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Instituto de Ciências Biomédicas de Abel Salazar
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Santos SD, Saraiva MJ. (2004). Enlarged ventricles, astrogliosis and neurodegeneration in heat shock factor 1 null mouse brain. Neuroscience. 126: 657-663.

Santos SD, Saraiva MJ. HSF1-KO mice expressing human mutant transthyretin V30M: a novel model for familial amyloidotic polyneuropathy affecting the peripheral nervous system. *In revision, Neuron.*

Santos SD; Cardoso I; Saraiva MJ. Ubiquitination in familial amyloidotic polyneuropathy. *In preparation.*

Santos SD; Gottesman M; Saraiva MJ. Functional roles of *Escherichia coli* TTR-like protein. *In preparation.*

Santos SD; Olsson A; Saraiva MJ. Investigation of stress as an environmental modulator on FAP progression in a transgenic mice model. *In preparation.*

Ao Rui,
Cujo enorme carinho esteve sempre comigo
durante todos estes anos de pesquisas
tornando esta tese possível!

... Tempo de chegar, de acontecer,
Sem eu nem tu em seu voraz fluir;
Tempo de mistério e de por-vir,
Tempo de criar, de poder ser.

Tempo de fluxos e símbolos vitais,
Labirinto carnal de conjunções,
Além do bem, do mal, das disjunções;
Tempo do tudo e do mais.

Tempo virtual, miscigenado
De origens e fins confluentes,
Tempo imenso e intenso de passagem,
Cintilações e mitos sem miragem.

Tempo que me toma e eu abraço
Sem ressentimento nem cansaço,
Traçando meus mapas, meus percursos,
Meus impasses e minhas aberturas,
Meu devires e minhas clausuras.

Fluir e traçar é todo o meu destino,
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Traçando meus mapas do incerto.

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Summary

Transthyretin (TTR) is one of many proteins described to be the causing agent of amyloid diseases. This protein is synthesized mainly by the liver and secreted to the blood where it circulates as a soluble homotetramer, being responsible for the transport of thyroxine and retinol bound to retinol binding protein. Mutations in TTR induce conformational changes that make the protein prone to aggregate forming amyloid, deposited on several tissues. Familial amyloidotic polyneuropathy (FAP) results from variant TTR deposition, which occurs systemically, though it is the peripheral nervous system that is prominently affected. Never was aggregated or fibrillar TTR detected intracellularly nor on the tissue of synthesis. The liver is then always spared from TTR amyloid. The reasons why at a particular time TTR leaves its circulating soluble conformation and aggregates in tissues is not completely understood. Protein conformation destabilization is accepted as the primary reason but other set of conditions are needed to explain the variability in clinical symptoms heterogeneity. These modulator factors may be related to genetic, environmental influence or tissue/circulating specific entities or even stochastic factors.

TTR deposition leads to compromised organ structure and function that is fatal. Peripheral nerves affected with TTR aggregates and amyloid show signs of oxidative stress, upregulation of inflammatory mediators and neurodegeneration through apoptosis. Intrinsic activated survival responses are not known and strategies to halt cellular death have not been established yet.

We proposed to study stress as an environmental factor influencing TTR pathogenesis. Transgenic mice expressing TTR Val30Met variant were submitted to a schedule of unpredictable chronic mild stress over one year, after which TTR deposition and amyloid formation were investigated. Except for a trend in anticipation of amyloid formation in stressed mice, no significant differences concerning aggressiveness of pathological signs were detected. One cannot exclude a stress influence on human patients and further studies should address this issue.

Next, our studies tried to establish a better transgenic mouse model for FAP. Previous characterized transgenic mice models do not recapitulate the whole

human FAP features since the peripheral nervous system is not affected, possibly related to interspecies differences or some protective pathway that is activated in the mice delaying the disease progression and sparing the nervous tissue. We crossed the available mice expressing human TTR variant Val30Met with mice knock-out for the heat shock transcription factor 1 (HSF1). This transcription factor is responsible for activation of heat shock proteins which in turn can function as molecular chaperones, anti-apoptotic molecules and other important functions to maintain cell homeostasis. The resulting mice were a success in the sense that TTR deposition was increased and extended to peripheral nerves, dorsal root and autonomic ganglia. This model opens new possibilities on FAP research, allowing for studies aiming to understand the biology of the disease, the investigation of appropriate drugs and to explore the heat shock response as an activated pathway in FAP. In fact, human individuals affected with TTR deposition presented an upregulation of hsps in neural and extra-neural tissues.

Another aim of this experimental project was the assessment of ubiquitination in FAP. Other neurodegenerative disorders were found to have a disregulation of the ubiquitin/proteasome system. Ubiquitin marks unfolded or damaged proteins to the proteasome where they are degraded which occurs during normal physiology, but when altered proteins exceed the ubiquitin capacity to eliminate them, they accumulate with noxious results to the cell, reflected as well in proteasome activity. This situation is easily understood when aggregated proteins accumulate intracellularly. However in TTR amyloidosis, aggregation is observed extracellularly. We investigated ubiquitin expression initially in the liver. We found no differences between normal and FAP liver despite the fact that a mutant with an altered structure is being synthesized, which is in accordance with the lack of intracellular aggregated TTR. Concerning tissues with TTR deposition, ubiquitin was found to be upregulated. Therefore this response can be elicited, indirectly, by TTR deposition, which might be a sign of the cell entering a death phase or an attempt of the cell to rescue itself from the negative effects caused by TTR deposition. A cellular model expressing TTR wild type and different variants showed a different pattern of ubiquitin adducts only when the expressed TTR was found aggregated in the medium. Again, ubiquitination appears to be indirectly influenced by extracellular misfolded TTR. The involvement of the receptor RAGE or other, is

probably important in the mechanism. Intracellularly, an increase in damaged proteins and alterations in signaling pathways occur that is reflected by an ubiquitin increase. This pathway seems to be altered in the early stages of FAP and maintained throughout the disease progression.

TTR is regarded as an amyloidogenic protein, even when no mutation is present. It is as well, a highly conserved protein throughout evolution. Homologous sequences to TTR were found in prokaryotes, named TTR-like, which could be related to evolutionary divergence. We proposed to study TTR-like from *E. coli* in terms of function, cellular localization and amyloidogenesis, in an attempt to study structural determinants related to aggregation. A knock-out mutant was generated and studied. Its growth behaviour dynamics were different in comparison to the parental strain. The lack of TTR-like resulted in compromised aerobic growth in conditions of low supply of nutrients and when nitrogen was present as an energy source. Moreover, enzymes which catalyze the degradation of pyrimidine nucleosides and their utilization as carbon and energy sources were found to be downregulated when TTR-like is absent, reinforcing its function during metabolic pathways.

E. coli TTR-like protein is a tetramer of identical subunits of approximately 14 kDa, secreted to the periplasmic space and has similar behaviour to eukaryotic TTR under conditions that normally dissociate the TTR tetramer to a monomer, albeit with a different behaviour as no dimer is observed. TTR-like at acidic conditions forms amyloid fibrils and can accelerate the rate of human TTR fibril formation by a seeding effect, which is not observed in human TTR fibrillogenesis. This seeding effect can possibly be useful in inducible models of the disease. Evolutionary TTR-like might be an ancestor of TTR whose functions have changed, though some biochemical characteristics were maintained, namely its amyloidogenic potential.

Overall, the project has drawn attention and enlightened the influence of the stress response in TTR pathogenesis.

Sumário

A transtirretina (TTR) é uma de muitas proteínas descritas como sendo a causa de doenças associadas a amilóide. Esta proteína sintetizada principalmente pelo fígado é secretada para o sangue onde circula como um homotetramero solúvel, sendo responsável pelo transporte da tiroxina e do "retinol binding protein" associado ao retinol (vitamina A). Mutações na TTR induzem mudanças de conformação que tornam a proteína susceptível de agregar originando amilóide, depositada em vários tecidos. A polineuropatia amiloidótica familiar (PAF) resulta da deposição de variantes de TTR que ocorre sistemicamente, embora o sistema nervoso periférico seja predominantemente afectado. A TTR sob a forma de agregados ou fibrilar nunca foi detectada intracelularmente, nem no tecido de síntese. O fígado é portanto, sempre poupado da agregação de TTR. As razões pelas quais numa dada altura a TTR deixa a sua conformação solúvel e agrega nos tecidos não são completamente compreendidas. A destabilização da conformação da proteína é aceite como razão principal, mas outras condições e circunstâncias são necessárias para explicar a variabilidade dos sintomas clínicos. Estes factores moduladores podem estar relacionados com uma influência genética ou ambiencial ou ainda factores tecidulares ou em circulação, ou até mesmo factores estocásticos.

A deposição de TTR compromete a estrutura e a função dos órgãos sendo fatal. Os nervos periféricos afectados com agregados de TTR e amilóide apresentam sinais de stress oxidativo, aumento de mediadores inflamatórios e neurodegeneração associada a apoptose. As respostas intrínsecas de sobrevivência activadas não são conhecidas e as estratégias para impedir a morte celular não foram também ainda encontradas.

Propusemos estudar o stress como um factor ambiencial que influencía o patogénese de TTR. Ratinhos transgénicos que expressam a variante de TTR Val30Met foram submetidos a um esquema de stress (unpredictable chronic mild stress) durante um ano, seguido de análises de deposição de TTR e formação de amilóide. Excepto uma tendência para a anticipação de formação de amilóide em ratinhos sujeitos a stress; nenhuma diferença significativa foi detectada

relativamente a agressividade de sinais patológicos. Não se deve excluir uma maior influência de stress em humanos FAP e mais experiências deverão ser dirigida neste sentido. Os estudos seguintes ambicionavam estabelecer um melhor modelo de ratinhos transgénicos para PAF. Os modelos transgénicos anteriormente caracterizados não recapitulavam todas as características humanas em PAF já que o sistema nervoso periférico não é afectado; relacionado possivelmente com diferenças de espécies ou a alguma via protectora activada nos ratinhos, retardando a progressão da doença e não afectando o tecido nervoso. Foram cruzados ratinhos transgénicos que expressam a variante humana TTR Val30Met com ratinhos knock-out para o factor de transcrição "heat shock factor 1" (HSF1). Este factor de transcrição é responsável pela activação das proteínas "heat shock" (hsps), que por sua vez funcionam como "chaperones", moléculas anti-apoptóticas e outras funções importantes para manter a homeostase da célula. Os ratinhos resultantes foram um sucesso já que a deposição de TTR foi aumentada e estendida aos nervos periféricos, DRGs e gânglios autonómicos. Este modelo abre novas perspectivas na pesquisa da PAF, permitindo estudos da biologia da doença, investigação de drogas apropriadas e exploração da resposta "heat shock" como um caminho activado em PAF. De facto, doentes humanos afectados com deposição de TTR apresentam um aumento de hsps em tecidos nervosos e outro tipo de tecidos.

Um outro objectivo deste projecto experimental foi o estudo do papel da ubiquitinação em PAF. Outras doenças neurodegenerativas apresentam uma desregulação do sistema ubiquitina/proteossoma. A ubiquitina marca proteínas danificadas ou com uma conformação alterada dirigindo-as para o proteossoma onde são degradadas. Quando as proteínas alteradas excedem a capacidade de eliminação pelo proteossoma, havendo uma acumulação com resultados nocivos para a célula, reflectido também na actividade do proteossoma. Esta situação é facilmente compreendida quando as proteínas agregadas se acumulam no interior da célula. Todavia, a amiloidose causada por TTR deriva de agregação extracelular. A expressão do ubiquitina foi inicialmente investigada no fígado. Não foi encontrada nenhuma diferença entre o fígado normal e o de PAF, apesar do facto de uma proteína mutante alterada estar a ser sintetizada no interior da célula, mas de acordo com a falta da acumulação intracelular de TTR agregada.

Relativamente a tecidos com deposição de TTR, a ubiquitina foi detectada com níveis aumentados. Assim, esta resposta pode ser induzida, indirectamente, pela deposição de TTR, o que pode ser um sinal da célula estar a entrar numa fase de morte ou uma tentativa da célula sobreviver aos efeitos negativos causados pelo deposição de TTR. Um modelo celular que expressa TTR wild type e diferentes variantes mostrou um padrão diferente de aductos de ubiquitina somente quando a TTR expressada agregava no meio. Uma vez mais a ubiquitinação é influenciada indirectamente pela presença extracelular de TTR. A participação do receptor RAGE ou outro, é provavelmente importante no mecanismo. Intracelularmente, ocorre um aumento de proteínas danificadas e alterações em vias de sinalização, que é refletido por um aumento de ubiquitina. Este mecanismo parece estar alterado nos estadíos iniciais de FAP e mantido durante a progressão da doença.

A TTR é considerada uma proteína amiloidogénica, mesmo na ausência de mutações. É também uma proteína altamente conservada durante todo a evolução. Sequências homólogas à TTR foram encontradas em organismos procariotas, chamados "TTR-like", podendo estas ser relacionadas com divergência durante a evolução. Foi proposto estudar a TTR-like de *E. coli* relativamente a função, localização celular e amiloidogénese, numa tentativa de estudar determinantes amiloidogénicos relacionados com agregação. Um knock-out para TTR-like foi gerado e estudado. A sua dinâmica de crescimento mostrou-se diferente comparativamente à estirpe original, wild type. A falta de TTR-like resultou num crescimento aeróbico comprometido em condições de nutrientes limitados e em presença de azoto como fonte de energia. Além disso, enzimas que catalizam a degradação de nucleósidos de pirimidina e a sua utilização como fonte de carbono e energia encontravam-se diminuídas devido à ausência de TTR-like, reforçando a sua função em vias metabólicas. A TTR-like de *E. coli* é um tetramero de subunidades idênticas secretado para o espaço periplasmático e tem um comportamento similar sob condições que normalmente dissociam o tetramero humano de TTR em monómero, contudo a TTR-like de *E. coli* mostra um comportamento diferente na medida em que nenhum dímero é observado. A TTR-like forma fibras em condições acídicas e acelera a amiloidogénese de TTR humana através de um efeito de "seeding", o que não se observa na fibrilogénese de TTR humana. Evolucionariamente, a TTR-like pode ser um ancestral de TTR

cujas funções mudaram, embora algumas características bioquímicas foram mantidas, tal como o seu potencial amiloidogénico.

Em suma, o projecto evidenciou e esclareceu a influência da resposta de stress na patogénese da TTR.

Résumé

La transthyrétine (TTR) est une des nombreuses protéines responsables de maladies associées à l'amyloïde. Cette protéine, synthétisée principalement par le foie, est sécrétée dans le sang où elle circule sous forme d'un homotétramère soluble, étant responsable du transport de la thyroxine et de la protéine de liaison du rétinol ("retinol binding protein") associée au retinol (vitamine A). Des mutations dans la TTR induisent des changements de conformation qui rendent la protéine capable de s'agréger en donnant naissance à de l'amyloïde, qui se dépose dans divers organes. La polyneuropathie amiloïdique familiale (PAF) résulte de la déposition systématique de TTR mutée dans différents organes, bien que le système nerveux périphérique soit majoritairement affecté. La TTR sous la forme agrégée ou fibrillaire n'a jamais été détectée en intracellulaire, ni dans l'organe où elle est synthétisée. Le foie est donc toujours épargné de l'agrégation de TTR. Les raisons pour lesquelles la TTR passe de sa conformation soluble à agrègée dans les organes ne sont pas complètement comprises. L'instabilité de la conformation de la protéine est acceptée comme la raison principale mais d'autres conditions sont nécessaires pour expliquer la variabilité des symptômes cliniques. Ces facteurs modulateurs peuvent être rapportés à l'influence génétique, environnementale ou encore à des facteurs spécifiques des organes ou de la circulation, ou même à des facteurs aléatoires. Le dépôt de la TTR compromet la structure et la fonction des organes ce qui leur est fatal. Les nerfs périphériques affectés par l'incorporation de TTR et de l'amyloïde présentent des signes de stress oxidatif, une augmentation des médiateurs inflammatoires et une neurodégénérescence associée à l`apoptose. Les réponses activées inhérentes à la survie ne sont pas connues et les stratégies pour empêcher le décès cellulaire n'ont jamais été élucidées non plus. Nous avons proposé d'étudier le stress comme un facteur de l'environnement qui a de l'influence sur la pathogénicité causée par la TTR. Des souris transgéniques qui expriment la variante de TTR Val30Met ont été soumises à un programme de stress modéré aléatoire et chronique (unpredictable chronic mild stress) pendant une année, suivi d'analyses des dépôts de TTR et des formations d'amyloïde.

A l'exception d'une tendance de précocité de formation de l'amyloïde chez les souris stressées, aucune différence significative n'a été détectée concernant l'agressivité des signes pathologiques. L'influence du stress chez des patients humains n'a pas été étudiée, néanmoins on ne peut pas l'exclure dans la FAP et plus d'expériences devront être menées sur ce sujet.

Les études suivantes tentaient d'établir un meilleur modèle à partir de souris transgéniques pour les malades atteints de PAF. Les modèles précédents ne reprennaient pas toutes les caractéristiques humaines de la PAF puisque le système nerveux périphérique n'était pas affecté chez les souris ; sans doute à cause de différences inter-espèces ou de la présence d'un mécanisme de défense activée chez les souris retardant la progression de la maladie et épargnant le tissu nerveux. On a accouplé des souris transgéniques qui expriment la variante humaine TTR Val30Met avec des souris knock-out pour le facteur de transcription "heat shock facteur 1" (HSF1). Ce facteur de transcription est responsable de l'activation des protéines "heat shock" (hsps), qui, à leur tour, fonctionnent comme des protéines-chaperon, ou comme des molécules anti-apoptotiques et qui ont d'autres fonctions importantes pour maintenir l'homéostasie de la cellule. Les souris résultantes ont été un succès puisque le dépôt de la TTR a été augmenté et élargi aux nerfs périphériques, aux ganglions dorsaux et ganglions autonomes. Ce modèle ouvre de nouvelles perspectives dans la recherche de la PAF, permettant des études de la biologie de la maladie, la recherche de drogues appropriées et l'investigation de la réponse "heat shock" comme un chemin activé dans la PAF. En fait, des malades humains affectés par le dépôt de TTR présentent une augmentation de hsps dans des tissus nerveux et non nerveux.

Un autre objectif de ce projet expérimental a été l'étude du rôle de l'ubiquitine dans la PAF. D'autres maladies neurodégénératives présentent un déséquilibre du système ubiquitine/protéosome. L'ubiquitine marque les protéines à conformation modifiée et les dirige vers le protéosome où elles sont dégradées. Quand la quantité de protéines modifiées dépasse la capacité d'élimination, une accumulation avec des résultats néfastes pour la cellule se produit, reflétée aussi dans l'activité du proteossome. Cette situation est facilement explicable car les protéines agrégées s'accumulent à l'intérieur de la cellule. Néanmoins l'amiloïdose causée par la TTR, se caractérise par de l'agrégation extracellulaire. L'expression

de l'ubiquitine a été initialement étudiée dans le foie. On n'a trouvé aucune différence entre le foie normal et celui PAF, malgré le fait q'une protéine mutante modifiée soit synthétisée à l'intérieur de la cellule, mais cette observation est en conformité avec l'absence d'accumulation intracellulaire de TTR comme protéine agrégée. Dans les tissus contenant des dépôts de TTR, l'ubiquitine présentait des niveaux augmentés. Cette réponse peut être induite, indirectement, par la précipitation de la TTR. Cela peut correspondre à l'entrée de la cellule dans une phase létale ou à une tentative de la cellule de survivre aux effets négatifs causés par le dépôt de TTR. Un modèle cellulaire qui exprime la TTR sauvage et différentes TTR mutées a montré une norme différente d'ubiquitine seulement quand la TTR exprimée s'agrègeait dans le milieu de culture. L' ubiquitination dans la FAP est influencée indirectement par la déposition extracellulaire de la TTR. La participation du récepteur RAGE ou d'autres, est probablement importante dans ce mécanisme. Dans la cellule, une augmentation de protéines préjudiciables et des modifications dans la signalisation se produisent et se reflétent par une augmentation de l'ubiquitine. Ce mécanisme semble être modifié dans les stades initiaux de la FAP et est maintenu pendant toute la progression de la maladie.

La TTR est considérée une protéine amyloidogène, même en l'absence de mutations. C'est aussi une protéine hautement conservée dans l'évolution. Des séquences homologues de TTR ont été trouvées chez des procaryotes, appelées "TTR-like", qui peuvent être rapportées à la divergence pendant l'évolution. Nous nous sommes proposés d'étudier la TTR-like d' *E. coli* à l'égard de la fonction, de la localisation cellulaire et de la capacité amiloïdogénique. Un knock-out pour la TTR-like a été produit et étudié. Sa dynamique de croissance s'est montrée différente comparativement à la lignée originale. L'absence de la TTR-like a eu pour conséquence une croissance aérobique compromise dans des conditions limitées en éléments nutritifs et en présence d'azote comme source d'énergie. En outre, des enzymes qui catalysent la dégradation des nucléosides de pyrimidine et leur utilisation comme source de carbone et d'énergie se trouvaient diminuées en l'absence de TTR-like renforçant leur fonction dans le métabolisme.

La TTR-like d'E. coli est un tétramère de sous-unités identiques d'environs 14kDa, sécrétée dans l'espace périplasmique, qui a le même comportement que la TTR eucaryote dans des conditions qui normalement dissocient le tétramère humaine

de la TTR en monomère. Néanmoins la TTR-like de *E. coli* montre un comportement différent dans la mesure où aucun dimère n'est observé. La TTR-like forme des fibrilles dans des conditions acides et accélère la formation d'amyloide de TTR humain par un effet d'amorce. D'un point de vue évolutif, la TTR-like peut être un ancêtre de la TTR dont les fonctions ont changé, bien que quelques caractéristiques biochimiques aient été maintenues, telle que leur potentiel amyloidogène.

En résumé, le projet a prouvé et a éclairci l'influence de la réponse du stress dans la pathogenèse de la TTR.

PART I

General introduction

GENERAL INTRODUCTION

General introduction

Familial amyloidotic polyneuropathy – FAP - is a neurodegenerative disease caused by variant transthyretin – TTR - tissue deposition in the form of aggregates and amyloid. Our research project aims at understanding factors that contribute to TTR amyloidogenesis, and to the pathological cascade.
We describe in the following sections several concepts that are the basis of the hypotheses formulated to develop the experimental research project.

1. Transthyretin

Transthyretin, shortly named TTR, is a plasmatic protein synthesized mainly by the liver (Soprano et al., 1985). The choroid plexus also produces TTR, though its regulation occurs independently from the liver (Dickson et al., 1986). Other organs also secrete minor amounts of TTR such as the retina of the eye (Cavallaro et al., 1990).
TTR was first identified in 1942 in the cerebrospinal fluid (CSF) (Kabat et al., 1942). The presence of TTR was soon discovered in plasma as well (Seibert and Nelson, 1942).
The name TransThyRetin reflects its dual physiological role as a transporter of thyroxine (Woeber and Ingbar, 1968) and the complex retinol-binding protein/retinol (Goodman, 1987).

1.1 TTR gene

TTR gene is localized in the long arm of chromosome 18 (Wallace et al., 1985) and is codified by a single copy gene. The gene spans 7.6 kilobases (kb) located at the region 18q11.2-q12.1 (Whitehead et al., 1984). It compromises four exons and three introns (A, B and C) (Tsuzuki et al., 1985). Exon 1, with 95 basepairs (bp)

encodes a leader peptide of 20 aminoacid residues (aa) and the first 3 aa of the mature protein; exon 2 (131 bp), exon 3 (136 bp), and exon 4 (253 bp) hold the coding sequences for aa residues 4-47, 48-92 and 93-127, respectively.

The introns (A, B and C) are 934 bp, 2090 bp and 3308 bp long, respectively. Introns A and C contain two independent open reading frames (ORF) with putative regulatory sequences but their function was unknown (Tsuzuki et al., 1985), and it was suggested that they may be responsible for expressing regulatory sequences. Later, it was determined that these intronic ORFs corresponded to unspliced or partially spliced TTR, that were not expressed as part of a larger transcript, or as an independent polypeptide (Soares et al., 2003).

Upstream of the transcription initiation site, several consensus sequences were found: a TATA box at position –30 to –24 bp followed by a GC rich region of approximately 20 bp, a CAAT box at position –101 to –96 bp and two overlapping sequences homologous to glucocorticoid responsive elements at positions –224 and –212 bp. A polyadenylation signal (AATAAA) was identified 123 bp downstream from the coding sequence (Sasaki et al, 1985). Two major regulatory regions were found in the mouse *ttr* gene: a promoter proximal gene, at approximately -150 bp relatively to the cap site and a distal enhancer sequence at -1.86 to -1.96 kb (Costa et al., 1986). By comparative analysis the same regulatory sequences were found in the human gene. The promoter region of the human *ttr* gene contains elements for hepatocyte-specific expression, including binding regions for HNF-1, C/EBP, HNF-3 and HNF-4. These regions are sufficient for normal hepatic expression but in the choroid plexus other upstream sequences are necessary (Costa et al., 1990; Yan et al., 1990). Transgenic mice showed that 600 bp sequences upstream of the *ttr* gene are sufficient for liver and yolk sac expression whereas upstream sequences of 6 kb are required for whole tissue specific synthesis and quantitatively normal expression (Yan et al., 1990; Nagata et al., 1995).

Transcription of the *ttr* gene results in ~ 0.7 kb mRNA that contains a 5'-untranslated region (26-27 nucleotides), the coding region (441 nucleotides), and a 3'-untranslated region (145-148 nucleotides) preceding the poly(A) tail (Mita et al., 1984; Soprano et al. 1985).

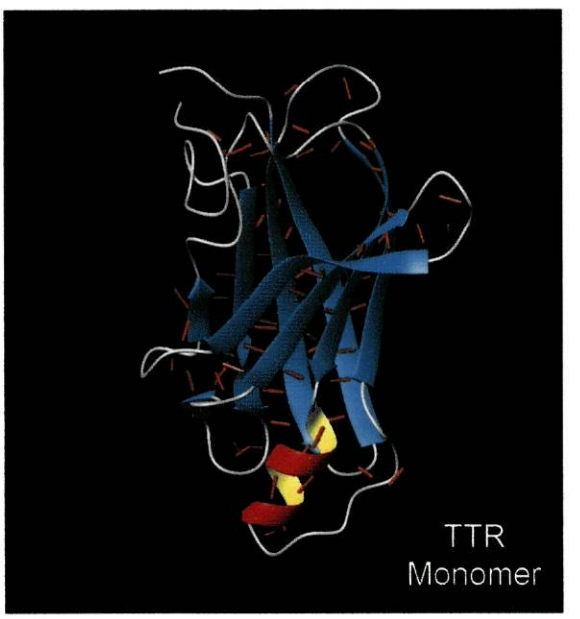
1.2 TTR structure

TTR is a tetrameric protein of four identical monomers, each with 127 aa (Kanda et al., 1974). The molecular mass of the monomer, based on the aminoacid composition is 13,745 Daltons (Da) (Soprano et al., 1985). Assembly of four monomers yields the tetramer with a molecular mass of 54,980 Da.

The three dimensional structure of TTR was performed by crystallographic and X-ray diffraction studies at 1.8 Å resolution (Blake et al., 1978). The dominant secondary structural element is the β-pleated sheet. Eight β-strands labeled A through H, interact in an anti-parallel fashion arranged in a topology similar to the classic greek key barrel with exception of strand A and G. Each monomer contains two β-sheets formed by strands DAGH and CBEF (figure 1 A). The strands are 7 to 8 residues long, except strand D, which has only 3 residues.

The single α-helix segment in the TTR monomer compromises residues 75 to 83 at the end of β-strand E (Blake et al., 1978).

A



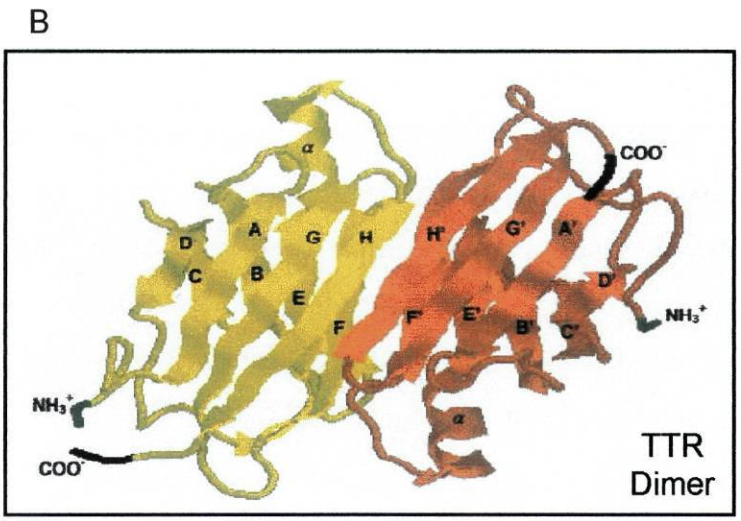


Figure 1-
Representation of (A): the TTR monomer (PDB entry 1ETB); and (B): the TTR dimer 3D structure. Each monomer contains two β-sheets composed of strands DAGH and CBEF, which extend to two 8-stranded β-sheets when two monomers associate forming a dimer (adapted from de la Paz et al., 1992).

Two monomers associate forming the dimer by hydrogen bonding between chains F and H of two β-sheets in each monomer. The strand arrangement within a dimer is DAGHH'G'A'D' and CBEFF'E'B'C' (figure 1 B). Strands A-H belong to one monomer whereas strands A'-H' represent the equivalent monomer in the other subunit.

Two dimers associate to form the tetramer, by dimer-dimer contacts occurring between two loops at the edge of the strand: AB loop of one dimer with the GH loop of the other dimer. The assembly of the tetramer with their inner eight-stranded sheets DAGHH'G'A'D' face to face, forms an open, central, hydrophobic channel, running through the molecule (Nilsson et al., 1971; Blake et al., 1978), where thyroid hormones bind.

The quaternary structure of TTR has the shape of a globular protein with an overall size of 70 Å x 55 Å x 50 Å (Blake et al., 1978). The two dimers are slightly rotated in relation to each other along the y axis (figure 2).

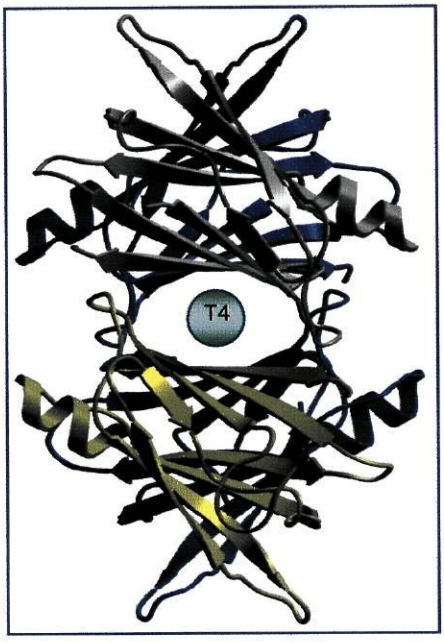


Figure 2-
The human TTR tetramer (PDB entry 1ETB; Hamilton et al., 1993). ● – T4 (thyroxine).

1.3 TTR expression

TTR is detected in the fetal blood very early during development, as soon as eight weeks after conception (Andreoli and Robbins, 1962). TTR synthesis in the embryonic liver appears, after the expression in the tela choroidea- the choroid plexus precursor (Murakami et al., 1989; Harms et al., 1991). Fetal TTR is probably originated from 2 sources: the pregnant woman through transplacental filtration (Gitlin et al., 1964); fetal hepatic synthesis, as shown by the presence of considerable amounts of TTR mRNA early in development of this organ (Makover et al., 1989).

The liver is responsible for more than 90% of TTR synthesis which is secreted into the blood. TTR plasma concentration is age dependent and in healthy newborns it is about half of that in adults (Stabilini et al., 1968; Vahlquist et al., 1975). Plasma TTR concentrations begin to decline after age 50 (Ingenbleek and De Visscher, 1979). TTR concentration in the serum varies from 170 to 420 μg/ml (Vatassery et al., 1991).

In addition to the liver, other sites of TTR synthesis have been identified in mammals: visceral yolk sac endoderm (Soprano et al., 1986, Sklan and Ross, 1987), retinal pigment epithelium (Martone et al., 1988), and choroid plexus epithelium (Aleshire et al., 1983). Other known extra-hepatic tissues with TTR expression include islets of Langerhans in the pancreas (Jacobson, 1989); pineal gland (Martone et al, 1993); and to a lower extent stomach; heart; spleen and skeletal muscle (Soprano et al., 1985).

Considerable amounts of TTR mRNA in specific CNS regions were observed (Aleshire et al., 1983) varying from 11 to 30% of the hepatic levels (Soprano et al., 1985; Schreiber et al., 1990). Another study revealed that the highest TTR mRNA concentration occurs in the epithelial cells lining the ventricular surface of the choroid plexus (Stauder et al., 1986) which secrete, at high rate, TTR into the cerebralspinal fluid (CSF) whose TTR concentration varies between 5 and 20 µg/ml (Vatassery et al., 1991). The choroid plexus is the major site of TTR synthesis if expressed as a ratio of tissue/mass. It corresponds to ~30-fold higher expression per gram of tissue than that found in liver (Weisner and Roething, 1983). Moreover TTR represents 20% of the total CSF proteins (Weisner and Roething, 1983).

1.4 TTR biological functions

TTR main biological functions include the transport of the thyroid hormone - thyroxine (T4) (Andrea et al., 1980) and retinol through the formation of a complex with retinol-binding protein (RBP) (Kanai et al., 1968).

1.4.1 Transport of thyroxine

Thyroid hormones are essential for development, growth, metabolism, fertility, cardiovascular system and brain function. Thyroid follicles produce mainly T4 which by deiodination in other tissues originate the biologically active hormone - triiodothyronine (T3) (Kohrle et al., 1991). Almost all of (99%) the circulating hormone is bound to three plasma proteins in man: thyroxine-binding globulin, transthyretin and albumin (Robbins, 1991), and in rodents to transthyretin and

albumin. In man, TBG shows a higher affinity constant for T4 (Ka = 1 x 10^{10} M^{-1}) than TTR (Ka = 7 x 10^{7} M^{-1}) and transports about 70% of the plasma T4. TTR transports about 15% of the hormone in the plasma and 80% in the CSF (Robins, 1991). Albumin presents the lowest binding affinity (Ka = 7 x 10^{5} M^{-1}) for T4 (Robbins, 1991; Loun and Hage, 1992). TTR has two binding sites for T4 located in the binding pocket formed by the central hydrophobic channel that runs through the molecule. They present negative cooperativity (Ferguson et al., 1975) implying that when the first thyroid hormone molecule occupies the first site, the affinity for the second molecule is highly reduced. As a result, the Ka estimated for the two binding sites for T4 are 1.05 x 10^{8} M^{-1} and 9.55 x 10^{5} M^{-1}, respectively (Andrea et al., 1980). The T4 binding channel possesses a charged region at the entrance, a hydrophilic central region and a hydrophobic patch which is adequate for the chemistry of the hormones. TTR also binds T3 but with lower affinity in mammals.

T4 can be taken up by the tissues from the free hormone pool (Mendel, 1989) or, as an alternative mechanism, the uptake can be mediated by the plasma proteins that bind this hormone (Pardrige, 1987). The role of plasma proteins in the delivery of hormones to target tissues has been controversial (Palha, 2002b). TTR has been proposed to mediate thyroid hormone transfer into the tissues, principally in the brain, across the choroid-plexus-cerebrospinal fluid barrier where TTR is the major thyroid hormone binding protein found (Hagen and Solberg, 1974). There are evidences suggesting that TTR might have an essential role in thyroid hormone transport into and within the brain: rat choroid plexus explants, *in vitro*, accumulate thyroid hormones from the surrounding media and when equilibrium is reached, the thyroid hormone intracellular/extracellular concentration ratio decreases when TTR media content is increased (Dickson et al, 1987). The same authors showed that after intravenous radioactive T4 injection, the radioactivity first appears in the choroid plexus, followed later by the brain. It was then proposed that T4 is transported from the blood into the choroid plexus where by itself or bound to TTR it is secreted into the CSF. Some *in vitro* and *in vivo* studies by Schreiber et al. (1990) further supported this hypothesis.

A possible mechanism is that, given its high lipophilicity, thyroid hormones should be able to cross the double lipid layer plasma membranes by passive diffusion, without the need of any plasma transport protein (Lein and Dowben, 1961).

Studies in a transthyretin-null mice strain have revealed that in fact, TTR is not essential for T4 entry into the brain and other tissues (Palha et al, 1997), nor for the maintenance of a euthyroid status, despite the fact of having reduced total T4 plasma levels (Palha et al, 1994). No other protein was found to replace TTR in the transport of T4 in the CSF, and while T4 levels were strongly reduced in the choroid plexus, in the cortex, cerebellum and hippocampus they were normal (Palha et al, 2000a). Moreover, a normal thyroid hormone distribution in this strain revealed that TTR is not required for the thyroid hormone homeostasis (Palha et al., 2002a). To further support these facts, albumin deficient mice (Mendel et al., 1989) and TBG deficient humans (Bartalena, 1993) show that tissue uptake of thyroid hormones is normal. These evidences are in accordance with the free hormone hypothesis implying that the free circulating hormone is taken up by the tissues. Possibly, TTR may have a storage role for T4 both in the plasma and CSF in normal physiological conditions, which might become important under conditions of increased hormone demand.

1.4.2 Transport of the complex retinol/retinol binding protein

Other described TTR role is the retinol transport through binding to retinol-binding protein (RBP) (Kanai et al., 1968). RBP is a 21 kDa monomeric protein comprising 182 aa residues (Rask et al., 1979). RBP is transported in the bloodstream, attached to TTR only when bound to retinol as a 1:1 molar protein complex (Goodman, 1984; Blaner, 1989). TTR association to RBP protects it from glomerular filtration and renal catabolism (Raz et al., 1970). RBP protects retinol from oxidation and prevents its plasma insolubility. Retinol or vitamin A is essential for vision, reproduction, growth, differentiation and metabolism. After retinol delivery to tissues, RBP shows a reduced affinity to TTR. Intracellularly, retinol is bound and transported to various enzymes for storage or metabolism (Sundaram et al., 1998). The conformational structure of RBP bound to retinol was determined by x-ray crystallography (Newcomer et al., 1984). Although TTR has four binding sites for RBP, two in each dimer, (Van Jaarsveld et al., 1973; Kopelman et al., 1976), under physiological conditions only one molecule is found bound to TTR due to the its limiting physiological concentration in the plasma. *In vitro*, TTR is capable of

binding two RBP molecules each establishing molecular interactions with one of the dimers and contacting with other monomer thus blocking another binding site (Monaco et al., 1995) (figure 3). The dissociation constants for the two RBP molecules are $1.9 \pm 1.0 \times 10^{-7}$ M for the first and 3.5×10^{-5} M for the second molecule.

Figure 3-
TTR complexed with RBP (violet molecules). (PDB entry 1QAB; Naylor and Newcomer, 1999).

The transmembrane transport of retinol has been controversial: three mechanisms were proposed. Some authors suggested that retinol would enter the cells by simple diffusion through the lipid bilayer (Fex and Johannesson, 1988; Noy and Xu, 1990). The second mechanism would account for the existence of membrane receptors that specifically interacted with RBP-retinol resulting in endocytosis of the complex. It was suggested that retinol was obtained from plasma not only through RBP but also via the RBP-TTR complex (Gjoen et al., 1987; Senoo et al., 1990). The third mechanism also involved a receptor and added a role for cellular retinol binding protein as an acceptor of retinol inside the cell whereas apo-RBP would remain outside the cell. RBP without retinol would lose its affinity to the receptor as well as to TTR, being filtered in the kidney and excreted. RBP, when bound to TTR, cannot bind simultaneously to the receptor (Sivaprasadarao and Findlay, 1994) and in agreement the binding sites of RBP with TTR overlap those needed for the membrane receptor binding.

In sum, in plasma, TTR complexed to RBP which in turn binds to retinol, may function as a reservoir of RBP-retinol, and after release of retinol to the cells, TTR frees more RBP-retinol.
It has been observed that both physiological ligands of TTR- RBP and T4, prevent its misfolding, thus functioning as a stabilizer interaction (Raghu and Sivakumar, 2004).

1.4.3 Other TTR functions

Transthyretin has been implicated in acute stressful conditions (Ingenbleek and Young, 1994) being directly and causally involved in the establishment of all stages of the stress response (Bernstein et al., 1989). TTR mRNA decrease during the acute-phase response and malnutrition (Wade et al., 1988). This regulation of expression occurs in the liver independently of the choroid plexus TTR synthesis (Dickson et al., 1986; Wade et al., 1988). This drop in TTR synthesis results in the release of its ligands that become available to the cells changing the equilibrium between bound and free T4 or retinol, but never to dangerous levels since the free hormone remains constant while the retinol levels are controlled by kidney filtration. In response to acute inflammation TTR mRNA decreases to a minimum of 25% of the normal level due to a change in the rate of synthesis and not because of an increase in the rate of degradation (Dickson et al., 1985).
During conditions of malnutrition TTR levels decline, reflecting the nutritional status (Ingenbleek and Young, 2002). In sum, this serum hepatic protein level is a useful indicator of severity of illness (Fuhrman et al., 2004).
TTR has been shown to bind with distinct compounds however with minor relevance. These interactions are poorly understood and not extensively studied. These molecules include: noradrenaline oxidation products (de Vera et al., 1988; Boomsma et al, 1991), pterins (Ernstrom et al, 1995), chicken lutein, hemin and hemoglobin (Martone and Herbert, 1993), polyhalogenated compounds (Brouwer, 1989; Cheek et al, 1999), retinoic acid (Smith et al, 1994) and plant flavonoids (Lueprasitsakul et al., 1990).

Other studies have shown that TTR can play a role in immunological processes (Burton et al., 1985). Besides, TTR is able to inhibit the secretion of interleukin I (Borish et al, 1992).

TTR can form stable complexes with amyloid β-peptide (Aβ) (Schwarzman et al, 1994) and interaction studies suggest that, in the CSF, TTR plays a key role in the modulation of Aβ aggregation, preventing A beta amyloid formation. In addition, TTR levels were reported to be significantly decreased in patients with Alzheimer`s disease (Riisoen, 1988; Serot et al, 1997; Merched et al, 1998) and depression (Hatterer et al., 1993). These data raised the possibility that amyloid fibril formation could be promoted in patients with late onset Alzheimer's disease by the lack of sufficient concentrations of TTR or by the presence of TTR variants. This later view is counteracted by the absence of TTR mutations in patients with Alzheimer's disease (Palha et al, 1996).

TTR has also been found associated to lipoproteins: Tanaka et al (1996) reported the presence of TTR not only in isolated low density lipoproteins (LDL) but also in high density lipoproteins (HDL). Very recently, it has been shown that TTR also associates with apolipoprotein AI (apo AI) suggesting that TTR and lipoprotein biology might be related (Sousa et al., 2000a).

Recently, TTR was described to have proteolytic activity and identified Apo AI as a substrate (Liz et al., 2004). TTR was able to process proteolytically Apo AI at the C-terminus. Moreover, it displayed a chymotrypsin-like activity. In the presence of RBP, its protease function was lost whereas when complexed to T4, only a minor decrease was detected.

1.5 TTR metabolism

Many studies have been performed regarding TTR metabolism and turnover. In humans the total body TTR turnover is 250-300 mg/m^2/day (Vahlquist et al., 1973). The biological half-life of TTR is about 2-3 days in humans (Socolow et al., 1965; Vahlquist et al., 1973), 22-23 hours in monkeys (Vahlquist, 1972) and 29 hours in rats (Dickson et al., 1982). The major sites of TTR degradation in the rat are the liver (36-

38%), followed by the muscle (12-15%) and skin (8-10%) (Makover et al., 1988). Other tissues of TTR degradation include the kidneys, adipose tissue, testes, and gastrointestinal tract which account each for 1-8% of body TTR degradation.

The mean fractional turnover of plasma TTR is 0.15/h, and that of the total body TTR 0.04/h. The turnover of cerebrospinal fluid TTR was estimated to be 0.33/h. Labelled TTR injected in CSF, besides being degraded at the CNS, it was also degraded in the major tissues of degradation described before. Thus, for both routes of injection of labelled TTR, the liver was the major site of TTR degradation, followed by the muscle and skin. No specific transfer and degradation in the nervous system was observed if labelled TTR was injected in the plasma.

TTR cellular uptake is not completely known. Distinct observations suggest that TTR internalization is receptor-mediated, both in human hepatoma cells (HepG2) (Divino and Schussler, 1990) and in chicken oocytes (Vieira et al., 1995). Megalin, a receptor implicated in the renal re-uptake of plasma proteins carriers of lipophilic compounds, was shown to play a role in uptake of TTR in kidneys (Sousa et al., 2000c). Binding/uptake experiments performed with immortalized rat yolk sac cells expressing high levels of megalin and with radiolabelled TTR, free and complexed with T4 or RBP, demonstrated that TTR was rapidly taken up by the cells. This uptake was strongly inhibited by a polyclonal megalin antibody and by the receptor-associated protein (RAP), a ligand for all members of the low-density lipoprotein receptor family (LDLr). With this work, TTR was shown to be a novel megalin ligand with potential importance in T4 transepithelial transport. Moreover, immunohistochemical analysis of kidney sections of megalin-deficient mice, were negative for TTR presence in intracellular vesicles of the proximal tubule cells.

TTR internalization mediated by a receptor was further explored by studying TTR uptake using hepatomas and primary hepatocytes (Sousa and Saraiva, 2001). This work showed direct evidence for TTR internalization by a specific receptor, forming a ~90 kDa complex that was inhibited by RAP. Also, a compromised uptake was observed with TTR bound to RBP (70% decrease) or to T4 (20% decrease).

All together, these results suggest a common pathway for TTR and lipoprotein metabolism.

1.6 TTR evolution

A thyroid hormone distribution network exists in larger mammals, including humans, the thyroxine-binding globulin, albumin and TTR. Three thyroid hormone-binding plasma proteins probably reflect an evolutionary advantage over a single protein. In smaller placental mammals, diprotodont marsupials and birds only albumin and TTR are present. In polyprotodont marsupials and reptilians it is albumin that performs this function in blood. Therefore, there has been an increase in extracellular thyroid hormone distribution capacity of the bloodstream during the evolution of the vertebrates (Richardson et al., 2002). Whereas for the brain the only thyroid hormone-binding protein synthesized is TTR (Chang et al., 1999).

The tissue distribution of TTR displays some differences in vertebrates: TTR is present in CSF of all vertebrates except in adult amphibians and fish. In birds and eutherians TTR is also present in the blood. In fish TTR exists only in the blood.

Evolution of TTR should have started in the choroid plexus of the stem reptiles 350 million years ago (Achen et al., 1993). Liver TTR synthesis is suggested to have been turned on much later, about 50 million years ago, and independently in the lineage leading to today's eutherians, diprotodont marsupials, polyprotodont marsupials, birds and fish (Schreiber et al., 1993).

During evolution the functional properties of TTRs have changed. The main differences include the tissue of synthesis (liver and/or choroid plexus) and binding affinities to the thyroid hormones T4 and T3.

