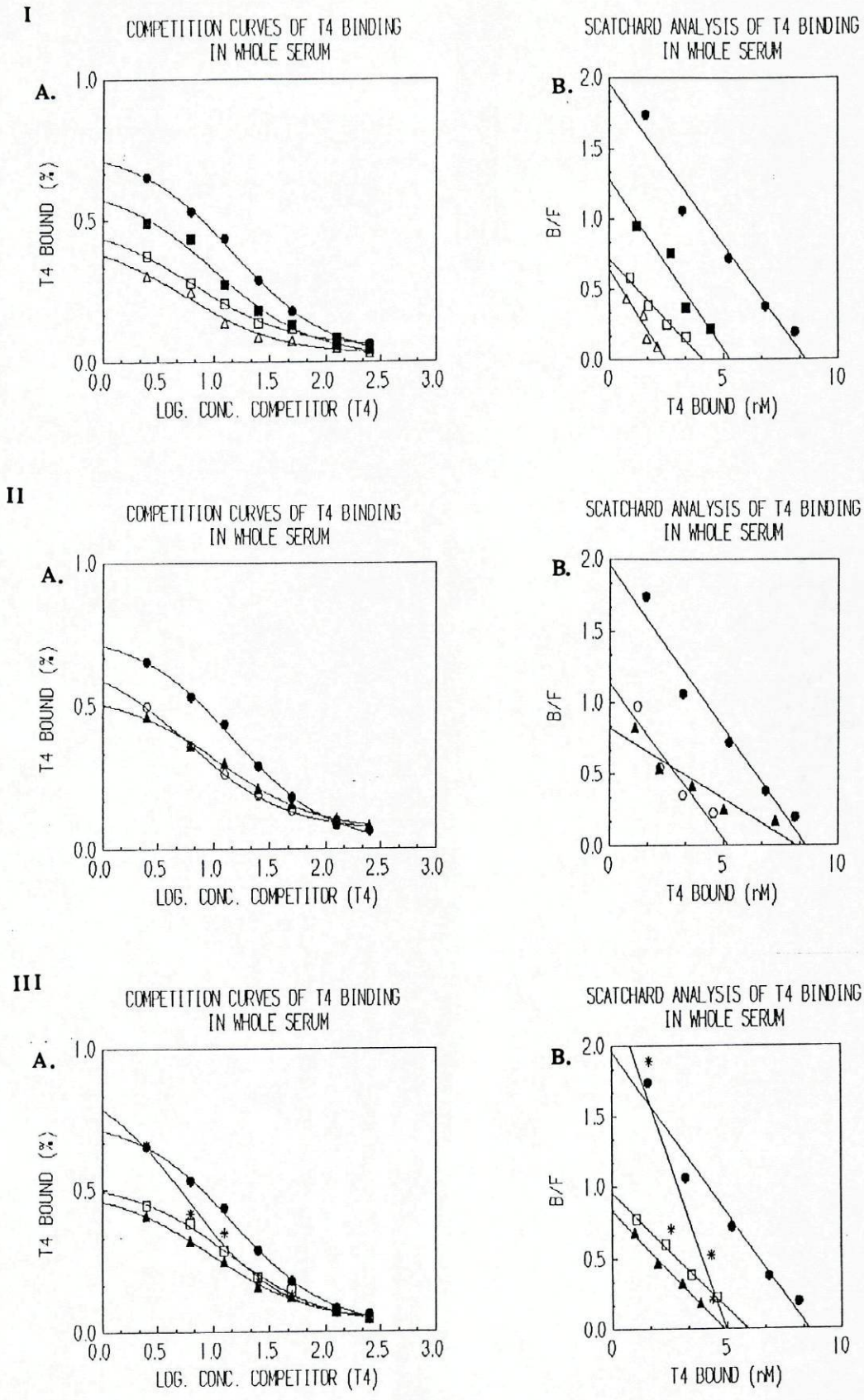


Fig. 1 - T_4 binding in whole serum from heterozygotic carriers of TTR variants. **A.** Competition curves and **B.** Scatchard analysis. The symbols are: ● Normal serum (all panels). **Panel I** - ■ TTR Ala 49, □ TTR Gln 89, △ TTR Ala 71; **Panel II** - ▲ TTR Met 111, ○ TTR Arg 102; **Panel III** - ✱ TTR Thr 109, ◻ TTR Pro 55, ▲ TTR Ile 68.

2. T_4 binding by isolated native TTR:

When we had enough available serum from the carriers of TTR variants (25-30 ml) we purified TTR from serum and assayed T_4 binding to the isolated protein. In Figure 2 we compare some of those isolated proteins, in the same manner as described above. Thus on the first pannel of Figure 2 we represent T_4 binding to isolated TTR from TTR Ala 49 and TTR Tyr 77 carriers and from a normal individual. In the middle pannel we compare TTR Leu 68, Arg 102 and Ile 64 with normal TTR. In the bottom pannel we present T_4 binding to TTR isolated from a carrier of TTR Pro 55 and from a normal individual. Taken together we can group the variants into five categories: (I) not differing from normal binding, and we included in this group TTR Tyr 77 and Leu 68; (ii) slightly above the normal (1.3 fold), found for TTR Ala 49 and Arg 102; (iii) higher than normal (3 fold) Thr 109; (iv) lower than normal (0.5), as found for Pro 55 and finally (v) very low, such as the case of TTR Ile 64.

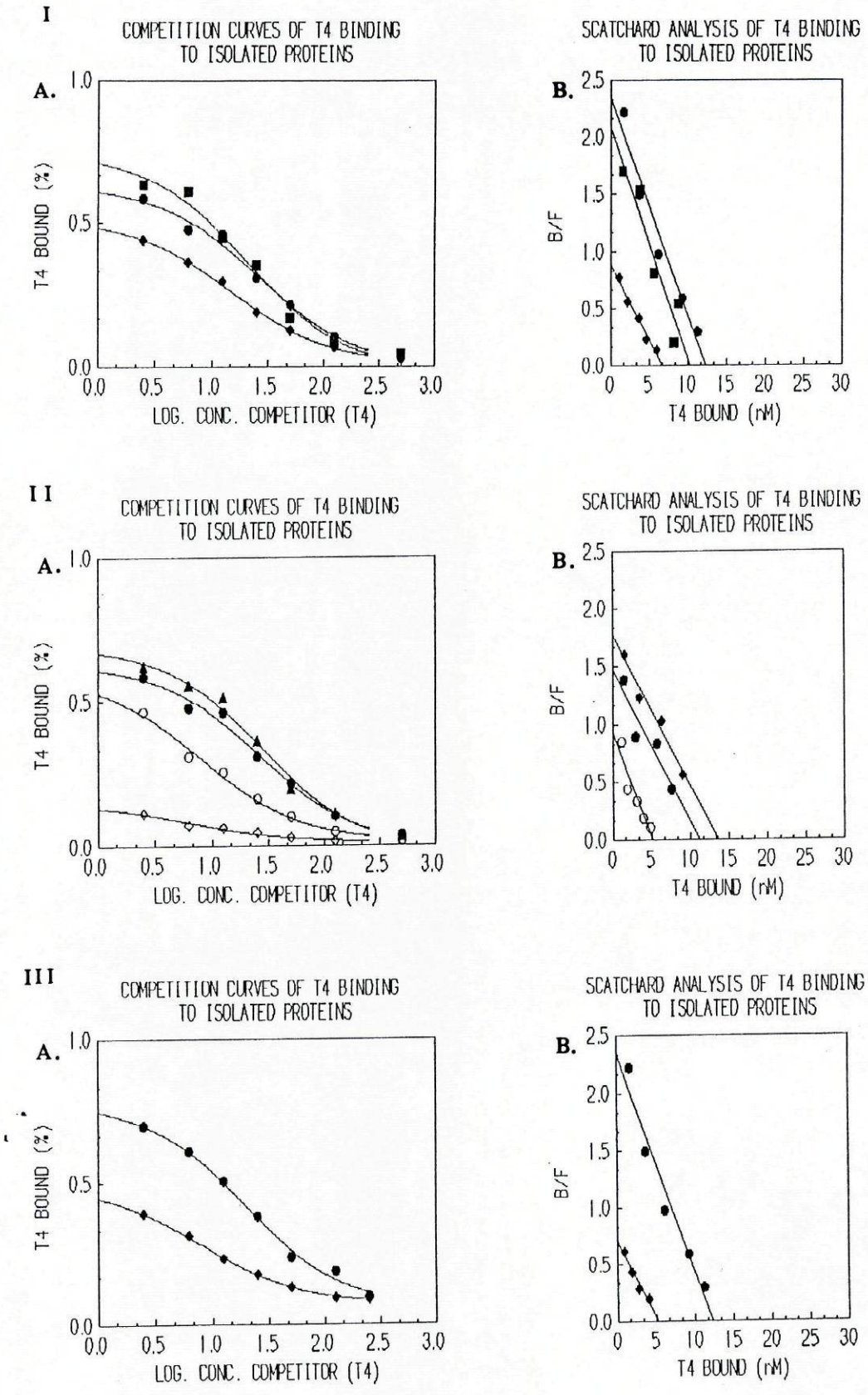


Fig. 2 - T_4 binding to isolated native TTR. **A.** Competition curves and **B.** Scatchard analysis. The symbols are: ● Normal serum (all panels). **Panel I** -■TTR Ala 49,◆TTR Tyr 77; **Panel II** - ▲ TTR Ile 68, Ö TTR Arg 102, ◊TTR Ile 64; **Panel III** - ◆ TTR Pro 55.

3. T_4 binding to recombinant TTR variants:

We studied T_4 binding to recombinant TTR variants. As in the previous cases, we grouped the results obtained in different pannels on Figure 3. Thus, on the top pannels, we compare recombinant TTR Ile 33, Ser 84 and Ile 122 with normal recombinant TTR. On the bottom pannels we present T_4 binding to recombinant TTR Pro 55, TTR Met 111 and normal recombinant TTR. All the recombinant variants tested presented lower affinity for T_4 binding than normal recombinant TTR. Thus, (i) Ile 122 presented an affinity of about 0.6 the affinity of the normal recombinant; (ii) Ser 84 has a lower affinity than Ile 122; (iii) Met 111 presented a very low T_4 binding affinity (0.2 the normal affinity); (iv) Ile 33 and Pro 55 presented virtually no T_4 binding.