The overall three-dimensional structure of TTR has not yet changed during vertebrate evolution (Richardson et al., 2002). The central channel which harbours the thyroid hormone-binding site is highly conserved in all TTR from distinct species (Power et al., 2000). Changes in amino acid residues have not occurred in the binding sites, but primarily on the surface of the molecule mostly within the first ten aminoacids from the NH_2-terminus, which becomes shorter and more hydrophilic (Aldred et al., 1997). The molecular mechanism was a stepwise shift in the 3´direction of the splice site at the border between exon 1 and exon 2. This has implications for T4 and T3 binding, since this part of the protein is close to the central channel where the two hormones bind, interfering with their accessibility. It is also possible that the change in the electrostatic potential of TTR (more

hydrophilic) favours binding from a neutral T3 to a negative T4 and probably that is why during evolution of TTR, affinities of T3 decreased whilst the binding of T4 increased (Chang et al., 1999). For eutherians (placental mammals), TTR preferentially binds T4 (Chang et al., 1999).

Characterization of TTR has been described for many species, especially in eutherians (placental mammals). Comparing human transthyretin to chicken transthyretin, the similarity of the amino acid sequences is 75% overall and 100% for the central channel containing the thyroxine-binding site (Duan et al., 1991). TTR gene expression was detected in avian choroid plexus and liver, which bound T3 with higher affinity than T4 (Chang et al., 1999).

In amphibians, TTR is synthesized during metamorphosis of tadpole liver but not in tadpole choroid plexus of the brain as documented for *Rana catesbeiana* (bullfrog) (Yamauchi et al., 1991) and *Xenopus laevis* (Prapunpoj et al., 2000). Adults lack TTR expression both in the liver and brain. The amphibian TTR has a higher affinity for T3 when compared to T4.

Amongst the marsupial species, some had albumin as their only thyroid hormone distributor protein, and others had TTR in addition to albumin. Those which had TTR in serum belonged to the Order Diprotodonta, and those which did not have TTR in their serum belonged to the Order Polyprotodonta (Richardson et al., 1994). Marsupial TTR binds T4 with higher affinity than T3. Similarly to the situation in eutherians, hepatic transthyretin synthesis in marsupials is under negative acute phase regulation (Richardson et al., 1994).

In reptiles, the TTR gene expression is observed in the choroid plexus of adult lizards, (*Tiliqua rugosa*) (Achen et al., 1993), crocodile (*Crocodylus porosus*) (Prapunpoj et al., 2002) and turtles (*Trachemys scripta*) (Richardson et al., 1997). In contrast to mammalian TTR, but similar to avian TTR, T3 is bound with higher affinity to TTR in crocodile (Prapunpoj et al., 2002) than T4, reflecting the longer NH_2-terminal region. The lizard TTR sequence is more similar to the avian TTR sequence than to the amphibian TTR sequence. Comparing aminoacid sequence, crocodile TTR is more similar to chicken than to lizard, it shares 90% conserved aminoacids. The alignment to other species shows that the β–strand A is highly conserved and, as already described, all the positions in the central channel, involved in thyroid hormone binding, are identical.

In *Sparus aurata* (fish) the liver is the main source of TTR and both T4 and T3 are bound to TTR although T3 binding appears to be higher (Santos and Power, 1999). The N-terminus is the same as in avian and reptile but has 3 additional aminoacids in comparison to eutherians, resulting in a more hydrophobic protein.

While in vertebrates the two main TTR ligands, T4 and RBP, represent an important role for the organism; in other eukaryotes and prokaryotes devoid of T4 and RBP, the presence of TTR raises questions concerning functionality. Such is the case of sequences, named TTR-like, homologous to transthyretin that have been documented. In fact, an increasing number of species ranging from bacteria, plants and animals with TTR-like sequences present in their genomes are emerging. Possibly, they represent an ancestor of TTR that evolved prior to the divergence of the vertebrates from the non-vertebrates (Richardson et al., 2000). 49 sequences belonging to the transthyretin-like family from 47 species have been documented (Eneqvist et al., 2003). A four aminoacid sequence motif at the C-terminus (Y-R-G-S) is common to the TTR-like family. Sequence similarities between TTR-like sequences and vertebrate TTR are higher for amphibian than reptilian, avian, marsupial or eutherian TTR sequences (Prapunpoj et al., 2000).

The TTR-like gene from *E. coli* is a 414 nucleotide sequence with a predicted signal sequence coded by the first 69 nucleotides (figure 4 A) and so this is a potential periplasmic protein. The TTR-like protein is composed by 137 aminoacids and after processing of the putative signal sequence the mature chain has 114 aa (figure 4 B). The predicted molecular weight of the mature protein is 13,012 kDa and the computed theoretical pI is 8.17 (http://www.expasy.org/). Its function and cellular localization in *E. coli* is however unknown.

The genomic context of the TTR-like gene reveals a putative transcriptional regulator, not yet classified (yedW), and a putative reductase (yedY) (figure 4 C). Orientation on the chromosome is clockwise.

A) TTR-like gene

*atgttaaagcgttatttagtactctcggtagcaacggcagcattttcattaccttctttggttaatgccg***cacaacaaaa cattcttagcgtgcacattttgaaccagcaaacaggaaaacccgctgctgacgtgacagtcactcttgaaa agaaggcggataacggctggttacaacttaataccgccaaaacagataaggatggacgaattaaggcac tgtggcccgagcaaactgcaactacgggcgattaccgtgtcgtatttaaaaccggggactatttcaagaaa caaaatcttgaaagtttcttccctgagatccccgttgaatttcatattaataaagtgaacgagcattatcatgtg cctttattacttagccaatatgggtattcaacctatcgtggcagttaa**

B) TTR-like protein sequence

1 Met Leu Lys Arg Tyr Leu Val Leu Ser Val Ala Thr Ala Ala Phe
16 Ser Leu Pro Ser Leu Val Asn Ala **Ala Gln Gln Asn Ile Leu Ser**
31 **Val His Ile Leu Asn Gln Gln Thr Gly Lys Pro Ala Ala Asp Val**
46 **Thr Val Thr Leu Glu Lys Lys Ala Asp Asn Gly Trp Leu Gln Leu**
61 **Asn Thr Ala Lys Thr Asp Lys Asp Gly Arg Ile Lys Ala Leu Trp**
76 **Pro Glu Gln Thr Ala Thr Thr Gly Asp Tyr Arg Val Val Phe Lys**
91 **Thr Gly Asp Tyr Phe Lys Lys Gln Asn Leu Glu Ser Phe Phe Pro**
106 **Glu Ile Pro Val Glu Phe His Ile Asn Lys Val Asn Glu His Tyr**
121 **His Val Pro Leu Leu Leu Ser Gln Tyr Gly Tyr Ser Thr Tyr Arg**
136 **Gly Ser**

C) Genomic context

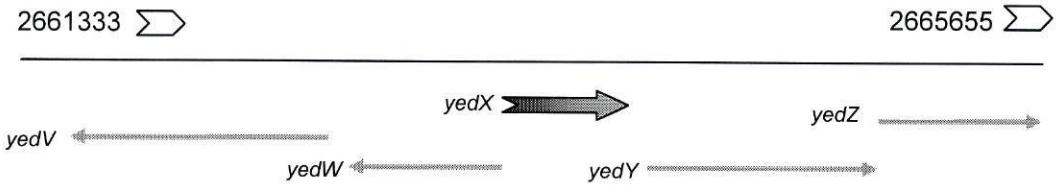


Figure 4-
A) Nucleotide sequence of TTR-like gene from *E. coli* K12: Italicized, grey nucleotides indicate the putative signal sequence (69 nt) and the following nucleotides in bold indicate the coding sequence of the mature chain. Source: http://www.ncbi.nih.gov/BLASTn.
B) Protein sequence of TTR-like from *E. coli* K12: 137 aa, showing the mature chain (bold, black aa) and the signal sequence (Italicized, grey). Source: http://www.ncbi.nih.gov/BLASTp.
C) Genomic address of TTR-like in the *E. coli* genome: 2036980-2037393 nt region. Neighbour genes are: *yedV*: putative 2-component sensor protein; *yedW*: putative 2-component transcriptional regulator; *yedX*: **TTR-like protein**; *yedY*: putative reductase and *yedZ*: hypothetical protein.

A BLAST search between *E. coli* TTR-like and other prokaryotic genomes revealed 11 different organisms with a TTR-like sequence present as shown in table 1.

Species	Protein	Expect	Identities %	Positives %	Gaps %
Salmonella typhimurium	TTR-like	1,0E-52	68	81	5
Caulobacter vibrioides (Caulobacter crescentus)	TTR-like	3,0E-18	41	60	4
Bacillus halodurans	Hypothetical TTR-like	3,0E-18	44	59	5
Mesorhizobium loti	Hypothetical TTR-like	5,0E-17	40	57	5
Sinorhizobium meliloti (Rhizobium meliloti)	Hypothetical TTR-like	7,0E-17	38	55	4
Ralstonia solanacearum	Hypothetical TTR-like	1,0E-14	35	53	4
Bacillus subtilis	TTR-like	2,0E-14	35	56	7
Pseudomonas aeruginosa	Hypothetical TTR-like	6,0E-13	32	54	4
Deinococcus radiodurans	Hypothetical TTR-like	3,0E-12	35	52	3
Brucella melitensis biovar Suis (Brucella suis)	Hypothetical TTR-like	4,0E-12	39	53	5
Brucella melitensis	Hypothetical TTR-like	4,0E-12	39	53	5

Table 1-
Prokaryotic organisms with protein homologies to TTR-like from *E. coli*. The expect value and the percentage of identities, positives and gaps obtained in comparison to *E. coli* TTR-like is shown. (Data collected in http://www.ncbi.nih.gov/BLAST).

Based on the Cluster of Orthologous Groups (COG) a tree with all known prokaryotic proteins is shown with the corresponding organisms (figure 5). Evolutionary, *E. coli* TTR-like is distant from *B. subtillis*, the only prokaryotic with a determined function for TTR-like.

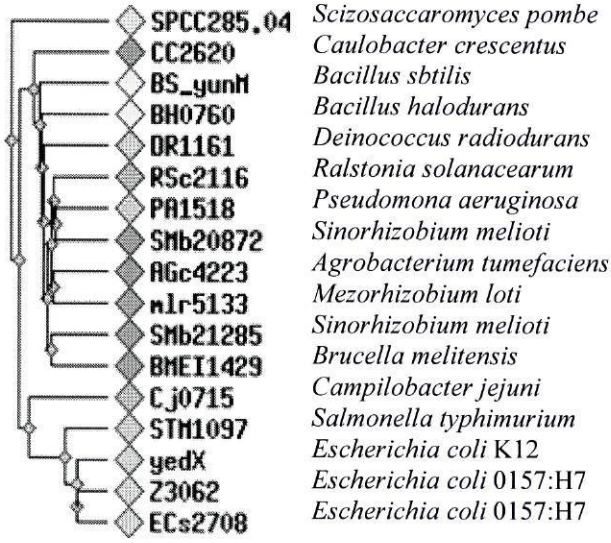


Figure 5-
Prokaryotic organisms with TTR-like present in their genomes. (Adapted from http://www.ncbi.nlm.nih.gov/COG/).

Performing a BLAST search between *E. coli* TTR-like sequence against eukaryotes results in 21 results (table2), including 3 TTR-like sequences from *C. elegans* with 35% identical and 57% similar aminoacids to *E. coli* TTR-like, *S. pombe* with 41% identical and 59% similar aa and *A. thaliana* presenting 34% identical and 49% similar aa to *E. coli* TTR-like. Also 18 TTR proteins from vertebrates were displayed being the most similar *G. gallus* (chicken) 39% identical and 49% similar in protein sequence and the least *S. macroura* (stripe-faced dunnart) 30% identical and 45% similar aa.

Species	Protein	Expect	Identities %	Positives %	Gaps %
Caenorhabditis elegans	Hypothetical TTR-like	6,0E-22	35	57	5
Schizosaccharomyces pombe	Hypothetical TTR-like	6,0E-16	41	59	6
Arabidopsis thaliana (thale cress)	TTR-like	1,0E-11	34	49	14
Gallus gallus (chicken)	Transthyretin	4,0E-11	39	49	1
Rattus norvegicus (Norway rat)	Transthyretin	2,0E-10	36	47	1
Crocodylus porosus	Transthyretin	5,0E-10	34	48	1
Mus musculus (house mouse)	Transthyretin	3,0E-09	34	45	1
Trachydosaurus rugosus (stump-tailed skink)	Transthyretin	3,0E-09	31	48	1
Ovis aries (sheep)	Transthyretin	4,0E-09	34	44	2
Bos taurus (cow)	Transthyretin	5,0E-09	35	45	1
Erinaceus europaeus (western European hedgehog)	Transthyretin	1,0E-08	29	43	1
Sorex araneus (European shrew)	Transthyretin	3,0E-08	32	46	1
Oryctolagus cuniculus (rabbit)	Transthyretin	5,0E-08	33	45	1
Sus scrofa (pig)	Transthyretin	5,0E-08	33	45	1
Homo sapiens (human)	Transthyretin	5,0E-08	32	43	1
Monodelphis domestica (gray short-tailed opossum)	Transthyretin	6,0E-08	33	46	1
Rana catesbeiana (bullfrog)	Transthyretin	1,0E-07	31	48	1
Macropus eugenii (tammar wallaby)	Transthyretin	2,0E-07	32	46	1
Petaurus breviceps (sugar glider)	Transthyretin	7,0E-07	31	46	1
Macropus giganteus (eastern gray kangaroo)	Transthyretin	9,0E-07	31	45	1
Sminthopsis macroura (stripe-faced dunnart)	Transthyretin	1,0E-06	30	45	1

Table 2-
Eukaryotic organisms with protein homologies to TTR-like from *E. coli*. (Data collected in http://www.ncbi.nih.gov/BLAST).

The search for homologous proteins to TTR-like in humans, by BLAST search, (http://www.ncbi.nih.gov) results transthyretin (TTR) with 32% homology (figure 6). Comparing to humans, TTR-like from *E. coli* shows 32% aminoacid identity and

43% aminoacid similarity, which is very high concerning the distant evolutionary relationship. Two gaps had to be introduced to obtain the best alignment.

```
gi|136464|sp|P02766|TTHY_HUMAN_  Transthyretin precursor (Prealbumin)
(TBPA) (TTR) (ATTR)
          Length = 147

 Score = 54.3 bits (129), Expect = 5e-08
 Identities = 35/107 (32%), Positives = 47/107 (43%), Gaps = 2/107 (1%)

Query: 29  LSVHILNQQTGKPAADVTV-TLEKKADNGWLQLNTAKTDKDGRIKALWPEQTATTGDYRV 87
           L V +L+   G PA +V V    K AD+ W    + KT + G +  L  E+    G Y+V
Sbjct: 32  LMVKVLDAVRGSPAINVAVHVFRKAADDTWEPFASGKTSESGELHGLTTEEEFVEGIYKV 91

Query: 88  VFKTGDYFKKQNLESFFPEIPVEFHINKVN-EHYHVPLLLSQYGYST 133
              T  Y+K   +  F     V F  N      Y +  LLS Y YST
Sbjct: 92  EIDTKSYWKALGISPFHEHAEVVFTANDSGPRRYTIAALLSPYSYST 138
```

Figure 6-
Protein-protein BLAST (blastp): *E. coli* TTR-like versus *Homo sapiens* protein database. The protein identified was human transthyretin with an e-value of $5e^{-08}$ and 32% homology. (Data collected in http://www.ncbi.nih.gov/BLAST).

When a comparison of the identity of aminoacids at the putative ligand binding sites was done (Eneqvist et al., 2003) they were almost entirely conserved.

The role for the TTR-like family has no consensus. For some organisms its function was studied. For example in *Bacillus subtilis*, TTR-like was proposed to be involved in the utilization of purines as a nitrogen source (Schultz et al., 2001). It seems that TTR-like is part of an operon (pucJKLM) required for urate oxidase (uricase) activity and inactivation of TTR-like (pucM) results in an uricase-defective phenotype. The other genes of the operon (pucJ and pucK) are highly homologous to xanthine permease and are probably responsible for uric acid transport. The other gene (pucL) shows aminoacid sequence identity to an uricase.

Caenorhabditis elegans has two TTR-like proteins or TRP (TTR related protein). These TRP genes are transcriptionally regulated during development but there is no phenotype associated with its absence as determined by double-stranded (ds) RNA-mediated interference (Eneqvist et al., 2003).

TTR-like from *Arabidopsis thaliana* (TTL) is involved in control of plant growth through association with the brassinosteroid-insensitive 1 receptor (BRI1) (Nam et al., 2004). Overexpression of this protein results in a phenotype resembling weak

bri1 mutants (that is, plant growth inhibition) and insertional mutations in the TTL gene promote plant growth and brassinosteroid sensitivity. Its C-terminal half shows 42% identity with TTR and the long N-terminal, essential for BRI1 interaction, is conserved among other known plant TTL. Although TTL is predicted to be a cytosolic protein it was shown in this study that it is a membrane associated protein. The genome of *E. coli* has already been sequenced (Blattner et al., 1997). A hypothetical protein homologous to transthyretin was identified and the gene named yedX. It codes for a protein with similar length to vertebrate TTR. Thirteen amino acid residues are 100% conserved across all TTR sequences. Of the 13 identical residues, seven are at the first or last residues of beta sheets, eight are in the core of the subunit, and three are in the A-B loop, probably responsible for the dimer-dimer interaction that forms the tetramer. Residues which are responsible for the monomer-monomer interactions are also conserved (Prapunpoj et al., 2000). A three-dimensional structure for *E. coli* TTR-like was created by homology model, based on human TTR (figure 7). The aminoacids insertions and deletion are situated exclusively at the N- and C-terminal ends, the surface exposed BC- CD-, DE-, the FG-loops, and the helix, while the AB and GH-loops comprising the dimer-dimer interface are well conserved. The resulting model shows differences at the hormone-binding site: some corresponding residues are conserved within the TTR-like family, but some are not similar, mainly those situated close to the C-terminus. This is where the lowest homology to TTR is found in contrast to the higher resemblance found within the TTR-like family. Regardless of the characteristics, E. coli TTR-like function is, to date, unknown.

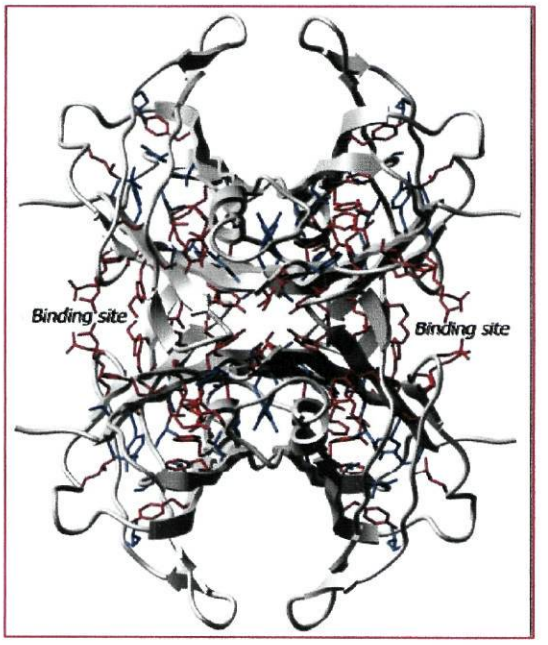


Figure 7-
Three-dimensional model structure of *E. coli* TTR-like based on human TTR (PDB accession code 1F41). Red residues: more than 80% identity; blue 80% similarity. (Enqvist et al., 2003).

Biochemical studies were performed by Enequevist and colleagues (Eneqvist et al., 2003) as an initial characterization of *E. coli* TTR-like protein. It forms a homotetramer as TTR does, however no binding to thyroid hormones was detected with the methodology used. While human TTR aggregates in the conditions used in the, E. coli TTR remains soluble.

1.7 TTR variants

An elevated number of TTR with aminoacid mutations is known (described in table 3). These variants result from point mutations in the polypeptide chain, except the case of a deletion of one aa residue at position 122. The majority of the TTR variants are associated with peripheral neuropathy, presenting often cardiomyopathy, carpal tunnel syndrome and vitreopathy.

Mutation	Codon change →		Predominant Clinical Features	Origin
Cys10Arg	TGT	CGT	PN, AN, Eye	Hungary
Leu12Pro	CTG	CCG	LM, PN, AN	UK
Asp18Glu	GAT	GAG	PN, AN	Columbia
Asp18Gly	GAT	GGT	LM	Hungary
Val20Ile	GTC	ATC	Heart	Germany
Ser23Asn	AGT	AAT	Heart	Portugal
Pro24Ser	CCT	TCT	Heart, CTS, PN	USA
Val28Met	GTG	ATG	PN, AN	Portugal
Val30Met	GTG	ATG	PN, AN, Eye	several
Val30Ala	GTG	GCG	Heart, AN	Germany
Val30Leu	GTG	CTG	PN, AN	Japan
Val30Gly	GTG	GGG	LM, Eye	France
Phe33Ile	TTC	ATC	PN, Eye	Poland
Phe33Leu	TTC	CTC	PN, AN	Poland
Phe33Val	TTC	GTC	PN, AN	UK
Phe33Cys	TTC	TGC	CTS, Heart	Italy
Arg34Thr	AGA	ACA	PN, Heart	Italy
Lys35Asn	AAG	AAC	PN, AN, Heart	France
Ala36Pro	GCT	CCT	PN, Eye	Greece
Asp38Ala	GAT	GCT	PN, Heart	Japan
Trp41Leu	TGG	TTG	Eye	Russia
Glu42Gly	GAG	GGG	PN, AN	Japan
Glu42Asp	GAG	GAT	Heart	France
Phe44Ser	TTT	TCT	PN, AN, Heart	Ireland
Ala45Asp	GCC	GAC	Heart	Italy
Ala45Ser	GCC	UCC	Heart	Sweden
Ala45Thr	GCC	ACC	Heart	Italy
Gly47Arg	GGG	CGG	PN, AN	Japan
Gly47Ala	GGG	GCG	Heart, PN, AN	Italy
Gly47Val	GGG	GTG	PN, AN, Heart	Sri Lanka
Gly47Glu	GGG	GAG	PN	Germany
Thr49Ala	ACC	GCC	Heart, PN	Italy
Thr49Ile	ACC	ATC	PN, Heart	Japan
Ser50Arg	AGT	AGG	PN, AN	Japan
Ser50Ile	AGT	ATT	Heart, PN, AN	Japan
Glu51Gly	GAG	GGG	Heart	USA
Ser52Pro	TCT	CCT	PN, AN, Heart	UK
Gly53Glu	GGA	GAA	LM, Heart	France
Glu54Gly	GAG	GGG	PN, AN	UK
Glu54Lys	GAG	GAA	PN, AN, Heart	Japan
Leu55Arg	CTG	CGG	LM, PN	Germany
Leu55Pro	CTG	CCG	PN, Heart, AN	Taiwan
Leu55Gln	CTG	CAG	AN, PN	USA
His56Arg	CAT	CGT	Heart	USA
Leu58His	CTC	CAC	CTS, Heart	Germany
Leu58Arg	CTC	CGC	CTS, AN, Eye	Japan
Thr59Lys	ACA	AAA	Heart, PN	Italy
Thr60Ala	ACT	GCT	Heart, CTS	Ireland
Glu61Lys	GAG	AAG	PN	Japan
Phe64Leu	TTT	CTT	PN, CTS, Heart	Italy
Phe64Ser	TTT	TCT	LM, PN, Eye	Italy
Ile68Leu	ATA	TTA	Heart	Germany
Tyr69His	TAC	CAC	Eye	Scotland
Tyr69Ile	TAC	ATC	CTS, Heart	Japan
Lys70Asn	AAA	AAC	CTS, PN, Eye	Germany
Val71Ala	GTG	GCG	PN, Eye	Spain
Ile73Val	ATA	GTA	PN, AN	Bangladesh
Ser77Phe	TCT	TTT	PN	France
Ser77Tyr	TCT	TAT	PN	Germany
Tyr78Phe	TAC	TTC	CPS, Heart	Italy
Ile84Ser	ATC	AGC	Heart, CTS, Eye	Switzerland

Mutation	Codon change →	Predominant Clinical Features	Origin
Ile84Asn	ATC AAC	Eye, Heart	Italy
Ile84Thr	ATC ACC	Heart, PN, AN	Germany
Glu89Gln	GAG CAG	PN, Heart	Italy
Glu89Lys	GAG AAG	PN, Heart	USA
Ala91Ser	GCA TCA	PN, CTS, Heart	France
Gln92Lys	GAG GCT	Heart	Japan
Ala97Gly	GCC GGC	Heart, PN	Japan
Ala97Ser	GCC TCC	PN, Heart	France
Ile107Val	ATT GTT	Heart, CTS, PN	Germany
Ile107Met	ATT ATG	PN, Heart	Germany
Ala109Ser	GCC TCC	PN	Japan
Leu111Met	CTG ATG	Heart	Denmark
Ser112Ile	AGC ATC	PN, Heart	Italy
Tyr114Cys	TAC TGC	PN, AN, Eye	Japan
Tyr114His	TAC CAC	CTS	Japan
Tyr116Ser	TAT TCT	PN, CTS	France
Ala120Ser	GCT TCT	Heart, PN, AN	Africa
Val122Ile	GTC ATC	Heart	Africa
Val122del	GTC loss	Heart, PN, CTS	Equador/Spain
Val122Ala	GTC GCC	Heart, Eye, PN	UK

Table 3-
TTR pathological mutations. AN- autonomic neuropathy; CTS- carpal tunnel syndrome; Eye- vitreous deposition; PN- peripheral neuropathy; LM- leptomeningeal amyloid; Heart- cardiomyopathy. (www.hotthyroidology.com: Saraiva, 2004).

Few other TTR mutations without pathogenic consequences have also been described in healthy people and are presented in table 4. Gly6Ser is present in 12% of the Caucasian population and can be considered as a polymorphism (Jacobson et al., 1995). Of great interest is the Thr119Met variant which occurs in 0.8% of the Portuguese and German population investigated (Alves et al., 1997).

Mutation	Codon change	Frequency*
Gly6Ser	GGT AGT	33/558
Met13Ile	ATG ATC	nd
Asp74His	GAC CAC	nd
His90Asn	CAT AAT	16/12,400
Gly101Ser	GGC AGC	nd
Pro102Arg	CCC CGC	1/8,000
Arg104Cys	CGC TGC	nd
Arg104His	CGC CAC	nd
Ala108Ala**	GCC GCT	nd
Ala109Thr	GCC ACC	1/10,000
Ala109Val	GCC GTC	nd
Thr119Met	ACG ATG	35/10,000
Pro125Ser	CCC TCC	nd

Table 4-
TTR mutations in healthy carriers. * Refers to mutant allele frequency. ** Silent mutation. nd- not determined. (www.hotthyroidology.com: Saraiva, 2004).

Compound heterozygotes, carriers of two different TTR mutations have also been reported (table 5). An extra non-amyloidogenic mutation can affect the phenotype, as is the case of the compound heterozygous TTR Val30Met/Thr119Met (Saraiva, 2001a). Thr119Met can be considered as a protective mutation since when expressed with Val30Met a more benign form of the disease is found. This variant is mainly associated with the Portuguese population while TTR Arg104His another protective mutation with the Japanese population (Terazaki et al., 1999). Carriers of the Val30Met and Arg104His have as well a very mild form of the disease with slow progression; serum from compound Arg104His/Val30Met carriers presents low T4 binding contrary to the situation of compound heterozygous for Val30Met/Thr119Met (Almeida et al., 2000).

Mutation	Frequency*
Gly6Ser/Val30Met	7/160
Gly6Ser Phe33Ile**	nd
Gly6Ser/Ala45Asp	nd
Gly6Ser/Ser77Tyr	nd
Gly6Ser/Tyr114Cys	nd
Gly6Ser/Thr119Met	nd
Gly6Ser/Val122/Ala	nd
His90Asn/Val30Met	nd
His90Asn Glu42Gly**	nd
His90Asn/Thr119Met	nd
Arg104His/Val30Met	nd
Arg104His/Thr59Lys	nd
Thr119Met/Val30Met	nd

Table 5-
TTR double mutations. * Refers to mutant allele frequency. ** Mutations on the same allele. nd- not determined. (www.hotthyroidology.com: Saraiva, 2004).

Most of these TTR variants are biochemically different from wild-type TTR, in the sense that their ligand binding affinities for T4 and RBP are altered (Almeida et al., 1997; Almeida et al., 2000), their tetrameric stability is compromised and sometime its conformation shows significant differences. For instance: biochemical studies reported with TTR Leu55Pro show lower tetramer stability when compared to the WT counterpart (McCutchen et al., 1993; Lashuel et al., 1998) and a very different crystal structure (Sebastião et al., 1998). Thyroxine binding studies showed a very low binding affinity for TTR Leu55Pro/TTR WT heterotetramers obtained from serum and inability of recombinant protein to bind T4 (Almeida and Saraiva, 1996). In contrast TTR Thr119Met shows, *in vitro*, an increased T4 binding affinity (Almeida et al., 1997) and a marked resistance against amyloid formation (McCutchen et al., 1995). Although there is no direct relationship between T4 binding properties and amyloidogenesis, it is know that they are both influenced by mutations that induce conformational alterations. A decreased catabolism of TTR Thr119Met is also found (Longo Alves et al., 1997) although explanations for this occurrence, such as a possible different affinity for receptors associated with its clearance, have not been conclusively proven.

2. Familial Amyloidotic Polyneuropathy

Associated to the pathological TTR variants is a neurodegenerative disorder: Familial Amyloidotic Polyneuropathy (FAP). It is a dominant hereditary disease characterized by systemic tissue deposition of aggregated and fibrillar TTR, affecting diverse organs though it is the peripheral nervous system that is specially affected. FAP is a progressive, life conditioning disorder that ultimately is fatal. The most common mutation found and more extensively investigated is a substitution from a Valine to a Methionine at position 30 - Val30Met. Worldwide diverse foci of populations with TTR pathological mutations are known. In Northern Portugal a considerable number of families affected with FAP exist, all having (to date) the Val30Met mutation.

2.1 FAP pathogenesis

Corino de Andrade first described FAP (Andrade, 1952) while investigating Portuguese patients with a peculiar form of peripheral neuropathy, characterized by extracellular deposition of amyloid fibrils. The first report relating immunologically TTR as the protein composing fibrils in FAP was in 1978, by Costa and colleagues (Costa et al., 1978). Six years latter, in 1984, Saraiva identified a Val30Met mutation in the protein isolated from Portuguese FAP patients (Saraiva et al., 1984). This variant was shown to be a biochemical marker for FAP (Saraiva et al., 1985) that resulted from a point mutation in the exon 2 of the TTR gene (Sasaki et al., 1984).

The amyloid deposits (figure 8) occur widely in the peripheral nervous system (PNS), including nerve trunks, plexuses, sensory and autonomic ganglia (Andrade, 1952; Said et al., 1984; Sobue et al., 1990). In the latter sites, they often form deposits around the small ganglion cells. In peripheral nerves, amyloid deposits affect the epineurium, perineurium and more prominently the endoneurium. In the endoneurium, TTR deposits are normally associated with Schwann cells, close to the membrane but without invading it or the cytoplasm (Coimbra and Andrade, 1971a,b; Guimarães et al., 1990; Adams and Said, 1996). Amyloid can also form

among collagen (figure 8). In some cases, amyloid deposits appear in endoneurial blood vessels which can affect blood supply. The primary change is segmental demyelination followed by axonal degeneration and neuronal loss. Most reports on FAP describe axonal degeneration starting in the unmyelinated and low diameter myelinated fibres and only in advanced pathology the heavy myelinated fibres are affected (Coimbra and Andrade 1971a,b; Thomas and King, 1974; Guimarães et al., 1990).

Amyloid deposits can be noxious to nerve fibres in several ways including mechanical and toxic effects on nerve fibres (Sousa and Saraiva, 2003). Compression by amyloid of the nervous tissue was addressed (Jedrzejowska, 1977; Sumino et al., 1983) but never clearly shown, neither did it explain the diffuse depletion of nerve fibres despite the fact that amyloid is small and focal and also the fact that the fibres first affected, which are unmyelinated, are those with higher resistance to compression. Nerve ischemia, secondary to lesion, is another possible hypothesis, though doubtful since impaired blood supply was not demonstrated. Last, the effect on dorsal root ganglia neurons or Schwann cells as come into attention as an indirect relationship to axonal loss. In ganglia, amyloid deposition occurs in the stroma in close contact with satellite cells (Hofer and Anderson, 1975; Sobue et al., 1990). The small diameter neurons in DRG are preferentially lost (Sobue et al., 1990; Toyooka et al., 1995). Efferent nerve fibres are greatly affected with amyloid deposition, whereas afferent roots are spared in accordance to the scarce amyloid found.

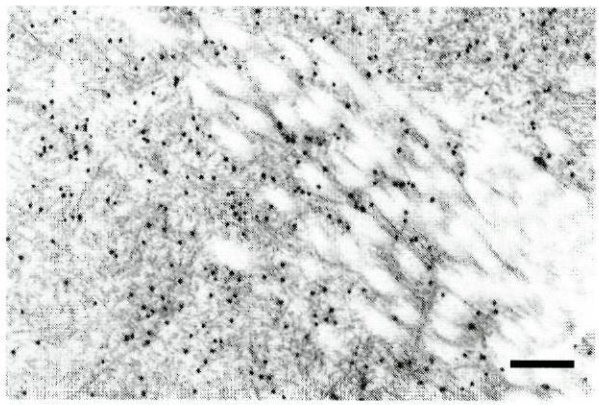


Figure 8–
Amyloid fibrils from a FAP nerve section.
Scale bar = 100 nm (kindly provided by Rui Fernandes, Molecular Neurobiology Unit, IBMC).

The original description of FAP also included the observation of aggregates (at the time of unknown origin), besides the fibrillar material (Coimbra and Andrade, 1971a,b) that were later evidenced by immunohistochemistry as reactive with anti-TTR antibody (Sousa et al., 2001b). Asymptomatic FAP patients, termed FAP 0 (Sousa et al., 2001b), display aggregated TTR in the nerves, which were negative for Congo red staining, the hallmark of amyloid. In subsequent stages (FAP 1-3, based on fibre counting), amyloid is identified and the degree of degeneration aggravates. The early aggregates are responsible for signs of toxicity and *in vitro* those are the species that can activate caspase-3 in cell culture. The mature congophilic fibrils are less toxic (Sousa et al., 2001b; Andersson et al., 2002; Reixach et al., 2004). In accordance, FAP 0 nerves already show signs of inflammatory stress having an increase in the synthesis of pro-inflammatory cytokines and macrophage colony stimulating factor as well as indication of oxidative stress with an upregulation of iNOS and nitrotyrosine, maintained at later stages of the disease FAP 1-3 (Sousa et al., 2001a,b). Oxygen free radicals (especially superoxide) by reacting with nitric oxide generate the highly reactive species, peroxynitrite, which modifies tyrosine residues in proteins. The presence of proteins with nitrated tyrosine residues due to nitrotyrosine function implicates oxidative damage to the protein; appreciable evidence implicating oxidative injury in the pathogenesis of this and other neurodegenerative diseases exists (Giasson et al., 2000).

The protective pathways that are activated to allow the nerve to survive in the initial stages of FAP, despite the toxicity inherent to the aggregated deposits, is however unknown.

Aggregated proteins may cause organ dysfunction by interacting with local receptors such as the receptor for advanced glycation endproduct- RAGE. RAGE is a multiligand member of the immunoglobulin superfamily. It binds certain products of nonenzymatic glycoxidation (Kislinger et al., 1999), Aβ fibrils (Yan et al., 1996), amphoterin (Hori et al., 1995) and S100/calgranulins (Hofmann et al., 1999). Ligand binding initiates signal transducing mechanisms. In FAP, RAGE seems to be relevant in cellular dysfunction since RAGE expression correlates with TTR deposits and is upregulated as the disease progresses (Sousa et al., 2000a).

Moreover, when TTR binds to RAGE, NF-kB is activated observed also when TTR is in a fibrillar form.

Recently, microarray analysis was performed on FAP salivary glands (Sousa et al., 2005). By comparison to normal control subjects, genes related to the extracellular matrix were overexpressed. These included biglycan and neutrophil gelatinase-associated lipocalin (NGAL). Also matrix metalloproteinase-9 (MMP-9), which is associated with NGAL, is increased in FAP and very interesting was the finding that *in vitro*, MMP-9 was able to degrade TTR aggregates and fibrils, only in the absence of serum amyloid P, a universal amyloid component. Probably, these extracellular-matrix remodeling components have a fundamental role in FAP neurodegeneration.

2.2 FAP clinical features

FAP patients show symptoms of impairment of temperature and pain sensation starting in the feet, and autonomic dysfunction leading to paresis, malabsorption resulting in malnutrition, emaciation and dysaesthesiae (Benson, 1991). Motor involvement occurs with disease progression causing wasting and weakness and a progressive loss of reflexes. Upper limbs involvement occurs later. At the end the patient may be confined to immobility. Other symptoms include electrocardiographic abnormalities, alternating constipation and diarrhea, nausea and vomiting, incontinence and sexual impotence.

Depending on the variants other clinical occurrences may be observed such as involvement of leptomeninges, the vitreous of the eye, appearance of the carpal tunnel syndrome - a compression of the median nerve, or occurrence of cardiomyopathy (Saraiva, 1995). This last characteristic is very common, since the heart is frequently involved in FAP. Clinically, the cardiomyopathy may present as an arrhythmia, electrocardiographic abnormalities and various conduction disturbances, heart block or heart failure (Staunder, 1991). Nephropathy and more rarely pulmonary implication (Lobato et al., 1991) and bone involvement (Allard et al., 1991) are other clinical features of FAP.

Nevertheless, if a pathological variant TTR is present the common event is the tissue amyloid deposition found, irrespective of the mutation.

2.3 Phenotypic diversity in FAP

Portugal has the higher number of affected families with FAP. The patient prevalence rate is $105x10^{-5}$ in the northern area (Sousa et al., 1995) and the gene carrier frequency has been estimated as 1 in 625 (Alves et al., 1997). The Val30Met mutation is the most common among FAP patients of Portuguese origin (Saraiva et al., 1984) resulting from a C-to-T transition at a CpG dinucleotide mutation hotspot region in the short D strand of the molecule (Serpell et al., 1996). Nevertheless, this variant has also been identified in patients of different origins: Sweden (Dwulet and Benson, 1984), Japan (Nakazato et al., 1984), Greece (Holt et al., 1989), France (Bhatia et al., 1993) and England (Bhatia et al., 1993). Within and among these populations, there is some heterogeneity in FAP, both in age of onset and clinical presentation. Depending on the geographic area, the mean age of onset varies from 30 to 60 years of age, with a progression to death within 10 to 20 years. Portuguese FAP populations have a median age of onset of 33.5 years (Sousa et al., 1995) although cases of late onset in TTR Val30Met related FAP have been observed in Portuguese patients (Ribeiro do Rosario et al., 1961; Saraiva et al., 1986). In the Japanese population, the first symptoms usually appear at the age of 35 (Ikeda et al., 2002). For Swedish kindreds the average onset is much later- at 56.7 years of age (Sousa et al., 1995). Swedish patients also differ from Portuguese patients by presenting frequent vitreous opacities. In French patients (Plante-Bordeneuve et al., 2003) a late age of onset is frequent (58.9 years). In the island of Maiorca, the mean age of FAP onset is 49 years of age (Munar-Qués et al., 1990). Moreover, the penetrance is incomplete and varies among families (Sakoda et al., 1983; Saraiva et al., 1986; Drugge et al., 1993; Holmgren et al., 1994; Sousa et al., 1995). The association between a later onset with a different geographical origin and the different penetrance of the gene suggests an interaction between genetic and environmental factors in the expression of FAP. It was proposed that sequences downstream of TTR, on the

noncarrier chromosome, affect the onset of symptoms through a trans-acting mechanism while regulatory flanking sequences did not seem to exert an influence (Soares et al., 2004).

FAP populations in Portugal show heterogeneity in phenotypic expression. A later onset of disease for women was observed (Sousa et al., 1995) and interestingly, but unexplained, was the precocity of first symptoms appearance from children with transmitting mothers (Sousa et al., 1990).

Originally, it was thought that all the patients carrying the TTR Val30Met mutation had a common founder of Portuguese origin. However, subsequent studies have shown at least three different haplotypes associated with this mutation (Yoshioka et al., 1989; Almeida et al., 1995). Haplotype I is associated to Portuguese descent and haplotype III in kindreds of non-Portuguese origin, which indicates multiple founders without evidence of natural selection (Reilly et al., 1995).

Members of the same family with the same mutation present different clinical manifestations, severity of the pathology and sometimes atypical symptoms. Moreover monozygotic twins carrying the amyloidogenic mutation Val30Met were studied presenting different characteristics regarding the age of onset, the duration of illness until death and the extent of sensorimotor syndrome. This was observed in monozygotic twins from Portugal, Sweden and Japan (Munar-Ques et al., 1999; Holmgren et al., 2004; Ando et al., 2000) and points towards an influence on FAP progression of non-genetic factors.

Another feature that was explored was the genetic anticipation found in genealogical investigations of Portuguese, Japanese (Tashima et al., 1995) and Swedish families (Drugge et al., 1993). In successive generations, the age when first symptoms appear, happens at progressively earlier ages, sometimes with increased severity. It was proposed by Soares and colleagues that triplet repeat expansions were the cause, but the results were not correlated (Soares et al., 1999). Recently, polymorphisms associated with genes encoding molecules involved in TTR function and TTR amyloid deposition were reported in Val30Met carriers of the Portuguese population (Soares et al., 2005). Also, it was proposed the existence of protective alleles at one or more interacting loci in the late-onset patients.

Usually, the carrier patients are heterozygous for mutant *ttr*, but few homozygous kindreds were found although not presenting an earlier or more severe form of the disease (Holmgren et al., 1988).

Besides the differences found in patients with the same mutation, different TTR variants, other than the Val30Met, can display the same or distinct clinical features. TTR Leu55Pro (Jacobson et al., 1992) and TTR Leu12Pro (Brett et al., 1999) are the most aggressive mutations, probably related to the high instability in terms of conformation. On the other hand, compound heterozygous individuals carrying the Val30Met and the Thr119Met mutations present a late onset and a more benign clinical course of the disease (Coelho et al., 1996) and so Thr119Met is regarded as a protective mutation.

Some TTR variants are associated with cardiomyopathy without neuropathy. This form of disease is named Familial Cardiac Amyloidosis (FAC), the two most common TTR variants associated with FAC are: TTR Val122Ile (Gorevic et al., 1989) and TTR Leu111Met (Nordvag et al., 1992). It is characterized by amyloid fibril deposition in the heart and a late age of onset. TTR Val122Ile is a common amyloidogenic mutation in individuals of African descent with a frequency of carriers of 2% (Jacobson, 1992). In the United States of America, 3.9% of the black population is heterozygous for this mutation, showing symptoms after the sixth decade of life. TTR Leu111Met shows an earlier age of onset, from 20 to 26 years and affects the Danish population (Ranlov et al., 1992). Associated also to amyloid deposition in the heart is the deposition of non-mutated TTR, that occurs in elderly people, named senile systemic amyloidosis (SSA). It affects to some extent 25% of people older that 80 years (Westermark et al., 1990). SSA reflects the intrinsic amyloidogenic property of TTR, which is more susceptible with age.

Besides the amyloidogenic potential of the transthyretin variant influencing the phenotypic expression of FAP, other modulator factors have for certain an important role in amyloid deposition and determine the specificity of tissue deposition localization. These candidates may include genetic background, environmental influence, stochastic events, tissue specific components or circulating factors.

2.4 Amyloidogenesis in FAP

Factors that induce TTR amyloidogenesis have been investigated by different laboratories for several years. Although much knowledge in this area was achieved, the exact mechanism that leads to TTR aggregation and amyloid formation in the tissues is still poorly understood. It is believed that several factors besides its inherent tendency to self-aggregate take part in the process that culminates in fibril formation.

Mechanistic hypothesis of amyloid formation were drawn and investigated. Here we describe three mechanisms that were proposed to induce TTR amyloidogenesis.

2.4.1 Hypothesis for FAP amyloidogenesis: the conformational hypothesis

A hypothesis was raised based on the formation of new structural conformations or exposure of new domains due to mutations. The structure of a protein is important in determining the amyloid formation process. Proteins with a high content of β–sheet secondary structure, as is the case of TTR, are more prone to form amyloid (Blake et al., 1978). Nowadays, it is currently accepted that TTR must undergo destabilization into a partially unfolded state, in order to self-assemble into fibrils. Mutations seem to accelerate this destabilization (Saraiva and Costa, 1991) that would instead occur at much slower rate in the absence of the mutation. In accordance, in SSA, amyloid deposition occurs late in life and is composed of non-mutated TTR (Westermark et al., 2003)

Alterations in the structural conformation lead to tetramer dissociation and formation of partially unfolded intermediates as the initiation step in the amyloidogenic cascade (see 2.5.1).

2.4.2 Hypothesis for FAP amyloidogenesis: the proteolytic hypothesis

Proteolysis has been viewed as another possible theory in FAP amyloidosis.
TTR peptides, in addition to the intact protein, have been found in amyloid fibrils from SSA and some FAP patients, raising the hypothesis that proteolysis could trigger fibril formation by releasing amyloidogenic fragments (Saraiva and Costa, 1991; Gustavsson et al., 1995). Different fragments released from cleavages were found. They varied according to the TTR variant involved: in SSA fibrils, the cleavage occurred at positions 46, 49 and 52 (Cornwell III, 1988); with TTR Val30Met, TTR Val122Ile and TTR Phe33Ile fragments resulted from cleavage at position 49 (Wahlquist et al., 1991; Gorevic et al., 1989; Pras et al., 1983; Nakazato et al., 1984); TTR Leu111Met carriers showed amyloid fibrils containing TTR molecules cleaved at positions 46, 49 and 59 (Nordlie et al., 1990; Hermansen et al., 1995).
Several studies were conducted in order to assess the capacity of TTR peptides to form amyloid fibrils. Most of the synthetic peptides that retained the ability to self-aggregate into fibrils represented β-strands in the tertiary structure of TTR and formed fibrils with the characteristic cross β-sheet arrangement (Gustavsson et al., 1991). The peptides displayed no predisposition to exclusively form β-strands structures in solution (Jarvis et al., 1994).
Nevertheless, Val30Met TTR fibrils do not always present the referred proteolytic fragments (Saraiva et al., 1984; Tawara et al., 1983) and thus, the role of proteolysis in TTR amyloidosis is somehow unclear and not fully explained and needs further confirmation to be established. The release of peptides may contribute to the initiation or progression of the pathology but is not the essential mechanism.

2.4.3 Hypothesis for FAP amyloidogenesis: Nucleation or seeding

A proposal of amyloid formation via a nucleation dependent oligomerization was made (Lansbury, 1997) in a similar manner as crystals grow. The ordered nucleus is formed after a lag phase. Following nucleation, the fibril growth occurs rapidly,

following a sigmoidal curve (Kisilevsky, 2000). This seeding event may be induced by administrating amyloid fibrils (Kisilevsky and Boudreau, 1983). It was demonstrated that TTR fragments with a fibrillar conformation induce *in vivo* murine AA amyloidosis (Johan et al., 1998), working as an amyloid enhancing factor.
Possibly, fragments proceeding from TTR proteolysis, in some cases, may provide a nidus for further and faster polymerization.
Although TTR associated amyloidosis was proposed to follow this pathway of nucleation event, some reports contradicted this theory. The study of a synthetic monomeric variant showed results that were not in accordance with a nucleation-dependent process (Hurshman et al., 2004). Moreover, *in vivo* administration of amyloid fibrils, extracted from the heart of a FAP TTR Val30Met patient, did not induce a precocious TTR amyloid deposition in transgenic mice expressing the human TTR Val30Met (Wei et al., 2004). It appears that the degree of inducibility of amyloid TTR is low and not compatible with a nucleation or seeding event.