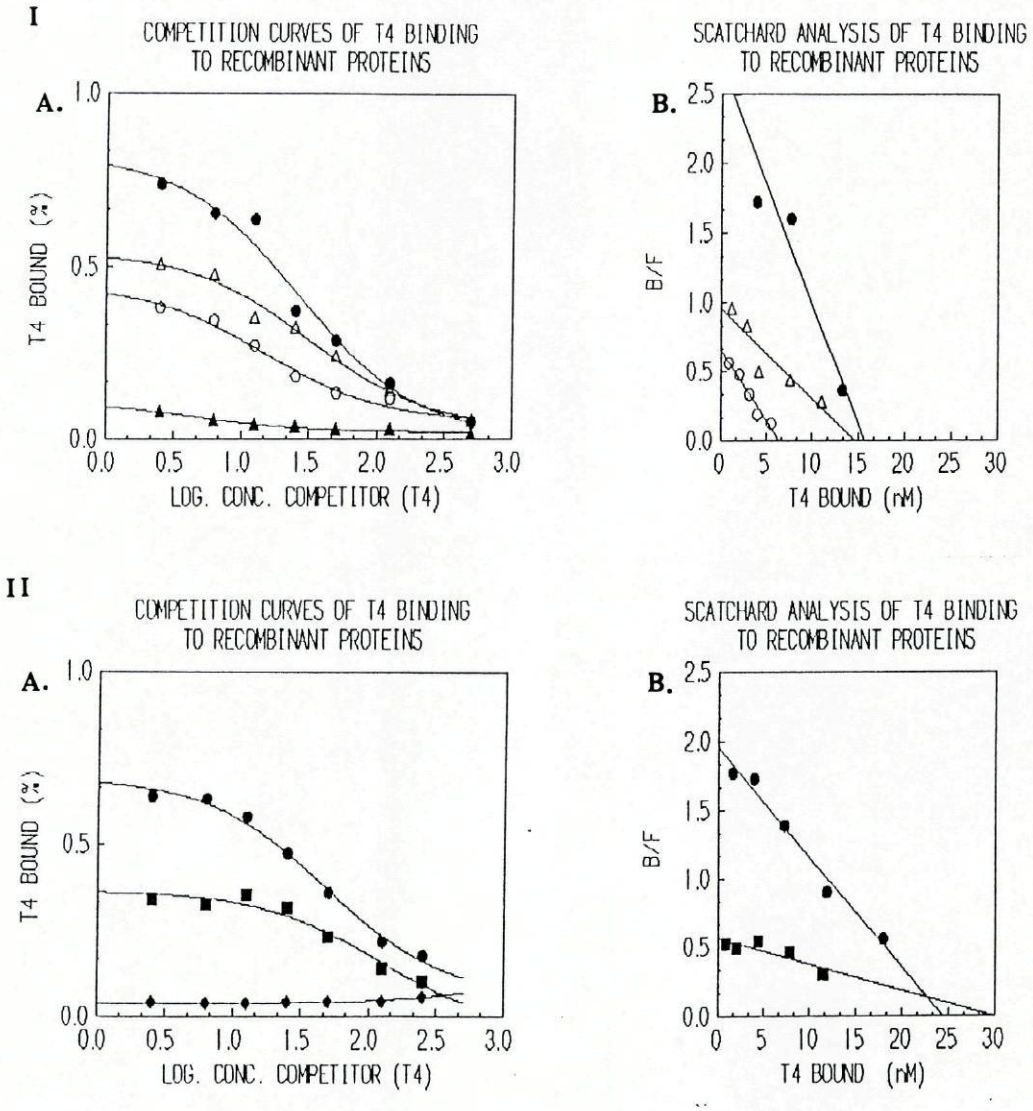


Fig. 3 - T_4 binding to recombinant TTR variants. **A.** Competition curves and **B.** Scatchard analysis. The symbols are: ● Normal serum (all panels). **Panel I** -ΔTTR Ser 84, ○TTR Ile 122, ▲TTR Ile 33; **Panel II** - ■ TTR Met 111, ♦ TTR Pro 55.

In table I we summarized the results obtained for the different assays presenting only relative binding affinities.

TABLE I - Relative T_4 binding affinity constants

Relative affinity	2.5	1.4	N	0.6	0.4	0.2	null
Whole serum	Thr 109		Ala 49 Ala 71 Arg 102	Pro 55 Leu 68 Gln 89 Met 111			
Native protein isolated		Ala 49 Arg 102	Tyr 77 Leu 68		Pro 55		
Recombinant protein				Ile 122	Ser 84	Met 111	Ile 33 Pro 55

From the results in Table I we can conclude that as in a previous section (section 4.5.), the relative values of the binding affinity constants are similar when we compare assays in whole serum with assays with the isolated proteins although we can see a slightly increase of binding affinity of TTR Ala 49 and TTR Arg 102 when isolated in comparison with the relative values of the assays in whole serum. On the other hand, isolated TTR Pro 55 shows a lower relative binding affinity constant when compared with in whole serum.

DISCUSSION

T_4 binds to TTR in a central channel where two binding sites are located. The structure of the binding site is well known and defined hydrophobic and hydrophilic regions in each site match the structure of T_4. Substitutions of amino acid residues lying in these binding sites may induce alterations of T_4 binding as is the case of TTR Thr 109 and TTR Met 119. In both cases there is an increase in the T_4 binding affinity constant. However, TTR Glu 54, a variant with an amino acid substitution located at the entrance of the binding channel does not alter the affinity for T_4 (Curtis *et al.,* 1994). On the other hand, amino acid substitutions in other regions of the molecule, and not directly involved in the binding channel, may originate differences in the T_4 binding affinity as happens, for instance, with

TTR Met 30 and TTR Ile 84 (Refetoff *et al.*, 1986). To characterize more TTR variants we assessed T_4 binding to TTR in whole serum and/or TTR purified from serum of individuals carriers of different TTR mutations. From the variants analysed only TTR Thr 109 presented an increased T_4 binding affinity of 2-3 times the value for normal TTR, as has been previously reported (Moses *et al.*, 1990; Rosen *et al.*, 1993). This variant has been extensively studied and the structural alterations induced by the substitution of a threonine for alanine have been determined by X-ray analysis of the recombinant mutant protein (Steinrauf *et al.*, 1993). This substitution at position 109 originates a slight widening of the binding site at the carbonyl oxygen of Thr 109 enhancing the formation of the T_4-TTR complex in TTR Thr 109. Thus we included this variant in our studies as an extra control sample.