2.5 TTR intermediate species in amyloidogenesis

TTR circulates in plasma as a soluble tetramer that aggregates in tissues, ultimately forming mature amyloid fibrils. In this process, intermediate TTR species are formed that attain a conformation different from the normal tetrameric fold. Different proposals where formulated to explain the mechanism.
Fibril formation *in vitro* opened the possibility to study the process by which native soluble TTR loses its normal fold and aggregates into fibrils, paving the way for better understanding the mechanism that culminates in amyloid formation *in vivo*. Different TTR species have been proposed as intermediates in the process of fibril formation.

2.5.1 TTR monomeric intermediates

In 1991, Gustavsson and colleagues (Gustavsson et al., 1991) first described the production of TTR amyloid-like fibrils when dissolving the protein in a 10% acetic acid solution, pH 2.3. At this pH a transition from a tetramer to a monomer would

occur, but a definite conclusion from the TTR modified intermediate was not reached. Colon and Kelly (1992) proposed the existence of a monomeric intermediate and hypothesized the lysosomal intervention due to the low range of pHs found. However, *in vivo*, TTR amyloid deposits are extracellular, arguing against this view that implies the formation of intracellular amyloid.

Further work in 1996 by Lai et al., suggested, rather than a rearranged tetrameric TTR, a monomeric modified intermediate implicated in fibril formation. It was obtained by the tetramer dissociation and it was dependent on both pH and concentration. Adequate conditions of pH and concentration were different depending on the TTR variant studied (McCutchen et al., 1995) resulting in a lower acid stability for variants with higher clinical severity. A study of inter-molecular interactions between monomers of TTR WT and of TTR Val30Met was developed based on the two-hybrid system. A markedly reduction of the mutant monomeric interactions was observed suggesting the destabilization of the tetramer in favor of the TTR Val30Met monomeric intermediate (Chen et al., 1996).

Other approaches were performed at physiological conditions to understand the process that takes place *in vivo*. Quintas et al., (1997) reported that TTR dissociates to a monomeric species upon dilution at pH 7.0 and at nearly physiological ionic strengths. Later, the same authors proposed that the monomers resulting from dissociation are non-native species that cannot re-associate into tetramers (Quintas et al., 1999). Based on protein unfolding experiments of tetrameric TTR, under physiological conditions, the non-native monomeric is formed, preceding amyloid fibril formation. This process is thus favored when an amyloidogenic mutation exists, destabilizing the tetramer (Quintas et al., 2001).

Taken together, these mechanisms are compatible with the real environment of the interstitial milieu.

2.5.2 TTR dimeric intermediates

In spite of the strong evidences supporting the involvement of monomeric species during TTR fibril assembly, several authors have questioned the monomer as the building block. A dimeric TTR intermediate structure has also been proposed to be responsible for fibril formation.

A work by Olofsson et al. (2001), reported the substitution of two aa in the hydrophobic core of TTR, leading to a mutant very prone to form amyloid. Prior to fibril formation, a stable dimeric species was detected at low temperature. However, a small population of monomers was also observed. The data is in good agreement with the findings by Serag et al. (2001) that suggest that the native dimeric interactions are preserved within the building block of amyloid fibrils.
Redondo et al., (2000) addressed the question of a possible dimeric intermediate, creating mutant dimmers covalently linked through disulphide bonds. It was show that these species were unable to polymerize into amyloid while disruption of the S-S bridges by a reducing agent, lead to amyloid formation. These data implied the need of a monomeric entity, and not dimeric species, prior to TTR fibril assembly.

2.5.3 TTR tetrameric intermediates

The contribution of tetrameric TTR in the process of fibril formation was investigated. First, it was proposed the contribution of both monomeric/dimeric intermediates as the building blocks of fibril formation (Blake and Serpell, 1996) based on the interpretation of X-ray diffraction analysis of fibrils. However with the same methodology Inoue et al., (1998) interpreted the results in a different manner suggesting that the existence of tetrameric intermediates was biophysically unlikely. Once again the monomeric intermediate was envisaged.
Ferrão-Gonçalves et al., (2000) proposed a hypothesis based on *in vitro* experiments under high pressure, which an altered tetramer could be responsible for TTR aggregation. However, the work did not present morphological evidence for fibril formation and showed no detailed structural analysis of the fibrils.
The structure of specially engineered TTR synthetic variants with 3 substitutions in the D strand: Gly53Ser, Glu54Asp, Leu55Ser, was resolved and an altered conformational arrangement with no tetramer dissociation was suggested as the building block of TTR fibrils (Eneqvist et al., 2000). These mutants polymerize at physiological conditions, originating high molecular weight aggregates with amyloid characteristics (Goldsteins et al., 1997). Therefore, the proposed model does not imply tetramer dissociation into monomers for fibril formation. However, it should be noted that this is a non-natural TTR variant.

2.6 Dynamics of TTR polymerization

The mechanism of TTR fibril assembly and its molecular elementary subunits components were investigated (Cardoso et al., 2002). TTR amyloidogenesis is a dynamic process: in the early stages, oligomers are the predominant species formed by acidification as revealed by electron microscopy studies. These entities are intermediate units for fibril formation. The mature fibrils formed are mostly 8 nm in diameter and can have lengths of up to 300 nm. Some other species were found: 4-5 nm or 9-10 nm wide fibrils and oligomers of various sizes Mass per length measurements indicated the TTR monomer as the basic forming unit of a protofilament. The protofilaments associate to form higher-order fibrils by lateral association of two or more protofilaments or instead elongate separately but maintaining a common protofilament substructure (Cardoso et al., 2002). Structural features and dimensions are similar either from *ex-vivo* amyloid (obtained from tissues) or produced *in vitro*. Minor variations of the dimensions occur, depending on the tissue extracted.

TTR amyloid displays the common characteristics of proteins attaining the fibrillar fold: they display a green birefringence under polarized light after staining with Congo red and a cross beta X-ray fiber diffraction pattern.

2.7 Other components in TTR amyloid deposits

Several other components, though not fibrillar, have been found associated with TTR amyloid fibrils in tissues. These include: amyloid P component, sulphonated glycosaminoglycans (GAGs), apoliprotein E (ApoE), several basement membrane components such as fibronectin, laminin and collagen type IV, and fatty acids.

These components are common to all fibril types and proposed to be implicated in amyloid formation inhibition (Wisniewski and Frangione, 1992); in preventing amyloid degradation; in enhancing fibrillogenesis or in increasing fibril stability.

The amyloid P component, a glycoprotein composed of a pair of noncovalently bound pentameric discs derivates from serum amyloid protein (SAP), a decameric plasma glycoprotein (Painter et al., 1992). Binding to fibrillar deposits occur in a

calcium–dependent manner (Pepys et al., 1979). Its presence in fibrillar deposits has been well documented (Pepys, 2001; Prelli et al., 1985) as well as for FAP fibrillar deposits (Kawamura et al., 1995; Rydh et al., 1998).

Other extracellular matrix components (ECM) including glycosaminoglycans (GAGs) and proteoglycans (PGs) have been demonstrated to be significant components of all amyloid deposits thus far analyzed (Magnus et al., 1991). Significant amounts of GAGs were detected in close association with purified myocardial amyloid fibrils (Magnus et al., 1991). The GAGs were identified as 50% chondroitin sulphate, 33% heparin/ heparan sulphate and 17% hyaluronan. It is also suggested that the proportion of different GAGs in the amyloid deposits may depend both on the organ or tissues affected and the type of fibrils. It was suggested by Inoue and colleges (Inoue et al., 1998) that TTR amyloid fibrils ultrastructure was a tight helical structure of chondroitin sulfate proteoglycan; a surface layer of heparan sulfate proteoglycan associated with TTR filaments located at the periphery.

It is clear that basement membrane and its constituents play a role in amyloidogenesis. Whether they enhance or inhibit fibrillogenesis is not clear and contradictory results have been reported.

A number of studies have revealed that apolipoprotein E was present in all types of amyloidoses (Westermark, 1998).

Another fibril component in FAP that is isolated with methanol extraction of amyloid, is a mixture of saturated and polyunsaturated fatty acids. It appears that they influence the stability of the fibrils (Hermansen et al., 1995).

2.8 Mice FAP models

The best approach for the *in vivo* study of a pathological condition is the generation of an efficient animal model that mimics the human condition. This methodology allows the elucidation of the underlying mechanisms of the disease process, the search of pharmacological agents that can prevent/retard the development of the pathogenesis. In order to acquire insights into amyloidogenesis occurring *in vivo*, different transgenic mice carrying the human *ttr* Val30Met gene were generated by

several groups as a model to study FAP. Microinjection of human *ttr* gene into fertilized eggs and the subsequent implantation into pseudopregnant females has been the strategy used to produce transgenic animals.

In 1986, the first FAP mouse model was generated (Sakaki et al., 1986). TTR Val30Met cDNA was expressed by an inducible heterologous promoter, the metallothionein (MT-promoter), in the hybrid background C57BL/6XC3H. These mice produced the transgene at very low amounts (2.5-12 µg/ ml). TTR mRNA was present in the liver, kidney, intestine, spleen, brain, heart and testis. After analysis, amyloid deposition was absent (Sasaki et al., 1989). As the authors suggested, plasma human TTR levels were probably not enough to induce amyloid deposition. Besides, only the liver and yolk sac, but not the choroid plexus expressed TTR, distinguishing these transgenic mice from the humans. Another attempt was performed by using the human promoter instead: the human *ttr* Val30Met cDNA plus a 600 bp upstream sequence that included the native TTR promoter (Yamamura et al., 1987). Again, low TTR serum concentration and absence of choroid plexus TTR synthesis were found. Shimada et al. (1989) compared the later strain (having human *ttr* Val30Met cDNA plus the 600 bp upstream sequence) with another strain having the cDNA of the human *ttr* Met30 ligated to the mouse MT-promoter plus the human TTR promoter, in the C57BL/6J background. Amyloid deposition related to TTR started at 15 months of age in the first strain and at 6 months of age in the second, beginning in the mucosa of the small intestine. With aging amyloid deposition aggravated and extended to other organs such as the renal glomeruli, heart and thyroid but never to the peripheral nerve. The MT-TTR Val30Met gene was expressed in various tissues as liver, brain, heart, skeletal muscle, kidney and lung. However, mRNA expression was not found in the small intestine suggesting that the deposited human TTR in this organ originates from the serum. In these animals TTR levels in serum were 10-60 µg/ml.

When a distal enhancer element in the mouse *ttr* gene was found (Costa et al., 1988) it was assumed that these sequences were required for the appropriate human TTR expression in the transgenic mice, and so another transgenic mouse was generated that had a 6kb upstream sequence to human TTR (Nagata et al., 1995). These mice showed similar TTR expression levels in the serum as found in man (200-600 µg/ml) and 10 times more than those mice having only the 0.6 kb

upstream region. TTR expression was not confined only to the liver and yolk sac but the choroid plexus was also found to express TTR. The characterization of amyloid deposition was performed later (Takaoka et al., 1997): amyloid deposition started at 9 months in the gastrointestinal tract (GI), cardiovascular system and kidneys and extends to other organs and tissues with advancing age. At the age of 24 months the pattern of amyloid deposition was similar to the autopsied cases of FAP except for the absence of TTR in the peripheral and autonomic nervous system. In 1993, the *ttr* knockout mice became available (Episkopou et al., 1993) and were crossed to the 6 kb-TTR Val30Met mice, generating animals lacking the endogenous *ttr* gene but carrying the human TTR Val30Met mutant (Maeda et al., 1996). The same pathological features were observed for these transgenic mice in a TTR-null background (Khono et al., 1997). Although the animals presented higher levels of circulating human Met30 than FAP patients, amyloid deposition was similar to the one observed for the 6 Kb-TTR Val30Met transgenics. Therefore, the presence of the mouse endogenous protein was not a factor interfering with amyloid deposition.

Differences in the penetrance of TTR deposition are observed when transgenic mice are bred and kept in normal pathogen-free conditions (SPF) in comparison to non-SPF conditions (Noguchi et al., 2002). Animals at variables ages present a decreased number affected with amyloid deposition in SPF conditions (Saraiva et al., 2004). These findings suggest the environmental factors as modulators of FAP.

Transgenic animals carrying mutations in human TTR, other than the Val30Met, have been generated: TTR Leu55Pro (Teng et al., 1995) and TTR Ile84Ser (Waits et al., 1995). Surprisingly, no amyloid deposits were found in these animals. As TTR Leu55Pro is a very amyloidogenic mutation a new attempt to generate mice expressing this variant was performed in a wt and KO background (Sousa et al., 2002). The transgene was expressed under control of the sheep metallothionein promotor and all the generated lines had the liver as the major site of TTR synthesis. Average plasma levels of TTR after one week of induction with zinc were approximately 150 µg/ml. TTR deposition and the presence of TTR fibrils were investigated by immunohistochemistry and Congo red binding, respectively. When compared to the TTR Val30Met mice, kept under the same conditions they presented earlier, non-fibrillar TTR deposition (1 months), detected in the GI tract

and skin. The cytotoxicity of these deposits was tested by nitrotyrosine immunostaining and a twofold increased level was found in tissues related to TTR deposition which are in accordance with the effects of cellular stress caused by TTR presence in human FAP tissues (Sousa et al., 2001b). However, again in this case, no deposition was found in peripheral nerve. This is the characteristic that distinguish these mice models from the human patients: the absence of TTR deposition affecting the peripheral nervous system. Another type of transgenic mouse was constructed which carried the human mutant TTR gene and the human serum amyloid P (SAP) gene. SAP was found associated with amyloid deposits and proposed to protect and contribute to the persistence of amyloid *in vivo* (Pepys et al., 1996). However, these mice had the same pattern of tissue amyloid deposition of previous characterized transgenic mice without SAP (Tashiro et al., 1991).

To study the process of age-related fibrillogenesis, a feature of senile cardiac amyloidosis, transgenic mice overexpressing human wild-type TTR have been produced. Eighty-four percent of C57Bl/6xDBA/2 mice older than 18 months developed TTR deposits primarily in the heart and kidney. In most animals, the deposits are non fibrillar and non-congophilic (Teng et al., 2001) however, twenty percent of the animals had TTR cardiac amyloid deposits identical to the lesions seen in SSA. Extraction of amyloid and non-amyloid deposits showed intact human TTR with no evidence of proteolysis or deposition of murine TTR. This work raised, for the first time, the hypothesis that the non-fibrillar material is either a pre-amyloid state or an alternate form of tissue deposition.

Figure 9–
Sv129 laboratory mouse. Adult, male animal.

2.9 Therapeutic strategies in FAP amyloidogenesis

Several therapeutic strategies have been adapted over the past years to prevent, retard or eliminate FAP. Furthermore, new efforts are emerging every year to improve previous strategies in order to effectively eliminate this disease.

Liver transplantation is to date, the most effective therapy in the sense that eliminates the organ that produces the pathogenic protein, replacing the synthesis of the causative agent in FAP by the normal protein (Lewis et al., 1994). The first liver transplantation for a FAP patient occurred in 1990 in Sweden. The procedure has then been used all over the world.

Holmgren et al., (1993) reported that amyloid deposits regressed after 1-2 years of transplantation. Stangou et al., (1999) referred to liver transplantation as producing improvements in autonomic function, gut symptoms, nutritional state, and general well being. However, improvements are not always observed, as recently reported for the progression of cardiomyopathy after liver transplantation in patients with FAP (Olofsson et al., 2002). Moreover, the peripheral neuropathy seems to recover very slowly. Regardless of the positive effects, this invasive therapy is associated with considerable risks for the patients. Besides, it cannot apply to all affected individuals.

Another possible approach is gene therapy to impede the synthesis of the mutant TTR, without liver transplantation. Two strategies in this area were explored: one focuses on inhibition of variant TTR mRNA expression by means of small interfering RNA or by ribozymes and the other is the repair of mutated TTR gene by antisense oligonucleotides or by chimaeraplasts.

TTR synthesis suppression was achieved in cultured cells expressing TTR by ribozyme degradation of TTR mRNA (Tanaka et al., 2001). Variant TTR production was down-regulated while the WT was unaffected. Adapting this methodology to the *in vivo* level poses problems in the appropriate delivery and the associated hazardness. Another attempt was performed with single-stranded oligonucleotides (SSOs). This approach by targeted gene repair is seen as a better strategy because the effect can last permanently. These experiments done by Nakamura and colleagues (Nakamura et al., 2004) resulted in the conversion of mutated Met30 TTR to WT TTR *in vitro*. In mice, endogenous TTR gene was replaced by

the murine TTR Val30Met gene. The level of gene conversion was however low. Nevertheless, it opens a promising method to halt the production of variant TTR.

SAP component is universally present in amyloid deposits (Prelli et al., 1985). It confers resistance to proteolysis, therefore preventing clearance of amyloid from tissues and it stabilizes amyloid fibrils (Tennent et al., 1995). Moreover, SAP knock-out mice show a delayed AA amyloid appearance (Botto et al., 1997). Taken together, SAP seems to have a role during amyloidogenesis and it could be a target in human amyloidosis treatment. The hypothesis of preventing SAP from interacting with amyloid was addressed and tested with a drug capable to compete for SAP binding. Experimental studies removed SAP from AA amyloid deposits in mice; and clinical trials resulted in a decrease of SAP plasma concentrations of patients with systemic amyloidosis of AL, AA and ATTR type (Pepys et al., 2002). Evidence of SAP depletion from tissues was observed by quantitative whole-body scintigraphy: SAP tracer was not retained in amyloid deposits of treated patients. This approach seems a possible treatment and further studies are warranted in FAP patients.

Other potential molecular strategies to delay or prevent TTR deposition/amyloid formation have also been proposed. These include the use of: 1) tetrameric stabilizers; 2) fibril inhibitors and 3) fibril disrupters. Figure 10 displays the step in the mechanism of amyloidogenesis where the strategy can interfere.

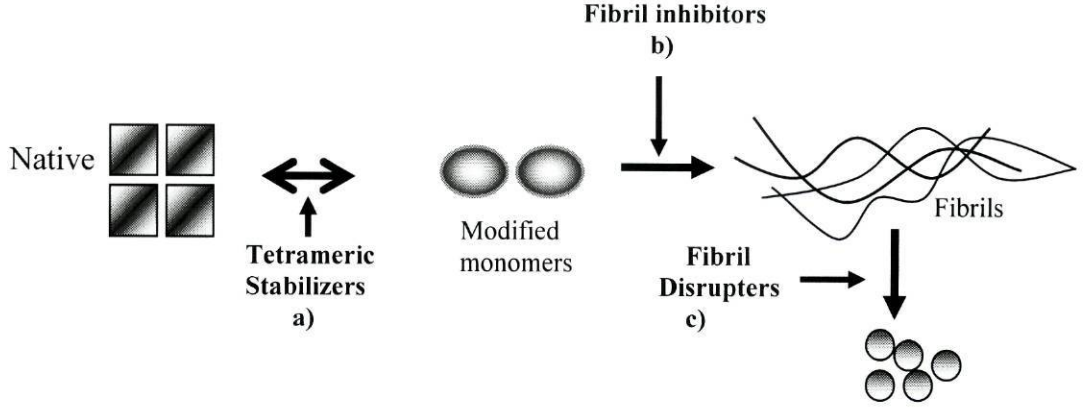


Figure 10-
Amyloid fibril assembly (possible pathway). For each step the proposed strategy is indicated. (Cardoso, PhD thesis, 2002).

The strategy a) would prevent the formation of the amyloidogenic intermediate by inhibiting tetramer dissociation. The potential use of small molecules that bind in the TTR channel, stabilizing the native fold of the protein has been explored: NSAIDs such as flufenamic acid (Peterson et al., 1998); diflunisal (Baures et al., 1998) and diclofenac (Oza et al., 2002) inhibit the conformational changes leading to amyloid formation as assessed by its ability to inhibit the denaturation occurring at acidic pH (Peterson et al., 1998; Klabunde et al., 2000). Their use *in vivo* may be compromised by the presence of other proteins with higher binding affinity, such as albumin and TBG, and so derivatives were developed that preferentially bind TTR such as an iodinated diflunisal derivative - IDIF (Almeida et al., 2004).

A different proposed approach was the intake of antioxidants to avoid oxidative stress. In this way, TTR tetramers and also monomers would maintain the reduced state providing an increased stability to TTR molecule as assessed by isoelectric focusing (Altland and Winter, 1996). Extrapolation of *in vitro* results regarding TTR stabilization needs caution since a compound binding and stabilizing the TTR tetramer, would not necessarily follow the same path *in vivo*.

Preventing fibril formation after the generation of the monomeric intermediate is another possibility. One of the possible therapies is to block the interaction and association of the intermediate structures of TTR into amyloid fibrils (strategy b), figure 10). This particular point of intervention has not been properly addressed due to the difficulty in identifying the species in question. It is accepted that amyloid fibrils result from the assembly of intermediate species with amyloidogenic potential. In the case of FAP, an altered monomer seems to be in the base of such process. In this sense, prevention of these altered monomers association would not allow fibril formation and elongation.

Therapeutic strategies can also aim at dissolving/disrupting mature amyloid fibrils (strategy c), figure 10). 4'-deoxy-4'-iododoxorubicin (I-DOX) is an example of such drug. Recently, I-DOX has been proven useful as a tool for disrupting TTR amyloid fibrils into amorphous TTR; however it could not solubilize the fibrils (Palha et al., 2000b). It strongly interacted with TTR amyloid fibrils but not soluble TTR. I-DOX cardiotoxicity is, however, a drawback for its use and efforts are under way to design non toxic analogues.

Other compounds such as tetracyclines and nitrophenols have been studied for their effect on amyloid fibril disaggregation (Cardoso et al., 2003). Doxycycline was a potential drug since it disrupted TTR fibrils originating non-toxic species.

3. Other amyloidogenic proteins

Amyloid show special tinctorial properties proceeding from its structure, regardless of protein nature. These include apple-green birefringence under polarized light after staining with Congo red (figure 11); staining with thioflavin S and thioflavine T, producing a yellow-green fluorescence (Vassar and Culling, 1959). Under light microscopy, amyloid is a homogeneous, hyaline and eosinophilic substance with conventional stains, both in fixed tissues and *in vivo*. Under electron microscopy, amyloid appear as bundles of straight or coiled fibrils, non-branched, 7-10 nm wide and variable in length (Blanden et al., 1966; Shirahama and Cohen, 1967; Glenner, 1980) and under X-ray diffraction, fibrils revealed to be ordered in the beta pleated sheet conformation (Eanes and Glenner, 1968; Bonar et al., 1969) with extensive antiparallel β-sheet strands with their axes running perpendicularly to the axis of the growing fibril (cross-β pattern) (Glenner, 1980; Lansbury, 1992).

Figure 11–
TTR amyloid deposit at the human nerve stained with Congo red and viewed under polarized light (from Sousa et al., 2001b).

Methods to isolate and purify these deposits were developed as well as the capability to solubilize the fibrils (Pras et al., 1968; Kaplan et al., 1999) which allowed the identification and characterization of the protein content. In 1971 the first amyloid-related protein was sequenced and characterized (Glenner et al., 1971), an immunoglobulin light chain. Since then, over 18 biochemically distinct proteins (table 6) were identified as having the ability to precipitate as amyloid fibrils (Tan and Pepys, 1994; Ghiso et al., 1994; Buxbaum and Tagoe, 2000). Examples are the Alzheimer's β-peptide and the prion protein.

Amyloid protein	Precursor	Systemic (S) or Localized, organ restricted (L)	Syndrome or Involved Tissues
AL	Immunoglobulin light chain	S, L	Primary Myeloma-associated
AH	Immunoglobulin heavy chain	S, L	Primary Myeloma-associated
$A\beta_2M$	β_2-microglobulin	S	Hemodialysis-associated
		L?	Joints
ATTR	Transthyretin	S	Familial Senile systemic
		L?	Tenosynovium
AA	(Apo)serum AA	S	Secondary, reactive
AApoAI	Apolipoprotein AI	S	Familial
		L	Aorta
AApoAII	Apolipoprotein AII	S	Familial
AApoAIV	Apolipoprotein AIV	S	Sporadic, associated with aging
AGel	Gelsolin	S	Familial (Finnish)
ALys	Lysozyme	S	Familial
AFib	Fibrinogen α-chain	S	Familial
ACys	Cystatin C	S	Familial
ABri	ABriPP	S	Familial dementia, British
ADan*	ADanPP	L	Familial dementia, Danish
Aβ	Aβ protein precursor (AβPP)	L	Alzheimer's disease, aging
APrP	Prion protein	L	Spongioform encephalopathies
ACal	(Pro)calcitonin	L	C-cell thyroid tumors
AIAPP	Islet amyloid polypeptide	L	Islets of Langerhans Insulinomas
AANF	Atrial natriuretic factor	L	Cardiac atria
APro	Prolactin	L	Aging pituitary Prolactinomas
AIns	Insulin	L	Iatrogenic
AMed	Lactadherin	L	Senile aortic, media
AKer	Kerato-epithelin	L	Cornea, familial
ALac	Lactoferrin	L	Cornea
*A(tbn)** tbn*		*L*	*Odontogenic tumors*

Proteins are, when possible, listed according to relationship. Thus, apolipoproteins are grouped together, as are polypeptides hormones.
*ADan comes from the same gene as ABri.
**A(tbn), to be named. The designation is waiting for a protein name.

Table 6-
Amyloid fibril proteins and their precursors in human# (from Westermark et al, in press).

The large number of distinct proteins with amyloidogenic potential and the similarity of the amyloid fibrils they form despite differences both in sequence and native structures, suggest the presence of common particularities that may contribute to their tendency to self-aggregate. In this regard, the high content in β-sheet structure, present in most amyloidogenic proteins, has been implicated as favoring fibril formation.

Theories for the events occurring *in vivo* have been proposed based on the knowledge of the *in vitro* results. Virtually, all types of fibrils can be produced from normal precursors if appropriate conditions of incubation *in vitro* are found for the specific protein (Chiti et al., 1999). Proteins unrelated to amyloid disorders are able to form fibrils *in vitro* at the correct conditions of pH, ionic strength and temperature. It appears that this is an inherent property from several proteins.

4. Other neurodegenerative diseases associated with protein misfolding and aggregation

The group of neurodegenerative diseases that are characterized by protein aggregation is extensive. Genetics have a causal role, such as the familial form of the disease, but sporadic occurrences have also been studied. In most instances, the mutations produce earlier-onset autosomal dominant disease, whereas the sporadic cases derive from precursors encoded by wild type genes and are found late in life.

Examples of neurodegenerative diseases associated to protein conformational alterations include: 1) Alzheimer's Disease (AD) associated by intra and extracellular deposition of Aβ peptides and intracellular hyperphosphorylated tau in the central nervous system; 2) Parkinson's disease (PD) resulting from intracellular neuronal Lewy bodies; 3) Huntington's disease (HD) due to extended polyglutamine sequences of huntingtin leading to intranuclear neuronal inclusions; 4) Amyotrophic lateral sclerosis (ALS) showing intracellular superoxide dismutase 1 (SOD1) inclusions in motor neurons of spinal cord; 5) transmissible spongiform encephalopathies or prion diseases with accumulation of an abnormal form of the

prion protein in the brain. Characteristics of these disorders overlap in many aspects; however each differs with respect to anatomical location and cell selectivity.

AD is one of the so-called conformational diseases characterized by protein deposits in the central nervous system associated with neurodegeneration (Thompson and Barrow, 2002). AD is a disorder of the elderly, affecting about 10% of persons over the age of 65 years (Evans et al., 1989). AD is characterized by the deposition of amyloid plaques and neurofibrillary tangles in the aging brain. Amyloid is composed of Aβ peptides that originate from cleavage of the amyloid β-protein precursor (AβPP) (De Strooper et al., 1999) and neurofibrillary tangles of phosphorylated tau found intracellularly (Brion, 1998). It predominantly affects the hippocampus and neocortex, leading to degenerating neurons and activated glial cells.

In the substantia nigra of patients with PD, α-synuclein, a very abundant presynaptic protein of unknown function, accumulates in neuronal inclusions, termed Lewy Bodies (Spillantini et al. 1997), in cell bodies and neurites of dopaminergic neurons (Mezey et al., 1998). In noninherited forms of PD, Lewy Bodies contain also an abnormal form of α-synuclein (Giasson et al., 2000).

The presence of nuclear protein inclusions in HD brain (Poirier et al. 2002) was recognized as the causative agent in this neurodegenerative disorder (Ross, 2002). Expanded polyglutamine [poly(Q)] tract of huntingtin produce intracellular aggregates and cause toxicity (Difiglia et al., 1997) specifically affecting neuronal populations of striatum and cerebral cortex, even though this gene is also expressed in other neurons and other cell types.

In ALS motor neurons are affected by protein inclusions leading to neuronal death. Mutations in superoxide dismutase lead to hereditary forms of ALS (Cleveland, 1999).

The prion-associated disorders are characterized by neuronal loss and protein aggregates, typically fibrillar, which include bovine spongiform encephalopathy (BSE or mad cow disease), human Creutzfeldt-Jakob disease (CJD).

The precise relationship between protein aggregation and cell dysfunction/death in these neurodegenerative diseases is central to many studies. Proteins abnormally

folded are a cause of the cell disorder, though the intermediates that play a role in the process are not widely known.

5. The stress response in neurodegenerative disorders associated with protein aggregation

Protein aggregation and amyloid formation are targets for the cellular defense mechanisms that should play a role in disease susceptibility. These involve the heat shock response (Auluck et al. 2002), chaperones (Hartl & Hayer-Hartl 2002), proteasomal degradation through ubiquitin targeting (Bence et al. 2001), or aggresome formation (Johnston et al. 1998). Integrity and functionality of defense mechanisms are key elements in the maintenance of the organism viability.

The two main protective strategies, hsps and ubiquitin/proteasome system that repair damaged proteins or selectively degrade them, are complementary mechanisms to eliminate threats for cell homeostasis and death. The dual purpose of protein quality control: 1) refolding by hsps of abnormal proteins and 2) the ability of the hsps and ubiquitin to initiate the destruction of these proteins appears quite important in the decision of rescuing proteins in one hand or preventing the waste of ATP in futile attempts to refold condemned proteins.

Factors that initiate mechanisms of defense are for example damaged proteins, and oxidative stress among others. Protection from neurodegeneration is not exclusively related to preventing protein aggregation, but also by clearance of molecules interacting inappropriately with transcription and signaling factors; by assisting intracellular trafficking and by blocking apoptotic cell death (Muchowski and Wacker, 2005).

5.1 Heat shock response

The heat shock response can be elicited by environmental stress, pathophysiological states besides the original definition with induced hyperthermia conditions. Specifically, these include oxygen-free radicals, aminoacid analogues,

transition heavy metals, ischemia, neuronal injury, oncogenes, viral and bacterial infection and others (Morimoto, 1998).

Proteins with an abnormal conformation, in cells, can activate two opposing processes: i) when there is a large, rapid, and overwhelming accumulation of unfolded proteins, apoptosis is induced or ii) if lower amounts of abnormal proteins are generated, then induction of the heat shock proteins (hsps) occurs leading to an enhanced cellular capacity to handle the threat, preventing disease progression and promoting cell viability. Whether the cells die or survive appears to depend on the net balance between these two responses (Gabai et al., 1998).

5.1.1 Heat shock proteins

Heat shock proteins are important to assist proper folding and prevent incorrect interactions between proteins. Also, they are able to impede protein aggregation by ensuring that proteins maintain their native conformation or instead promote refolding of proteins that were damaged. Besides these important functions in cytoprotection from abnormal proteins, hsps also have roles in protein targeting, degradation in the proteasome, transport across cellular membranes and signal transduction (Hartl and Hayer-Hartl, 2002). Different classes of hsps are presently viewed as important protective modulators of cell survival, neuronal or not, and protectors from apoptotic signaling pathways (Parcellier et al., 2003). So, they have a broad repertoire of pathways were they interfere. Their essential functions are reflected by a high content present in normal cells: 1% of the total protein content (Ellis et al., 1989).

Many different hsps are characterized, concerning their specific protective function. Experimental data is available on the importance of hsps on apoptosis related to neurodegenerative pathologies. Hsp27 has been shown to play a role in cellular repair and neuroprotective mechanisms. In AD human brain, hsp27 interacts with hyperphosphorilated tau in neurofibrillary tangles and in cell culture decreases its levels and diminishes cell death (Shimura et al., 2004). Hsp27 upregulation has also been implicated in sensory and motor neuronal survival after peripheral nerve crush and its absence in apoptotic cell death (Benn et al., 2002). Polyglutamine associated toxicity inherent to intracellular inclusions of huntingtin, result in

neuronal cell death and production of reactive oxygen species (ROS). In the presence of hsp27, ROS production is prevented (Wyttenbach et al., 2002), moreover neurons are protected from degeneration by hsp70 (Warrick et al., 1999). Hsp70 can also reduce aggregates in PD (Klucken et al., 2004). Moreover, hsp70 was shown to interfere with apoptotic cell program by inhibiting the release of cytochrome c from mitochondria and by preventing the processing of procaspases 9 and 3 (Mosser et al., 2000). α B-crystallin, a small heat shock protein is able to disaggregate intermediate filament-based glial inclusion bodies (Head and Goldman, 2000) present in astrocytes of Alexander's disease patients. In ALS, the chaperoning system was also involved in protective cell mechanisms against neurodegeneration (Bruening et al., 1999). It is also interesting the results obtained with cells infected with the mutant prion protein that showed reduced capacity to induce hsps, which increased their vulnerability to prion-induced apoptosis (Tatzelt et al., 1995).

Therefore, an immense data regarding hsps and protection from protein aggregation related disorders is available.

The hsps may facilitate also the ubiquitination and degradation of abnormal proteins that cannot refold (Bercovich et al., 1997) and prevent JNK activation and thus apoptosis (Gabai et al., 2001).

5.1.2 Heat shock transcription factor 1

There are 4 different heat shock transcription factors (HSF) which play a role in spontaneous hsps expression during embryogenesis, organogenesis, development and post-natal growth (Pirkkala et al., 2001). As important, is the fundamental role in the regulation of the stress response accomplished by HSF1, the main stress inducible transactivator of heat shock proteins (hsps) (Sarge et al, 1991; Nakai et al, 1997). The inactive form of HSF1 exists in a complex with hsp90 (Zou et al., 1998). When activated, hsp90 releases HSF1 that is able to attain an activated structure that translocating to the nucleus where, in a homotrimeric, phosphorylated form, binds to heat shock elements driving hsps synthesis (figure 12).

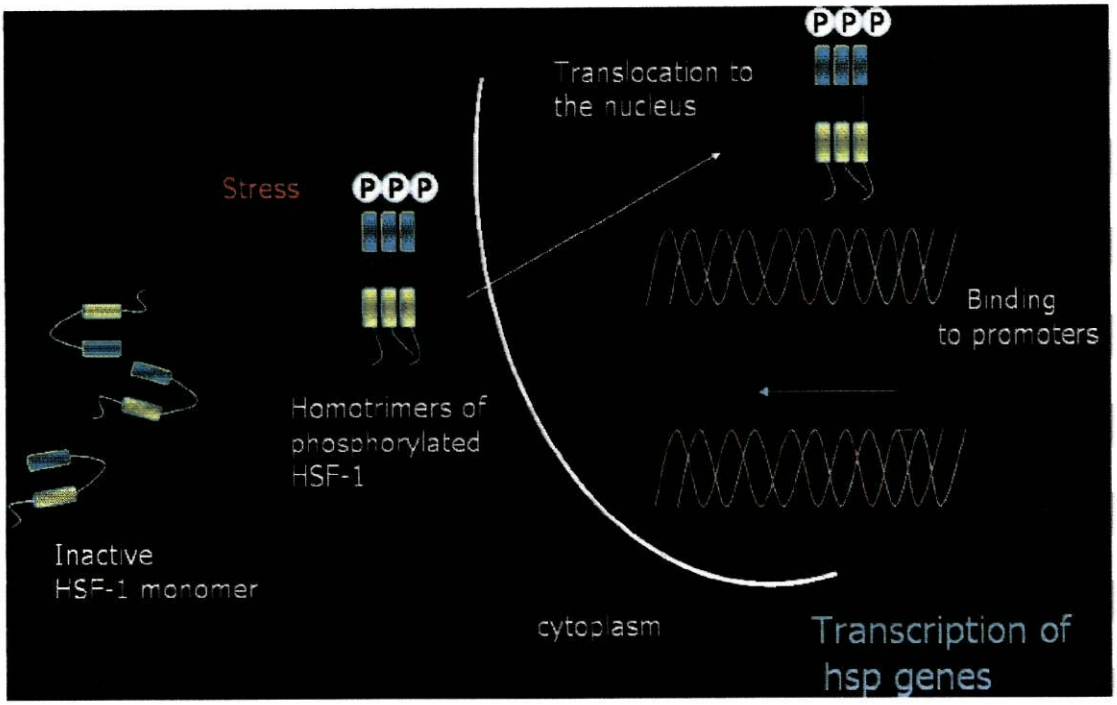


Figure 12-
Pathway for HSF-1 activation induced by a stress stimulus. Transcription of hsps genes is the goal of HSF-1 activity.

5.2 Ubiquitin/Proteasome system

AD; PD, HD and ALS are all neurodegenerative disease associated with the Ubiquitin Proteasome System (UPS), when inclusions co-localizing with ubiquitin are found and proteasomal degradation is altered (Sherman and Goldberg, 2001). Moreover, mutations in ubiquitination enzymes are associated with neurodegeneration (van Leeuwen et al., 1998; Shimura et al., 2000; Fischer et al., 2003). Taken together, these evidences are in accordance with the view that UPS is an important intervening factor in neurodegeneration.

The protein-degrading system of the ubiquitin and proteasome network, which may act in collaboration with chaperones (Esser et al., 2004), are important anti-stress mechanisms (Hershko and Ciechanover, 1998; Layfield et al., 2003) in the sense that proteins potentially dangerous to the cell can be eliminated by this pathway.

5.2.1 Ubiquitin in cell biological functions

Protein ubiquitination is essential for intracellular surveillance against misfolded and denatured proteins; membrane or secretory proteins that fail to fold in the ER; proteins that fail to bind cofactors; free subunits of multimeric complexes or oxidant-damaged proteins (Sherman and Goldberg, 2001). This quality control system is able to efficiently eliminate proteins that are a threat for cell survival.

The presence of misfolded proteins, if it occurs in the endoplasmic reticulum (ER) elicits the unfolded protein response (UPR) and the heat shock response if it occurs in the cytosol. Both of these cell responses are capable of inducing the polyubiquitin gene (Mitch and Goldberg, 1996) as well as formation of ubiquitin conjugates (Fujimuro et al., 1997; Kaneko and Nomura, 2003).

How unfolded or damaged proteins are recognized and selectively degraded is now starting to be clarified. The selective process to eliminate misfolded or damaged proteins, sparing other essential proteins, is achieved by enzymes for ubiquitin conjugation. In this process (figure 13), the ubiquitin molecule is first activated by one of the three types of enzymes involved in this process, the enzyme, E1 in an ATP requiring reaction. After, ubiquitin is transferred to one of the cell's ubiquitin-carrier proteins (E2s). An ubiquitin-ligase (E3) recognizes the target protein to be degraded and together with E2 performs a covalent linkage to a lysine residue of the activated ubiquitin (figure 13). The selectivity in this pathway results mainly from hundreds of different available E3 in mammals.

The protein marked with a chain of four or more ubiquitins is committed for rapid breakdown by the very large proteolytic complex, the 26S proteasome (Coux et al., 1996; Hershko and Ciechanover, 1998).

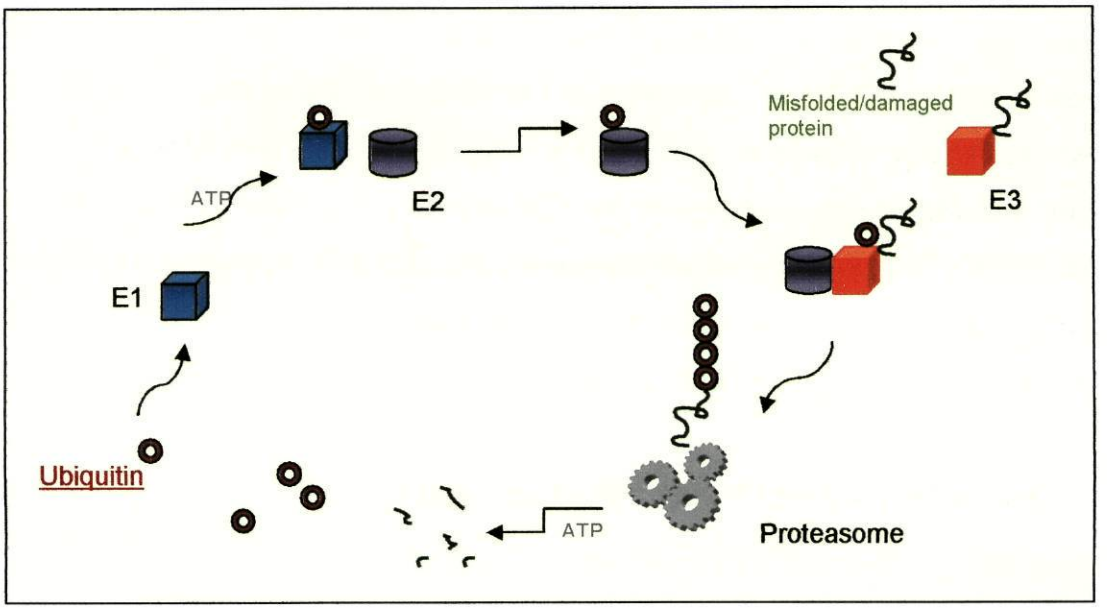


Figure 13-
Pathway for protein degradation, mediated by ubiquitin.

The 26S proteasome consists of two 19S regulatory complexes situated at either end of the 20S core proteasome, a hollow, barrel-shaped structure, within which proteins are degraded, isolated from the surrounding cytosol. Substrates first bind via their ubiquitin chains to the 19S regulatory complex that is responsible for substrate recognition and transport into the core particle (Voges et al., 1999). This structure contains a ring of 6 ATPase molecules, which unfold globular proteins allowing the translocation to the 20S proteasome for proteolysis (Benaroudj and Goldberg, 2000). After protein digestion, the regulatory complex releases free ubiquitin molecules, which can then be re-used in the degradation of other proteins. The proteasome does not cut a protein like typical proteases, but instead completely degrade proteins to small peptides (Kisselev et al., 1999), which are rapidly hydrolyzed to amino acids by cytosolic peptidases.

5.2.2 Ubiquitin targeting besides proteasome degradation

Ubiquitin has an essential role in protection from unwanted proteins by directing them to degradation by the proteasome. Other functions which are proteasome-

independent such as cell cycle progression; cell proliferation; cellular differentiation; endocytosis; apoptosis; transcriptional regulation; modulation of cell surface receptor; protein transport; regulation of immune and inflammatory reaction; DNA repair and stress responses are mediated by ubiquitin as well (Weissman, 2001; Aguilar and Wendland, 2003; Bach and Ostendorff, 2003; Reed, 2003; Schnell and Hicke, 2003). These tightly regulated events are directed by monoubiquitination or by poly-ubiquitin chains connected to a different lysine residue of ubiquitin from that in the UPS mechanism (Hicke, 2001).

5.2.3 Ubiquitin and programmed cell death

The UPS can be turned on by physiological but also pathological signs.
The involvement of the ubiquitin/proteasome system (UPS) in apoptosis has been discussed as an important mechanism (Jesenberger and Jentsch, 2002). Intracellular and extracellular stimuli can trigger programmed cell death in a higly regulated manner. The regulation of apoptosis by UPS is due to ubiquitination of many components of the cellular apoptosis machinery such as the Bcl-2 protein family (Li and Dou, 2000) and p53 (Haupt et al., 1997) which has a proapoptotic function. The NF-kB pathway is also regulated by ubiquitination when the precursor protein is processed in a precise way by the proteasome to form the mature form (Palombella et al., 1994 and Orian et al., 1995) and also when IkB is phosphorylated and ubiquitinated (therefore degraded) which releases NF-kB that can translocate to the nucleus where it activates survival genes involved in protection from apoptosis (Deng et al., 2000). Besides this role in regulation, ubiquitin is also necessary for the intracellular degradation of proteins which are consequent upon apoptosis.
Another evidence of the UPS and apoptosis comes from results in inhibiton of the proteasome in different cell lines which impairs proteolytic activity and activates the cell death programme. (Shinohara et al., 1996; Drexler, 1997). So these accumulating evidences indicate an anti-apoptotic activity of ubiquitination to enhance cell survival.

5.2.4 Ubiquitin in cell pathology

The ability of unfolded proteins to activate apoptosis has probably an important role in neurodegenerative diseases when accumulation of aggregated proteins occurs. Intracellular inclusions of denatured proteins are characteristic features of many neurological diseases, such as Bunina bodies in Amyotrophic Lateral Sclerosis, neurofibrillary tangles in Alzheimer's disease, Lewy bodies in Parkinson's disease, insoluble form of the prion protein (scrapie) in Prion disease and inclusions of expanded polyglutamine-containing proteins in polyglutamine disorders. All these inclusions contain components of the ubiquitin-proteasome degradative pathway and also molecular chaperones, which represent the two main systems that protect eukaryotic cells against the misfolded proteins. These intracellular inclusions reflect a failure of the proteasome activity to remove the abnormal proteins which might explain the neurodegeneration that characterized this process (Ding et al., 2002; Dimcheff et al., 2003; Keck et al., 2003; McNaught et al., 2003). Other confirmations of the relationship between UPS and neurodegenerative diseases comes from findings of mutations in enzymes of the ubiquitin pathway that cause neurological disorders (Layfield et al., 2003) as for example such parkin, an E3 ubiquitin ligase, associated with Parkinson disease (Dawson and Dawson, 2003) and in the ubiquitin itself (Ubiquitin^{+1}) implicated in sporadic late-onset forms of Alzheimer´s disease (Van Leeuwen et al., 1998).

Ubiquitin has undoubtedly been related to disorders of intracellular protein aggregation as a mechanism of defense and regulation. Amyloidoses are extracellular disorders also caused by protein aggregation, but these proteins do not aggregate before they are secreted inside the cells. Instead, the aggregation occurs in the extracellular space. Familial amyloidotic polyneuropathy belongs to the group of amyloidoses, showing extracellular TTR deposition. Protein aggregation occurs in different tissues but never in the main organ of synthesis, the liver. With this scenario, what mechanism of defense can be elicited when deposition of aggregated protein is extracellular? If aggregation is extracellularly, as is the case of FAP, apoptosis might be induced through an extrinsic pathway possibly by intermediate receptors as for example the death receptors FAS and TNF (Becker et al., 1997; Li et al., 2002). It can be suggested also a role for

ubiquitin and hsps in this process, not directly, since a physical separation exists, but indirectly as a defence mechanism.

6. Stress and aging in neurodegenerative disorders

The fact that neurodegenerative diseases usually take decades of life before onset, implies that the organism is able to deal successfully with a mutant or a potentially dangerous protein for many years. With aging, the cell's mechanisms of defense possibly become deficient and insufficient to prevent their accumulation and toxic consequences, triggering pathogenic cascades.

In accordance with such view, is that senescence is accompanied by a poor response to stressors and diminished production of stress proteins in a variety of tissues (Snoeckx et al., 2001). Of particular interest in this regard are various observations, indicating that aged organisms and senescent cultures of mammalian cells are less able to induce hsps in response to protein-damaging conditions (Rattan and Derventzi, 1991) and the magnitude of responses to heat shock decreases in aged organisms (Heydari et al., 1994). The ability of HSF1 to induce hsps synthesis declines with age, not due to a decreased HSF1 synthesis, but because of its lower DNA binding ability (Heydari et al., 2000). With aging, cells also seem to have a reduced capacity for protein degradation (Shang et al., 1997). Such changes in the cell's proteolytic capacity, and the increase in unfolded molecules, should further limit the capacity of the aged organism to cope with a mutant protein or other abnormal folded polypeptides and may thus allow the onset of neurodegenerative diseases. The defense pathways become important when the cell faces the consequences of a stress. If stress accelerates the progression of a disease, due to an abnormal protein with intra- or extracellular aggregation, then impairment of the system against stress would accelerate even further the disease. Failure of the systems that eliminate peptides before they aggregate to form cell-damaging precipitates, plus failure of the aggregate-dissolving mechanisms drive to disease development, in most instances, by an age-associated decline of normal defense functions.

Oxidative stress has been implicated in neurodegenerative diseases (Contestabile, 2001) as well as in aging. Moreover, exposure to oxidative stress increases the synthesis of hsps as part of a protective network, but this response is diminished with age (Locke and Tanguay, 1996; Muramatsu et al., 1996). The effect of oxidative stress and aging include also the reduced activity and levels of the proteasome, suggesting a compromised cell viability (Keller et al., 2000b).
The close association between aging, deficits in defense mechanisms and neurodegeneration imply an indirect consequence of stress and aging on neurodegeneration after the protein misfolding primary cause.

7. Concluding remarks

Modulating factors influencing TTR amyloidogenesis are not completely understood. FAP is affected by TTR deposition due to conformational disturbances, but besides the primary cause, other influences are suspected to have a fundamental role in the onset of the disease and the pathological process. The environment is a reasonable hypothesis, though its importance alone or together with other factors is not known.
The first approach of experimental studies encompasses the use of animal models that mimic FAP. These were proven to be an important tool: mice synthesize a functional human variant TTR and show systemic TTR deposition either as aggregates and ordered amyloid fibrils. However, TTR deposition in the nervous system has never been observed in various available mice models. This is the most important feature of FAP in humans. The generation of an improved mice model that recapitulate the whole human pathology within its life period is therefore of great importance on the investigation of the amyloidogenic pathway.
In humans, TTR deposition elicits biological responses and cellular alterations that ultimately culminate with death by apoptosis. The complete mechanism is now starting to be clarified. RAGE receptor has been identified as a cell surface binding site for TTR in pathological conditions. This binding probably results in intracellular alterations. Other receptors besides RAGE may have a role in the disease process, that result in noxious consequences for cellular function and homeostasis

Inflammation and oxidative markers are known to be upregulated in FAP tissues. Other mediators may be part of this process as a cellular response or even as an activated mechanism involved in cell survival. Taken together, further investigations are needed to understand the entire pathological cascade associated with TTR amyloidogenesis.

PART II

Research project

OBJECTIVES

Objectives

The main aim of this project was to search for some modulators of *in vivo* amyloidogenesis and pathways triggered in TTR pathogenesis.
Additionally, TTR was investigated in a lower organism in terms of amyloidogeneicity.

Specifically these included:

1) Investigation of stress as an environmental factor affecting FAP, either as a factor accelerating or anticipating the disease or aggravating its development. Chapter 1.

2) Generation and characterization of a transgenic mouse model for Val30Met, compromised in the heat shock response. Chapters 2 and 3.

3) Investigation of ubiquitin involvement in FAP. Chapter 4.

4) Characterization of *E. coli* TTR-like protein. Chapter 5.

CHAPTER 1

Investigation of stress as an environmental modulator on FAP progression in a transgenic mice model.

Investigation of stress as an environmental modulator on FAP progression in a transgenic mice model.

Sofia Duque Santos[1,2]; Anna Olsson[3]; Maria João Saraiva[1,2]

[1]Molecular Neurobiology Unit, Institute for Molecular and Cell Biology, Porto, Portugal; [2]Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Portugal; [3]Animal Science and Welfare, Institute for Molecular and Cell Biology, Porto, Portugal.

Corresponding author: Maria João Saraiva; Molecular Neurobiology; Instituto de Biologia Molecular e Celular. Rua do Campo Alegre 823. 4150-180 Porto. Portugal.
Tel. 351-226074900; Fax 351-226099157. e-mail: mjsaraiv@ibmc.up.pt

Running title: Stress in familial amyloidotic polyneuropathy

Abstract

Familial amyloidotic polyneuropathy (FAP) belongs to the group of disorders named amyloidoses. It has a systemic predominance range but it is the peripheral nervous system that is prominently affected.

The primary cause of FAP is known: mutations in transthyretin protein lead to decreased conformation stability that alter its solubility making the protein prone to aggregate and form amyloid fibrils in tissues.

Screening of diverse families in different populations reveals that the expression of this pathology is a complex process, clearly involving the contribution of other unidentified factors besides the TTR genetic mutation. Study of these modulator factors is very important to understand the pathogenesis of FAP and a way to envisage strategies for ameliorating symptoms or preventing FAP onset.

The study of transgenic mice has proven to be a crucial tool for understanding FAP pathogenesis. We chose 3, 6 and 12 months transgenic mice expressing the human TTR V30M to search for a possible influence of an environmental adverse stimulus - unpredictable chronic mild stress - in the progression of FAP. Stress treatments altered the body weight, food and water consumption of the males, while with females the results were not significantly different. TTR serum levels were not altered during the course of experiments, due to stress. Corticosterone levels were significantly higher at 6 months; while TTR non-fibrillar deposition was similar in control and stressed mice. Amyloid was observed at 6 months (2/12) and 15 months (5/11) while control animals were amyloid free. We conclude that the employed stress stimulus was sufficient to enhance amyloid deposition.

Introduction

Familial amyloidotic polyneuropathy is a hereditary disease caused by mutations in transthyretin which result in its conformational disturbances leading to aggregation and amyloid formation. While TTR should be a plasmatic protein, in FAP, TTR is found insoluble deposited in distinct tissues, though it is the peripheral nervous system that is prominently affected. Amyloid deposits are detected in the peripheral nervous system with common spatial distribution pattern of neuronal and fibre loss. The unmyelinated fibres are first affected while the myelinated begin degeneration later.

Genetic analysis of FAP patients revealed heterogeneity in the different TTR mutations found in various populations around the world. The mutational alteration more frequent occurs at position 30, exchanging a valine for a methionine. In Northern Portugal, Japan and Sweden a higher number of FAP families affected by TTR Val30Met are found, mostly heterozygous for the TTR gene.

The clinical symptoms (Steen and Ek, 1983; Saraiva, 2001b) initiate at adult age with a progressive sensory motorneuropathy starting in the lower extremities with paresis, sometimes dysesthesias and inability to discriminate temperature, touch and pain. The symptoms in the upper extremities may occur with the carpal tunnel syndrome, a compression neuropathy of the median nerve. The pathology often progresses over 10 to 20 years, at the end confining the patient to immobility. Disturbance of gastrointestinal function is observed with malabsorption, constipation alternating with diarrhea, nausea, vomiting and emaciation. Heart arrhythmia and male sexual impotence are also common features. Sometimes the vitreous of the eye is affected.

While these characteristics are similar between different affected individuals, closer studies on these populations reveal different disease expression within and among families from the same or different countries. Members of the same family with the same mutation present different clinical manifestations, severity of the pathology and sometimes atypical symptoms. Moreover monozygotic twins carrying the amyloidogenic mutation Val30Met were studied presenting different characteristics regarding the age of onset, the duration of illness until death and the extent of

sensorimotor syndrome. This was observed in monozygotic twins from Portugal, Sweden and Japan (Munar-Ques et al., 1999; Holmgren et al., 2004; Ando et al., 2000). This points towards an influence on FAP progression besides modifier genes that is, non-genetic factors involved in FAP pathology.

Depending on the geographic area the mean age of onset varies from 30 to 60 years of age. In Portugal, the mean age of onset is 33.5 years (Sousa et al., 1995). In Japan, the first symptoms usually appear at the age of 35 (Ikeda et al., 2002). In Swedish population, FAP patients develop the pathology at 56.7 years (Sousa et al., 1993). In French patients (Plante-Bordeneuve et al., 2003), a late age of onset is frequent (58.9 years). A study developed in Portuguese and French families revealed a higher penetrance at the age of 80 for the first population whereby the risk for mutation carriers of being affected is higher and earlier in Portuguese than in French population. Differences are also found between Japanese and Swedish patients not only in the age of onset but also in the average of survival after the first symptoms appear. Moreover the penetrance is incomplete and varies among families (Sakoda et al., 1983; Saraiva et al., 1986; Drugge et al., 1993; Holmgren et al., 1994; Sousa et al., 1995). There are reported cases of early or late onset Val30Met associated FAP, appearing before or after 50 years of age, respectively. Besides this age difference, also the clinical occurrences are different, namely concerning nerve fibre pathology (Sobue et al 2003; Koike et al., 2004). Unmyelinated fibres are affected more severely in the early- than late-onset cases, while the contrary is observed for myelinated fibres. In the late-onset cases, myelinated fibres of variable length are reduced and in the early-onset cases, small myelinated fibres are more affected than large ones. FAP appearance is most common before the fifth decade of life and is also better characterized. The siblings with late-onset FAP pathology have usually probands displaying symptoms late in life. Besides, a higher frequency of males affected with late onset was found but remain unexplained (Misu et al., 1999). Other difference concerns the duration of the pathology, which is invariably short in late onset.

Another feature that was explored was the genetic anticipation found in genealogical investigations of Portuguese, Japanese (Tashima et al., 1995) and Swedish families (Drugge et al., 1993). In successive generations, the age when first symptoms appear, happens at progressively earlier ages, sometimes with

increased severity. It was proposed by Soares and colleagues that triplet repeat expansions were the cause, but the results were not correlated (Soares et al., 1999). Recently, polymorphisms associated with genes encoding molecules involved in TTR function and TTR amyloid deposition were reported in Val30Met carriers of the Portuguese population, and proposed to influence FAP. Also, it was proposed the existence of protective alleles at one or more interacting loci in the late-onset patients (Soares et al., 2005).

Until date, many efforts of different laboratories were done to achieve an effective therapy and various approaches were tested but no cure was obtained with success. The only strategy with results is the liver transplant which substitutes the organ that synthesises the mutant transthyretin. However, this is not the solution for every patient. Other approaches without limitations and less invasive will have to be implemented so as every affected individual may effectively be treated. For that reason it is important to understand what causes TTR systemic deposition besides the known genetic mutation.

The study of transgenic mice expressing human TTR Val30Met has been promising in our and other laboratories in order to achieve a wider understanding of FAP pathogenesis since this protein is expressed under a functional form and presents systemic deposition in different organs, although they do not manifest the peripheral neuropathy.

Previous evidences showed that transgenic mice expressing human TTR Val30Met showed different frequency and extent of tissue TTR amyloid deposition depending on the housing conditions: in specific pathogen-free environment (SPF) there were fewer animals presenting amyloid and the extent was also diminished (Noguchi et al., 2002; Saraiva et al., 2004). This effect was explained by differences in the gut flora. Animals proceeding from this colony and kept at our facilities did not present however a similar phenotype in terms of the number of affected mice. Before these studies, it was already proposed that the lowest occurrence of amyloid, unrelated to TTR, happened in a pathogen-free facility where conditions of stress were minimized (Lipman et al., 1993).

Stress environmental stimulus was proposed to be a factor that influences the progression of the disease. Therefore we investigate, using a transgenic mice model for FAP, if stress could facilitate and/or accelerate the process of

amyloidogenesis in FAP. We chose diverse stress stimulus such as impairment of the circadian rhythm, continuous noise, food and water abstinence, social disruption and limited space in a randomly and unpredictable schedule over one year and analysed its effects in terms of TTR deposition, amyloid formation and oxidative stress.

Materials and methods

Mice

Transgenic mice for human TTR Val30Met, named hTTR, bred and kept in our facilities were separated in 2 groups at the age of 2 months - one to be submitted to unpredictable chronic mild stress and the other to serve as a control. The stress group was subdivided in 3 to be sacrificed after 3, 6 and 12 months of stress conditions (n=12 for each age). The control group was sacrificed after 12 months of experiments (n=12). Each group was composed of 6 males and 6 females.

6 grouped mice were maintained in cages with 530 cm^2 of floor area, under controlled temperature and humidity. Except for some of the stress conditions, mice were under a 12 hours light/dark cycle and laboratory chow and water were available ad libitum.

Stress conditions

All mice were subject to the same stress schedule starting at the same age.

Six different treatments were applied to the stress group randomly so that they were unpredictable to the animal. These included: 1) food and water deprivation, 2/3) perturbation of the light/dark period, 4) wet bedding, 5) social isolation and 6) limited space. 1) Food and water deprivation was applied by removing the laboratory chow and the water for 12 hours either during the light or the dark period. 2/3) The photoperiods were inversed for 12 hours: 2) the disturbed light period was applied by covering the cage over the day so that the light period is omitted; 3) the disturbed dark period was achieved by placing the mice in a new location with lights on and with ventilation noise, overnight for 12 hours. 4) Wet bedding occurred by adding water to the sawdust bedding during 6 hours, either in the morning or the afternoon. 5) Social isolation was imposed for 2 days, where animals were separated from their groups and placed alone in a 335 cm^2 cage, after this period they were grouped again. 6) For limited space conditions, grouped mice were transferred from a 530 cm^2 cage to a 335 cm^2 cage for one week.

The wet bedding procedure was used in the first 3 months; social isolation and limited space conditions were applied in the 9 last months of experiments. All other

stress conditions were performed during the whole course of the experiment. None of the stress conditions were applied together and there were never more than two subsequent days with disturbance. 15 to 17 treatments selected randomly were applied each month, one treatment per day. Treatments to be applied during the night time started at 20.00 h. Treatments to be applied during the light period started early in the morning or afternoon. Animals were left undisturbed and allowed to rest in home cage for 1 or 2 days before the following treatment.

Data register

Body weight, laboratory chow and water consumption was recorded every week. Behaviour and physical differences such as body shape, healthy status and state of the fur were detected by usual and daily contact.

Organ collection

After 3, 6 and 12 months of stress conditions and data collection, mice groups were sacrificed.

Weights were recorded just before sacrifice. Blood was collected, centrifuged at 3000 rpm for 15 minutes and the serum stored at -70ºC. The liver was extracted and weighted. Other organs extracted to paraffin blocks and to store at -70ºC were: stomach, esophagus, small and large intestine, pancreas, skin, kidney, spleen, salivary glands, eye, tongue, heart, lung, reproductive system (testis or uterus), brain, spinal cord and sciatic nerve.

Tissue analysis

Congo red staining was performed as described by Puchtler and Sweat (1965) and visualized under a polarized light. For immunohistochemistry, tissue paraffin slides were deparafinated and hydrated. Endogenous peroxidase was destroyed before blocking with 1% BSA, 4% fetal bovine serum in PBS for 1 hour at 37ºC. Anti-TTR diluted in blocking buffer (1:1000, DAKO) was incubated in a humidified chamber, overnight (o/n) at 4ºC. Corresponding secondary antibodies used were biotin-conjugated (1:20 Sigma), followed by extravidin labelling (1:20, Sigma). Each was incubated for 30 minutes at 37ºC. The reaction was developed with 3-amino-9-ethyl

carbaxole: AEC (Sigma) or 3, 3´- diaminobenzidine: DAB (Sigma), counterstained with haematoxylin and mounted with an aqueous mounting medium from DAKO.

TTR quantification

TTR was quantified from the different groups by sandwich ELISA using anti-TTR (1:500, DAKO) as the capture antibody and anti-TTR HRP linked (1:500, Binding Site) as the detection antibody. TTR standards were made from recombinant human TTR with concentration ranging from 300 ng/ml to 15 ng/ml. Mouse serum samples to analyse were diluted 1:2000 in PBS. Positive reaction was developed with ABTS (2, 2'-Azino-bis 3-Ethylbenzthiazoline-6-Sulfonic Acid) and absorbance read at 405 nm in a microplate reader.

Corticosterone quantification

Mouse serum samples obtained after sacrifice were stored at -70 ºC until analysis of corticosterone levels. This was accomplished with a corticosterone EIA kit (Cayman) according to supplier's instructions.

Results

Stress was proposed to be an environmental factor influencing FAP. This was experimented in mice transgenic for human TTR Val30Met that were submitted to unpredictable chronic mild stress for different periods of time. Stress induction is readily achieved in rodents but since the mice have an increased capacity to adapt to adverse situations, a randomly schedule of stress treatments were followed and during the experiment new situations were included so as to minimize the expectancy developed by continuous treatments (table 1). These alterations are below the limit of a situation that can develop clinical problems such as immunological responses, not associated with the genetic alteration.

Stress treatment	Duration
Food deprivation	12 hours
Water deprivation	12 hours
Change in the dark period + noise	12 hours
Change in the light period	12 hours
Wet bedding	6 hours
Social isolation	2 days
Limited space	1 week

Table 1-
Stress treatments applied to the mice and the corresponding period of time. Wet bedding was used only in the first 3 months, social isolation and limited space in the 9 final months of experiments.

Body weight

Weight was measured weekly. A small increase in body weight was detected in male hTTR mice submitted to stress stimulus in comparison to control mice not submitted to stress (chart 1). The pattern depicted, related to the stress stimulus, was the wide variations in body weight, especially at older ages, that were on the other hand maintained rather constant in control mice.

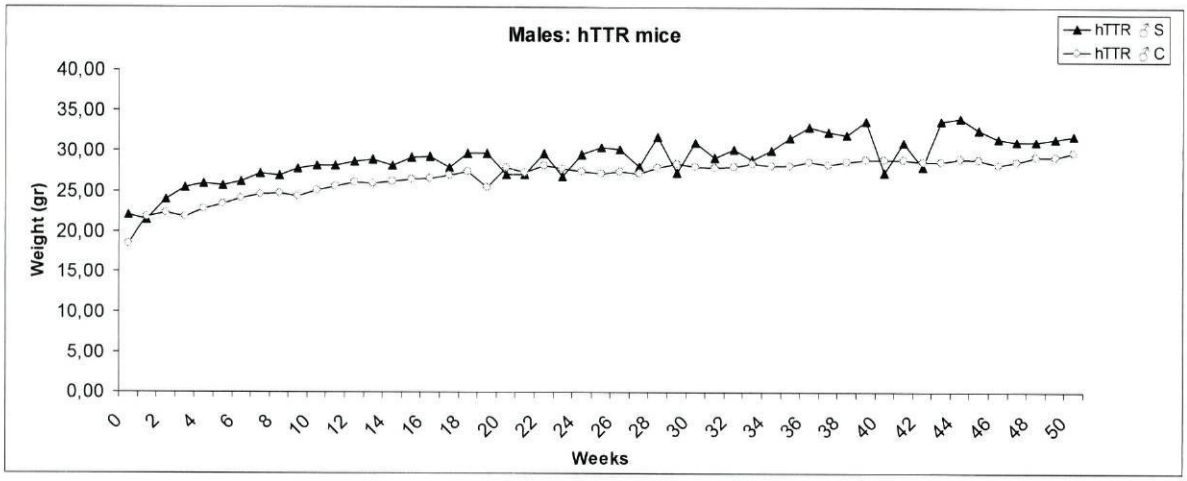


Chart 1-
Mean body weight of male hTTR mice measured weakly for 12 months. S- stress; C- control.

Concerning females, similar values were obtained between stress and control mice (chart 2). Females hTTR showed occasionally a decrease on their mean weight, contrary to the increase observed for male mice. However, these variations of the body weight were not as sharply or as frequently as with male mice.

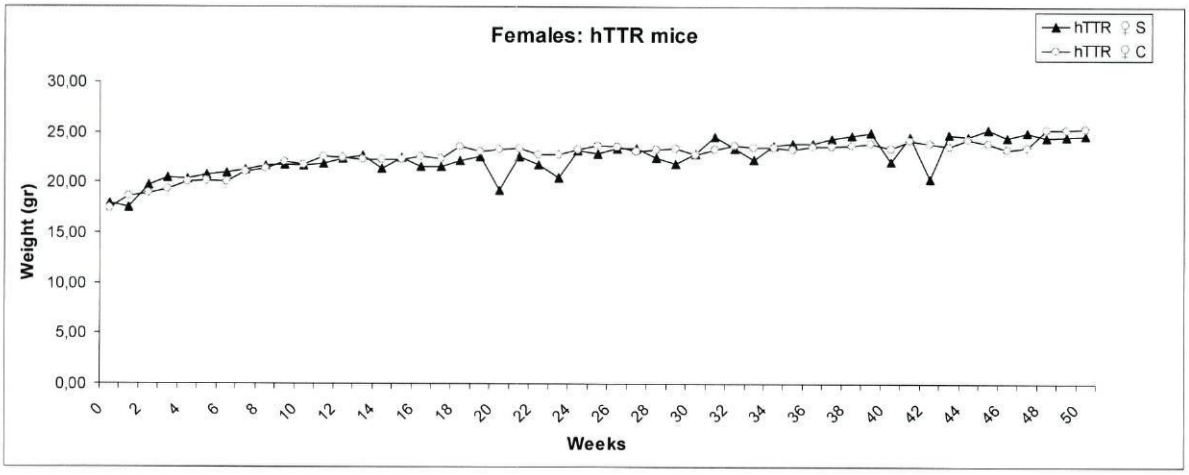


Chart 2-
Mean body weight of female hTTR mice measured weakly for 12 months. S- stress; C- control.

Food and water consumption

Food consumption was analysed in terms of the body weight and the results were identical for both stress and control mice for both sexes (chart 3 and 4). Since no significant variations were observed for males (chart 3) or females (chart 4) the

differences on the body weight observed, namely the increased body weight for males and the wide variation during the experiments reflected the different food consumption throughout the course of the experiments.

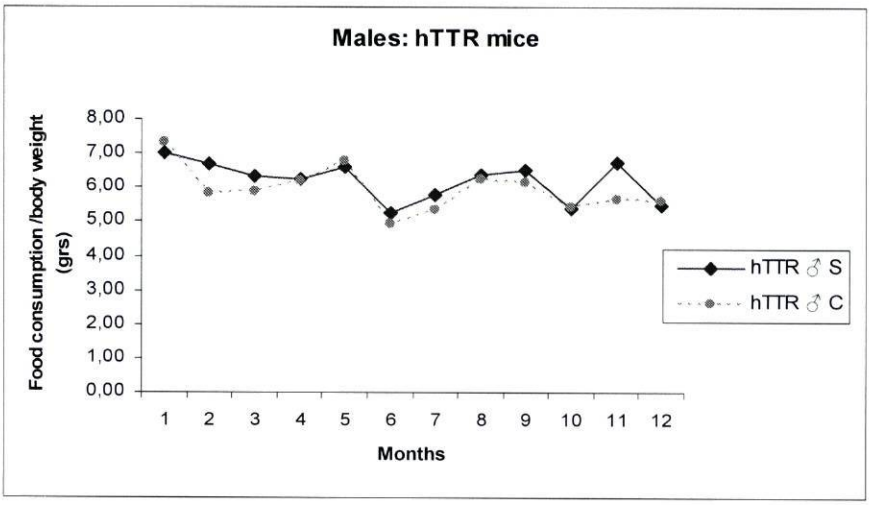


Chart 3-
Food consumption in terms of the body weight of male mice measured weakly for 12 months. The monthly mean is shown. S- stress; C- control.

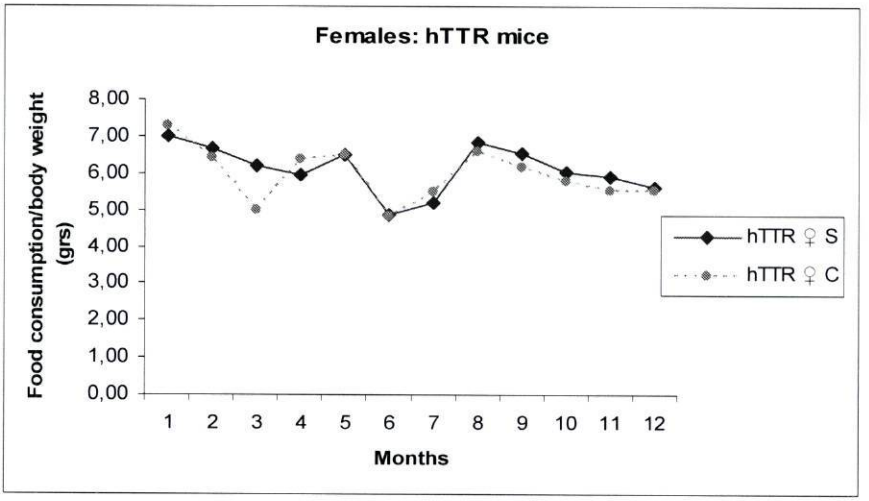


Chart 4-
Food consumption in terms of the body weight of female mice measured weakly for 12 months. The monthly mean is shown. S- stress; C- control.

Concerning the water drunk, the hTTR male mice submitted to stress were the group that had a higher consumption in comparison to unstressed mice (chart 5). This can be explained by a higher metabolism occurring in accordance to the higher food consumption and reflected higher body weight.

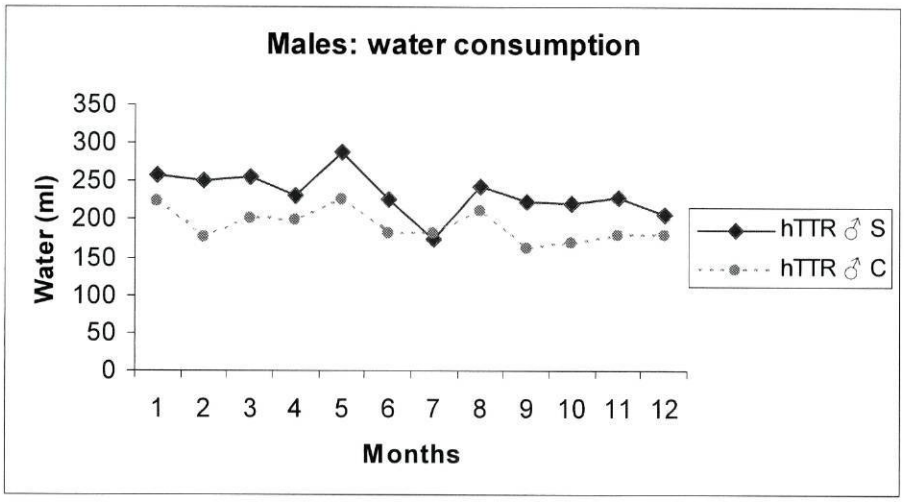


Chart 5-
Water consumption of male mice measured weakly for 12 months. The monthly mean is shown. S- stress; C- control.

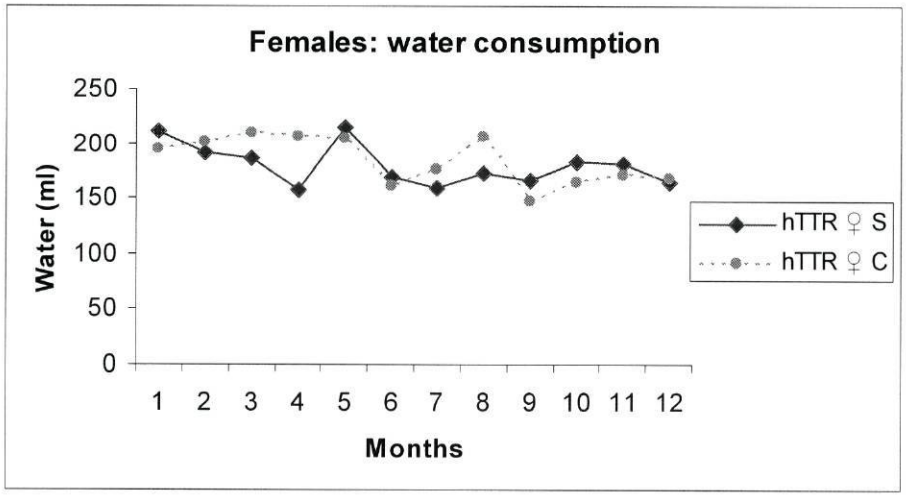


Chart 6-
Water consumption of female mice measured weakly for 12 months. The monthly mean is shown. S- stress; C- control.

Stress group mice characteristics

Besides the weight, food and water register, mice were observed daily in terms of social behaviour and interactions; body shape; health status and state of the fur. Male mice did not show an increased aggression due to being faced with stress. There was one group of 6 male mice from the stress treatment group that had, in the beginning, one animal presenting wounding in the back and tail due to fighting. After a few weeks this mouse recovered without any further wounds. In terms of amyloid and TTR deposition there was no difference in comparison to the other mice proceeding from the same group. One female from the stress group was found sick after 5 months of stress showing immobility and lack of interest for handling. At sacrifice (1 month later) this female had an enlarged heart and spleen. Air was found in the GI. This mouse did not show differences regarding subsequent tissue analysis.

The state of the fur in the stress group was not affected during the course of the experiments in comparison to control group. Mice submitted to stress lost their vibrissae but not facial hair, which can be sometimes observed in mice not subject to stress stimuli as well.

Liver

Since the liver protein expression is controlled in a precise regulated manner and is strongly influenced by the physiological condition of the organism, we measured the weight of the liver at sacrifice and normalized it to the body weight. There were no detectable differences between transgenic mice submitted to stress (chart 7), either males or females.

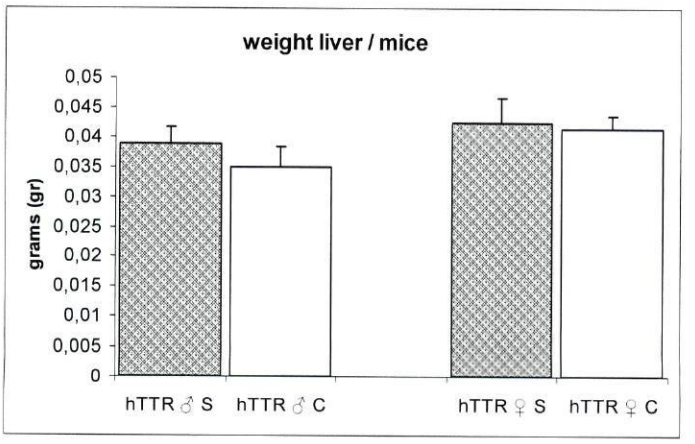


Chart 7-
Liver weight of the stress and control group, related to the body weight. S- stress; C- control.

Corticosterone levels

We analysed the corticosterone levels in the different strains following the stress schedule in comparison to control animals (chart 8).

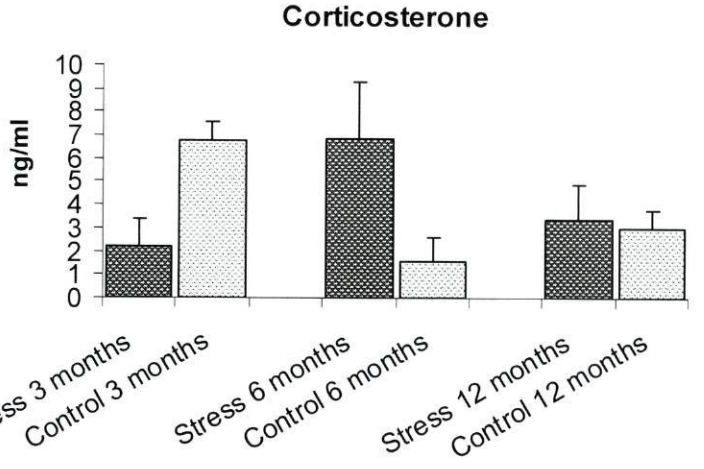


Chart 8-
Corticosterone serum levels of the experimental groups: stress and control. Mean measurements at different time points: 3, 6 and 12 months of treatments.

The pattern of corticosterone levels was different throughout the analysed time points. The only increase for the stress group was observed at 6 months, though the same would be expected in the stress groups of stress at 3 and 12 months.

The rise of corticosterone levels in the organism should in principle confirm the extent of the induced stress. Many different factors influence the levels of corticosterone; besides being upregulated by a stress situation, it follows a daily circadian rhythm which is higher in the morning. The serum was obtained either in the morning or the afternoon depending on the time when the mice were sacrificed. This gives some variability in the results obtained. Moreover, a physiological interference of aging in the stress response should also be taken into account, which would explain why 6 months group had a response while 12 months group don't; although it does not explain what happened with the 3 months group. Other factor that would not allow the values to reflect the stress conditions imposed, was the interval of time between the last stressor and the time of sacrifice which varied from 2 to 3 days. During this time the mice may have recovered from the last treatment.

TTR quantification

TTR protein was quantified in the serum by ELISA. In comparison to control mice no differences were obtained for the stress group, neither among the stress mice, evaluated at different periods of the stress treatments (chart 9).

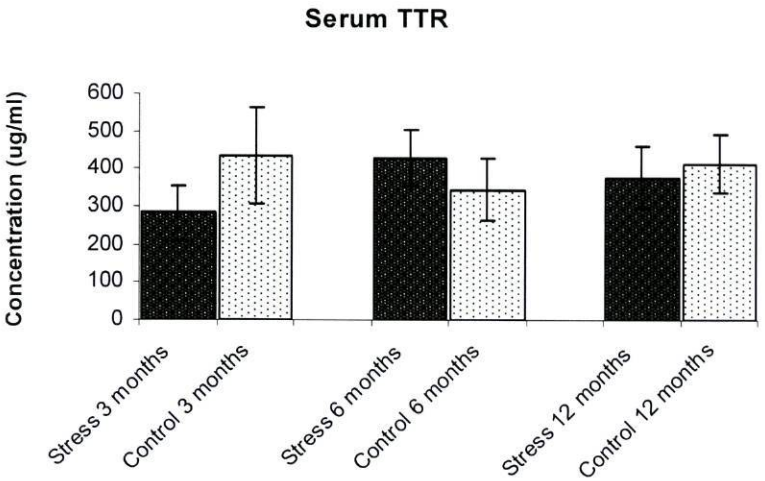


Chart 9-
TTR serum quantification measured by ELISA.

Amyloid and TTR deposition

Amyloid in the gastrointestinal tract was evaluated in the stress groups. The statistics of animals Congo red positive are displayed in table 2.

	3 months		*6 months*		*12 months*	
	Stress	Control	Stress	Control	Stress	Control
CR	**0/12**	**0/18**	**2/12**	**0/19**	**5/11**	**0/21**
Stomach	0/12	0/18	1/12	0/19	4/11	0/21
Intestine	0/12	0/18	2/12	0/19	1/11	0/21

	3 months		*6 months*		*12 months*	
	Stress	hTTR	Stress	hTTR	Stress	hTTR
IHC	**12/12**	**15/18**	**12/12**	**13/19**	**11/11**	**19/21**
Stomach	11/12	14/18	8/12	8/19	10/11	11/21
Intestine	10/12	10/18	8/12	5/19	9/11	8/21

Table 2-
Amyloid and TTR deposition analysed by Congo red (CR) and immunohistochemistry (IHC) respectively of the gastrointestinal tract: stomach and intestine.

At the age of three months none of the animals had TTR deposited as fibrils as it happens for the control group. At the age of 6 months some small percentage of the animals had Congo red positive TTR deposits either in the stomach or intestine (figure 1); while the control group did not develop amyloid. At 12 months the number of animals affected with amyloid deposition increased (5/11), while the control group was free of positive amyloid staining. Previous reports on this strain analysed at 13-18 months showed however an appreciable number of animals Congo red positive in the GI: 13 in 27 animals analysed (Sousa et al., 2002) which is close to the percentage of amyloid positive animals in the 12 months stressed mice. If a differentiation is made regarding the sex of the animal affected with amyloid, we see a preferential involvement of male mice in comparison to females: at 6 months, 2 male mice showed amyloid in the GI, while none of the females presented Congo red positive staining. At 12 months, 3 male mice displayed amyloid while 2 females were amyloid positive.

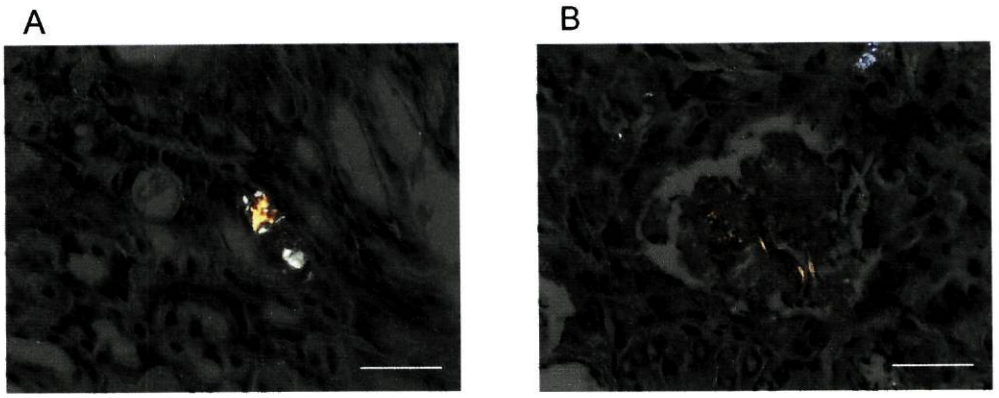


Figure 1-
Congo red binding in the stomach of 6 months (A) and 12 months (B) stressed mice. Scale bar 30 μm.

Regarding TTR assessed by immunohistochemistry, a small increase was detected in the stress mice: at the age of three months every animal have TTR deposition in the GI while the control group display a smaller percentage of mice (table 2). At older ages (12 months) the difference becomes similar between stress and control mice regarding the percentage of animals with TTR deposition in the GI. It appears that these small differences are not enough to be significantly related to the stress stimulus since it is just a slight increase in the percentage of positive animals for TTR deposition in comparison to the control group (table 2). Comparing the extent and amount of the TTR deposition in the GI, the observations were similar between the control and the stress group (figure 2), so mice faced with chronic mild stress, that were affected with TTR deposition did not present a higher amount of TTR deposited in tissues.

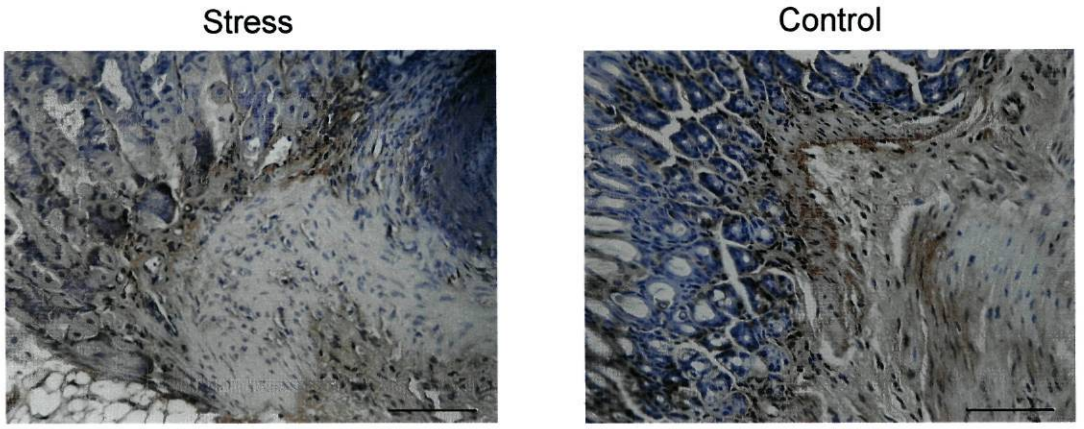


Figure 2-
TTR deposition in the stomach of stress and control mice. Scale bar 30 μm.

Additional studies of tissue TTR deposition included the peripheral nervous system. Animals with 12 months of age did not display positive TTR immunostaining (data not presented), indicating that, as previously reported, TTR aggregation was not extended to the peripheral nervous system.

Discussion

FAP, as a neurodegenerative disorder resulting from a genetic mutation in transthyretin, presents a wide spectrum of characteristics among and within populations such as incomplete penetrance, differences in clinical expression and sex ratio, and wide interval of ages of onset. TTR mutations are unquestionably responsible for FAP amyloidogenesis but clearly not sufficient for clinical expression. Possible explanations that support the discordant phenotypic expression include genetic background and/or environmental differences.

We proposed to assess the effect of chronic mild stress in triggering and influencing amyloidogenesis in FAP, using transgenic mice expressing the human TTR variant Val30Met.

It was a long time ago when it was investigated the effect of social interaction and wounding in amyloidosis (Page and Glenner, 1972). The observation that the submissive male, with wounds due to fighting, had a 100% incidence of amyloid in different organs was very interesting. Dominant mice were spared as were most of the females. A possible relationship between chronic inflammation and infection to amyloidosis of AA type was underlined in that study. Our male mice were not aggressive to each other and fighting was normal, not creating serious wounds. The only male mice affected with this behaviour, one of the submissive mice, did not present increase amyloid or TTR deposition. The only female that was probably sick did not show accelerated amyloid. In sum, if there is an effect of wounding and immunological response in FAP amyloidogenesis, our studies did not allow to determine this relationship. Of interest to know was the experiments performed in differently grouped mice related to spontaneous amyloidosis, many years ago. It was observed that many male mice caged together had more amyloid than when one male was grouped together with several females (Ebbesen, 1967). While our experimental outline did not embrace this situation – males mixed with females, we found that male mice had more amyloid than females that is consistent with the Ebbesen study. Or instead, the results are related to a gender influence on amyloidosis (Shtrasburg et al., 2004).

Other environmental factors influencing amyloid formation can be found in the literature. For example, isolation stress accelerated amyloid plaque formation in a mouse model of Alzheimer's disease (AD) (Dong et al., 2004), that was accompanied by a decrease in hippocampal proliferation. Inducing proliferation in hippocampus by drug administration, during the stress treatment, did not reduce amyloid, suggesting that stress had a role in the disease progression. Other evidence is the effect of food deprivation in the increase of tau hyperphosphorylation that compose neurofibrillary tangles, one hallmark of AD (Yanagisawa et al., 1999). These stressors were imposed to our experimental group, as well as others discriminated in the materials and methods section. Generally, no effects due to chronic mild stress were found on TTR deposition, but an effect on fibrillar deposition was seen, as it was observed for other experiments on different pathologies. What is important to have in mind is that the same stress regimen can have different effects depending on the strain (Ducottet and Belzung, 2005) and thus different results could be altered in the Val30Met kept in different backgrounds.

Unpredictable chronic mild stress is a long term continuous stress occurring in a randomly manner. The stress reaction is usually accompanied by various physiological responses. Among other manifestations, differences in the hormonal secretory pattern are observed resulting from activation of the pituitary-adrenal axis and sympathetic nervous system (Tsigos and Chrousos, 2002). The initial phase of activation stimulates the release of hormones such as corticosterone that have widespread systemic effects on metabolic, immunological and behavioural variables (De Kloet, 2004). Steroid hormone receptors are modular in structure. They have one ligand binding domain that in an inactive state is bound to heat shock proteins. Ligand binding releases the large multiprotein complex of chaperones thus activating the receptor (Muller et al., 2002). Although the primary goal of corticoids is protective, excessive elevation is detrimental. It can induce pathophysiological conditions, such as dysfunction of the immune system and neurodegeneration in aging and human affective disorders (Chrousos and Gold 1992; McEwen, 2000). Also, long term exposure to corticosteroids decreased the clearance of β-amyloid (Harris-White et al., 2001). On the other hand, they are important in terminating the stress response preventing damage caused by

excessive stimulation of neuroendocrine and immunological agents. After the response is complete, they return to basal levels. Our results only showed this elevation of corticosterone, due to stress treatments, for the 6 months group. This fact may be explained by different hypothesis. The knowledge that the increase is transient and does not subsist indefinitely is one possibility. Therefore, after a stressor, the levels may have been increased, but at the time we analysed them, after sacrifice, the values were close to the basal levels. Moreover, the long lasting effects of a stress stimulus when continuously repeated, probably diminish with repeated treatment and the magnitude of the response decreases across treatments, but for this the rise should have been observed at 3 months and not at 6 or 12 months, as was the case. Other possible explanation may come from the adaptation to the daily pattern of life which included one or two days of rest followed by some unpredicted disturbance, that is, a stress experience might sensitize the animal to further stressors that are not new for them and may be predicted. The timing of repeated stressors is also crucial to the experimental results (De Boer et al., 1990). Multiple stressors encountered over prolonged periods of time may not have an impact as do stressors separated from short periods. We could not impose such schedule for 12 months, since it would induce immunological adverse responses not associated with FAP. Therefore, the intervals of stressors may have reduced the effects on the mice, not resulting in the expected earlier and more extensive amyloidogenesis. Taken together, there are many variables that should be taken into account when analysing the corticosterone values.

Unpredictable chronic mild stress induces a depression-like behaviour in mice and affects the fur due to reduction of self-grooming (Mineur et al., 2003) which is important for body hygiene, thermoregulation, social communication and antibacterial protection (Spruijt et al., 1992). The fur state of the stress group was not different from the control group. This made us to suspect that the stressors were not inducing a visible effect on the mice. But the vibrissae were absent in the stress group and this was an indication of stress.

We also analysed differences related to liver weight induced by stress. The liver is the organ most affected in the acute phase response. The acute phase response is an orchestrated attempt to restore homeostasis in different situations: malnutrition,

inflammation, acute stress. Protein synthesis is augmented, mainly in the liver (Biolo et al., 1997) which is the main organ of TTR production. The synthesis of TTR is independently regulated in the liver and choroid plexus (Dickson et al., 1986). TTR is downregulated in the acute phase response (Qian et al., 1995). In fact, TTR was proposed to be directly and causally involved in the establishment of the stress reaction (Ingenbleek et al., 1999). Cytokines are known to have a role in the decreased levels of TTR which in turn lead to an increase in T4 and retinol, important for the injured body (Ingenbleek and Young, 1994). Thyroid hormones are important for corticosterone release in immobilization stress (Rodriguez et al., 2003) and retinol for the activation and modulation of the host defence systems (Yamamoto, 1991). IL-6 is one such cytokine involved in this regulation (Heinrich et al., 1990; Bartalena et al., 1992). This also occurs in situations of malnutrition. The liver also undergoes shifts in synthesis during malnutrition and inflammation (Wade et al., 1988; Ingenbleek and Bernstein, 1999). An optimal nutritional status confers a metabolic advantage so that the stressed body is able to develop appropriate responses of higher magnitude and longer duration. For these reasons the liver was studied in terms of the size which could indicate some possible damage, related to the stress condition. No differences in the weight of the liver were found indicating that the experiment did not affect this organ.

Our investigation of stress influence as an environmental modulator in FAP resulted in no differences in the onset, progression and tissue distribution of TTR deposits but an anticipation of amyloid deposition in transgenic mice. However, it is not straightforward to transpose this to humans since stress is a very complex situation that involves many mechanisms. And if in one hand humans are very complex and subjective, mice can obviously adapt better to an adverse situation than would humans. So variables present in humans may never be achieved in mice. So far, the conclusion can be applicable to mice; careful epidemiological studies in humans might establish a possible correlation between environmental factors, such as stress and its entire dimension with FAP pathology.