TTR Arg 102 and TTR Ala 49, when isolated from serum, presented a slightly higher affinity binding constant as compared to isolated normal TTR. However, this difference does not seem significant, but we do not have enough number of determinations to compare them statistically. Also, TTR Leu 68, that presented a slightly lower T_4 binding affinity when studied in whole serum, was considered normal when isolated from serum. However, the T_4 binding affinity of TTR Pro 55 was consistently lower in both whole serum and isolated protein; if tested (no serum was available to test), Ile 64 and Tyr 77 should also present a lower affinity of binding than normal TTR. We have previously mentioned that the relative binding affinities of the TTR variants in whole serum were similar to the ones obtained for the isolated protein, but we did not exclude the possibility that other factors or conditions in serum may interfere differently with each variant. Furthermore, we used only one serum sample from each individual carrier of a TTR variant thus, the variability between each individual must be considered. These facts do not invalidate the results presented here since our main interest was to compare the variants and to proceed with other binding and structural studies in case we found differences.

We analysed T_4 binding to different natural TTR variants including amyloidogenic and non - amyloidogenic and we could not relate the amyloidogenicity with the respective T_4 binding. This can be due to the fact that we have been studying heterozygous forms of the protein, which contain also the normal monomer, and which are probably more stable than

the equivalent homotetramers, where a more pronounced effect of the mutation was observed. According to this hypothesis, the natural homozygotic TTR variants known presented lower T_4 binding (Ile 122 - Rosen *et al.,* 1993) or even no affinity for T_4 binding (TTR Met 30 - Rosen *et al.,* 1993) than the correspondent heterozygotic variants. This is also true for the amyloidogenic recombinant variants studied here TTR Pro 55 and TTR Met 111.

Considering the other recombinant TTR variants studied, TTR Ile 122 and TTR Ile 64 presented low T_4 binding affinity. Though no other report of T_4 binding affinity for recombinant TTR Ile 122 is available, Rosen *et al.* (1993) reported a similar result for TTR Ile 122 in homozygotic carriers of this mutation. However TTR Ile 122 from heterozygotic carriers presented an affinity similar to normal TTR. Noteworthy is the specially low affinity for T_4 binding found for mutations Ile 33, Pro 55 and Met 111. Recombinant TTR Met 111 showed low T_4 binding affinity that may be due to an alteration of the binding cooperativity similarly to that found for Asn 90 mutation, referred to in the previous section (section 4.5.). A lower T_4 binding affinity to TTR was also observed in whole serum from a Met 111 heterozygotic carrier, although the effect was not so evident.

The reason why the Met 111 mutation and other mutations such as Ile 33 and, in particular, Pro 55 bind T_4 poorly can only be answered by X-ray studies. In a way, TTR Pro 55 presents a behaviour similar to that of TTR Met 30 since in the heterozygous form it binds less T_4 than normal TTR and in the homozygous form it does not present specific binding of T_4. Like the Met 30 mutation, Pro 55 does not interact directly with T_4, and both are not localized in the T_4 binding channel. Thus, the alteration of T_4 binding must be due to tertiary and/or quaternary structure modifications. X-ray studies of homozygous TTR Met 30 revealed that the substitution of a bulkier methionine residue for valine at position 30 induces a shift of sheet DAGH into the central cavity and a movement of sheet CBEF out in the solvent. Movement in the DAGH result in changes of the shape of the central cavity resulting in a poorer fitting of the T_4 molecule. Therefore, contrary to Thr 109 that increases T_4 binding due to a direct interaction with T_4, TTR Met 30, not located in the channel, induces conformational changes in the channel. Furthermore, although the Met 30 substitution occurs in strand B, from the outer sheet, it induces structural changes that affect residues which belong to ß-strands A and D of the inner sheet. The region composed by C and D strands

has been suggested to be a hot spot region for amyloidogenic mutations and it is believed that this is due to changes in the conformation of the TTR molecule (Serpell & Blake, 1994). Concerning TTR Pro 55, its tridimensional structure is not yet known but it is predictable that a substitution of a proline for a leucine residue should have a pronounced effect in the structure of the protein since it is well known that proline residues are ß-strand breakers. Besides, TTR Pro 55 occurs in ß-strand D and, according to Kelly and Landsbury (1994), it could originate a rearrangement of this region in an only loop including strands C and D. How these changes affect thyroxine binding it is still to clarify but it has been suggested that this mutation may alter the quaternary structure of TTR and consequently it would induce an altered T_4 binding.

To assess possible alterations of the TTR Pro 55 quaternary structure McCutchen *et al.* (1993) studied the stability of this variant in acidic conditions. They suggested that an altered quaternary structure would lead to instability of the tetramer, in specific conditions, favouring amyloidogenesis through a modified intermediate (monomer or dimer). Since TTR Pro 55 is associated with a particularly aggressive form of amyloidosis (Jacobson *et al.*, 1992b; Yamamoto *et al.*, 1994) they related the degree of amyloidogenecity with the acid stability of the TTR tetramer and suggested that all the amyloidogenic TTR variants should have an altered tetrameric stability.

To further study this question more recombinant variants, in particular non-amyloidogenic variants, should be analysed concerning T_4 binding, acid stability of the tetramer and, finally, the structure should be defined by X- ray crystallography of the variants.

5. CONCLUSÕES, IMPACTO E PERSPECTIVAS

5. Conclusões, impacto e perspectivas

Com a identificação de novas variantes de TTR, incluindo variantes amiloidogénicas (neuropáticas ou cardiomiopáticas) e variantes não amiloidogénicas contribuimos para o aumento do número das variantes de TTR conhecidas e dos síndromes clínicos a elas associados. Esta crescente heterogeneidade clínica e molecular da TTR leva-nos a afirmar a necessidade cada vez maior de um diagnóstico cuidado e preciso da mutação associada a cada patologia numa determinada família. Por isso, neste trabalho, relevamos a importância da implementação de métodos de diagnóstico simples para estudos familiares. Os métodos utilizados foram análise de RFLPs e hibridização com sondas específicas de alelo (ASOs), para mutações já caracterizadas, e sequenciação de DNA quando não há qualquer indicação da mutação envolvida. Recentemente, o rastreio de mutações por análise de SSCPs aplicada ao gene da TTR (Torres *et al., in press*) possibilita a detecção de uma elevada percentagem de mutações facilitando o posterior diagnóstico específico da mutação.

Para a caracterização funcional e estrutural das variantes de TTR realizamos estudos de ligação de T_4. Apesar das variantes amiloidogénicas estudadas não se localizarem no canal de ligação de T_4, a maior parte delas apresenta menor afinidade para a T_4. As diferenças de afinidade de ligação de T_4 a estas variantes de TTR demonstram a existência de alterações conformacionais. O canal de ligação de T_4 tem uma estrutura onde existem pequenas concavidades que se destinam a alojar os átomos de iodo presentes na molécula de T_4 permitindo uma interacção forte entre o ligando e a proteína. Alterações conformacionais nestas regiões, ainda que pequenas, poderão modificar a interacção dos átomos de iodo com a proteína alterando a afinidade desta para a T_4.

As variantes não amiloidogénicas localizadas no canal de ligação de T_4 podem alterar (aumentar ou diminuir) ou não afinidade para a T_4. Estes resultados levam-nos a concluir que a substituição de um aminoácido na molécula de TTR é suficiente para alterar a interacção da TTR com a T_4 quer isso se deva a alterações da estrutura terciária e/ou quaternária.

Os estudos de ligação efectuados, possibilitam uma análise mais fácil de um grande número de variantes de TTR. Entre estas incluem-se as proteínas de indivíduos heterozigotos e heterozigotos compostos, tornando possível a avaliação comparativa do efeito global da ocorrência de um monómero normal e um mutado ou de dois monómeros mutados. Por exemplo, a análise de TTR dos heterozigotos compostos demonstrou um efeito de aumento da afinidade para a T_4 devido à presença do monómero mutante TTR Met 119, independentemente do outro monómero presente.