ACKNOWLEDGEMENTS
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CHAPTER 2

Enlarged ventricles, astrogliosis and neurodegeneration in Heat Shock Factor 1 null mouse brain

Enlarged ventricles, astrogliosis and neurodegeneration in Heat Shock Factor 1 null mouse brain

Sofia Duque Santos[1,2], Maria João Saraiva[1,2]

[1]Molecular Neurobiology Unit, Institute for Molecular and Cell Biology; [2]Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Portugal.

Corresponding author: Maria João Saraiva; Molecular Neurobiology; Instituto de Biologia Molecular e Celular. Rua do Campo Alegre 823. 4150-180 Porto. Portugal.
Tel. 351-226074900; Fax 351-226099157. e-mail: mjsaraiv@ibmc.up.pt

Running title: Brain abnormalities in HSF 1 null mouse

Abstract

Heat shock transcription factors mediate the regulation of the organism physiological maintenance and adaptation. We investigated the morphology and cellular expression of selected genes in brains of transgenic mice lacking the heat shock transcription factor 1, HSF1, the main transactivator under stress conditions. All HSF1 null mice displayed major brain morphological alterations: the lateral ventricles were markedly enlarged and the white matter reduced, as in ventriculomegaly. Heterozygous mice for the HSF1 gene also had these abnormalities albeit to a lower extent in comparison to the wild type, indicating a gene dosage effect. Cell loss, vacuolisation, amorphous eosinophilic cytoplasm and pyknotic nucleus were evident in the white matter, especially in periventricular regions. These areas also exhibited astrogliosis and neurodegeneration. The expression of heat shock protein hsp27 was up-regulated whereas αB-crystallin was down-regulated in different areas of HSF1 null mouse brain in comparison to control mice.

These data implicate HSF1 in maintaining the post-natal mammalian brain under non-stress conditions.

Introduction

Heat shock transcription factor 1 (HSF1) is the main stress inducible transactivator of heat shock proteins (Hsps) among the 3 mammalian HSFs (Sarge et al, 1991; Nakai et al, 1997). Hsps are highly conserved proteins that function as molecular chaperones preventing protein unfolding while restoring homeostasis. Their constitutive expression varies during embryogenesis, early postnatal growth and during adult life concerning protein levels and tissues (D´Souza and Brown, 1998). Accumulating evidence supports the general notion that the HSF1 regulatory pathway is required also under non-stressed conditions (Pirkkala et al, 2001), during reproduction, development and differentiation, albeit such effects are only partially characterized. HSF1 null mice were generated and reported viable to adulthood by Xiao et al (1998) but exhibited defects in the choriallantoic placenta, prenatal lethality, female infertility, elimination of the classical heat shock response and exaggerated tumor necrosis factor α when challenged by endotoxin. Elimination of HSF1 also resulted in a reduced cardiac expression of Hsp27 and αB-crystallin while hsp60 and hsp90 expression was unaltered (Yan et al, 2002). Homozygous embryos had no distinguishable morphological or microscopic alterations in soft tissues including the brain. However, a detailed analysis of the adult HSF1 null mouse brain is lacking. Interestingly, studies of HSF2 null mouse showed brain differences during embryonic stages and variable brain phenotypes during post-natal development (Kallio et al, 2002; McMillan et al, 2002; Wang et al, 2003). We report here analysis and characterization of the adult HSF1 null mouse brain.

Materials and methods

Animals

HSF1 null mice were kindly provided by Dr Ivor Benjamin from the Department of Internal Medicine, The University of Texas Southwestern Medical Center, Dallas, USA.

The experiments were carried out in 3 month old mice (n=10), male and female homozygous for HSF1 gene as determined by PCR, back-crossed into Sv129 background for 8 generations from the original Balbc x Sv129 hybrid strain (Xiao et al, 1998). Sv129 mice served as controls (n=6). For morphological studies we also used 1 month old mice homozygous (n=3) and heterozygous (n=3) for HSF1 gene.

The animals were housed in a controlled temperature room that was maintained under a 12 hour light/dark period. Water and food were available ad libitum. All animal experiments were carried out in accordance with the European Communities Council Directive.

RT-PCR analysis

To assess the differential expression of αB-crystallin, hsp27 and GFAP in different areas of the brain, 4 control and 4 HSF1 knock-out mice were sacrificed, the brain extracted and dissected. Hippocampus, striatum, cortex (including the external capsule), medulla oblongata and cerebellum were isolated and homogenised in trizol buffer (GibcoBRL). Total RNA was extracted according to supplier's instructions. cDNA was synthesized by using superscript first-strand synthesis system for RT-PCR with oligo (dT) primer (Invitrogen). Primers for αB-crystallin, hsp27 and GFAP were designed as follows: alpha B-crystallin (sense)-cctgttggagtctgacctcttc, (anti-sense)- gtttccttggtccattcacagt. Hsp27 (sense) acgtcaaccacttcgctccggagg, (anti-sense) cttggctccagactgttcagacttcgctgac. GFAP (sense) ctccttgtctcgaatgactcct, (anti-sense) caacctttctctccaaatccac. Normalization was performed with beta-actin primers. The amplification products were quantified with ImageQuant software. Statistical analyses were performed by a two-tailed, paired Student's t-test. The significance level was set at $p<0.05$.

Protein extraction and Western blots

In order to analyse protein levels for GFAP, brain tissue proteins from hippocampus, striatum, cortex (including the external capsule), medulla oblongata and cerebellum of four control and four HSF1-KO mice were extracted by homogenising tissues at 4°C in suspension buffer (NaCl 0.1 M; Tris-Cl pH 7.6, 0.01M; EDTA pH8.0, 0.001 M and complete protease inhibitor cocktail), followed by sonication for 30 seconds in Laemmli Buffer (SDS 4%, Tris-Cl pH 6.8, 0.12 M, glycerol 20% and DTT 0.2 M). The homogenates were centrifuged at 4°C for 5 minutes at 5000 g.

Proteins were quantified by BIO-RAD protein assay (BIORAD) and by optical density at 280 nm. 100 μg of each protein extract was resolved in a 12% polyacrylamide gel (SDS-PAGE) after 5 minutes boiling, transferred to a nitrocellulose membrane and probed against GFAP (Santa Cruz, dilution 1:800). Goat IgG-HRP was employed as the secondary antibody at a 1:10000 dilution. The blot was developed using ECL-plus chemiluminescent reagent (Amersham) and exposed to an X-ray film. Protein expression was quantified using the Scion program and the significance of the differences was determined with a two-tailed, paired Student's t-test. Statistical significance was assessed when $p<0.05$.

Brain tissue preparation

Mice were anaesthetised at the time of killing and transcardially perfused with 4% paraformaldehyde in PBS. The brain was fixed for 2 hours with 4% paraformaldehyde in PBS, left in 30% sucrose overnight at 4°C, and frozen. 30 μm sections were cut on a sliding microtome cryostat at –20ªC. Sections were collected in PBS and stored in cryoprotective solution at –20°C until use.

Tissue analysis

Nissl staining was performed with 0.1% thionin for 10 minutes at room temperature. Fluorojade B (Histochem Inc) staining was done according to Schumed and Hopkins, 2000.

For immunohystochemistry, brain coronal sections were incubated in blocking buffer (5% calf serum in PBS-T) for 2 hours at room temperature, followed by overnight incubation with the primary antibody in blocking buffer at 4°C. Primary

antibodies included: goat anti-GFAP (Santa Cruz Biotech, dilution 1:400); goat anti-Hsp27 (Santa Cruz Biotech, dilution 1:600); rabbit anti-αB-crystallin (Calbiochem, dilution 1:1000); and mouse anti-nitrotyrosine (Zymed Laboratories Inc., 1:3000). The sections were incubated with the corresponding biotinylated secondary antibody for 1 hour at room temperature and then with extravidin (Sigma, dilution 1:20) for 45 minutes at room temperature. Vector M.O.M. immunodetection kit was used to detect reactivity of mouse anti-nitrotyrosine according to the supplier's instructions. The reaction was developed with 3,3´diaminobenzidine as chromogen. Free-floating sections were mounted on 1% gelatine coated slides, air dried for 48 hours at room temperature and counterstained with thionin. Semi-quantitative analyses of immunohistochemistry were performed with Scion image program that measures the area occupied by immunohistochemical substrate colour's in pixels. Each brain section was analysed in two different selected areas.

RESULTS

We analysed brains of HSF1 null animals of 1 and 3 months of age in comparison to control mice. All HSF1 null mice displayed major brain morphological alterations in comparison to normal mice, the lateral ventricles were markedly enlarged and the white matter reduced (as documented in figure 1A, right and left panels). There is no thinning of the cerebral cortex and stenosis of the Sylvius aqueduct. The third ventricle was just slightly larger than normal and the size of the fourth ventricle was unaltered. No other developmental morphological alterations were detected including the hippocampus and cerebellum. The changes detected were not different between 1 and 3 months old animals meaning that the abnormalities found occur either pre-natally, after embryonic day 18.5, or early post-natally before 1 month of age. We then analysed heterozygous mice for HSF1 and found that the enlarged lateral ventricles were smaller than the null mice but still larger than wild type animals, as shown in figure 1A (middle panel), suggesting that the brain abnormality found is influenced in a gene-dependent manner.

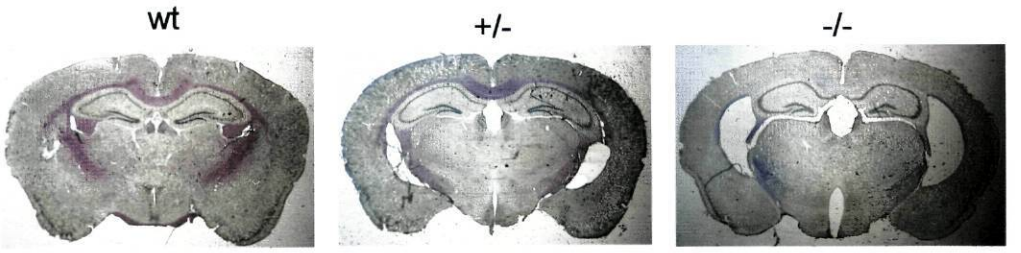
Fluorojade-B was used to investigate neurodegeneration. Staining of neurons from caudate putamen (striatum) (figure 1B, right) indicated neurotoxicity and aberrant neuronal activity that was absent in the wild type control brain.

By using Nissl staining, spongiosis of HSF1 null tissues in caudate putamen, external capsule and corpus calosum was evident (figure 1C i), with pyknosis of the nucleus and amorphous cytoplasmic eosinophilia.

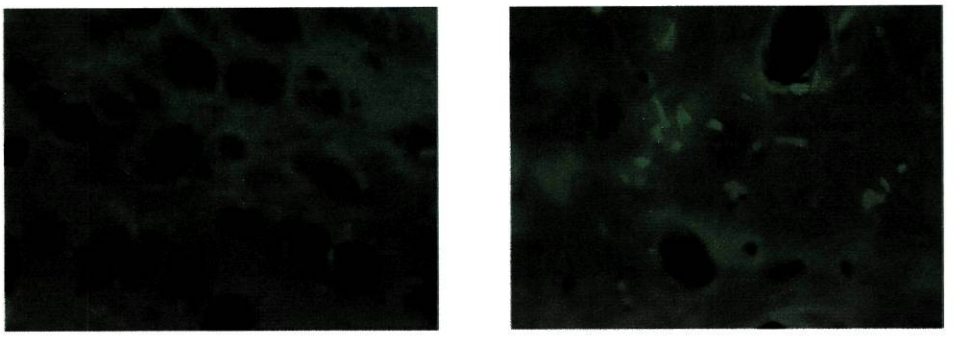
Glial fibrillary acidic protein (GFAP) reactivity was increased in HSF1, particularly in striatum and external capsule tissues in comparison with control tissues as shown by immunohistochemistry (figure 1C ii). Reactive astrocytes were star shaped and with longer processes when compared to normal control brain. Semi-quantification of GFAP expression was performed by RT-PCR and Western blot in hippocampus; striatum; cortex; medulla oblongata and cerebellum. An over-expression of GFAP in HSF1-KO mice brain was evident (figure 1C iii and iv) particularly in hippocampus, striatum, cortex, and cerebellum that are in agreement with the histochemical results.

Figure 1

A.



B.



C.

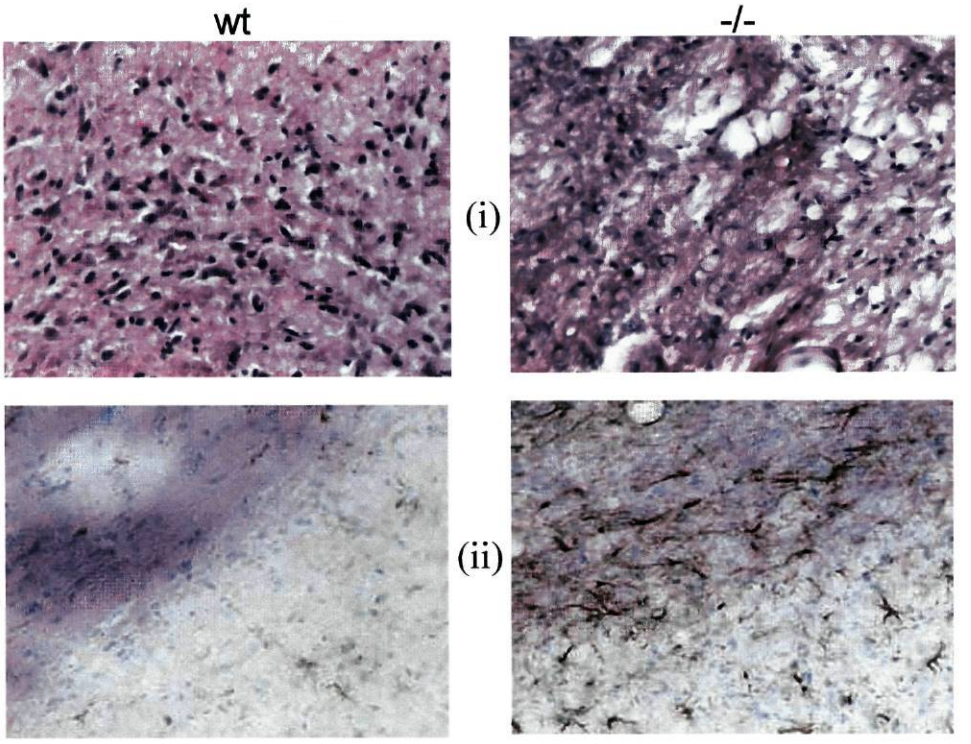


Figure 1C.

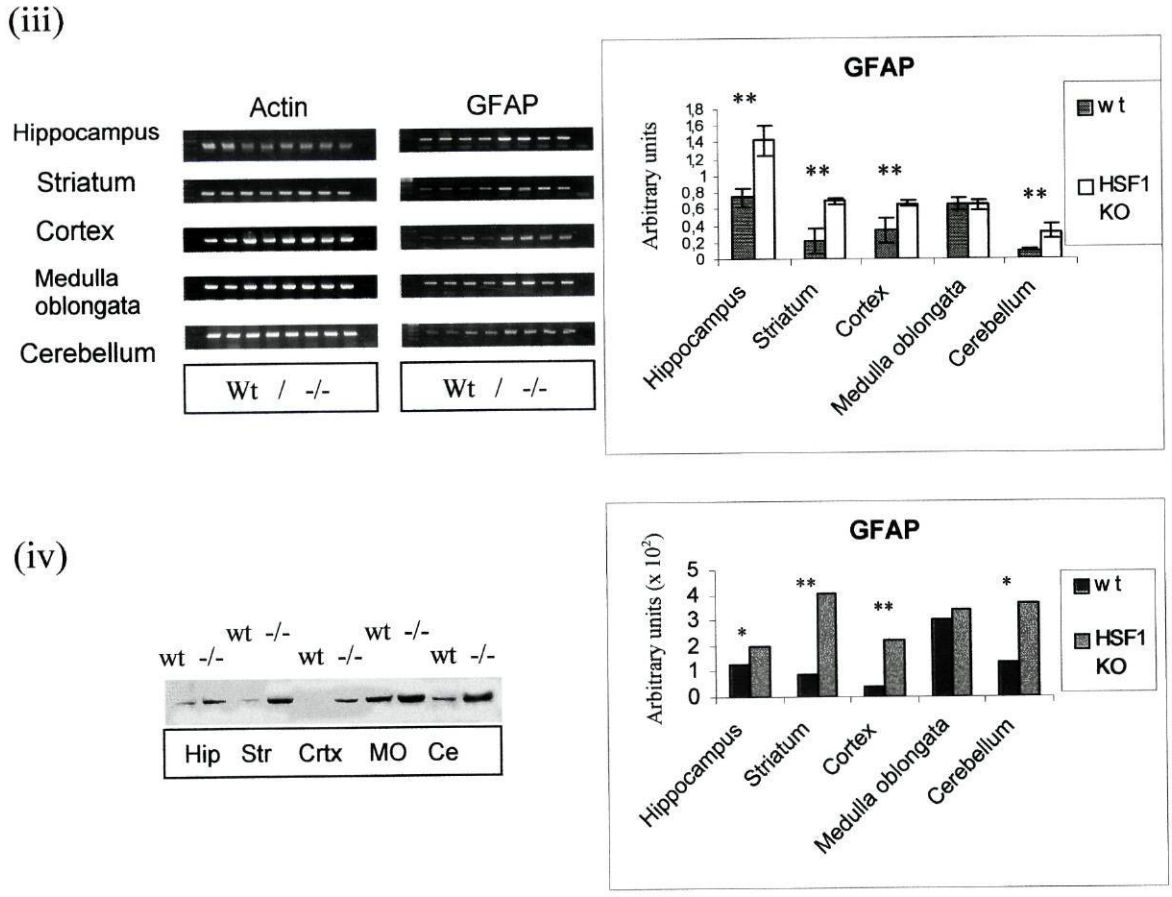
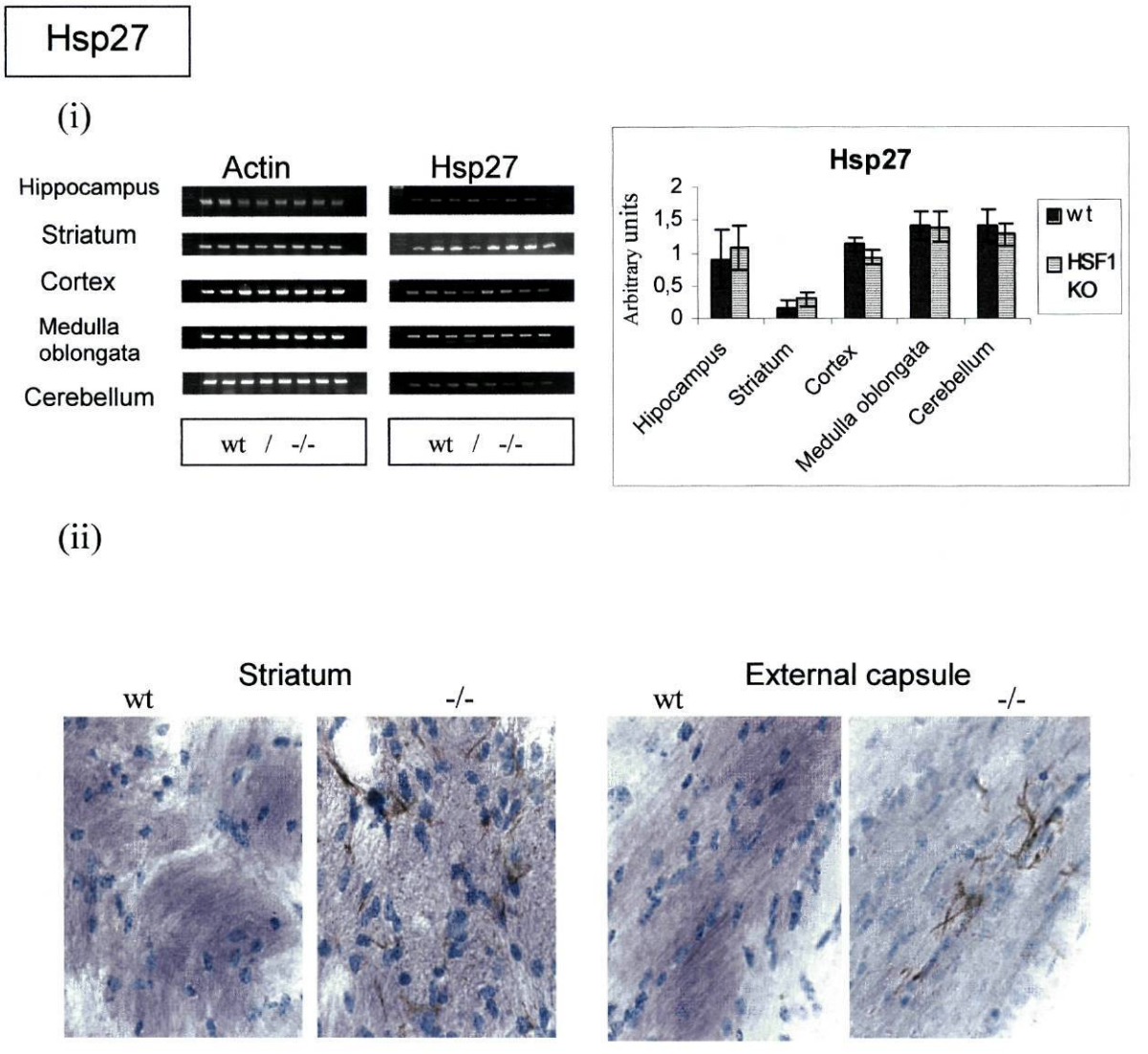


Figure 1-
Brain abnormalities of HSF1 null mouse
A- Enlarged lateral ventricles. Thionin counterstaining of brain coronal sections: wild type mice (left, n=6), heterozygous (middle, n=3) and homozygous (right, n=10).
B- Neurodegeneration. Fluorojade staining of caudate putamen neurons in homozygous mice (right, n=5) versus control animals (left, n=4). Magnification 100X.
C- Gliosis in homozygous (right, n=10) mice versus wild type animals (left, n=6).
(i) Spongiosis. Haematoxylin and eosin counterstaining of striatum. Magnification 50X.
(ii) Astroglyosis. Anti-GFAP staining of the external capsule. Magnification 50X.
(iii and iv) Semi-quantitative analyses of GFAP by RT-PCR (iii) and Western blot (iv); n=4 for each group of wt and HSF1 KO (-/-) animals. Quantitative analyses with Image Quant program for RT-PCR and with Scion Image program for protein blots (Student's t-test *, $p<0.05$; **, $p<0.02$).

Two genes under the control of HSF1, belonging to the small hsp family, were next studied: Hsp27 and αB-crystallin. While performing comparative hsp27 expression by RT-PCR no significant alteration was found in HSF1 null animals (figure 2 i). However, when we quantified hsp27 expression by semi-quantitative immunohistochemistry, there was a higher expression of hsp27 by astrocytes in the

external capsule and striatum of HSF1 null mice (figure 2 ii, right figures), whereas neurons of the medulla oblongata and cerebellum presented the same intensity of staining in the 2 strains of mice (fig 2 ii right chart). The discrepancy between hsp27 total RNA and protein is explained by the presence of other cell types in homogenates that dilute the differences in expression observed in specific cell types by immunohistochemistry. Taken together, these data indicates that Hsp27 might be regulated by different mechanisms in distinct cell types of the central nervous system.

Figure 2



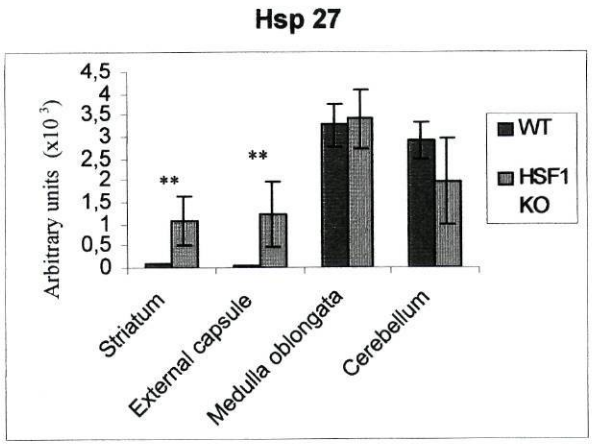


Figure 2-
Hsp27 expression.
(i) RT-PCR of hippocampus, striatum, cortex, medulla oblongata and cerebellum from wild type control animals (wt, n=4) and HSF1 KO mice (-/-, n=4). Quantitative analyses with ImageQuant program.
(ii) HSP27 immunohistochemistry of external capsule and striatum of HSF1 KO mice (-/-, n=6, right side) versus control mice (wt, n= 5, left side). Magnification 100X. Chart: Hsp27 quantification with Scion image program. (Student's t-test **, $p<0.02$)

RT-PCR was carried out as to examine a possible differential expression of αB-crystallin. In HSF1 null mice a significant decrease was found for cerebellum, and less strongly for medulla oblongata as in figure 3 i. By immunohistochemistry, αB-crystallin expression was considerably decreased in HSF1 mice comparatively to control mice (figure 3 ii), namely in the external capsule, medulla oblongata and cerebellum specifically in the central medulla of white matter and the inner granular cell layer. Noteworthy was the lower αB-crystallin expression in cerebellum where HSF1 levels were reported to be higher (Brown and Rush, 1999). The difference in the medulla oblongata and cerebellum observed by immunohistochemistry that was not detected by RT-PCR might be explained by the reason pointed out above, i.e. the cells expressing αB-crystallin identified by immunohistochemistry might be underrepresented in the brain extracts.

Figure 3 Alpha B-crystallin

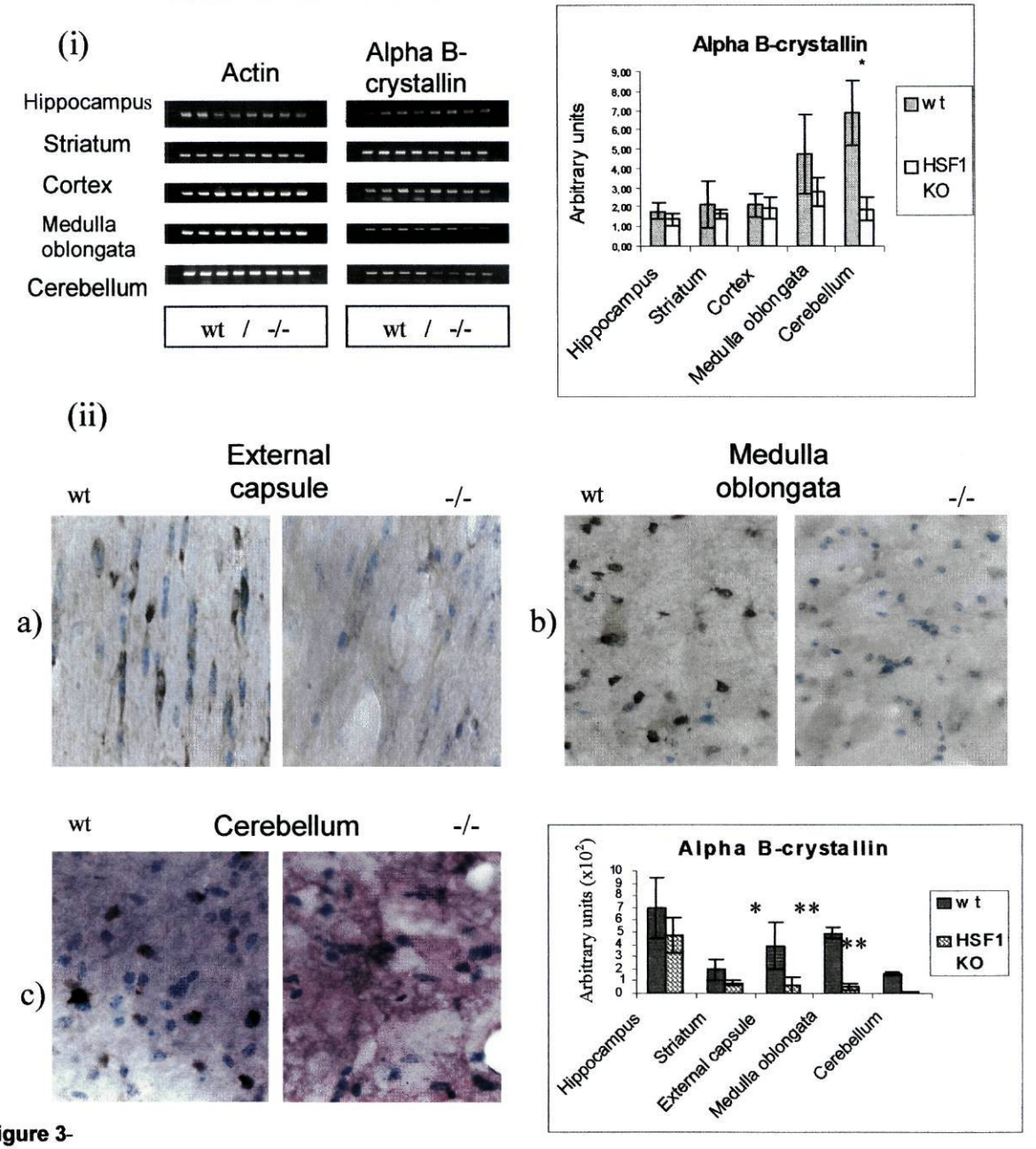


Figure 3-

(i) RT-PCR of αB-crystallin in hippocampus, striatum, cortex, medulla oblongata and cerebellum from wild type control animals (wt, n=4) and HSF1 KO mice (-/-, n=4, right side). Quantitative analyses with ImageQuant program. (Student's t-test *, $p< 0.05$)

(ii) αB-crystallin immunohistochemistry of: (a) external capsule, magnification 150X; (b) medulla oblongata, magnification 100X and (c) cerebellum, magnification 100x of HSF1 KO mice (-/-,n=6) and wild type mice (wt, n=5). Chart: αB-crystallin quantification with Scion image program. (Student's t-test *, $p<0.05$; **, $p<0.02$)

Nitrotyrosine staining, indicative of oxidative modification, was indistinguishable between HSF1 null and wild type mice (data not shown), meaning that redox homeostasis is unchanged, neither as a consequence nor as a cause of these abnormalities.

DISCUSSION

Besides the well-known involvement of HSF1 under stressful conditions, our data in HSF1 null mice supports its significance in the central nervous system under physiological conditions. HSF1 suppression resulted in brain with enlarged lateral ventricles (ventriculomegaly), astrogliosis and neurodegeneration in specific areas.
Ventriculomegaly refers to an enlargement of the ventricles resulting probably from an atrophy of the brain parenchyma, brain tissue destruction (which is in accordance with the white matter reduction observed) or insufficient brain growth to fill the voided space, rather than impaired cerebrospinal fluid dynamics that is associated with hydrocephally. Animal models have been studied that show ventriculomegaly such as the knock-out mice for the enzyme adenosine deaminase (Turner et al, 2003) or an induced ventriculomegaly such as by A1 adenosine receptor activation in rats (Turner et al, 2002) or by hypoxia conditions (Ment et al, 1998). We exclude the term hydrocephalus for characterizing HSF1 null mouse brain since there is no stenosis of the Sylvius aqueduct, no thinning of the cerebral cortex and as well as no characteristic enlarged domed shaped head.
In HSF2 null mice model studies, conflicting data have arisen: a study describes HSF2 null brain as normal (McMillan et al, 2002), whereas other indicates abnormalities in the adult brain (Kallio et al, 2002) and yet some other authors detected brain alterations during embryonic development and variability during adult stages (Wang et al, 2003). The existence of multiple HSFs can account for pleiotropic functions and for differences in the different phenotypes found in HSF2 null mice. Accumulating evidence is indicating that there are distinct target genes for different HSFs although a complex regulation must not be excluded. While HSF2 knock-out mice have morphological observations by the two last studies that related it to hydrocephalus, our histological observations of HSF1 null mouse brain did not reveal hydrocephalic brain neither obstruction of CSF flow between the ventricles, but showed cell loss with the appearance of vacuoles, eosinophilic cytoplasm and piknotic nucleus, particularly in periventricular areas and an extensive white matter decrease that can relate HSF1 KO mice to ventriculomegaly.

HSF1 expression is different in the brain during different developmental stages, and it does not parallel HSF2 expression (Walsh et al, 1997; Brown and Rush, 1999). We have not performed studies in embryonic stages as this aspect was addressed by others: until E18.5, the HSF1 null brain is normal (Xiao et al, 1999) and so the brain abnormalities now found occur after, but at very early stages before 1 month of age.

We started by addressing alteration in gene expression by investigating Hsp27 and αB-crystallin. These genes were previously shown important in protecting cells against stress and apoptosis and have been involved in cellular repair (Kamradt et al, 2001 and Salvador-Silva et al, 2001). Our data shows that the expression of these 2 small hsps is altered in non-stress conditions between normal control mice and HSF1 knock-out mice, with overexpression of Hsp27 in some areas of the brain while others have the same basal expression, and down-expression of αB-crystallin in specific brain areas of HSF1-KO. This is in contrast with a reduced Hsp27 expression in cardiac tissues from HSF1 null mouse (Yan et al, 2002), reflecting tissue specific transcriptional regulation for heart and brain Hsp27 but not for αB-crystallin, as cardiac tissues from HSF1 null mice also had a lower expression of this chaperone. Our results suggest that Hsp27 expression is probably regulated in brain by transcription factors other than HSF1. The higher Hsp27 expression found in HSF1 null mouse brain external capsule and striatum most probably did not reach the level necessary to avoid cell damage.

αB-crystallin is present in astrocytes and oligodendrocytes and by binding to caspase-3 prevents cell death (Kamradt et al, 2001). It also disaggregates GFAP inclusions (Koyama et al, 1999). Therefore, down-expression of αB-crystallin in HSF1 mice might be associated with the observed gliosis and neurodegeneration.

Taken together, our data indicates that, under non-stress conditions, the basal expression of hsps is influenced by HSF1 in the central nervous system and implicates its role in maintaining the normal brain during mammalian post-natal development The mechanisms of regulation of gene expression underlying the abnormalities found need to be addressed in the future.

Most of the HSF1 target genes are probably unknown and their influence in the normal cell function remains to be elucidated. In view of this, a wide comparative tissue analyses of gene expression in HSF1 null mice under normal and stress conditions tissues is warranted using this and other HSF null mice strains.

ACKNOWLEDGEMENTS

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CHAPTER 3

HSF1-KO mice expressing human mutant transthyretin V30M: a novel model for familial amyloidotic polyneuropathy affecting the peripheral nervous system

HSF1-KO mice expressing human mutant transthyretin V30M: a novel model for familial amyloidotic polyneuropathy affecting the peripheral nervous system

Sofia Duque Santos[1,2], Maria João Saraiva[1,2]

[1]Molecular Neurobiology Unit, Institute for Molecular and Cell Biology; [2]Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Portugal.

Corresponding author: Maria João Saraiva; Molecular Neurobiology; Instituto de Biologia Molecular e Celular. Rua do Campo Alegre 823. 4150-180 Porto. Portugal.
Tel. 351-226074900; Fax 351-226099157. e-mail: mjsaraiv@ibmc.up.pt

Running title: Familial amyloidotic polyneuropathy mouse model

Abstract

Familial amyloidotic polyneuropathy (FAP) is a hereditary neurodegenerative disease affecting specially the peripheral nervous system (PNS). The cause of this life threatening pathology is the tissue deposition of mutant transthyretin (TTR) in the form of amyloid fibrils, presenting early non-fibrillar TTR deposits in the pre-clinical phase and signs of inflammatory and oxidative stress. The FAP mouse models characterized so far do not recapitulate the whole features when compared to humans, since the peripheral and autonomic nervous systems are spared. We have generated a new transgenic mouse model expressing the human TTR V30M crossed into a heat shock transcription factor 1 null background which present more extensive and earlier non-fibrillar TTR deposition in distinct organs; furthermore, deposition is found in the peripheral (dorsal root ganglia and nerve) and autonomic nervous systems, not observed in the described FAP mouse models. Similarly to asymptomatic carriers of V30M, inflammatory and oxidative stress was detected in the PNS. The results suggest that genes under HSF1 control are involved in FAP pathogenesis. In fact, upregulation of hsp70 and hsp27 was detected in FAP tissues. The novel transgenic mouse model here described is of utmost importance for testing therapeutic strategies to fight the disease and serves as a model to address the influence of the stress response in protein misfolding-related diseases.

Introduction

Several hereditary mutations in human plasma transthyretin (TTR) have been identified and related to familial amyloidotic polyneuropathy (FAP). The most common variant TTR is the substitution of a valine for a methionine at position 30 – TTR V30M (Saraiva et al., 1984), resulting in a destabilized protein that easily aggregates, deposits in tissues and forms amyloid. It is a systemic pathology with the peripheral nervous system (PNS) specially affected: nerve fibres, plexuses, sensory and autonomic ganglia (Coimbra and Andrade, 1971a,b). Initially Congo red negative non-fibrillar TTR aggregates are deposited extracellularly in close proximity to Schwann cells and collagen, before assembling into mature amyloid fibers – FAP 0- (reviewed in Sousa & Saraiva 2003). These aggregates were shown to be more toxic in cell culture as compared to native fibrils as studied by Sousa et al. (2001b), and corroborated later by other studies (Andersson et al., 2002 and Reixach et al., 2004). The unmyelinated fibres are first affected while the myelinated fibres begin degeneration later.

Activation of NF-kB has been observed in FAP nerves (Sousa et al., 2000b) and salivary glands (Sousa et al., 2005) involving activation of the receptor for advanced glycation end products – RAGE- (Sousa et al., 2001a), triggering inflammatory and oxidative pathways that culminates with neuronal loss. Thus, TTR deposits as a cellular stress, result in increased protein expression of pro-inflammatory mechanisms, such as tumor necrosis factor-alpha (TNF-α), interleukin1-beta (iL1–β) in the axons of affected individuals (Sousa et al., 2001a) and elevated nitration, measured by nitrotyrosine (3-NT).

Until now, there is no effective therapy for this neurodegenerative disease that ultimately leads to death. The search for pharmacological agents that can prevent/retard the development of FAP as well as the knowledge of the underlying mechanisms of pathogenesis encompasses the study of animal models, which have proven to be crucial tools. Several groups have generated transgenic mice carrying the human *ttr* V30M gene. Sakaki et al., (1986) have expressed this protein by an inducible heterologous promoter, the metallothionein (MT-promoter), in the hybrid background C57BL/6XC3H. These mice produced the transgene at

very low amounts, and as they suggested, it was probably not enough to induce amyloid deposition. Only the liver and yolk sac but not the choroid plexus expressed TTR, distinguishing these transgenic mice from the humans. Another attempt was performed by using the human promoter instead: the human *ttr* V30M plus a 600 bp upstream sequence that included the native TTR promoter (Yamamura et al., 1987). Again, low TTR serum concentration and absence of choroid plexus TTR synthesis were found. Shimada et al. (1989) compared the inbred strain of mouse C57BL/6 with the human *ttr* V30M plus the 600 bp upstream sequence with the strain having the structural gene of the human TTR V30M ligated to the MT-promoter. Amyloid deposition related to TTR started at 15 months of age in the first strain and at 6 months of age in the second, beginning in the mucosa of the small intestine. With aging amyloid deposition aggravated and extended to other organs but never to the peripheral nerves.

When a distal enhancer element in the mouse TTR gene was found (Costa et al., 1988) it was assumed that these sequences were required for the appropriate human TTR expression in the transgenic mice, and so another transgenic mouse was generated that had a 6kb upstream sequence to human TTR (Nagata et al., 1995). These mice showed the same TTR expression levels in the serum as found in man and 10 times more than those mice having only the 0.6 kb upstream region. TTR expression was not confined only to the liver and yolk sac but the choroid plexus was also found to express TTR. The characterization of amyloid deposition was performed later (Takaoka et al., 1997): amyloid deposition started at 9 months in the GI tract and at the age of 24 months the pattern of amyloid deposition was similar to the autopsied cases of FAP except for the absence of TTR in the peripheral and autonomic nervous system. The same pathological features were observed when these transgenic mice were backcrossed to a TTR-null background (Kohno et al., 1997). In our laboratory, transgenic mice expressing the human TTR-L55P, one of the most aggressive human variant TTR found in nature, were generated (Sousa et al., 2002). TTR deposition and the presence of TTR fibrils were investigated by immunohistochemistry and Congo red binding, respectively. When compared to the TTR V30M mice, they present earlier, non-fibrillar TTR deposition (1-3 months). Later, amyloid was detected in the GI tract and skin. The cytotoxicity of these deposits was tested by nitrotyrosine immunostaining and a

twofold increased level was found in tissues related to TTR deposition which are in accordance with the effects of cellular stress caused by TTR presence in human FAP tissues. However, again in this case, no deposition was found in peripheral nerves.

TTR synthesis and deposition are asynchronous in time and space: the liver and the choroid plexuses of brain are the main organs responsible for its synthesis but deposition occurs extracellularly at distant sites. Interestingly, intracellularly no aggregate inclusions were ever found in the liver neither in other affected organ in FAP. So the hypothesis that there is a compromised function in the quality control during TTR synthesis can be excluded. But the question of which factors are intervening in the extracellular aggregation remain poorly explored. TTR intrinsic propensity to acquire a pathological conformation is probably not the sole factor. One possible defence mechanism in harsh extracellular conditions is the stress response; therefore, disruption of this pathway could be affected or affect TTR amyloidosis.

To test this hypothesis we generated a new mouse strain by crossing mice lacking the main heat shock transcription factor involved in the regulated synthesis of chaperones (HSF1), and transgenic mice expressing the human TTR V30M.

These mice display many pivotal characteristics absent in the previous mouse models such as TTR deposition in the peripheral and autonomic nervous systems, high levels of oxidative stress in tissues related to the presence of aggregated TTR, and upregulation of pro-inflammatory cytokines in the PNS. This model represents a close approach to the study of FAP and opens a new window for the involvement of the stress response in this neurodegenerative disease, never addressed before.

Material and Methods

Animals

HSF1-KO mice were a kind gift from Dr Ivor Benjamin from Utah Health Sciences Center, USA (Xiao et al., 1999). By mating HSF1-KO mice in the Sv129 background with transgenic mice for human TTR V30M carrying the human homologous promoter (Nagata et al., 1995) for more than 9 generations we obtained hTTRV30M/HSF1-KO, labelled **hmTTR/HSF1-KO** mice. By crossing HSF1-KO mice with the mouse line carrying a null mutation at the endogenous *ttr* locus and expressing the human mutant *ttr* gene 6.0-hV30M described by Kohno et al., (1997) we obtained TTRV30M x TTR-KO/ HSF1-KO, labelled **hTTR/HSF1-KO**.

The animals were housed in pathogen-free conditions, in a controlled temperature room, maintained under a 12 hour light/dark period. Water and food were freely available. All animal experiments were carried out in accordance with the European Communities Council Directive.

Since HSF1-KO homozygous females are infertile (Xiao et al, 1999; Christians et al., 2000) all crosses were performed between homozygous males and heterozygous females. The offspring was analysed by PCR to determine homozygoty or heterozygoty for HSF1 using two pairs of primers, one pair annealing within the area deleted in the HSF1-KO mice (Xiao et al., 1999) constituted by the sense primer at the intron 4 (5′-TCTCCTGTCCTGTGTGCCTAGC-3′) and the anti-sense primer at intron 6 (5′-CAGGTCAACTGCCTACACAGACC-3′) and another pair of primers, the sense primer annealing at the introduced *neo* cassette of the HSF1-KO mice (5′-AGGACATAGCGTTGGCTACCCGTG-3′) and the anti-sense annealing at the end of the *neo* cassette and after, in the *hsf1* gene. In the case of hTTR/HSF1-KO, primers were designed for the disrupted exon 2 of mouse TTR: 5′-CTTGTCTCCTCTGTGCCCAG-3′; 5′-GGTGGGCTTTCTACAAGCTT-3′, sense and anti-sense primers respectively (Episkopou et al., 1993). The presence of human TTR was tested by an ELISA procedure for plasma TTR with a polyclonal antibody to human TTR (DAKO) as a capture antibody and an anti-human TTR coupled to HRP as a detection antibody, as described earlier (Sousa et al., 2002).

Heterozygous HSF1-KO mice, transgenic for human TTR V30M with and without endogeneous mouse TTR are labelled **hmTTR/HSF1-het** and **hTTR/HSF1-het**, respectively.

Tissue analysis

Mice were anesthetised intraperitoneally and sacrificed. These included: hmTTR/HSF1-KO (3, 6 and 12 months of age), hTTR/HSF1-KO (3 and 6 months of age), hTTR/HSF1-het and hmTTR/HSF1-het (3 months of age each strain). Stomach, esophagus, small and large intestine, liver, pancreas, skin, kidney, spleen, salivary glands, eye, tongue, heart, lung, reproductive system (testis or uterus) brain, spinal cord, sciatic nerve and dorsal root ganglia (DRG) were collected, fixed in neutral buffered formalin and embedded in paraffin. The same tissues were collected and frozen immediately in dry ice and stored at -70°C until use. Congo red staining was performed as described by Puchtler and Sweat (1965) and visualized under polarized light microscopy. For immunohistochemistry, tissue paraffin slides were deparaffinated and hydrated. Endogenous peroxidase was destroyed before blocking with 1%BSA, 4% fetal bovine serum in PBS for 1 hour at 37°C. Primary antibodies, diluted in blocking buffer, were incubated in a humidified chamber, overnight (o/n) at 4°C except anti-nitrotyrosine and anti-hsp70 antibodies which used 1 hour at room temperature before incubation o/n at 4°C. Primary antibodies used included: rabbit anti-human TTR (1:1000, DAKO); rabbit anti-mouse TTR (1:1500, produced from recombinant mouse TTR); rabbit anti-nitrotyrosine (1:1000, Chemicon); goat anti-TNF-α (1:25, Santa Cruz Biotechnology, Inc.); goat anti iL1-β (1:25, Santa Cruz Biotechnology, Inc.); mouse anti-MCP-1 (1:1000, Chemicon); mouse monoclonal NCL-MACRO (1:100, NOVOcastra); rabbit anti-RAGE (1:50, Santa Cruz Biotechnology, Inc.); rabbit anti-NF-kB (1:500, Santa Cruz Biotechnology, Inc.). Corresponding secondary antibodies used were biotin-conjugated (1:20 Sigma), followed by extravidin labelling (1:20, Sigma). Each antibody was incubated for 30 minutes at 37°C. Mouse monoclonal antibodies were performed using the MOM kit (Vector) according to the supplier's instructions. Anti-nitrotyrosine, anti-RAGE and NCL-MACRO were used after high temperature antigen unmasking technique with sodium citrate buffer. The reaction was developed with 3-amino-9-ethyl carbaxole, AEC (Sigma) or 3,3´-diaminobenzidine DAB (Sigma), counterstained with haematoxylin and mounted with an aqueous

mounting medium from DAKO. In some control experiments, the primary antibody was replaced by blocking buffer; control analysis of TTR in the PNS included pre-absorption of the primary antibody with human recombinant TTR as described before (Sousa et al., 2002).

Semi-quantitative analysis of immunohistochemical slides was done using Scion image quant program that measures the area occupied by the substrate reaction colour and normalized to the total image area. Each animal tissue was evaluated in four different areas. Mean values of % occupied area are displayed with the corresponding standard deviation. Statistical analyses were performed by a two-tailed, paired Student's *t*-test. The significance level was set at $p<0.02$ (*) and $p<0.002$ (**).

The presence of human TTR in mice parasympatic ganglia of the stomach and intestine was studied by immunofluorescent staining with rabbit anti-human TTR (1:1000, DAKO) and mouse anti-PGP9.5 (1:600, Biogenesis) as the primary antibodies; secondary antibodies were, respectively: Alexa Fluor 488 anti-rabbit and Alexa Fluor 568 goat anti-mouse, at a dilution of 1:1000 in blocking buffer, 30 minutes at room temperature, protected from light. After washing with PBS, slides were mounted with vectashield (Vector) and visualized in a Zeiss Cell Observer System microscope (Carl Zeiss, Germany) equipped with filters for FITC and rhodamine like dyes.

Tissue TTR extraction

A method developed by Kaplan (1994) was employed to extract deposited aggregated and/or fibrillar TTR in selected tissues (skin, stomach and nerve) of some mice, as indicated in the results section.

To perform the experiment in nerve, we pooled sciatic nerves from 4 different mice (summing approximately 50 mg of tissue). For skin and stomach we used 100-150 mg of tissue. These tissues were homogenized on ice with 1 ml of PBS using a glass rod and centrifuged for 10 minutes at 4ºC, 14,000 rpm. This wash was repeated three more times to remove blood and soluble TTR. The pellet was then incubated with 1 ml of acetonitrile (20%), trifluoroacetic acid (0.1%) mixture to extract aggregated TTR, three times, for 1 hour each, with agitation at room temperature. The supernatants were pooled, lyophilized and then resuspended in 30 to 100 µl of SDS buffer. After boiling 5 min the aggregates extracts were loaded

on a 15% SDS polyacrylamide gel. A Western blot was probed for human TTR (anti-TTR, 1:1500, DAKO) and developed with super signal west pico (Pierce).

Hsp70, hsp27 expression

We studied the expression of hsp70 and hsp27 not only in transgenic mice tissues but also in humans (normal and FAP subjects) in parallel. Human skins derived from biopsies of normal individuals (n=4) and FAP patients positive for the presence of human TTR and negative for amyloid (+/-, n=7) and positive for human TTR and amyloid (+/+, n=6) (as described in Sousa et al., 2004). Human nerves studied included a control group (n=7), FAP 0 (n=6), FAP 1 (n=5), FAP 2 (n=4) and FAP 3 (n=3). The classification results from morphometric studies of nerve fibres: FAP 0- the least affected nerves (no nerve degeneration, no amyloid but already some TTR deposited), FAP 1- discrete reduction in the number of nerve fibres and some fibrils present, FAP 2- evident reduction of nerve fibres and amyloid through the nerve tissue and FAP 3- numerous nerve fibres severely affected and reduced and extensive amyloid deposition (Sousa et al., 2001b). The biopsy specimens from FAP patients, asymptomatic carriers and normal controls (near relatives of FAP patients who ultimately turned out not to be FAP carriers) were available at the Hospital Geral de Santo António, Porto, Portugal, since this material was obtained as part of the clinical diagnosis and evaluation of FAP, prior to the current use of molecular diagnostic methods. Immunohistochemical analyses were carried out using goat anti-hsp27 (1:100, Santa Cruz Biotechnology, Inc.) and goat anti-hsp70 (1:25, Santa Cruz Biotechnology, Inc.) following the same procedures as described in the tissue analysis section.

Hsp70 expression was also investigated in mice by Western blotting of tissues extracts; tissues were minced with a razorblade and homogenised on ice with a glass rod, in 500 µl of lysis buffer that contained EDTA, EGTA, MOPS, Triton, PMSF and proteases inhibitors (cocktail set III, Calbiochem). Total homogenates were centrifuged at 14,000 rpm for 30 minutes and supernatants separated by SDS-PAGE and immunoblotted for hsp70 (1:100, Santa Cruz Biotechnology, Inc.). The same amount of protein was loaded as visualized by β-actin immunoblot (1:5000, Sigma).

Results

TTR/HSF1-KO mice

We analysed hmTTR/HSF1-KO and hTTR/HSF1-KO. The crosses were performed between homozygous (*hsf* $^{-/-}$) males and heterozygous (*hsf* $^{+/-}$) females (since homozygous females are infertile) for HSF1. The number of *hsf* $^{-/-}$ versus *hsf* $^{+/-}$ newborns were evaluated which did not follow a Mendelian distribution. There is a sharp decrease in the number of *hsf* $^{-/-}$ born: of 241 hmTTR/HSF1-KO mice analysed, 58 were *hsf* $^{-/-}$ (24.1%). The prevalence was *hsf* $^{-/-}$ males: 43 to 15 females. The *hsf* $^{+/-}$ obtained were half males half females so the difference in *hsf* $^{-/-}$ male versus *hsf* $^{-/-}$ female was not due to a different number of born males compared to females but instead a probable higher detrimental effect for the lack of HSF1 in females during embryogenesis. The analysis of hTTR/HSF1-KO mice showed an even higher decrease in the number of born *hsf* $^{-/-}$: of 230 newborns analysed 17 were homozygous (7.4%), 11 males and 6 females. The lack of HSF1 expression combined with the absence of mouse TTR increases the negative effect on homozygous *hsf1* viability. Expression of human TTR does not prevent the negative effects of mouse TTR absence. Again, the heterozygous male or female number was equivalent. The comparison was also done with the control experimental group: HSF1-KO mice. From the 229 newborns analysed, 34 were homozygous males and 17 homozygous females (22.3% total homozygous). The mean number of pups born in each offspring was similar in the different strains analysed: 5.0 (±3.0) newborns in HSF1-KO strain, 6.0 (±2.1) in hmTTR/HSF1-KO and 5.1 (±2.6) in hTTR/HSF1-KO. However these values are lower in comparison to the number of pups born in wild type mice, approximately 10 to 12 pups (Suckow et al., 2001) which is a result of the decreased number of homozygous mice obtained in all HSF1-KO mice strains. Homozygous mice were smaller at birth in comparison to heterozygous in all strains studied. During development and in adulthood no muscular atrophy or gait disturbance was detected.

TTR and amyloid deposition in extra-neural tissues

a. Penetrance

We studied TTR and amyloid deposition in four strains of mice at different ages: hmTTR/HSF1-KO, hTTR/HSF1-KO, hmTTR/HSF1-het and hTTR/HSF1-het. Immunohistochemistry against TTR was performed in distinct organs as described under materials and methods. Table 1 displays the number of animals with TTR deposition in the different strains regarding skin and GI (gastrointestinal tract: stomach and intestine), as these were main targets in the previous TTR V30M transgenics kept under the same conditions (Sousa et al., 2002).

Strains	3 months		6 months		12 months	
	Skin	GI	Skin	GI	Skin	GI
hmTTR/HSF1-KO	9/15	15/15	8/12	12/12	6/7	7/7
hTTR/HSF1-KO	3/5	4/6	5/8	8/8		
hmTTR/HSF1-het	9/12	12/12				
hTTR/HSF1-het	8/10	10/10				

Table1-
Skin and GI deposition of TTR in different strains of TTR/HSF1 mice – Penetrance of deposition as assessed by immunohistochemistry.

In all TTR/HSF1-KO strains, the GI tract presented deposition at all ages analyzed; ranging from 3 to 12 months. hmTTR/HSF1-KO mice at 3 months of age were 100% positive for human TTR (n=15). This fact contrast to the previously described hmTTR mice in the wild type background in which 75% of mice showed positive reaction for human TTR in the GI tract (n=16). With advancing age, 100% of hmTTR/HSF1-KO mice continued to present TTR deposition in the GI tract (n=12 for 6 months and n=7 for 12 months). In 3 month old hTTR/HSF1-KO mice, TTR deposition occurred in the GI tract in 67% of the animals (n=6) which does not differ substantially from to hTTR mice in the wild type background (78% in 18 animals). However, 6 month old animals were 100% positive for human TTR immunostaining (n=8). This value is distinct and much higher from what was described in hTTR mice of the same age where 42% of the 19 animals analysed had TTR in the GI tract. Thus, both strains of TTR/HSF1 mice with and without endogeneous mouse

TTR, show a more extensive TTR deposition in the GI tract related to HSF1 disruption.

When TTR/HSF1-het mice were tested, a high penetrance of TTR deposition in the GI tract and skin was seen (table 1). All animals heterozygous for HSF1 at the age of 3 months either expressing or not expressing endogenous mouse TTR in conjunction with human TTR V30M had the intestine and stomach affected with TTR deposition. Therefore, the incomplete removal of HSF1 with reduction of its expression in heterozygous mice is sufficient to produce detectable increase in tissue TTR deposition in comparison to animals with endogenous HSF1.

The skin was also evaluated as a site of major deposition and found not so extensively affected as the GI tract. The phenotype of TTR/HSF1-KO mice was markedly different from TTR V30M transgenic mice in a wt background for HSF1 characterized in our laboratory. While TTR deposition in the latter animals (hmTTR) occurred in skin only over 12 months, affecting only 4% of mice, hmTTR/HSF1-KO mice showed TTR deposition in the skin as early as 3 months, in 60% of the animals characterized (n=15). This deposition was more extensive with advancing age and affected progressively a higher number of animals at 6 and 12 months: 66 and 86% respectively (table1). Moreover, hTTR/HSF1-KO mice display TTR deposition in the skin at the age of 3 months in contrast to hTTR mice with HSF1 which had skin TTR deposition after.

The overall observation indicates that HSF1 absence influences TTR oligomerization in tissues resulting in an earlier, extensive and systemic TTR deposition in comparison to hmTTR or hTTR mice. TTR was also observed in other extra neural tissues analyzed, as described under methods. With the exception of the tongue and kidney, tissues did not present as extensive deposition as in the case of stomach, esophagus, intestine and skin. For instance, kidneys from hmTTR/HSF1-KO and hTTR/HSF1-KO strains were all positive for TTR in the different ages evaluated (100% positive) as assessed by immunohistochemistry. The tongue, more specifically the muscle is affected with TTR deposition in all hmTTR/HSF1-KO mice (n=19). Approximately half of the animals analyzed had TTR deposits at all ages in the other extra-neural tissues.

Amyloid formation was evaluated as well in the four strains of mice by Congo red staining: amyloid was detected in the stomach of two 6 month old hTTR/HSF1-KO

mice (n=12) and in one animal in the small intestine of the same group of animals. With increasing age, amyloid deposition was not extended to other organs. Since in hTTR mice in the wild type background amyloid is not visible before 6 months, it seems that HSF-1 disruption does not accelerate fibril formation but can accelerate non-fibrillar deposition.

b. Organ deposition

Figure 1A shows representative TTR immunolabelling in extra-neural tissues, namely in the skin, in a blood vessel of pancreas, in the stomach, intestine, and kidney from TTR/ HSF-1 mice with (+/-) and without (-/-) TTR deposition. The described deposition pattern applies to the 4 different strains analyzed. In the skin, TTR deposition occurs contiguous to sebaceous glands and close to collagen fibres at the reticular layer of the dermis; it also occurs close to sweat glands and in the connective tissue proximal to the hair follicle. In blood vessels (shown, a blood vessel from pancreas), immunoreactive human TTR is seen at the adventia. In the stomach, deposition is evident mainly in the *muscularis mucosae* and to a lesser extent in the corion of the mucosa. The *muscularis mucosae* in intestine is also a target for deposition, as well as the core of the intestinal villi. In kidney, glomeruli and the medullar area were infiltrated with TTR. The areas of deposition in the documented mice organs are similar to TTR deposition described in FAP. The same applied for other organs; for instance, salivary glands show TTR immunoreactivity around the glandular acini and associated with excretory ducts and blood vessels between the lobes.

As in FAP, deposition did not affect the liver, except near blood vessels.

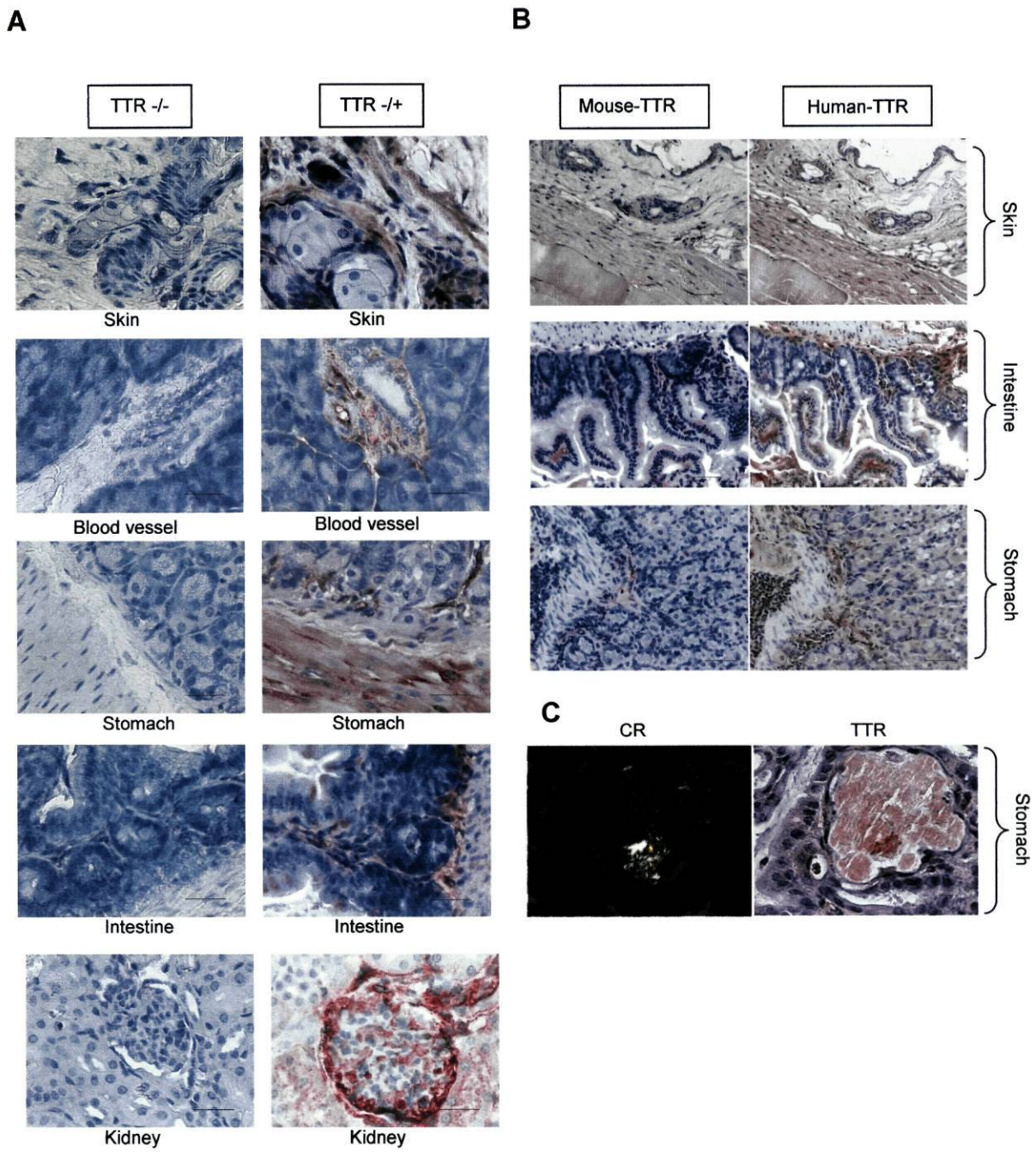


Figure 1 - TTR deposition in extra-neural tissues of TTR/HSF1 mice

A. Human TTR deposition in different tissues of hmTTR/HSF1-KO mice. From top to bottom: skin, blood vessel, stomach, intestine and kidney. Scale bar: 30 µm. Negative tissues (-/-, without deposition); positive tissues (-/+, with deposition). For explanation, see text.

B. Mouse TTR deposition. Left pictures represent immunolabelling for mouse TTR (red) in skin, intestine and stomach (from top to bottom). Right pictures are corresponding areas positive for human TTR (brown). Scale bar 50 µm.

C. Amyloid in the stomach, detected by Congo red binding (left picture). At the right is the corresponding area, immunoreactive for human TTR antibody. Scale bar 30 µm.

Mouse TTR was also observed to deposit along with human variant TTR in the strains with the endogeneous protein (hmTTR/HSF1-KO), but to a lesser extent as documented in figure 1B; however this observation might be due to a different affinity of the anti-mouse TTR antibody for the different hybrid tetramers composed of mouse and human subunits which circulate in transgenics for human TTR in a wild type background (Sousa et al., 2002).

Figure 1C, left panel, shows amyloid in mucosa of the stomach, in-between the gastric glands, as assessed by the green birefringence viewed under polarized light, after Congo red binding. The nature of amyloid deposition was shown to be human TTR by immunohistochemistry (figure 1C, right panel).

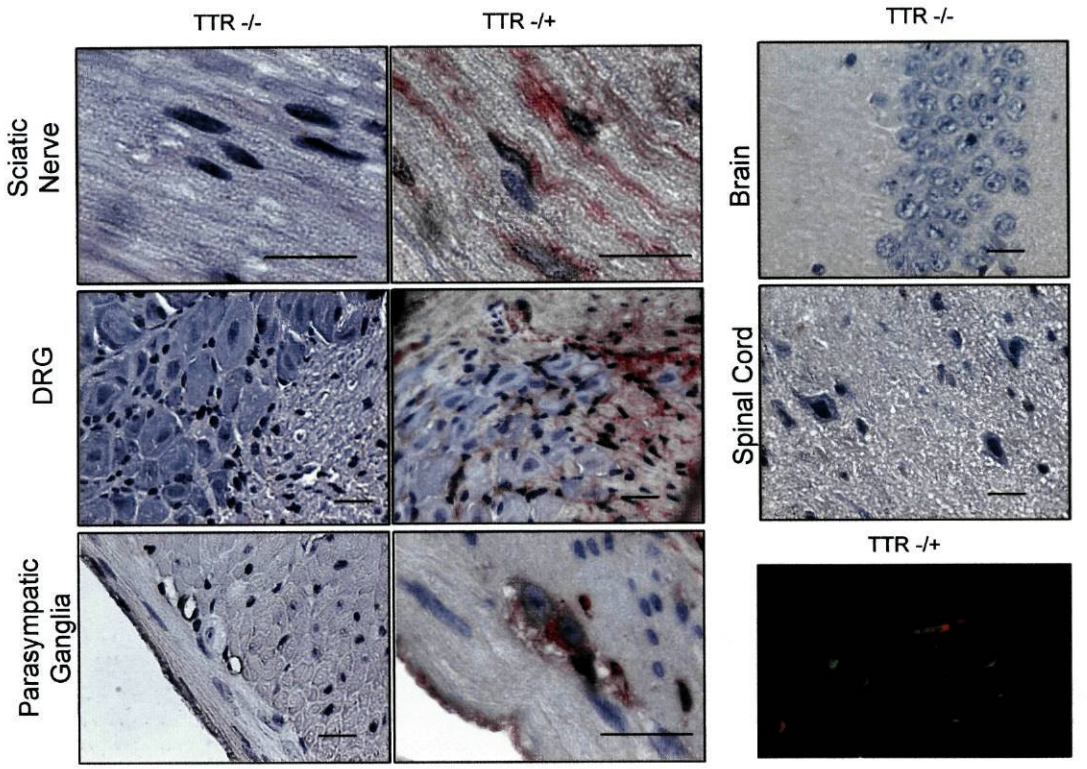
TTR and amyloid deposition in the nervous system

While analysing the central (CNS), peripheral (PNS) and the autonomic nervous systems of this new transgenic mouse model, we observed, for the first time, TTR deposition in the sciatic nerve, DRGs, and autonomic ganglia, similarly to what is observed in human FAP patients. TTR was deposited extracellularly in the connective tissue between nervous fibres either myelinated or unmyelinated of the sciatic nerve and close to Schwann cells (figure 2 A). In the ganglia, namely in the DRG neurons, TTR is observed around the cell bodies in close contact with satellite cells and in the fascicle of nerve fibres. The right panels of figure 2A are photomicrographs of CNS tissues that lack TTR deposition: brain and spinal cord. This distribution is in accordance to the human situation as the CNS is spared in the vast majority of V30M patients.

We also investigated the autonomic nervous system, particularly the parasympatic system. Part of parasympatic ganglia between smooth muscle layers of stomach and intestine presented deposition, around neurons and their satellite cells, as investigated by double labeling with a neuronal marker (Figure 2A, right picture at the bottom); when TTR was found to be present in the parasympatic ganglia, the *muscularis mucosae* next to it had always positive reaction for TTR while the contrary was not seen. Such situation is in accordance with a later deposition in the autonomic nervous system. This is another evidence that TTR deposition also affects the autonomous nervous system in this transgenic mouse model as it is observed in FAP patients. The table represented in figure 2A compares numbers of

animals with TTR deposition in DRG and sciatic nerve in the 4 strains of TTR/HSF1 mice, at different ages. In these tissues, amyloid formation was never detected, but TTR aggregates were observed with penetrance of deposition increasing with age. Protein aggregates were extracted from hmTTR/HSF1-KO nerve, skin and stomach of TTR positive (-/+) and negative (-/-) animals as assessed by immunohistochemistry. The results confirmed the immunohistochemical data, i.e., a TTR immunoreactive band appeared only in TTR positive animals.

A



Strains	3 months		6 months		12 months	
	DRG	Sciatic Nerve	DRG	Sciatic Nerve	DRG	Sciatic Nerve
hmTTR/HSF1-KO	11/15	5/15	9/12	7/12	6/7	4/7
hTTR/HSF1-KO	4/6	1/6	6/8	3/8		
hmTTR/HSF1-het	4/12	2/12				
hTTR/HSF1-het	6/10	1/10				

B

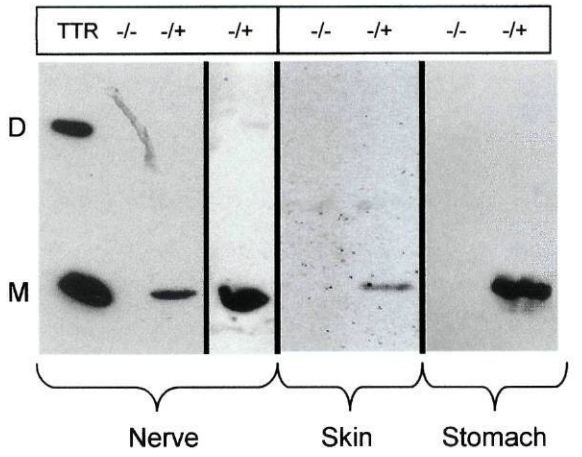


Figure 2 - TTR deposition in CNS, PNS and autonomic nervous systems of TTR/HSF1-KO mice

A. *Left 2 rows*: optic microscopy demonstrating the presence (-/+) and absence (-/-) of TTR deposits in the sciatic nerve, DRG and parasympatic ganglia of stomach of hmTTR/HSF1-KO mice. *Right picture at the bottom*: parasympatic ganglia of a TTR positive intestine labelled by double immunofluorescence (merging image) for TTR (red) and Pgp9.5 (green). *Right upper row*: absence of TTR deposits in CNS, namely in the brain (hippocampus) and spinal cord.
Scale bar 20 µm.
The table represents the penetrance of TTR deposition in sciatic nerve and DRG in the different strains of TTR/HSF1 mice.
B. Extracted aggregates from nerve, skin and stomach of hmTTR/HSF1-KO mice, negative (-/-) and/or positive (-/+) for human TTR by immunohistochemistry analyzed by Western blotting using anti-human TTR. TTR: standard human recombinant TTR; M-TTR monomer; D- TTR dimer.

Oxidative stress

Tyrosine nitration, a marker for oxidative stress in FAP (Sousa et al., 2001a) was evaluated by semi-quantitative immunohistochemistry in the GI tract of hmTTR/HSF1-KO mice with and without TTR deposition and compared with age-matched hTTR mice in the wild type background. Mice without TTR deposits did not stain for nitrotyrosine, whereas mice with deposition did. However, a statistically significant higher nitrotyrosine staining was evident for hmTTR/HSF1-KO versus controls (hmTTR mice) at all ages studied (3, 6 and 12 months) which is documented in figure 3. The same results were observed in hTTR/HSF1-KO when compared to age matched hTTR mice (3 and 6 months). Thus, HSF1 not only accelerates TTR oligomerization but also affects oxidative damage, a situation found in FAP. We found no correlation between TTR deposition and nitrotyrosine staining in sciatic nerve and DRG (not shown) probably related to the extent of TTR deposition in these tissues.

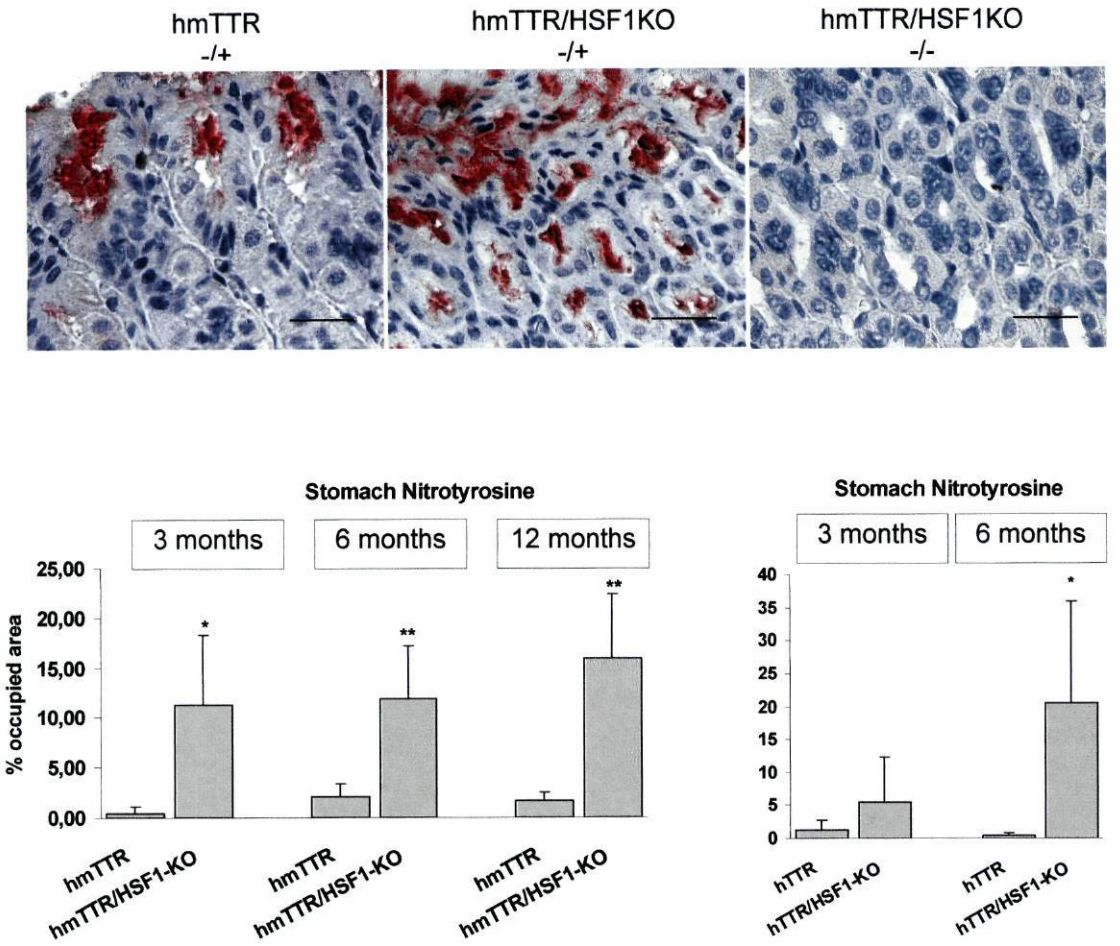


Figure 3 - Oxidative stress in stomach of TTR/HSF1 mice
Nitrotyrosine staining of stomach: *Left picture*: hTTR mice, positive for human TTR (-/+); *middle and right pictures*: age matched hmTTR/HSF1-KO mice, positive (-/+) and negative (-/-) for human TTR. Scale bar 30 µm. *Charts*: comparative semiquantitative analyses of nitrotyrosine immunolabelling in TTR positive age matched stomachs, at different ages, using the scion image quant program. *Left chart*: comparison between hmTTR and hmTTR/HSF1-KO mice *Right chart*: comparison between hTTR and hTTR/HSF1-KO mice. * $p<0.02$, ** $p<0.002$.

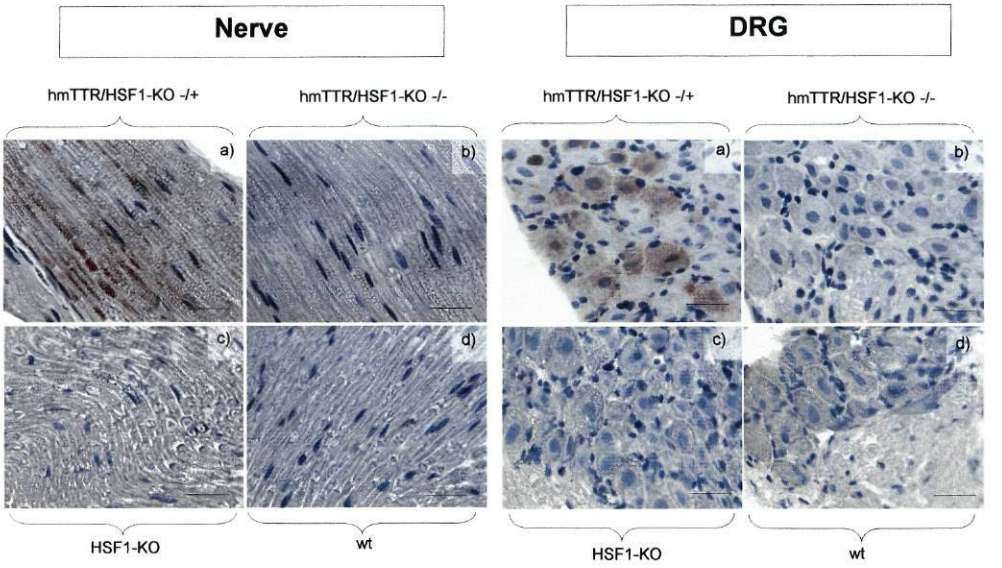
Inflammatory pathways

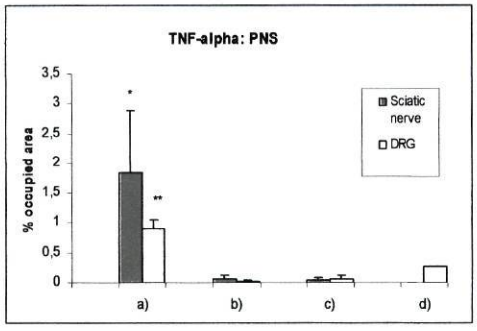
The pro-inflammatory cytokines, TNF-α and iL1-β, known to be up-regulated in human FAP nerves were evaluated by semi-quantitative immunohistochemistry in 6 months old hmTTR/HSF1-KO sciatic nerves with and without TTR deposition (figure 4). TNF-α levels (figure 4 A) are markedly increased in mice having deposited TTR in the sciatic nerve ($p<0.02$) in comparison with nerves without deposition. TNF-α values obtained for the control groups (HSF1-KO and sv129 wt mice) were similar to hmTTR/HSF1-KO sciatic nerves negative for TTR, meaning

that the increase observed for TNF-α is related exclusively to TTR deposition in peripheral nerve. The same situation was evident in DRG, i.e., an increase in TNF-α levels was evident in neurons from ganglia presenting TTR deposition (figure 4A). Concerning iL1-β, an increase on expression is clearly observed in sciatic nerve with TTR deposition versus sciatic nerve without deposition (figure 4B); nerve from wild type animals did not label with anti-iL1-β whereas in HSF1-KO nerve a slight staining was detected, probably related to HSF1 disruption. DRG with TTR deposition also showed a clear increase in iL1-β levels in neurons as compared with animals without deposition (figure 4B).

Taken together, expressions of these cytokines (TNF-α and iL1-β) are indicative of ongoing inflammatory pathways related to TTR deposition in the PNS in a similar fashion as in asymptomatic carriers of the V30M mutation that present non-fibrillar deposition.

A - TNF-alpha





B - iL1-beta

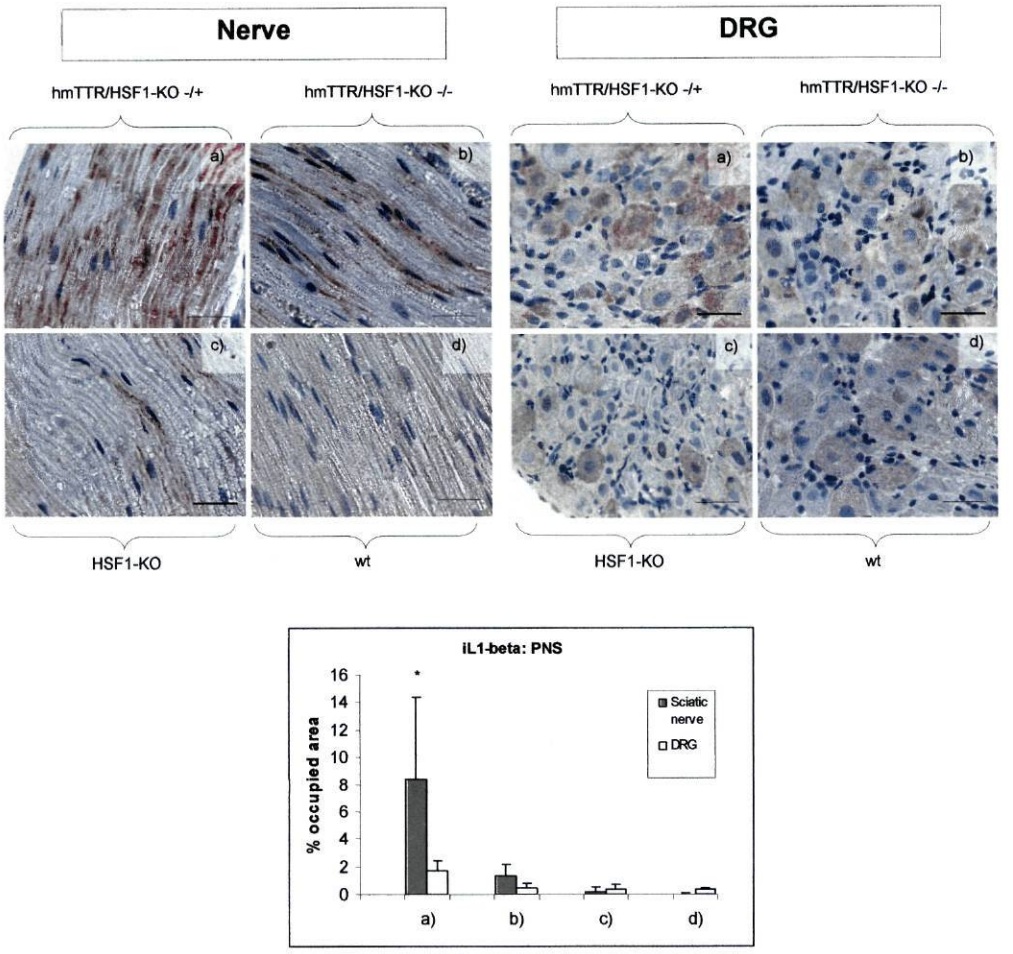


Figure 4 - Pro-inflammatory cytokines in the PNS of TTR/HSF1-KO mice
TNF-alpha (A) and iL1-beta (B) expression in sciatic nerve (4 panels on the left of each figure) and DRG (4 panels on the right of each figure) of: a) hmTTR/HSF1-KO positive (-/+) for human TTR (n=5); b) hmTTR/HSF1-KO negative (-/-) for human TTR (n=5); c) HSF1-KO mice (n=7); d) wild type mice (n=6); Scale bar 30 µm. *Charts*: semiquantitative analysis of TNF-alpha expression and iL1-beta in the sciatic nerve and DRG, using Scion image quant program, showing a significative increase in their expression in tissues with TTR deposition . * $p<0.02$, ** $p<0.002$.

The expression of other proteins related to inflammation previously tested in FAP tissues were evaluated in hmTTR/HSF1-KO sciatic nerve and DRG, namely, RAGE, NF-kB, MCP-1 and NCL-MACRO (macrophage marker). In humans, it is known that deposition of TTR V30M in nerve triggers NF-kB activation and that RAGE is involved in this pathway; furthermore RAGE upregulation is observed at sites of TTR deposition (Sousa et al, 2000b and 2001a). TTR/HSF1-KO nerves and DRG without TTR deposition never showed increased expression of RAGE and/or NF-kB

translocation to the nucleus, while on average half of the TTR positive nerves and DRG displayed an increase in RAGE expression (figure 5), and NF-kB translocation (figure 5) to the nucleus as it is seen in FAP patients.

Levels of MCP-1 were not up-regulated in hmTTR/HSF1-KO sciatic nerve and DRG with or without the presence of TTR (data not shown) as humans in the pre-symptomatic phase of the disease. The same applied for the macrophage marker, NCL- MACRO, showing no evidence for macrophage increase and migration.

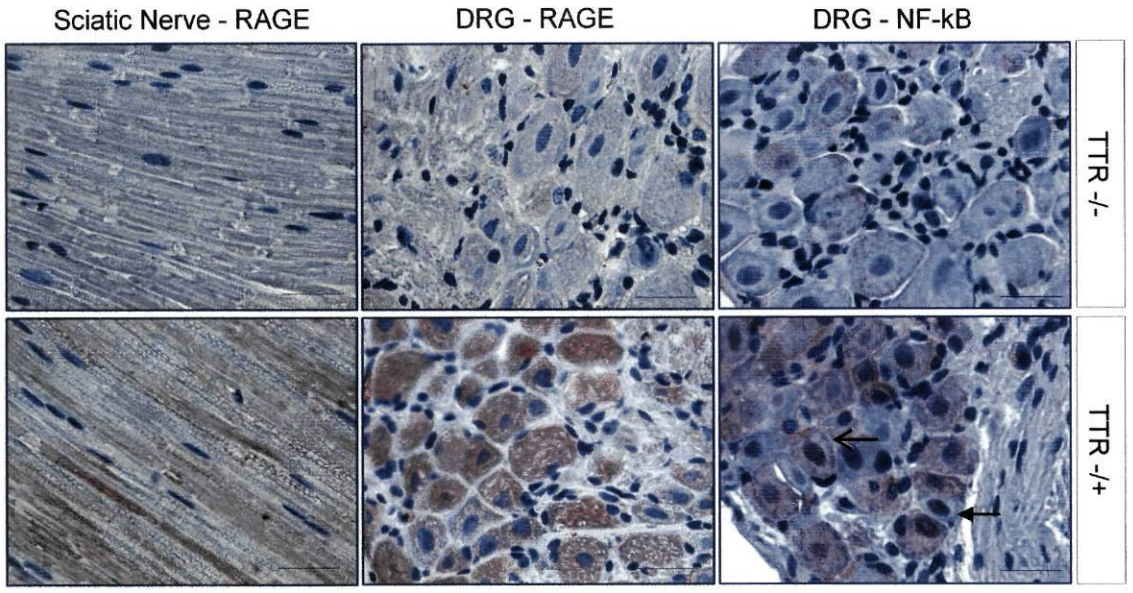


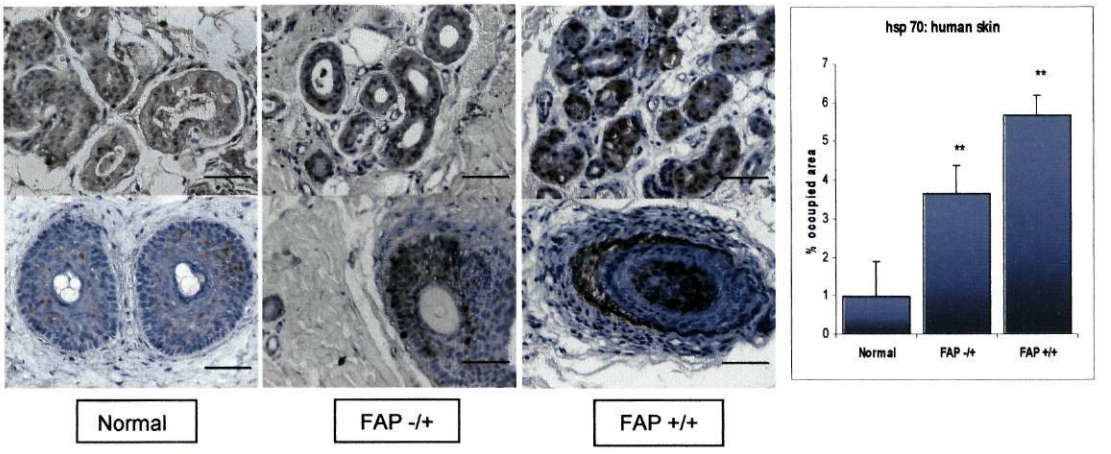
Figure 5 - RAGE expression and NF-kB activation in the PNS of TTR/HSF1 mice
Left and middle panels: Increased expression of RAGE in sciatic nerve and DRG of hmTTR/HSF1-KO mice without (TTR-/-) and with (TTR-/+) deposition. *Right panels*: activation of NF-KB in tissues with deposition. Arrows indicate translocation to the nucleus. Scale bar 30 µm

Stress response

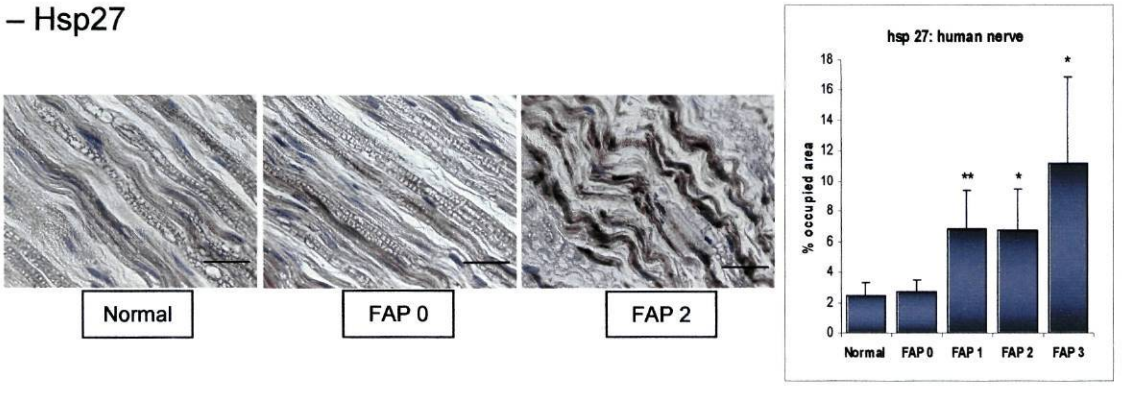
One of our main aims was to determine if a stress response was involved in FAP. Since no data is available in these patients concerning this aspect, we started by selecting 2 heat shock proteins involved in the stress response, hsp70 and hsp27, to investigate their expression in human skin and nerve biopsies with TTR deposition either of the non-fibrillar (FAP 0/ TTR -/+) and fibrillar types (FAP 1-3/ TTR +/+) as compared to control biopsies without deposition. Skins from affected individuals (figure 6A) showed an expressive increase in hsp70 both in the hair follicle (bottom pictures) and also in the sweat glands (top pictures) in comparison

to control human skin. This rise is even more pronounced in FAP +/+ skins, as shown in the quantification chart of figure 6A.

A – hsp70



B – Hsp27



C

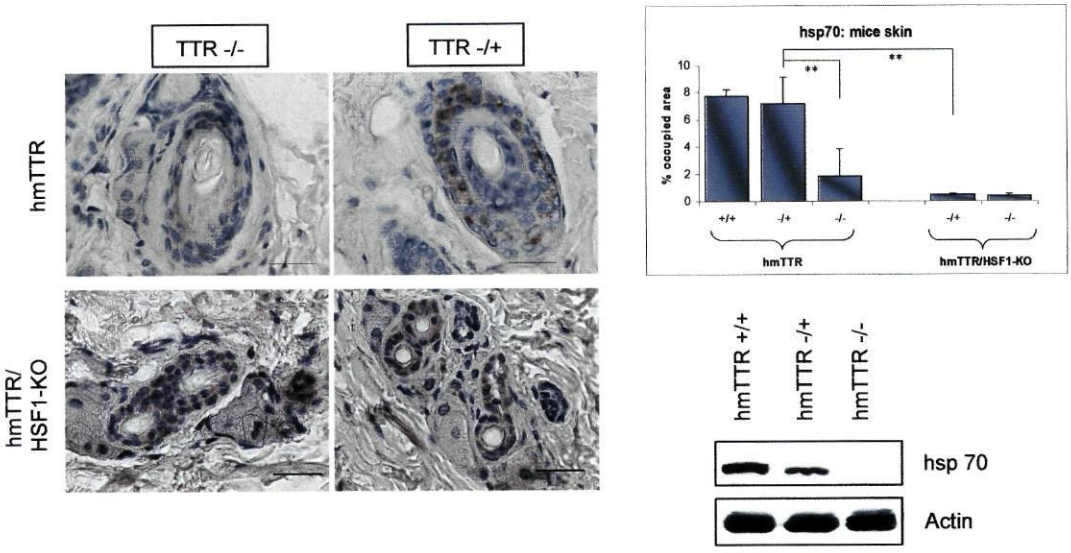


Figure 6 - Chaperones expression and FAP
A. Hsp70 skin expression in humans: top pictures are sweat glands and bottom pictures hair follicles. From left to right: normal; PAF +/- and PAF +/+ skin respectively. The semiquantitative analysis of sweet glands is represented in the chart as the % of occupied area by immunolabeling, standard deviation and probabilistic significance (**, $p<0.002$). Scale bar 30 µm
B. Hsp27 nerve expression in humans: left picture shows a normal nerve, middle picture a FAP 0 nerve and right picture a FAP 2 nerve. The chart displays the semiquantitative values of hsp27 immunolabelling in different stages of the disease (FAP0 to FAP3) in % of occupied area plus standard deviation. * $p<0.02$, ** $p<0.002$. Scale bar 30 µm
C. Hsp 70 skin (hair follicles) expression in TTR/HSF1 mice: top pictures represent skin from hTTR mice and bottom pictures from hmTTR/HSF1-KO, without (TTR -/-) and with (TTR -/+) deposition. Scale bar 30 µm. The chart represents a semiquantitative analysis of hsp70 expression in the skin hair follicles of the referred mice. ** $p<0.002$. *Right bottom*: Western blot analysis of skin from hTTR mice probed for hsp70 and actin.

Next we analyzed hsp70 in FAP nerves but no correlation was drawn with deposition (data not shown). When we analysed hsp27 expression in nerve comparing normal and FAP tissues at different stages (FAP 0-3), a comparable signal was observed in the axons of normal and FAP nerves with non-fibrillar TTR deposition (figure 6B); however in the more advanced stages (FAP 1-3) up-regulation of hsp27 was evident. This interesting result suggests that the extracellular TTR deposition is capable of exerting an intracellular effect involving the stress response.