Embora os estudos de ligação que realizamos se limitassem a estudos de interacções das variantes de TTR com a T_4, parece-nos importante prosseguir com estes estudos utilizando outros ligandos, nomeadamente compostos polihalogenados bifenílicos (PHBs). Apesar de conhecidos os efeitos tóxicos destes compostos, existem ainda dúvidas em relação ao mecanismo que induz essa toxicidade. A analogia estrutural dos PHBs com a T_4 permite que estes se liguem à TTR no canal de ligação da T_4. Pensa-se que a interação dos PHBs com a TTR influencia a ligação da TTR ao RBP o que justificaria a diminuição dos níveis de vitamina A verificados em ratinhos expostos a PHBs (Brouwer & van den Berg, 1986). Assim, o estudo das interacções destes compostos com as diferentes variantes de TTR poderá, por um lado, demonstrar uma maior ou menor toxicidade destes compostos para os indivíduos portadores dessas mutações e, por outro lado, reflectir algum efeito protector da mutação em relação à proteína normal que justifique a sua selecção.

Outro dos pontos não explorados neste trabalho e que será alvo de estudos futuros é a interacção destas proteínas mutantes com a proteína de ligação ao retinol (RBP). O mecanismo de entrada do retinol nas células não está perfeitamente esclarecido embora se saiba ser mediado por receptores de RBP na membrana celular das células alvo (Sivaprasadarao & Findlay, 1988). Alguns dos resíduos de aminoácido envolvidos na interacção com o receptor parecem estar também envolvidos na interacção do RBP com a TTR, parecendo improvável que o receptor de RBP interactue com o complexo TTR-RBP. A ligação do RBP à TTR é mais forte do que ao receptor e deste modo é possível que a TTR condicione a entrada de retinol nas células. Alterações de afinidade do RBP à TTR podem afectar a disponibilidade do retinol para as células (Sivaprasadarao & Findlay, 1994).

Outras vertentes de investigação, em perspectiva, são a interacção destas variantes com receptores de TTR e péptido ß. Estudos recentes em oócitos de galinha mostraram evidências para a endocitose de TTR mediada por receptores (Vieira *et al.*, 1995). A ligação de variantes de TTR a esses receptores poderá contribuir para a elucidação de aspectos estruturais dessa interacção. Estes estudos podem também reflectir funções de TTR não conhecidas já que se sabe que a TTR não é essencial para a entrada de T_4 nas células (Palha *et al.*, 1994) o mesmo podendo ser verdade em relação ao retinol (Wei *et al.*, 1995). O estudo detalhado da interacção da TTR com o péptido ß contribuirá para a definição das regiões de cada uma das moléculas do complexo envolvidas na interacção. Estes estudos poderão sugerir uma solução terapeutica na doença de Alzheimer.

A localização dispersa das várias substituições de aminoácidos na molécula de TTR não nos permite determinar regiões específicas para a localização das variantes amiloidogénicas ou não amiloidogénicas. Contudo, neste trabalho verificamos que as diferentes substituições de aminoácido introduzem diferentes alterações estruturais e, consequentemente, funcionais na molécula de TTR , como foi demonstrado pelos estudos de ligação de tiroxina. Salientamos assim a importância de estudos futuros a outros ligandos para a interpretação da estrutura tridimensional das variantes. Sendo de aceitação geral que a amiloidogénese é um processo multifactorial, as diferentes alterações conformacionais das variantes podem afectar preferencialmente um ou outro dos processos envolvidos na amiloidogénese. Assim, a formação de amilóide pode ser devida a exposição de regiões da molécula envolvidas em interacções com outras moléculas como por exemplo proteases, componentes da matriz extracelular (GAGs, Pgs, Apo E) e/ou chaperones moleculares. Por outro lado, estas modificações podem alterar a estabilidade da molécula em condições específicas ou interferir em processos de desnaturação ou outros envolvidos na formação de fibras. A interferência específica com um ou outro destes aspectos em particular, poderá ser responsável pela diversidade de expressão clínica associada a estas variantes.

Finalmente, gostariamos de salientar que este trabalho e as possíveis linhas de investigação futura que deixamos em aberto demonstram a importância das variantes

moleculares naturais e sinteticamente produzidas para estudos de estrutura e função das proteínas e de patologia molecular.

6. BIBLIOGRAFIA

BIBLIOGRAFIA

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