The results led us to investigate the expression of these selected hsps in the skin and nerve of transgenic mice for human TTR V30M in the wild type versus the HSF1-KO background. A rise in hsp70 expression was observed in the skin of

hTTR mice with TTR non-fibrillar (-/+) and fibrillar amyloid (+/+) deposition, as compared to mice without deposition (-/-), which is documented in the pictures of figure 6C and in the quantification chart. The same finding was evident when hsp70 expression in skin was examined by Western blot analysis (figure 6C); thus hsp70 level was higher in skin with amyloid and TTR deposition as compared to skin without deposition. In contrast, in hmTTR/HSF1-KO skin, there was no relationship between TTR deposition and hsp70 levels, that is, the level of hsp70 was maintained at background levels in both cases, with (-/+) and without (-/-) deposition as depicted in figure 6C and quantification chart, a reason for not observing a signal in Western blots (not shown). Therefore, the presence of non-fibrillar and fibrillar aggregates induces a stress response in tissues that do not synthesize TTR, a response regulated by HSF1.

Hsp27 was also investigated in nerve of hmTTR/HSF1 mice with and without deposition. As expected, no difference in expression levels was found.

DISCUSSION

Protein misfolding diseases result to a certain extent from the incapacity of the cell to stay below the dangerous limits of intra and/or extracellular accumulated aggregated proteins as in Alzheimer´s disease, Parkinson´s disease, familial amyotrophic lateral sclerosis, Huntington´s and other polyglutamine related disorders. The proteins involved are predisposed to aggregate, forming protofilaments and eventually amyloid fibrils. In the case of FAP, extracellular accumulation of aggregated TTR affects mainly the PNS, leading to neurodegeneration. The most common mutation associated with FAP is V30M, in kindreds with age of onset of clinical symptoms in the third to fourth decade of life. The typical clinical features are early impairment of temperature and pain sensations in the feet and autonomic dysfunction leading to paresis, malabsorption, emaciation and death. Although originally regarded as a rare disease, it is now becoming clear that many kindreds exist worldwide. The mechanism by which the various TTR mutations lead to amyloid aggregation has been the focus of research for two decades. Development of therapeutic strategies in FAP entails, among others, not only the elucidation of molecular mechanism leading to fibril formation but also the cellular/tissue effects produced by TTR deposition. Whilst the former aspect has gained some insights due to intense research at the *in vitro* level, the latter issue has not been properly addressed, in part due to the lack of appropriate animal models with deposition in the PNS.

TTR is a tetrameric serum protein of four identical subunits of 14 kDa synthesized mainly in the liver and choroid plexus of the brain (Soprano et al., 1985). Each monomer contains two β-sheets that interact to form a dimer. Amyloidogenic mutations appear to reduce the stability of the tetrameric structure. All of these structural studies have associated the amyloidogenic potential of TTR with weak interactions between its subunits. Indeed, stability studies have shown a relationship between the amyloidogenic potential of TTR, and a decrease in tetrameric stability (Quintas et al., 2001). These studies suggest that amyloid fibril formation by some TTR mutants might be triggered by tetramer dissociation to a compact non-native monomer with low conformational stability, which results in

partially unfolded monomeric species with a high tendency for ordered aggregation into amyloid fibrils.

The failure to generate good FAP animal models with PNS deposition has been attributed to different reasons; a plausible explanation is that nerve barriers might be more tight in rodents than in humans not allowing access of mutant TTR to the nerve; in this regard, the two major sites of TTR synthesis are the liver and the choroid plexus of the brain that are respectively the sources of TTR found in the plasma and in the cerebrospinal fluid (CSF) (Soprano et al., 1985). Therefore the deposited TTR in the PNS of FAP patients may derive from the plasma pool and/or from the CSF pool of the protein. Recently, we reported *de novo* TTR amyloidosis in recipients of FAP livers demonstrating that in individuals where the liver is the sole source of mutated TTR, the protein is able to deposit in the connective tissue of the endoneurium and skin, suggesting that the plasma pool of the protein is sufficient for PNS involvement in FAP (Sousa et al., 2004). However, one cannot exclude the contribution of CSF TTR to deposition in DRG and nerve. In view of the demonstration of TTR deposition in peripheral nerve in the TTR/HSF1 here described, the nerve barrier constraint for rodent FAP models does not apply.

Another explanation has considered environmental factors as modulators of deposition and/clinical disease. This is based on the observation that monozygotic twins with FAP were discordant for age of onset and clinical manifestations (Munar-Ques et al., 1999). Differences in the penetrance of TTR deposition have been observed in TTR V30M transgenic mice when animals are bred and kept in normal versus pathogen free conditions (SPF) (Noguchi et al., 2002, Saraiva et al., 2004). Factors like inflammation, oxidative stress are thus likely to aggravate TTR deposition. Tissue changes associated with TTR deposition have been investigated at the RNA and protein levels in tissues from asymptomatic carriers of TTRV30M (FAP 0) and from FAP patients. At the membrane level, at least 1 receptor is involved in signaling cascades triggered by the presence of TTR deposits which is RAGE. At the nuclear level, at least the NF-kB transcription factor is involved since it has been clearly observed to be overexpressed and translocated into the nucleus in FAP tissues (Sousa et al., 2005) and is responsible in part for the activation of inflammatory and oxidative stress pathways so far investigated; these pathways

can be either a consequence of toxicity from intermediate species/amyloid, or be players in TTR deposition.

The dramatic increase in the expression of hsp70 and hsp27 found in FAP skins and nerves respectively is indicative of the involvement of a stress response; in an earlier study, we found that the expression of hsp27, as well as alpha B-crystallin is affected in the brain of HSF1-KO mice, which present as main features enlarged ventricles, astrogliosis and neurodegeneration at peri-ventricular areas (Santos and Saraiva, 2004). The expression studies both on clinical samples and mice (TTR transgenics versus TTR/HSF1) are suggestive that absence/reduction of HSF1 might result in the impairment for responsiveness of specific hsps to signals deriving from the presence of aggregated TTR in the external milieu aggravating deposition. The intracellular molecular signaling mechanisms involved in such a scenario merit certainly further dissection as well as the possible intervention of an extracellular chaperone on protein aggregation which cannot be ruled out at this point.

Many studies have pointed towards an important role of hsps during neurodegeneration as protective effectors by interacting with aberrantly folded proteins to prevent aggregate formation. In fly and mouse models of polyglutamine and Parkinson´s diseases many reports show the protective influence of chaperones in neurodegeneration. For instance, overexpressing hsp70 in a drosophila model of Huntington´s disease suppresses the aggregation and toxicity of intracellular inclusions (Warrick et al, 1999) and in Parkinson´s disease model, the expression of a dominant negative hsp70 exacerbates the neurodegenerative process (Auluk et al., 2002). And for example, in primary cell culture of neurons expressing beta-amyloid 42 (Abeta42, involved in Alzheimer´s disease) the virally mediated hsp70 overexpression rescued neurons from the toxicity associated with Abeta accumulation (Magrane et al., 2004). Although being different pathologies, they share the fact that aggregated proteins are involved and hsps are beneficial elements in the process. It is of scientific knowledge that with age there is a compromised ability to activate HSF1 and hsps decrease their activity (Heydari et al., 2000). For instance, hsp70 induction is compromised in senescent cells (Liu et al., 1991) and probably that is why protein conformational pathologies appear as age-related diseases. Probably the chaperone temporal expression changes

differently during aging when comparing a normal organism and a disease affected one. The disease might accelerate the normal progressive decline activity of hsps and the loss of control in homeostasis, and so, increase the organism susceptibility to aggressions. Also, the referred compromised transcriptional activity of HSF1 with ageing might in its turn contribute to different pathologies associated with age.

In the case of FAP, an hypothetical direct interaction between hsps and abnormally folded TTR would have to occur extracellularly, and in this respect the molecular machinery involved is highly unknown. As for the role of hsps in intracellular signaling a great deal of research has been performed in cancer, infectious, vascular diseases, transplant rejection (for a review see Pockley 2001) but not yet in TTR amyloid research. The stress response has been reported to interfere with inflammatory processes to attenuate its mechanism of injury. Inflammatory stimulus induces expression of TNF-α and iL1-β cytokines which can be prevented by activating HSF1 (Singh et al., 2000; Xie et al., 2002), and by increasing hsp70 synthesis (Ding et al., 2001) or hsp27 (Park et al., 1998) which are under direct control of HSF1. Both pro-inflammatory cytokines are known to increase during FAP pathogenesis (Sousa et al., 2001a) which is also observed in the TTR/HSF1 mouse model here described. Normally, in inflammation a network of cytokine production occurs, with inflammatory cytokines first regulating the production of additional inflammatory cytokines followed by the production of anti-inflammatory cytokines consequently turning-off inflammation. In fact, we saw previously in FAP nerves increased expression of the anti-inflammatory cytokine iL-10 (after FAP1 stage) that occurs concomitantly with the overexpression of the pro-inflammatory cytokines iL-1β and TNF-α (after FAP 0 stage). Hsps might have an additional anti-inflammatory role, contributing to the progression of the disease.

Experimental data is also available on the importance of hsps on apoptosis: for hsp27, its upregulation has been implicated in sensory and motor neuronal survival after peripheral nerve crush and hsp27 absence in apoptotic cell death (Benn et al., 2002); besides, hsp70 was shown to interfere with apoptotic cell program by inhibiting the release of cytochrome c from mitochondria and by preventing the processing of procaspases 9 and 3 (Mosser et al., 2000). Hsps could act in FAP also at this level.

The TTR/HSF1-KO mouse model analyzed so far recapitulates the initial stages of FAP; similarities worth stressing are: early GI involvement, lack of deposition in liver and CNS, same organ anatomical sites for deposition either for extra-neural, PNS and autonomic nervous system, lack of an immune/inflammatory infiltrate despite the production of pro-inflammatory cytokines by PNS cells, common known signaling pathways and age dependent penetrance of deposition. The age range analyzed so far did not show amyloid in PNS or other organ, except for the GI tract in 3 animals; FAP has adult onset, with typical onset between 25-35 years of age; however, a certain percentage of V30M carriers present a late onset, after their sixties (in Portugal, about 10% of carriers). It is expected that older TTR/HSF1 mice give evidence for fibrillar TTR deposition that will allow to extend studies on signaling and neurodegeneration mechanisms not available in humans. For instance, in FAP it has been hypothesized that the loss of sensory and autonomic neurons may function as the pathological background for the preferential degeneration of unmyelinated and small myelinated fibers (Thomas and King, 1974). Sural nerve biopsies, on which most of the pathological analysis in FAP has been performed, represent a restricted portion of the PNS, and it is clearly possible that TTR deposits in ganglia, or more proximally in nerve trunks, that might be responsible for distal nerve fiber loss. The possibility to analyze DRG with deposition in this mice model, presenting early signs of increase in inflammatory markers is of much value, and permits a close evaluation of events occurring in sensory and autonomic neurons responsible for neurodegeneration.

Finally, this much awaited model is necessary for evaluation of effective therapeutical drugs and approaches to fight the disease, including targets to the PNS; so far, potential drugs have been tested *in vitro* and encompass small compounds to prevent aggregate formation (Almeida et al., 2004), to disrupt fibrils (Cardoso et al., 2004). Other avenues entail signaling pathways involving RAGE or other mediator. The manipulation of the stress response can be viewed now as a new potential pharmacological drug in FAP. In other protein aggregation pathologies related to intracellular deposition, the induction of the stress response was used with success to prevent neurodegeneration, as in the case of the ALS mice model (Kieran et al., 2004).

New strategies to improve the inherited organism capacity to respond to the presence of an altered conformation protein are of crucial importance to postpone / prevent an inevitable disease for those carrying an amyloidogenic TTR mutation.

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CHAPTER 4

Ubiquitination in familial amyloidotic polyneuropathy

Ubiquitination in familial amyloidotic polyneuropathy

Sofia Duque Santos[1,2]; Isabel Cardoso[1]; Maria João Saraiva[1,2]

[1]Molecular Neurobiology Unit, Institute for Molecular and Cell Biology; [2]Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Portugal.

Corresponding author: Maria João Saraiva; Molecular Neurobiology; Instituto de Biologia Molecular e Celular. Rua do Campo Alegre 823. 4150-180 Porto. Portugal.
Tel. 351-226074900; Fax 351-226099157. e-mail: mjsaraiv@ibmc.up.pt

Running title: Ubiquitination in FAP

Abstract

The mechanisms developed by the cells to acquire protection from the deleterious effects of misfolded proteins are currently well characterized. One essential mechanism is the ubiquitin-proteasome system (UPS) which has been related to neurodegenerative disorders of intracellular protein aggregation. We have studied the UPS in familial amyloidotic polyneuropathy (FAP), a neurodegenerative disorder caused by extracellular deposition of mutant transthyretin (TTR). We found a relationship between protein aggregation/extracellular TTR deposition and ubiquitination. The studies were conducted in TTR synthesizing and non-synthesizing tissues from affected individuals and from transgenics mice models for FAP. By using a Schwannoma cell line secreting different TTR amyloidogenic variants we measured ubiquitination and proteasome activity and found no significative differences in these parameters between wt TTR and the most common TTR variant associated with FAP, TTR Val30Met; however in cells secreting a more amyloidogenic TTR variant, TTR Leu55Pro, ubiquitin inclusions, alterations in cytoskeleton and decreased proteasome activity in the insoluble fraction were detected, possibly related with aggregates found in conditioned media.

The overall results are indicative of a function of deposited TTR as an external stimulus to an intracellular response involving the UPS.

Introduction

Protein ubiquitination is essential for intracellular surveillance against misfolded and denatured proteins, e.g., membrane or secretory proteins that fail to fold in the ER, proteins that fail to bind cofactors, free subunits of multimeric complexes or oxidant-damaged proteins (Sherman and Goldberg, 2001). The knowledge that more than 30% of total newly synthesised proteins attain an incorrect structure leading to ubiquitination (Schubert et al., 2000) and proteasomal degradation, characterizes this system, the ubiquitin-proteasome system (UPS) as an extraordinarily effective quality control mechanism.

The ability of proteins to activate apoptosis has probably an important role in neurodegenerative diseases when accumulation of aggregated proteins occurs. Intracellular inclusions of denatured proteins are characteristic features of many neurological diseases, such as Bunina bodies in Amyotrophic Lateral Sclerosis, neurofibrillary tangles in Alzheimer's disease (AD), Lewy bodies in Parkinson's disease (PD), insoluble form of the prion protein (scrapie) in Prion disease and inclusions of expanded polyglutamine-containing proteins in polyglutamine disorders. All these inclusions contain components of the ubiquitin-proteasome degradative pathway and also molecular chaperones, which represent the two main systems that protect eukaryotic cells against these misfolded proteins. These intracellular inclusions reflect a failure of the proteasome activity to remove the abnormal proteins which might explain the neurodegeneration that characterizes this process (Ding et al., 2002; Dimcheff et al., 2003; Keck et al., 2003; McNaught et al., 2003). Other confirmations of the relationship between UPS and neurodegenerative diseases come from findings of mutations in enzymes of the ubiquitin pathway that cause neurological disorders such as the case of Parkin, an E3 ubiquitin ligase, associated with PD (Dawson and Dawson, 2003).

These neurological disorders present protein aggregation inside the cells, but what mechanism of defence is elicited when deposition of aggregated protein is extracellular distant from the local of synthesis as in amyloidoses? One such example is Familial amyloidotic polyneuropathy (FAP) where mutant forms of transthyretin (TTR) are deposited extracellularly in different tissues in particular in

peripheral nerve, being TTR Val30Met the most common variant found (Saraiva et al., 1984) whereas TTR Leu55Pro is a rare variant that leads to more aggressive clinical symptoms (Jacobson et al., 1992; Yamamoto et al., 1994). The biological activities of TTR are not impaired by any of the mutations characterized so far: it is normally synthesised by the liver and the choroid plexus of brain, secreted to the plasma where it functions as a transporter for the thyroid hormone T4 and to bind the complex retinol binding protein-retinol preventing its glomerular filtration. The catabolism of TTR occurs mainly in the liver. Protein aggregation occurs in different tissues but never in the main organ of synthesis, the liver, and intracellular TTR inclusions have never been described in FAP tissues even for highly amyloidogenic mutations. The consequences of TTR deposition involve oxidative damage, activation of inflammatory responses, induction of the NF-kB pathway and apoptosis (Sousa and Saraiva, 2003). We have shown the heat shock response as one mechanism of defence activated during the progression of FAP (chapter 3).

To date, no information is available on the UPS response in FAP, either at TTR synthesizing and non-synthesizing tissues. We addressed this issue by studying ubiquitination in tissues from affected individuals and from transgenic mice models for FAP and by using a Schwannoma cell line secreting different TTR amyloidogenic variants.

Materials and methods

Tissue samples

a) Human

Skin biopsies from normal individuals (n=6) and sural nerve and skin biopsies from asymptomatic carriers, with TTR non-fibrillar (-/+; n=9) and fibrillar (+/+; n=6) were available at the Hospital Geral de Santo António, Porto, Portugal. This material was obtained as part of the clinical diagnosis and evaluation of polyneuropathy, prior to the current use of less invasive methods. These tissues were fixed in neutral buffered formalin, embedded in paraffin, and cut into 4 µm sections. Liver samples were obtained from autopsies performed at the Instituto de Medicina Legal do Porto.

b) Mice

Transgenic mice expressing either human TTR Val30Met or TTR Leu55Pro and without mouse TTR (Kohno et al., 1997; Sousa et al., 2002) were investigated. Mice not expressing endogenous TTR- mouse TTR KO (Episkopou et al., 1993) were used as a control. Skin and stomach were dissected after anesthetising the animals intraperitoneally and sacrifice. Tissues were fixed in neutral buffered formalin, embedded in paraffin, and cut into 4 µm sections. Skin tissues were also immediately frozen in dry ice and stored at -70ºC until use.

Immunohistochemistry

Tissue paraffin slides were deparafinated and hydrated. Endogenous peroxidase was destroyed before blocking with 1% BSA, 4% fetal bovine serum in PBS for 1 hour at 37ºC. Primary antibodies were mouse anti-ubiquitin (1:600, Santa Cruz Biotechnology, Inc) and rabbit anti-human TTR (1:1000, DAKO), and slides were incubated in a humidified chamber, overnight (o/n) at 4ºC. Human and mice tissues tested for ubiquitin were treated for epitope exposure in boiling citrate buffer, for two times, 5 minutes each in the microwave. The extra-3 kit (SIGMA) was used as the detection method and substrate reaction colour was visualized with 3-amino-9-ethyl carbaxole, AEC (Sigma) or 3,3´diaminobenzidine DAB (Sigma). Immunohistochemistry of mice samples with the monoclonal antibody ubiquitin was

performed using the MOM kit (Vector) according to supplier's instructions. The reaction was developed as described, counterstained with haematoxylin and mounted with an aqueous mounting medium from DAKO.

Semi-quantitative analysis of the positive immunostaining was determined with the Scion programme that measures the area occupied by the substrate colour in pixels. Statistical significance was determined using the student's t-test with a minimal p value of <0.02 required for significance.

Immunofluorescence

Mouse anti-ubiquitin (1:600, Santa Cruz Biotechnology, Inc), rabbit anti-TTR (1:1000; DAKO) antibodies were evaluated in human and mice tissue paraffin slides. Secondary antibodies were donkey anti-rabbit IgG- Alexa Fluor 488 and goat anti-mouse IgG- Alexa Fluor 568, diluted 1:1000 in blocking buffer, 30 minutes at room temperature, protected from light. After washing with PBS, slides were mounted with vectashield (Vector) or vectashield with DAPI (Vector), and visualized in a Zeiss Cell Observer System microscope (Carl Zeiss, Germany) equipped with filters for FITC and rhodamine like dyes.

Total protein extracts

Mice skin tissues (approximately 100 mg) were minced with a razorblade and homogenised on ice with a glass rod in 500 µl of lysis buffer that contained EDTA, EGTA, MOPS, Triton, PMSF and proteases inhibitors (cocktail set III, Calbiochem). Total homogenates were passed through a 26 Gauge syringe and centrifuged at 14,000 rpm for 30 minutes. Supernatants were separated for immunoblot analysis. Proteins were quantified with Bio-Rad protein assay (Bio Rad) using BSA as standards. The same amount of protein was loaded in a 12% SDS-PAGE, transferred to a nitrocellulose membrane, incubated for 15 minutes in boiling water for epitope exposure, blotted and probed for ubiquitin (1:500, Santa Cruz Biotechnology, Inc). Blocking was done with 10% skimmed milk o/n at 4°C. As determined by β-actin immunoblot (1:5000, Sigma) the same amount of protein was loaded.

Tissue aggregates extracts

Aggregated and fibrillar protein was extracted from mice skin with the Kaplan's method (Kaplan et al., 1994). Briefly: 100-150 mg of skin were homogenized on ice with 1 ml of PBS using a glass rod and centrifuged for 10 minutes at 4ºC, 14000 rpm. This step was repeated three more times to remove the blood and soluble components. The pellet was then incubated with 1 ml of acetonitrile (20%), trifluoroacetic acid (0.1%) mixture, three times, for 1 hour each with agitation at room temperature. The supernatants were pooled, lyophilized and then resuspended in 50 µl of 1x SDS buffer. After boiling 5 min, 25 µl of the extracted aggregates were loaded on a 15% SDS polyacrylamide gel. A western blot was performed with anti-TTR antibody (1:500, DAKO) and developed with supersignal west pico chemiluminescent substrate (Pierce). After stripping with re-blot plus solution (Chemicon), the membrane was reprobed with antibody anti-ubiquitin (1:500, Santa Cruz Biotechnology, Inc) and developed using the same procedure.

Cell culture

A rat Schawnn cell line expressing TTR wt, TTR Met30 and TTR Pro55, under the control of an inducible methalothionein promoter were stably co-transfected in the laboratory (Cardoso, PhD thesis, 2002). These and untransfected cells were cultured in DMEM Dulbeco´s medium supplemented with 10% fetal bovine serum and the antibiotics penicillin/streptomycin (0.1 mg/ml) and G418 (1 mg/ml). TTR expression was driven by adding 0.1 mM of zinc solution for 3 days. Cells grown on a lab-tek chamber slide (Nalgene Nunc International) were washed twice with PBS and fixed for 30 minutes at room temperature with 2% paraformaldehyde in order to perform immunocitochemistry. 2.5 x 10^6 cells, grown a 10 Ø dishes (Nalgene Nunc International), were harvested for total protein extracts, after washing twice with ice cold PBS. Lysis buffer was added and cells were scraped and collected. The lysate was centrifuged at 14,000 rpm for 30 minutes at 4ºC. Proteins in the supernatant fraction were quantified and stored at -20ºC until analysis by western blotting. Quantification of positive ubiquitin reaction by western blotting was done using the Scion image quant and normalized for actin.

Isolation and purification of TTR

TTR wt was produced in an E. coli expression system. Protein isolation and purification was performed as described by Furuya et al., 1991, using preparative gel electrophoresis after ion exchange chromatography.

Preparation of TTR species

Soluble TTR was obtained after isolation and purification. TTR was filtered with 0.2 μm filters just before use.

Aggregated TTR was prepared by incubating the protein at a concentration of 2 mg/ml in 0.05 M sodium acetate (pH 3.6) for 72 hours at room temperature. The sample was then centrifuged at 14000 rpm for 30 minutes and the pellet resuspended in filtered PBS.

Fibrils of TTR were initiated by the same process of aggregated TTR and then further incubated at 37ºC and allowed to form and grow for 15 days.

Oligomers of TTR were made by incubating the protein with stirring at room temperature for 8 days. TTR was at a concentration of 0.25 μg/ml.

Proteasome inhibition

Cells grown as described above, were treated with the 20 μM proteasome inhibitor-MG132 (Calbiochem) for 12 hours. Control experimental group received the equivalent DMSO volume, for the same period of time. Cells were collected for western blot analysis of the total protein extracts or fixed for immunocitochemistry.

Immunocitochemistry

Cells fixed on a labtek were washed in PBS and incubated in blocking buffer (4% FBS; 1% BSA) for 30 minutes at 37ºC. Primary antibodies were added at 4ºC for an o/n. These included: anti-human TTR (1:1000, DAKO); anti-ubiquitin (1:500, Santa Cruz Biotech), and anti-vimentin (1:100, DAKO). After washing twice with PBS for 15 minutes, corresponding secondary immunofluorescent antibodies were incubated for 30 minutes at room temperature. Immunofluorescence staining was visualized in a Zeiss Cell Observer System microscope (Carl Zeiss, Germany) equipped with filters for FITC and rhodamine like dyes.

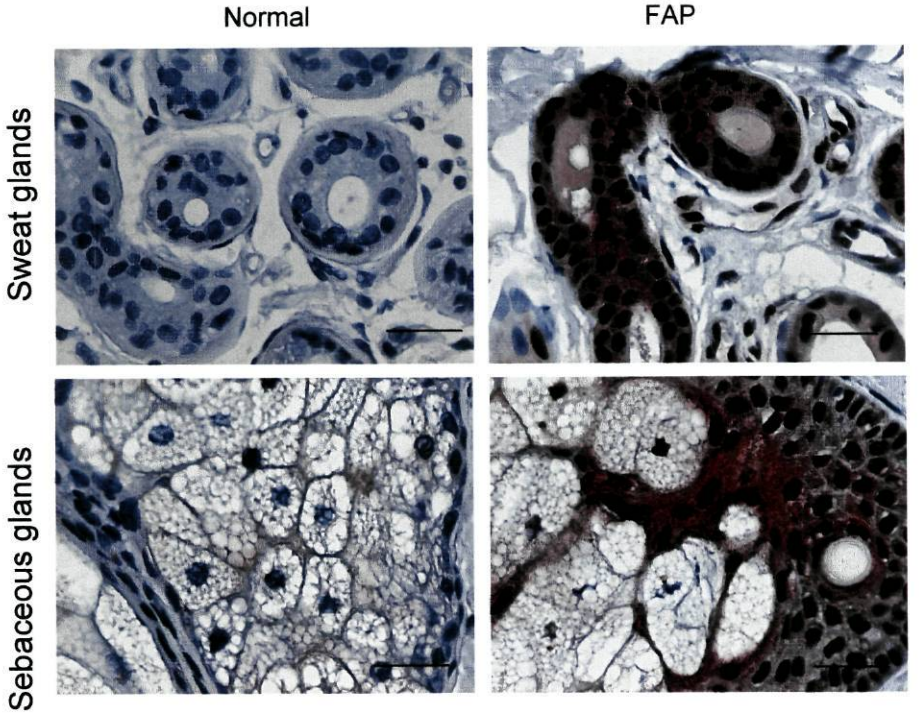
Proteasome activity

The rat schwanomas, described above, were collected with proteasome lysis buffer (10mM Tris pH7.5, 1 mM EDTA, 2 mM ATP, 4mM DTT and 20% glycerol). Cells extracts were centrifuged for 30 minutes at 14,000 rpm and the supernatant (soluble fraction - SF) was separated from the pellet (insoluble fraction - IF). This last fraction was resuspended in 100 µl of lysis buffer and protein content in both fractions determined as described above. 20 µg of the SF and 40 µg of the IF were used for the determination of the proteasome activity. The assay buffer (50 mM Tris pH8.0, 1 mM EDTA, 1 mM ATP and 50 µM of the fluorogenic substrate) was added to perform 400 µl of total reaction volume. The fluorogenic substrate- Suc-LLVYMCA, (Calbiochem) used belongs to the class of chymotrypsin substrate III. To measure the background activity in control parallel reactions the proteasome inhibitor MG132 was added at a concentration of 40 µM. The reaction was performed in the dark, at 37°C for 30 minutes and stopped by adding 800 µl of ice-cold water and incubating 10 minutes on ice. The proteasome activity was measured with 1/10 of the reaction volume, in a spectrofluorometer apparatus (Jasco FP- 770) at an emission of 380 nm and an excitation of 460 nm.

Results

Ubiquitin increased expression in human FAP skin

We started our studies by analysing ubiquitin levels in the main TTR synthesizing organ, the liver. Comparison between normal and PAF patients expressing TTR Val30Met showed no differences in this tissue (not shown). Ubiquitin was investigated by semi-quantitative immunohistochemistry as well in skin biopsies of FAP patients with overt amyloid deposition (labelled +/+) and in skin biopsies with aggregated non-fibrillar TTR deposits (labelled -/+) from carriers of Val30Met, in comparison with normal control biopsies. A clear, significative difference was observed: thus, while normal control skin stained for ubiquitin at background levels, both types of clinical samples from FAP patients and carriers of Val30Met revealed an upregulation of ubiquitin expression (figure 1A, showing a normal and an asymptomatic carrier skin biopsy). This up-regulation was observed in sweat and sebaceous glands and in epidermis.



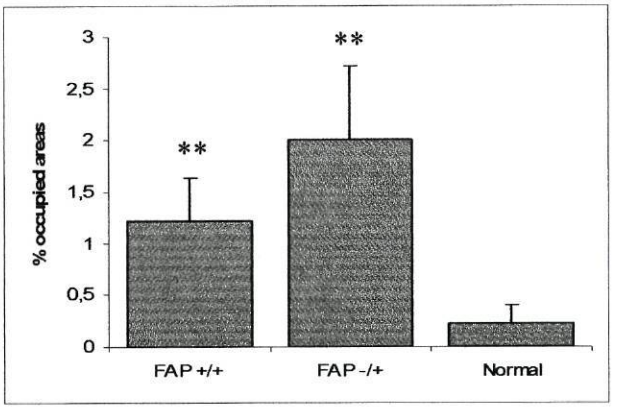


Figure 1A-
Optical immunohistochemistry for ubiquitin in human skin showing sweat glands (top pictures) and sebaceous glands (bottom pictures). Scale bar 30 µm. The chart displays the percentage of occupied area by the substrate colour in human FAP skin (+/+) n=6; human FAP skin (-/+) n=9 and human control skin (n=6). ** $p< 0.001$.

We investigated as well the relationship between tissues affected with aggregated TTR and tissues affected with amyloid deposition composed of TTR fibrils. Regarding this question a small difference was detected with an increase in -/+ skin in comparison to +/+ skin (figure 1A, chart), so this response seems to occur early in FAP being more robust in the initial phase and maintained during the course of the disease.

We also examined ubiquitin expression in the peripheral nerves of FAP patients but no direct correlation could be drawn in the nervous system, relating TTR deposition and ubiquitin expression. Instead, blood vessels located in FAP patients nerves, regardless of its classification, displayed ubiquitin increased expression that was not observed in normal individuals (not shown). It is possible that this effect occurs in dorsal root ganglia (DRGs) close to neurons where extracellular deposition occurs.

By double immunofluorescence we examined if TTR co-localized with ubiquitin. We could not co-localize ubiquitin with TTR in the skin but instead the tissues close to TTR deposits present an up-regulation of ubiquitin expression as it is shown in the sweat glands close to TTR deposits (figure 1B).

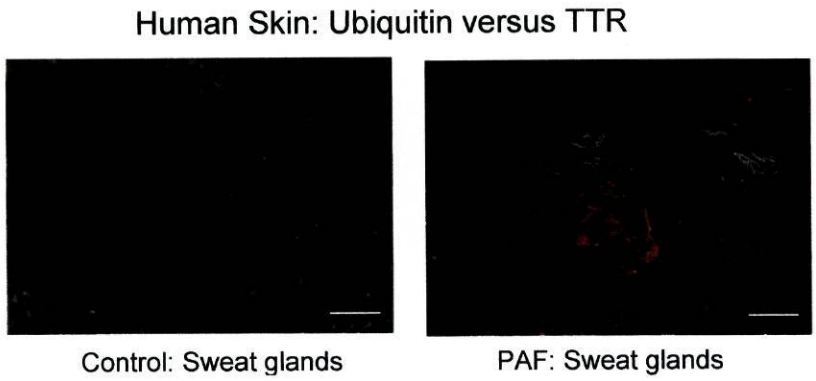


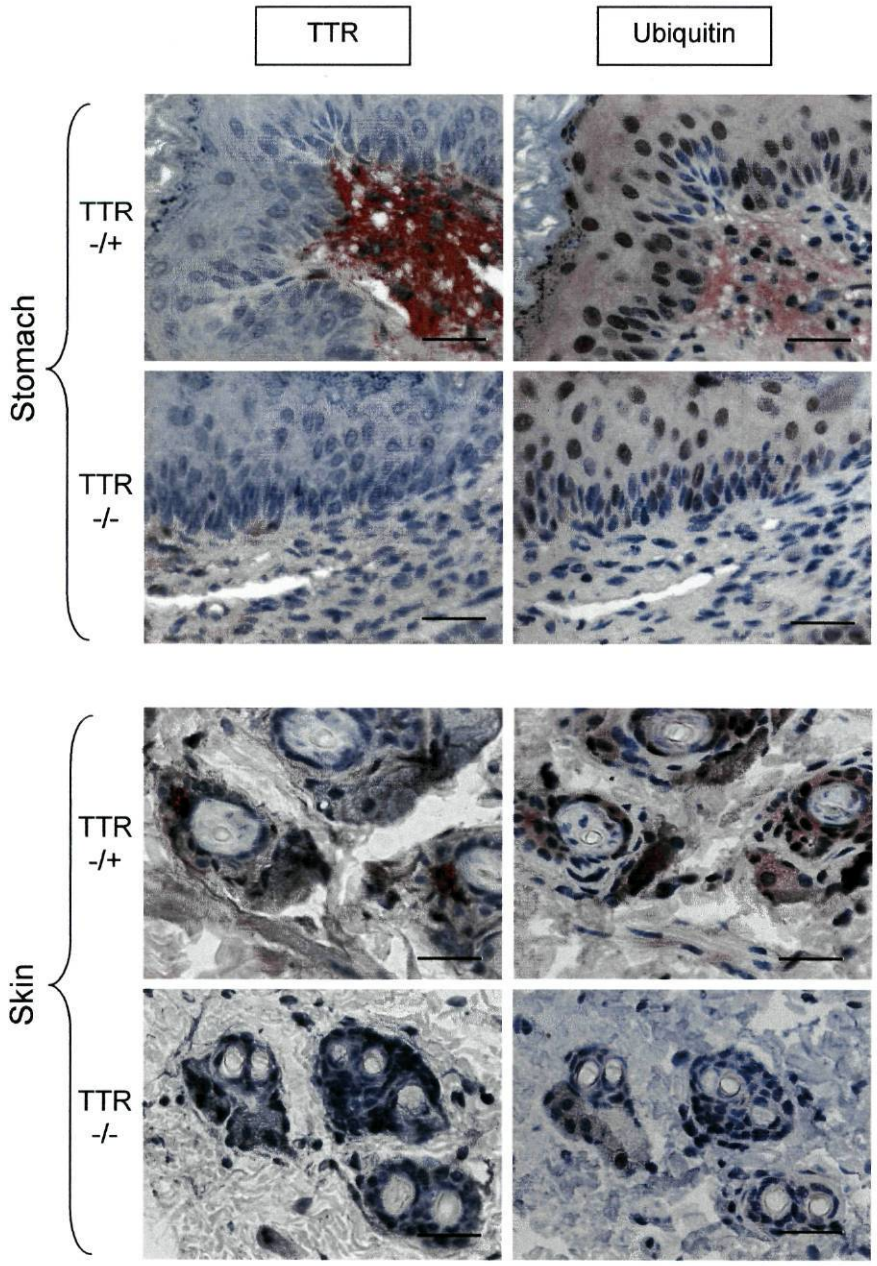
Figure 1B-
Immunofluorescence in human skin - sweat glands – for TTR (green) and ubiquitin (red). Scale bar 30 μm.

Ubiquitin can recognize misfolded proteins with the auxiliary enzymes of the ubiquitin pathway; since skin does not synthesize TTR, and ubiquitin is localized intracellularly and does not co-localize with TTR, these results indicate that ubiquitination occurs as a consequence of the presence of extracellular aggregates, eliciting an UPS response.

Ubiquitin expression in TTR transgenic mouse.

Transgenic mice for TTR Val30Met present systemic TTR deposition in different organs, including the skin and gastrointestinal tract. We investigated if these mice skin had an increased ubiquitin expression, close to TTR deposits, as humans do and extended the studies to stomach, which is a main organ affected by TTR deposition in this model.

Analyses were performed by immunohistochemistry and up-regulation of ubiquitin expression was observed when TTR deposition occurred (figure 2A, upper panels). Ubiquitin up-regulation was also observed for another transgenic mouse model for FAP expressing TTR Leu55Pro, a variant that leads to more aggressive clinical characteristics in humans (Jacobson et al., 1992; Yamamoto et al., 1994) and in mice as well (Sousa et al., 2002). Comparing the two strains of mice, ubiquitin up-regulation related to TTR deposition was more pronounced in the TTR Leu55Pro mice (figure 2A, lower panel and chart), probably reflecting the higher cytotoxic effect of tissue TTR Leu55Pro deposition in mice than TTR Val30Met (Sousa et al., 2002). In both cases, ubiquitin up-regulation occurred close to TTR deposits.



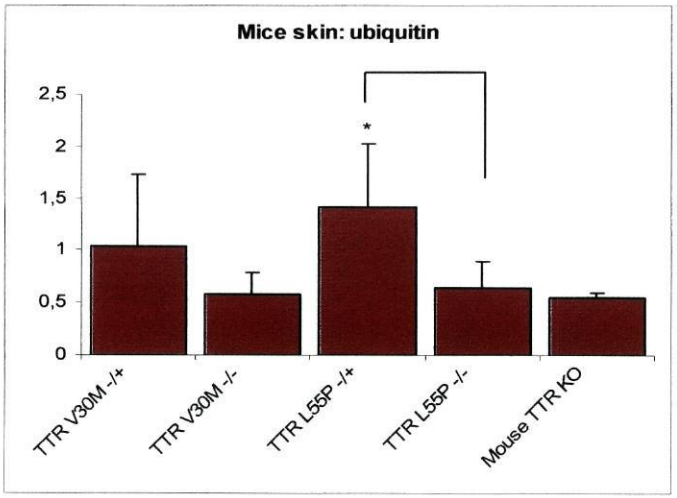


Figure 2A-
TTR immunoreactivity (left pictures) in stomach and skin and corresponding areas with immunostaining for ubiquitin (right pictures). Scale bar 30 µm. Bottom chart shows the semiquantitative analysis of ubiquitin of the skin in different strains of mice: TTR Val30Met -/+ (n=5), -/- (n=4); TTR Leu55Pro -/+ (n=8), -/- (n=3); Mouse TTR KO (n=5). *p< 0.002.

By contrast, in liver of both Val30Met and Leu55Pro mice that synthesize TTR but do not present deposition, no difference in ubiquitin level was detected in comparison to the wild type mice (data not shown).

The increased ubiquitin expression between mice with and without TTR deposition was confirmed by subsequent experiments. By Western blot analyses for ubiquitin we analysed total proteins extracts from Leu55Pro and Val30Met tissues with (-/+) and without (-/-) deposition in comparison with TTR null mice skin (figure 2B). We observed a higher ubiquitin staining in transgenic mice with TTR deposition in comparison to animals without deposition or control TTR null mice, particularly for high molecular weight ubiquitinated aggregates.

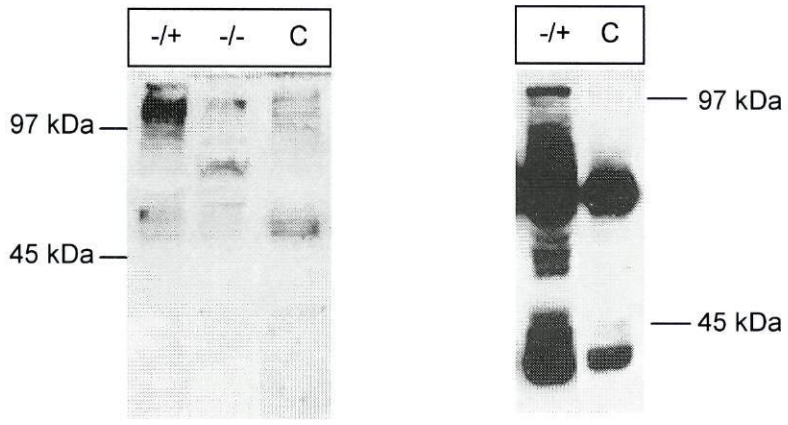


Figure 2B-
Mice skin total protein extracts probed for ubiquitin. Left blot: Val30Met mice with (-/+) and without (-/-) deposition; C- control mTTR-KO mice. Right blot: Leu55Pro mice with (-/+) deposition and control mTTR-KO mice.

To address the question if this increased ubiquitin expression was directly influenced by the presence or absence of TTR deposition we used the Kaplan's method (see materials and methods) to extract TTR aggregates from tissues and probed the extracts for TTR and reprobed for ubiquitin after. The resulting pattern was different from a tissue without and with deposited TTR (figure 2C), the latter showing an ubiquitin ladder with high molecular weight proteins conjugated to ubiquitin which is clearly absent in tissue without TTR deposition.

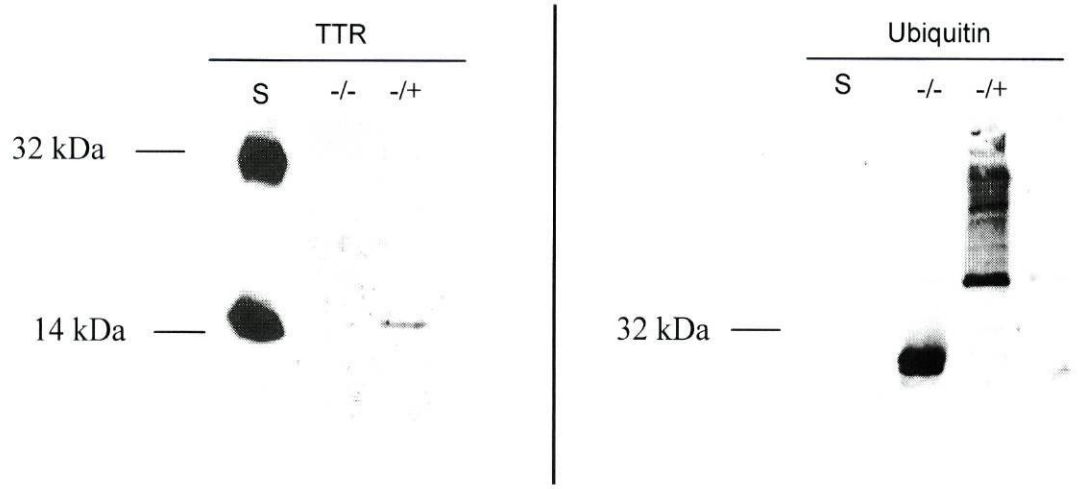


Figure 2C-
Aggregates extracts from Val30Met mice skin with (-/+) and without (-/-) deposition by the Kaplan´s method analysed by anti-human TTR western blot, followed by anti-ubiquitin western blot as described under materials and methods. S- standard human recombinant TTR.

UPS response in cells expressing TTR amyloidogenic variants

With the aim to understand the meaning of the different ubiquitin levels observed in humans and mice, we cultured mammalian cells – a rat Schwann cell line - transfected with wild type TTR and the two studied TTR variants: Val30Met and Leu55Pro. Furthermore, the effect on ubiquitination after proteasomal inhibition was compared in cells expressing the different TTR variants. In the absence of proteasome inhibition, different ubiquitin levels in these cells were detected (figure 3A upper part and chart). RN22 cells expressing TTR Leu55Pro showed the highest effect in ubiquitin expression: an increase in high molecular weight conjugates in comparison not only to control (RN22), but also to TTR wt and TTR Val30Met expressing cells. Interestingly, higher free ubiquitin was present in Leu55Pro expressing cells thus suggesting that protein aggregates do not deplete the free ubiquitin pools and possibly lead to an increase in ubiquitin synthesis instead.

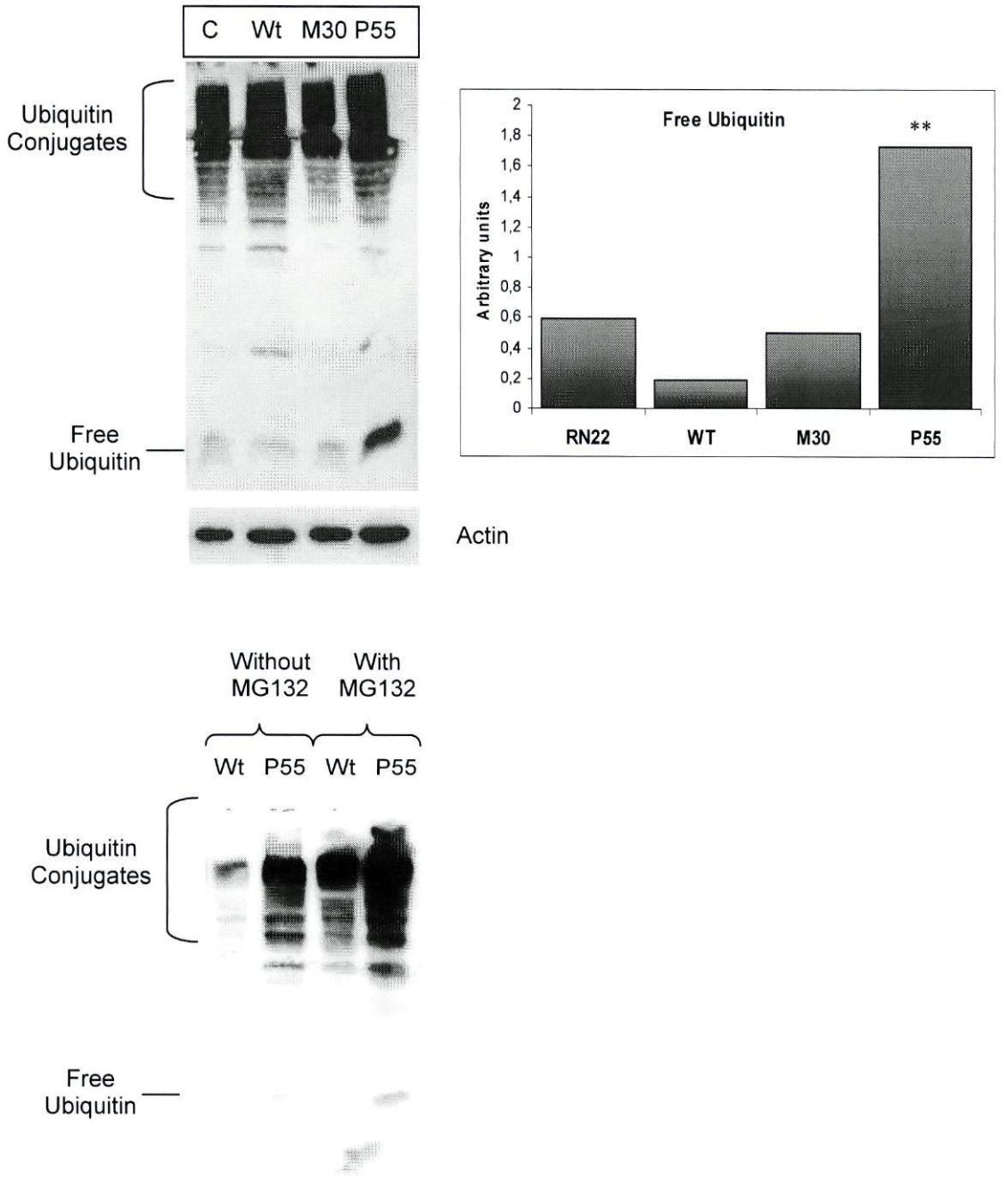


Figure 3A–
Upper part: Anti-ubiquitin western blot of total protein extracts from RN22 cells not expressing TTR (C) and expressing TTR WT (Wt), Val30Met (M30) and Leu55Pro (P55). Results were obtained from 3 independent experiments. Chart: quantification of free ubiquitin levels using scion image quant program. ** $p < 0.002$.
Bottom part: Anti-ubiquitin western blot of RN22 cells expressing TTR wt or TTR Leu55Pro with and without addition of proteasome inhibitor MG132.

The ubiquitin conjugates did not stain for TTR by western blotting (not shown) indicating that proteins other than TTR are ubiquitinated. The results obtained with

Leu55Pro cell protein extracts are in accordance with the pronounced effect observed in Leu55Pro mice tissues (described above). The results of Val30Met cells are not as significative as those with Val30Met transgenics.

In this cell model, TTR is secreted to the medium and while TTR Val30Met does not aggregate in the media, TTR Leu55Pro does. The latter forms small aggregates, as studied by a filter assay that retains aggregated and fibrillar proteins while excluding soluble ones (Cardoso, PhD thesis, 2002); thus, the noticeable effect on UPS between Val30Met and Leu55Pro producing cells reflect extracellular TTR aggregate formation that occurs exclusively in Leu55Pro expressing cells.

Next, the effect of proteasome inhibition on ubiquitination of wt and Leu55Pro synthesizing cells was investigated, as this variant showed the highest difference related to ubiquitin expression. The fate of ubiquitinated proteins is degradation by the proteasome; inhibition of the proteasome induces cellular protein accumulation. After proteasome inhibition by MG132, we detected the expected increase in ubiquitin bands (figure 3A, bottom part) for both variants. Moreover, increased free ubiquitin was present in RN22 Leu55Pro as described before, both without and with the addition of MG132, which suggests that the increase in ubiquitin conjugated protein aggregates up-regulates ubiquitin synthesis, that become available for further ubiquitination.

Immunocytochemistry was followed so as to analyse TTR and ubiquitin expression (figure 3B) Double immunofluorescence showed no co-localization of TTR with ubiquitin. We used transfected RN22 cells that direct TTR synthesis to the extracellular space. We detected TTR in all transfected cells, albeit not in a punctuated/aggregated form. An increase in ubiquitin was detected in cells expressing Val30Met and Leu55Pro TTR. This last variant showed ubiquitin intracellular inclusions that morphologically resembled aggresomes (figure 3B, arrows). Occasionally, these round ubiquitin inclusions were detected in RN22 Val30Met cells but when found were smaller. The same cells were investigated after inhibiting the proteasome. As visualized by immunocitochemistry, proteasome inhibition increased the ubiquitin detected inside the cells (figure 3B right panels), even for untransfected cells, but ubiquitin round inclusions were never observed, except with the Leu55Pro variant, making this protein more vulnerable to the effects of the proteasome inhibition.

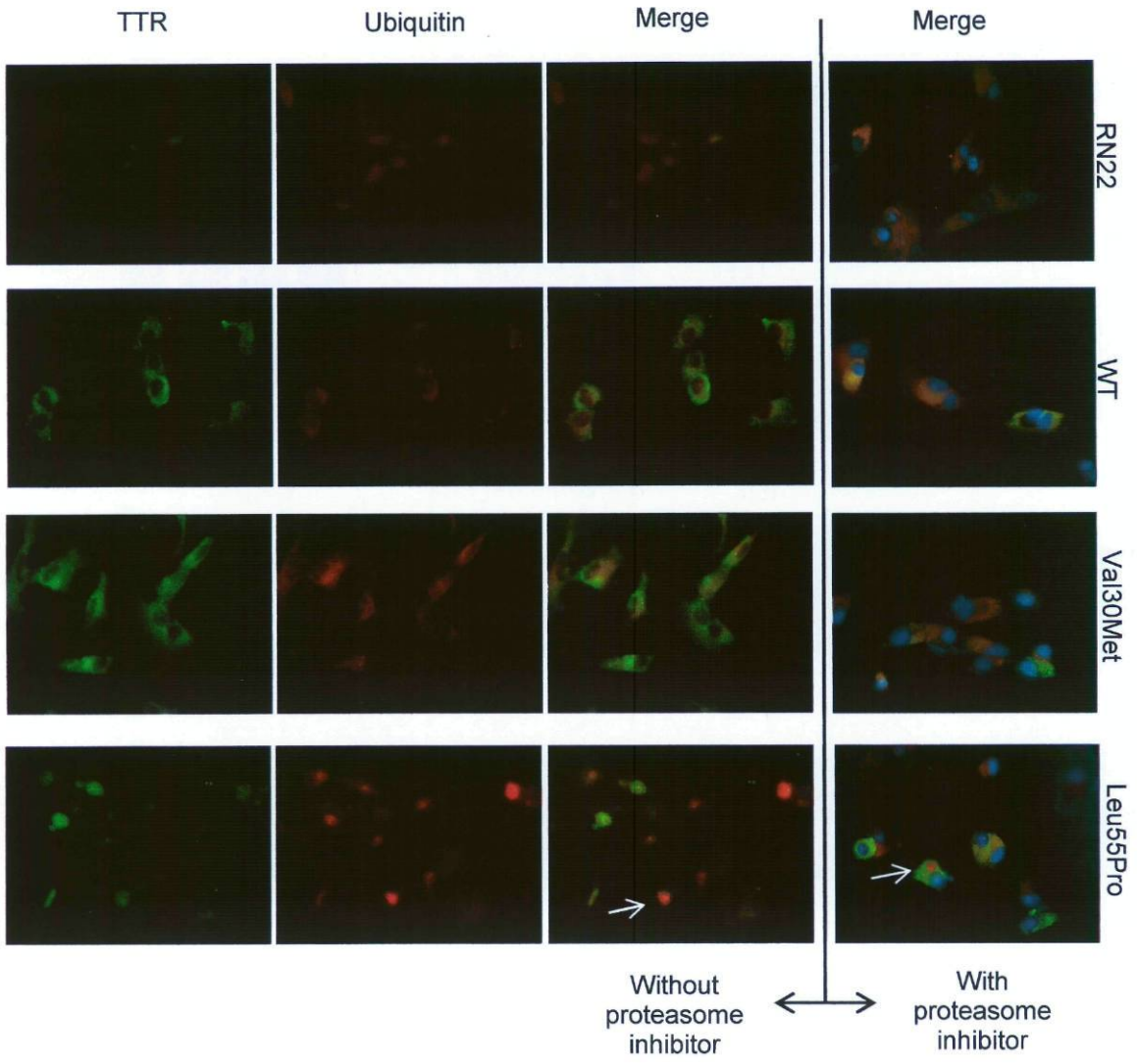


Figure 3B–
Double Immunofluorescence of TTR (green) and ubiquitin (red) of RN22 cells without expression of TTR (RN22) and expressing TTR Wt, Val30Met and Leu55Pro. Left 3 panels are cells grown without proteasome inhibitor, in the right panel are cells in the presence of proteasome inhibitor.

Proteasome activity was investigated in RN22 cells expressing wt, Val30Met and Leu55Pro TTR and compared to non-transfected cells. No differences were observed in proteasome activity for the soluble fraction (figure 4); however, a significative decrease in activity was found for the insoluble fraction in the case of Leu55Pro synthesizing cells. We assume an impairment of the proteasome due to the influence of extracellular aggregate formation, which is absent from other RN22transfected cells.

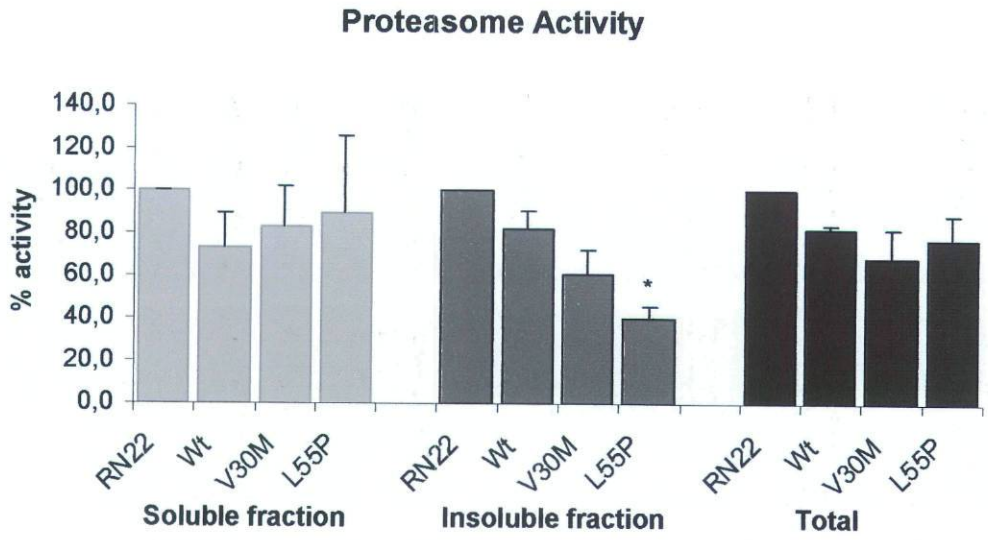


Figure 4–
Measurement of the proteasome activity in the soluble fraction, insoluble fraction and total (soluble + insoluble fractions) in different conditions: untransfected RN22 cells (100% activity), and transfected with plasmids for wt, Val30Met and Leu55Pro. Results were obtained from 3 independent experiments; $p < 0.05$.

Aggresomes are intracellular amorphous protein inclusions (Johnston et al., 1998) that contain ubiquitin and chaperones, associated with proteins unable to be degraded by the proteasome. Its formation is accompanied by a redistribution of the intermediate filament – vimentin. Analysis of the cytoskeleton network in this cell model was performed with vimentin, a component of the cytoskeletal system that appears as elongated bundles of filaments in normal cells. When intracellular protein inclusions are formed, as described for example in Huntigton's disease, it appears as a ring-like structure surrounding the aggregated protein. The study of vimentin in RN22 cell line resulted in very interesting results for RN22 Leu55Pro both in the presence and in the absence of proteasome inhibition. These cells presented a distribution of vimentin distinct from RN22 and from RN22 wt or RN22 Val30Met (figure 5) as vimentin appear to be pushed away to the cell periphery and in some cells with perinuclear redistribution. Inhibition of the proteasome activity leads to a reorganization of the vimentin intermediate filament network and its collapse in all RN22 cells (not shown). However, in RN22 Leu55Pro cells, once again, vimentin appears in the cell periphery. In RN22 Val30Met this pattern could be observed as well but not so expressive as with RN22 Leu55Pro.

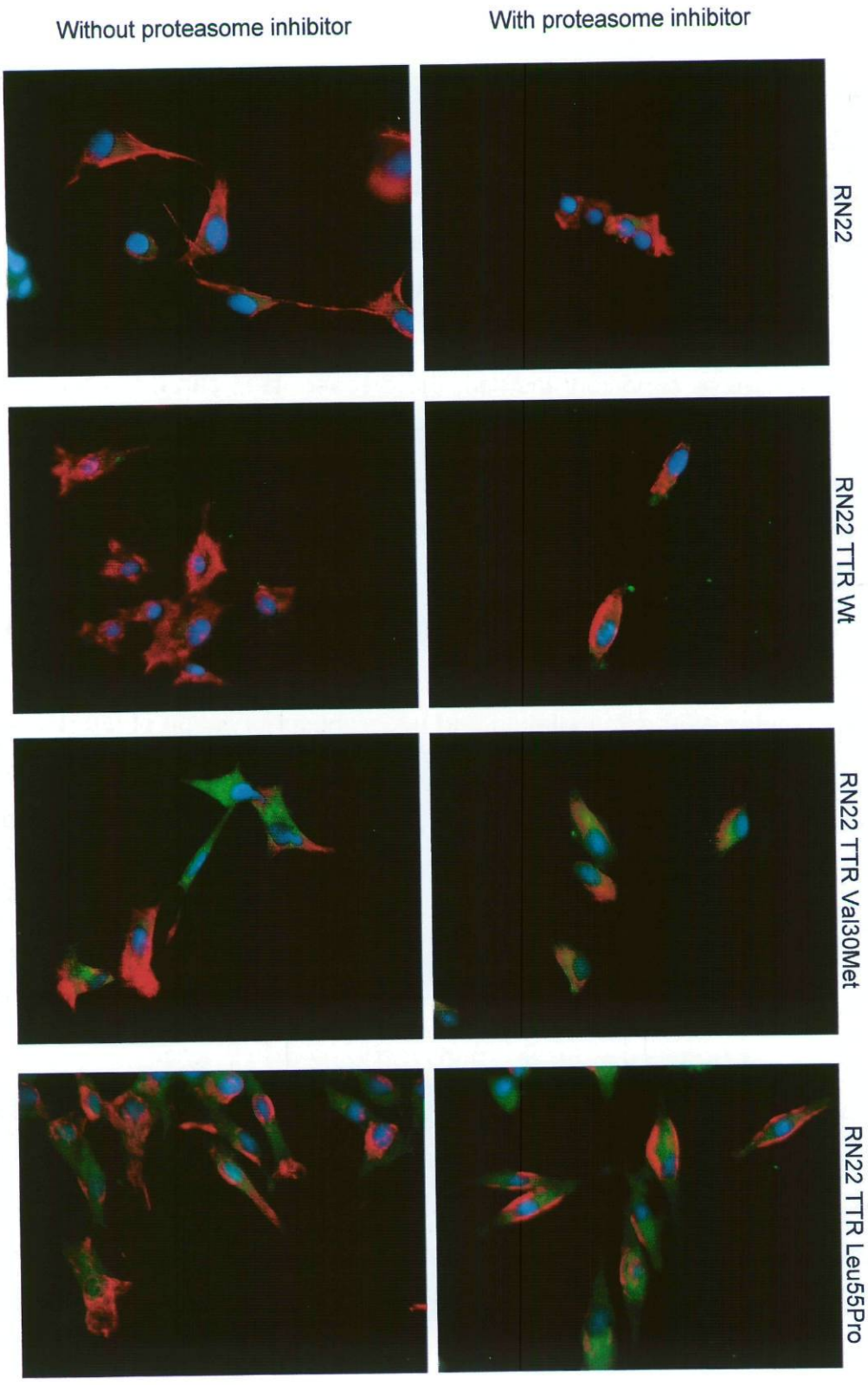


Figure 5–
Analysis of TTR (green) versus vimentin (red) in RN22 cells without expression of TTR (RN22) and expressing TTR Wt, Val30Met and Leu55Pro. Blue represents the nucleus as revealed by DAPI staining. Left rows are immunofluorescence of cells not treated with MG132. whereas right rows are cells treated with MG132.

Discussion

Ubiquitin is unquestionably involved in the interplay between molecular cell biological and molecular cell pathological mechanisms. Findings in healthy and diseased cells have indicated its vital role in cell homeostasis.

In pathological conditions, questions have arisen if an ubiquitin higher expression is a result of the disease itself or can instead contribute to its initiation or progression: is it a cause or a consequence? If ubiquitin function, as a primary event, is impaired it can lead to a disease condition; instead, the disease itself can lead to ubiquitin impairment, as a secondary response. The former situation has been analysed with findings linking mutations in the UPS to neurodegenerative diseases (Kitada et al., 1998; Cummings et al., 1999), as in autossomal recessive juvenile parkinsonism a result of mutations in Parkin, a ubiquitin-protein ligase (Kitada et al., 1998) or mutations in the presenilin or the ub^{+1}. The second hypothesis is strengthened with many reports over the last years. In AD, ubiquitin is associated with tau protein in neurofibrillary tangles after accumulation and hyperphosphorylation of tau (Bancher et al., 1989). Moreover impaired proteasome function in defined brain regions of AD affected individuals (Keller et al., 2000a) and defective ubiquitination (López Salon et al., 2000) has been studied. Likewise, some (but not all) Lewy bodies in PD are ubiquitin positive (Spillantini et al., 1998; Gomez-Tortosa et al., 2000) and proteasome function is compromised (McNaught and Jenner, 2001). In a cell line model of Huntigton´s disease aggregation is the cause of ubiquitination and inhibition of the UPS (Bence et al., 2001). Therefore, it is more likely that ubiquitination is not an initiating event in protein aggregation related pathologies but a later response to aggregation that is common in these diseases. In sum, pathologies related to protein misfolding, as in Huntington´s disease, aggregates are inhibitors of the UPS as are molecular products that result from its inhibition occurring intracellularly. FAP is also a protein misfolding disorder but aggregation happens extracellularly. An upregulation of ubiquitin expression was observed in tissues related to TTR deposition, while in liver synthesising TTR wt or amyloidogenic variants, but not having TTR deposition, no increase in ubiquitin was observed. This physical separation between extracellular TTR aggregates and

intracellular ubiquitin impedes a direct correlation between the UPS and TTR aggregation, but might be explained by an indirect influence through cellular membranes and its receptors.

Ubiquitin expression is ubiquitously detected in different tissues and in distinct situations; in the FAP tissues analysed, ubiquitin up-regulation was observed close to TTR deposition, and tissues with deposition had overt protein ubiquitination. Nevertheless, different tissues might respond differently, depending on the cell type involved.

The findings obtained with TTR Leu55Pro variant in mice and cells were more pronounced than those with TTR Val30Met. Analysis of the structure, binding properties, stability and amyloidogenicity of both variants, shows that TTR Leu55Pro has a decreased stability of the tetramer towards dissociation into monomers and confers a lower affinity to thyroxine, which binds in the channel that runs through the tetramer (Almeida and Saraiva, 1996). This variant also shows a greater propensity to amyloid formation *in vitro*. Crystallographic studies of the structure of the Leu55Pro variant revealed major conformational alterations (Sebastião et al., 1998). The conformational changes observed in these "aggressive' mutations may resemble intermediate forms in the process of amyloidogenesis. So, comparing TTR Val30Met and TTR Leu55Pro, the latter is much more amyloidogenic and the clinical manifestations are more aggravated.

The higher influence on the UPS by TTR Leu55Pro correlates well with its biochemical and pathological characteristics. Therefore, we suggest that in FAP, components of the cell quality control system in cells expressing TTR are not impaired, but the extracellular deposition of TTR leads to the UPS response.

The implication of the UPS for FAP pathology is broad and may involve oxidative injury and apoptosis. It is known that oxidative stress and concomitantly expression of ROS species causes inhibition of the proteasome (Keller et al., 2000b) and that oxidized proteins are recognized and degraded by the proteasome but severe oxidative injury leads to an inhibition of proteasome activity and protein accumulation (Shringarpure et al., 2000). Studies in tissues from FAP patients have shown an increase in oxidative pathways such as nitrosylation of proteins and increase in nitric oxide synthase; this increased cellular oxidative stress may lead to the ubiquitination observed in tissues and in cells exposed to TTR oligomers. In

FAP patients, oxidant levels were altered due to TTR deposition (Sousa et al., 2001a) and these levels in turn may influence the UPS.

On the other hand, it was established that the programmed cell death is activated in FAP through caspase-3 (Sousa et al., 2001b). Furthermore, it is known that inhibition of the proteolytic function activates the programmed cell death (Drexler, 1997) so the proteasome can be implicated in protection against cell death through apoptosis.

Extracellular signals are continuously an input to the inside of the cell resulting in diverse responses. A plausible hypothesis is that when TTR functions as a pathological signal by extracellular deposition it induces an UPS response, as a defence response for survival that eventually might turn pathological due to the degree of cellular damage. Ubiquitin appears as a mediator in FAP pathogenesis between TTR extracellular deposition and a higher susceptibility towards stressors. UPS would function as a means of the cell to respond to extracellular insults. If effective or not, should depend on the degree of aggression.

The receptor and signaling pathway(s) involved in this process remain to be clarified. RAGE is a possible mediator for the ongoing response by UPS in FAP. This receptor is a multiligand member of the immunoglobulin superfamily with a broad repertoire of ligands, including amyloid-associated proteins. Soluble and fibrillar TTR binds RAGE (Sousa et al., 2000b). In FAP, RAGE seems to be relevant in cellular dysfunction since its expression correlates with TTR deposits and is upregulated as the disease progresses (Sousa et al, 2000b and 2001a). Moreover, when TTR binds to RAGE it triggers inflammatory and oxidative pathways and NF-kB activation as well, which culminates with neuronal death. Ubiquitin as intermediate in this mechanism could interfere in a protective response to delay the pathology.

ACKNOWLEDGEMENTS

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CHAPTER 5

Functional roles of *Escherichia coli* TTR-like protein

Functional roles of *Escherichia coli* TTR-like protein

Sofia Duque Santos[1,2]; Max Gottesman[3]; Maria João Saraiva[1,2]

[1]Molecular Neurobiology Unit, Institute for Molecular and Cell Biology; [2]Instituto de Ciências Biomédicas de Abel Salazar, University of Porto, Portugal; [3] Institute of Cancer Research, College of Physicians and Surgeons of Columbia University, New York, USA.

Corresponding author: Maria João Saraiva; Molecular Neurobiology; Instituto de Biologia Molecular e Celular. Rua do Campo Alegre 823. 4150-180 Porto. Portugal.
Tel. 351-226074900; Fax 351-226099157. e-mail: mjsaraiv@ibmc.up.pt

Running title: *E.coli* TTR-like

Abstract

Homologous proteins are widely found in different organisms and in most cases they share the same function. In *Escherichia coli* (*E. coli*) a protein that is 32% homologous to human transthyretin (TTR) was identified and named TTR-like. This protein was also described in other prokaryotes and in lower eukaryotes.

Human TTR is a plasma transporter of thyroxin (T4) and retinol, this last one through association with retinol binding protein. It is an extremely well described protein at the biological, biochemical and molecular levels, whereas TTR-like proteins are not. We generated an *E. coli* TTR-like knock-out by homologous recombination obtaining a mutant with phenotypic variations. Although TTR-like is not essential to the survival of the organism since the knock-out is viable, it has a role during aerobic metabolism and in conditions of nitrogen as an energy source. Moreover, enzymes which catalyze the degradation of pyrimidine nucleosides and their utilization as carbon and energy sources were found to be downregulated when TTR-like is absent, reinforcing its function during metabolic pathways. We found other proteins differentially expressed in the *E. coli* TTR-like KO that were mainly located in the periplasm. This is the predicted localization of the TTR-like protein due to the existence of a signal peptide in its sequence. In accordance, we confirmed its presence in the periplasmic space.

TTR-like protein was also produced for biochemical characterization. Prokaryotic TTR-like protein is a tetramer of identical subunits of approximately 14 kDa and presents similar behaviour under conditions that normally dissociate the TTR tetramer to a monomer, albeit with a different behaviour as no dimer is observed. Interestingly, we found an interaction between TTR and TTR-like. TTR-like is able to form amyloid fibrils by acidification as does human TTR wt and can accelerate the rate of human TTR fibril formation. An endogenous ligand candidate for TTR-like in prokaryotes was not investigated, though one of the natural ligands for human TTR, namely T4 did not present specific binding to TTR-like. Nevertheless, TTR-like opens the possibility for the search of other ligands and allows, as well the investigation of structural-functional characteristics.

Moreover, TTR-like might be an ancestor of TTR whose divergence should now start to be clarified.

Introduction

In almost all vertebrates, transthyretin (TTR) is responsible for the transport of RBP complexed with retinol (vitamin A) and for the appropriate tissue distribution of thyroid hormones - thyroxine (T4) and 3, 3´, 5-triiodothyronine (T3), important for growth, development and metabolism.

TTR should have evolved in the choroid plexus of the stem reptiles 350 million years ago (Achen et al., 1993). Liver TTR synthesis is suggested to have been turned on much later, about 50 million years ago, and independently in the lineage leading to today's eutherians, diprotodont marsupials, polyprotodont marsupials, birds and fish (Schreiber et al., 1993).

During evolution, the overall three-dimensional structure of TTR has not changed much during vertebrate evolution (Richardson et al., 2002). Aminoacid differences between species have occurred on the surface of the molecule. Most of the changes are located primarily within the first ten aminoacids from the NH_2-terminus, which becomes shorter and more hydrophilic (Aldred et al., 1997), while in the binding sites no changes were detected among species. Therefore, the central channel which harbours the thyroid hormone-binding site is highly conserved in all TTRs (Power et al., 2000). While in one hand TTR structure has not changed, in the other, its functional properties have changed during evolution. The main differences include the tissue of synthesis (liver and/or choroid plexus) and the decrease of T3 affinity in favour of T4 (Chang et al., 1999).

Comparing human TTR to chicken TTR, the similarity of the amino acid sequences is 75% overall and 100% for the central channel containing the thyroxine-binding site (Duan et al., 1991). The homology between mouse and human TTR sequences is considerably high: only 25 substitutions of 127 aa that compose mammalian TTR have occurred. 24 of these substitutions are located in the outer surface (Wakasugi et al., 1986), implying that the regions corresponding to functional domains are highly conserved between the two species.

An increasing number of prokaryotes and lower eukaryotes with functional sequences homologous to TTR are emerging, named TTR-like. A four aminoacid sequence motif at the C-terminus: Y-R-G-S is common to all TTR-like proteins

(Eneqvist et al., 2003). The hypothesis that TTR-like would be an ancestor of TTR (Richardson et al., 2000) imply that this sequence possibly results from divergent evolution. TTR-like has a higher sequence similarity with amphibian than with other vertebrates such as reptilian, avian, marsupial or eutherian TTR sequences (Prapunpoj et al., 2000).

Organisms with characterized TTR-like protein include: *Arabidopsis thaliana*; *Caenorhabditis elegans*; *Bacillus subtilis*.

TTR-like from *A. thaliana* (TTL) was identified as a functional protein, involved in control of growth through association with the brassinosteroid-insensitive 1 receptor (BRI1) (Nam et al., 2004). In this study, TTR-like was shown to be a membrane associated protein.

Caenorhabditis elegans has two TTR-like proteins or TRP (TTR related protein) (Eneqvist et al., 2003). These TRP genes are transcriptionally regulated during development but there is no phenotype associated with its absence as determined by double-stranded (ds) RNA-mediated interference. Recombinant protein forms tetramers, as does TTR from vertebrates (Richardson et al., 2005).

In *Bacillus subtilis*, TTR-like was proposed to be involved in the utilization of purines as a nitrogen source and inactivation of TTR-like results in an uricase-defective phenotype (Schultz et al., 2001).

Studies were performed by Eneqvist and colleagues as an initial characterization of *E. coli* TTR-like protein (Eneqvist et al., 2003); no functional studies were included but structural and biochemical issues were addressed. It is a homotetramer of 50 kDa approximately, however binding to natural ligands of human TTR, in particular to thyroid hormones, was not detected with the methodology used. Amyloid formation by partial acid denaturation was accomplished with no success: while human TTR forms amyloid in the conditions used in the study, *E. coli* TTR-like does not.

In this work we present studies performed with TTR-like from *E. coli* so as to enlighten us about its role and importance. We investigated its biological role by generating a knock-out mutant for the TTR-like gene. In order to perform its biochemical characterization, the protein was recombinantly expressed, isolated and purified. Moreover, a possible relationship to TTR evolution is discussed.

Materials and methods

Generation of the *E. coli* TTR-like mutant by homologous recombination

a) Construction of linear DNA sequence for homologous recombination

The chloramphenicol-resistant cassette – CAT - was obtained by standard PCR conditions using primers for CAT with a 5' end homologous to flanking regions of the TTR-like gene thus generating a linear DNA product with CAT flanked by TTR-like homology harms, named CATTR. Primer sense was: 5'-gatataaacatgttaaagcgttatttagtactctcggta/aaaatgagacgttgatcggcacg-3' (TTR-like/CAT promoter); primer anti-sense was: 5'-agcagagatctgcttttacatatttatgcattaactgcc/accagcaatagacataagcgg-3' (TTR-like/CAT sequence). The template DNA was a CAT cassette. The PCR program used compromised an initial 5 minutes denaturation step at 96°C, then 25 cycles of 1 minute at 96°C, 90 seconds of primer annealing at 60°C and 3 minutes of primer extension at 72°C followed by a final extension step of 10 minutes at 72°C. The PCR product of 750 bp was extracted from an agarose gel with a gel extraction kit (Qiagen) following manufacture's instructions.

b) Generation of the *E. coli* knock-out mutant

DY378 *E. coli* strain was chosen for obtaining the TTR-like knock-out. DY378 is sensitive to chloramphenicol to allow selection of recombinants with integrated DNA; it has a Rec A^- background and contains a λ prophage harbouring the recombinant genes: exo, beta and gam, under control of a temperature sensitive λ CI repressor. Thus it is a defective prophage without the lytic genes that can protect and recombine the introduced DNA.

To prepare competent cells for introducing the CATTR by electroporation, *E. coli* DY378 was grown in Luria-Bertani (LB) medium at 32°C, overnight (o/n). The culture was diluted 50 fold in LB medium and grown until optical density at 600 nm (OD_{600}) reached 0.6-0.7. 10 ml of the culture was heated to 42°C, to supply recombination functions, and shacked vigorously for 15 minutes. The induced culture as well as a control uninduced culture were cooled in ice water slurry, swirling for 15 minutes and centrifuged at 8000 rpm for 10 minutes at 4°C. The pellet was resuspended in 1 ml of ice-cold sterile dH_2O and spun for 20 seconds at

top speed. This procedure was repeated 3 times. The final pellet was resuspended in 100 µl of sterile H_2O and half of this preparation corresponding to approximately 10^4 cells, was used for electroporation. 100-300 µg of the purified linear CATTR sequence was mixed with 50 µl of competent *E. coli* cells on ice and transferred to a pre-cooled electroporation cuvette of 0.2 cm. The negative control (uninduced culture) had the same treatment. Electroporation was carried out in a BioRad gene pulser. The settings were: 2.5 kV, 25 µF and 200 Ohms with a time constant of 4.5 ms. The electroporated cells were diluted in 1 ml LB and incubated for 1.5 hours at 30ºC. The electroporated cells were then plated on a LB/chloramphenicol (LB/cm) plate and incubated o/n at 30ºC. Diluted cultures were plated on LB to determine cell viability. These experiments were based on a technique developed by Yu and colleagues (Yu et al., 2000)

Selection of the *E. coli* TTR-like KO

The recombinants which integrated the CATTR sequence by homologous recombination were selected on a LB/cm plate at 30ºC. 18 recombinants (9 small colonies and 9 big colonies) were chosen and re-plated in another LB/cm plate. Colony PCR was performed in these recombinants using primers for CATTR and for TTR-like gene. *E. coli* amplifying the CATTR sequence but not the TTR-like sequence was selected and DNA extracted to perform a Southern blot. DNA was digested with BamH I (which has a frequency of recognition site in the genome of 140 bp) and Pvu II (whose frequency of restriction cut in the genome is 410 bp) and transferred from an agarose gel to a Hybond-N^+ membrane (Amersham). 2 x 10^6 cpm of each ^{32}P-labelled probe was added per ml of hybridization solution (QuickHyb, Stratagene). The radioactive signal was detected by exposing the membrane to an autoradiography film.

Transduction with P1 phage

An E. coli N99 TTR-like KO was generated for phenotyping studies by transduction of CATTR with P1 phage grown on DY378 TTR-like. The selected TTR-like KO *E. coli* DY378 was grown in LB medium at 30ºC. 100 µl of the o/n culture was inoculated in 10 ml of LB and grown until OD_{600} reached 0.3. P1 phage was added to the culture with 10 µl of 1M $CaCl_2$ and grown for different periods of time: 3, 5, 6

hours and o/n. Chloroform (100 µl) was added and the mixture shacked for 15 minutes at room temperature and centrifuged. The supernatant was used for infecting N99 wild type *E. coli*. N99 was grown o/n in LB, centrifuged and resuspended in sterile dH_2O. $MgSO_4$ (10 mM), $CaCl_2$ (5 mM) and different amounts (5-50 µl) of P1 phage, obtained previously, were added to 100 µl of N99. The treated culture was incubated for 30 minutes at 37°C. 1 ml of LB and sodium citrate (5 mM) was added and incubated for 1 hour at 37°C. The mixture was centrifuged and plated in selection chloramphenicol plates.

Phenotypic characterization

Growth rates of N99 wt and N99 TTR-like KO were studied in different growth media. A 5 ml preculture medium was inoculated with a colony of N99 wt or N99 TTR-like KO *E. coli* and grown o/n at 37°C. The OD was measured at 600 nm and the cultures diluted in 50 ml of medium to reach an OD_{600} of 0.022 and allowed to grow at 37°C. Cell concentrations, as a measure of growth rate were determined by OD_{600}. The preculture was either LB or minimal (M9) medium. The culture media tested were: LB, LB supplemented with 0.2% glucose, M9, M9 with 10 times lower glucose (0.02%), M9 without casamino acids and M9 supplemented with succinate.

Protein profile analysis

Total and periplasmic proteins were extracted from a steady state culture of N99 wt and from N99 TTR-like KO *E. coli*. Periplasmic proteins were obtained by osmotic shock. Briefly, the culture was centrifuged at 4000 rpm for 15 minutes; the pellet was resuspended in 20% sacarose with 0.001 M EDTA and shacked for 10 minutes. After a second centrifugation, the pellet was resuspended in ice cold Tris 0.01 M pH 8.0 and shacked for another 10 minutes. The supernatant was then isolated after 50 minutes centrifugation at 4°C, for analysis of the periplasmic proteins. Total proteins were obtained after adding 1X SDS Laemmli sample buffer, boiling 5 minutes and centrifuged. Equal amounts of proteins were loaded on a 15% SDS-PAGE gel and stained with coomassie blue. For 2D analysis, periplasmic proteins were isolated from N99 TTR-like KO and N99 wt, treated with the 2D Clean up Kit (Amersham) and proteins dissolved in isoelectric focusing (IEF) buffer. IPG strips, 17 cm (BioRad), pH ranging from 3-10, were hydrated in IEF buffer o/n at rt. The

IEF was run in a Multiphor plate (Amersham) and the second dimension in a 10% acrylamide gel on Ettan Dalt six apparatus. Proteins were visualized by Sypro Ruby (Molecular Probes) fluorescent staining.

RT-PCR of proteins differentially expressed in N99 TTR-like KO

N99 wt and N99 TTR-like KO were grown in LB medium for 4 hours at 37°C after inoculation with an o/n culture. RNA was extracted from 10^7 cells of N99 wt and N99 TTR-like KO using Trizol (Invitrogen) and treated with DNase RNase free. cDNA was produced using an Invitrogen kit according to supplier's instructions. Primers for thymidine phosphorylase were: sense: 5'-gtacgctcgacaaactggaatc-3' and anti-sense: 5'-catacgaccaaagacttctgcc-3'. Primers for deoxyribose-phosphate aldolase were: sense: 5'-tacggtaaccaacttcccacac-3' and anti-sense: 5'-ccatatcacggatcacttccat-3'.

Different amounts of template were used for RT-PCR amplification. In order to normalize the amount of PCR product we chose the house keeping gene 18S. Primer sense: 5'-gagtttgatcctggctcag-3' and primer anti-sense: 5'-gtattaccgcggctgctg-3'.

Cloning of TTR-like with and without the signal peptide

TTR-like with and without the signal sequence named ss TTR-like and mc TTR-like respectively, both tagged with a His sequence at the C-terminus, were cloned in a pET-21a vector (Novagen) in the Nde I and Hind III cloning sites. Primers for ss TTR-like cloning were designed as to include the signal sequence of TTR-like, primer sense: 5'-gtccatatgttaaagcgttattta-3'; and for the mc TTR-like, primers started after the signal sequence, at the beginning of the mature protein, TTR-like primer sense: 5'-gctcatatggcacaacaaaacatt-3'. The anti-sense primer (used for both clonings) corresponded to the C-terminus of TTR-like, but without the stop codon in order to include the His tag of the plasmid, primer anti-sense: 5'-gataagcttactgccacgataggt-3'. Primers sense contained the recognition site for Nde I, and the anti-sense for Hind III.

After cloning, the plasmids were transformed into XL1-blue *E. coli* and colonies selected with ampicillin. Plasmids were extracted from colonies and sequenced.

Cellular localization of TTR-like

a) Expression of ss TTR-like and mc TTR-like

Plasmids containing the cloned sequences were transformed in BL21 *E. coli*. Ss TTR-like and mc TTR-like proteins were expressed, after induction with IPTG, for 4 hours. Total and periplasmic proteins were extracted as described before. Proteins extracted were loaded on a 15% SDS-PAGE gel and either stained with coomassie blue or transferred to a nitrocellulose membrane (Hybond-C, Amersham) for western blot anti-His tag (1:3000, Amersham).

b) Transmission electron microscopy - TEM

Ss TTR-like and mc TTR-like expression was induced as above. 1 x 10^9 cells were used for TEM. Cells were centrifuged for 5 minutes at 5000 rpm and washed twice with 500 µl of PBS. The final pellet was resuspended in 2.5% glutaraldehyde for 30 minutes at 4°C. Negative staining was executed as follows: *E. coli* samples were adsorbed to glow-discharged carbon-coated collodion film supported on 200-mesh copper grids for 1 minute. After washing with dH_2O, samples were stained with a 0.75% uranil acetate solution. Grids were visualized with a Zeiss microscope, operated at 60 kV.

Cloning and purification of TTR-like with Flag tag

TTR-like was cloned with a Flag tag at the C-terminus in a pET 3a vector (Novagen) in the Nde I and Hind III cloning sites. Primers were designed for TTR-like sequence (without the signal peptide) having the Nde I recognition sequence in the primer sense and the flag sequence plus the Hind III recognition site in the primer anti-sense. Primer sense was: 5'-gggaattccatatggcacaacaaaacattcttagcgt-3'. Primer anti-sense was: 5'-cgcggatcctcacttgtcgtcatcgtctttgtagtcactgccacgataggttga-3'. Plasmids with the TTR-like sequence were transformed into BL21 pLyss *E. coli* and grown in LB. Induction was accomplished with IPTG at 30°C o/n. After cell lysis by sonication the supernatant was purified by ion-exchange chromatography with diethylaminoethyl (DEAE)-cellulose (Whatman), dialyzed, lyophilised and further purification was accomplished by a native preparative Prosieve agarose (FMC) gel electrophoresis. After electrophoresis, the TTR-like band was excised and

electroeluted in an Elutrap system (Schleider and Schuell) in 38 mM glycine, 5 mM Tris pH 8.3, overnight at 50 V (4ºC).

TTR-like identification was accomplished by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS), in an Applied Biosystems model 494. Briefly, TTR-like protein band was excised from a 15% SDS-PAGE gel stained with coomassie blue. Proteins in the gel band were reduced by incubation with 0.01 M dithiothreitol in 0.1 M Tris pH 8.5 buffer, alkylated with 0.015 M iodoacetamide, 0.1 M Tris pH 8.5 and prepared for trypsin digestion by washing once with 25% acetonitrile in 0.05 M Tris pH 8.5, and twice with 50% acetonitrile in 0.05 M Tris pH 8.5. After destaining, gel bands were digested with trypsin and analyzed on a Perspective Voyager DE-RP mass spectrometer in a linear mode (Henzel et al., 1993).

TTR-like was analysed by high performance liquid chromatography (HPLC), performed on a Protein Pak 125 column (Waters), coupled to a high-precision pump (P-302) and a halocrome-280 UV detector (Gilson). The eluent used was 200 mM sodium phosphate buffer pH 7, at a flow rate of 0.4 ml/min. 400 μl fractions were collected for 60 minutes. Protein standards used for calibration were: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and lysozyme (14 kDa).

TTR-like tetramer stability

TTR-like was analysed in a SDS-PAGE (15% acrylamide) after being subject to the different treatments: dilution in 1) 1x SDS buffer with β-mercaptoethanol and boiling for 5 minutes; 2) 1x SDS buffer with β-mercaptoethanol, without boiling; 3) 1x SDS buffer without β-mercaptoethanol and boiling for 5 minutes and 4) 1x SDS buffer without β-mercaptoethanol and without boiling. The gel was stained with coomassie blue.

TTR-like binding to human TTR

a) Native electrophoresis

Equal molar amounts of TTR-like and human TTR (3.6 mM) were incubated in Tris 25 mM, pH 7.0, for 8 hours or for 3 days at 4ºC, separated on a native PAGE and

transferred to a PVDF membrane (Hybond-P, Amersham). A western blot was performed using antibodies against human TTR (1:1500, DAKO) and against the Flag peptide, as described before.

b) HPLC analysis

Both proteins with the same concentration and with a total amount of 60 µg each were separated by HPLC as previously described. 100 µl of selected fractions were transferred to a nitrocellulose membrane by dot blot and probed for Flag peptide (as described before) and for human TTR.

TTR-like fibril formation

a) Thioflavin-T binding

TTR-like protein was dialysed against H_2O and quantified. 1mg/ml of protein in 100 µl of 50 mM sodium acetate buffer at different pHs was used to produce fibrils. The pH range was: 3.6; 4.6; 5.6 and 6.6. The solutions were incubated at RT for 72 hours. TTR-like at pH 3.6 was also incubated for the same period of time at 37°C. Fibrils were identified by binding to Thioflavin-T, Th-T (Fluka) in 50 mM Glycine buffer, pH 9.0. Excitation spectra were recorded with a Jasco FP-770 spectrofluoremeter to confirm amyloid like properties with the characteristic excitation maxima at 450 nm upon Th-T binding.

b) Dot blot filter assay

Soluble (500 ng) and fibrillar TTR-like (100 ng) were applied onto a 0.2 µm pore cellulose acetate membrane filter (Schleicher & Schuell) which retains aggregated and fibrillar but not soluble protein. A manifold system (Gibco BRL) under vacuum was used. The filter was washed 3 times with PBS and processed for TTR-like immunodetection with anti-flag antibody, as described before. Detection was performed with Super Signal West Pico (Pierce).

c) TEM

TTR-like fibril formation was induced at pH 3.6 for 5, 10 and 15 days and visualized with TEM by negative staining as described before. Measurement of TTR-like fibrils width was made from photomicrographs using a magnifying glass with a built-in calibrated rule.

Seeding

TTR-like fibrils were induced by incubation in 50 mM sodium acetate buffer, pH 3.6, for 10 days. Th-T binding was tested to confirm fibril formation. Fibrils were sedimented by centrifugation for 20 minutes at top speed and resuspended in 1/10 of the initial volume in PBS. 4 µg of TTR-like fibrils were added to 100 µg of soluble human TTR wt or soluble TTR-like and Th-T fluorescence was measured at different time points. This methodology was also tested for soluble TTR-like incubated with human TTR wt fibrils as seeding.

T4 binding assay

Radiolabelled ^{125}I-Thyroxine (T4) (Amersham) was incubated with: recombinant *E. coli* TTR-like (10 µg); human TTR (5 µg) and mouse TTR (5 µg) and with 5 µl of human or mouse serum. Incubation was carried out at room temperature for 1 hour. The samples were mixed with 4 µl of glycerol and glycine acetate pH 8.0 and loaded on a native glycine-acetate 8% polyacrylamide gel. A parallel sample of human TTR with bromophenol blue was included so as follow albumin migration. Electrophoresis was finished when albumin band reached 2 cm from the wells. T4 bound proteins were visualized by autoradiography.

T4 competition assay

T4 was purified in a 1 ml Sephadex LH-20 (SIGMA) column to separate free iodine from labelled ^{125}I-T4. Free iodine was eluted with 0.1 M HCL. The column was washed with 2 volumes of H_2O and labelled ^{125}I-T4 was eluted with 0.25% ammonia/methanol. ^{125}I-T4 was evaporated to dryness under nitrogen and redissolved in 0.1 M Tris, 0.1 M NaCl and 1 mM EDTA, pH 8.0. T4 competition assay was performed as described elsewhere (Almeida and Saraiva, 1996): 50 000 cpm of ^{125}I-T4 were incubated with 100 µl of 30 or 60 nM of TTR-like in the presence of different amounts of cold T4: 20 µM, 6 µM, 0.6 µM, 0.2 µM, 0.06 µM or 0.02 µM for an o/n at 4ºC. Control experiment was performed with 30 nM of recombinant human TTR. All the assays were performed in duplicate. T4 binding was measured in a γ–counter after separation of free from bound T4 in 1 ml of Bio-gel P6 (BioRad) columns.

Results

Generation of the TTR-like KO

In order to determine TTR-like functional role in *E. coli* a knock-out mutant was generated. An *E. coli* strain - DY378 - with Gam, Exo and Beta recombination functions was chosen for the homologous recombination procedure. A CAT cassette flanked by TTR-like sequences was introduced into DY378, generating a KO mutant for TTR-like. The strategy followed is summarized in figure 1.

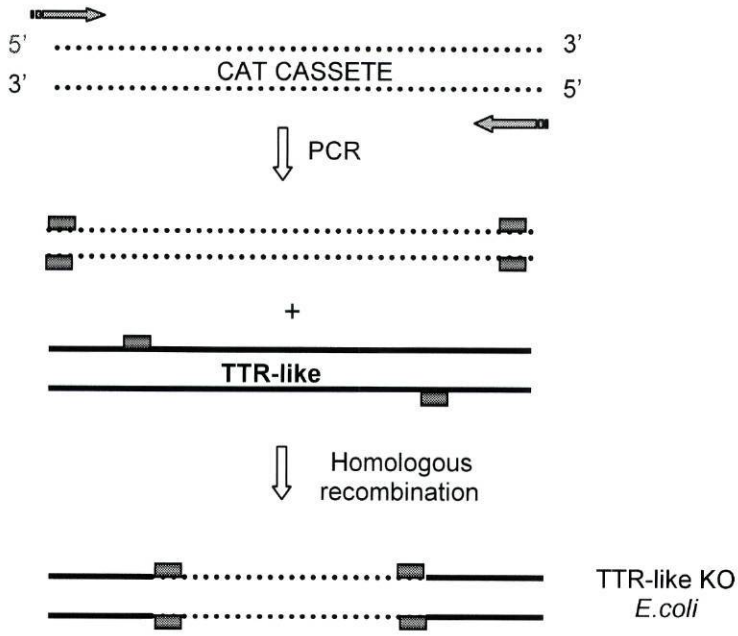


Figure 1-
Strategy designed for generation of the *E. coli* TTR-like mutant: a linear CAT cassette flanked by TTR-like sequences was obtained by PCR and introduced into *E. coli*. By homologous recombination, a TTR-like *E. coli* mutant was generated.

The TTR-like KO was selected by chloramphenicol resistance resulting in a high percentage of big sized colonies. Some small colonies of recombinants were also isolated; these did not grow in LB/chloramphenicol (LB/cm) medium and in LB plates without antibiotic returned to normal size. The disruption of the TTR-like gene was confirmed by PCR using primers for the CAT cassette and for the TTR-like gene (figure 2). Some small colonies had TTR-like gene and these should correspond to CATTR integration in the genome other than the TTR-like gene or the KO with the wild type present in a duplication of the region or finally chloramphenicol spontaneous resistant colonies.

Figure 2-
PCR of DNA from *E. coli* wt and from TTR-like KO *E. coli* with primers for CAT sequence and TTR-like gene.

E. coli colonies amplifying the CAT cassette but not the TTR-like gene were selected. Next, a southern blot with the CATTR sequence (used to generate the mutants) as probe 1 and the TTR-like gene as probe 2 was done. Figure 3 shows the signal displayed for each probe by the *E. coli* KO and the *E. coli* wild type. *E. coli* KO had signals for the CAT probe (middle picture) confirming that the CAT cassette was introduced in the DNA and no signal for the TTR-like gene is observed (right picture), meaning that the CAT probe was introduced correctly.

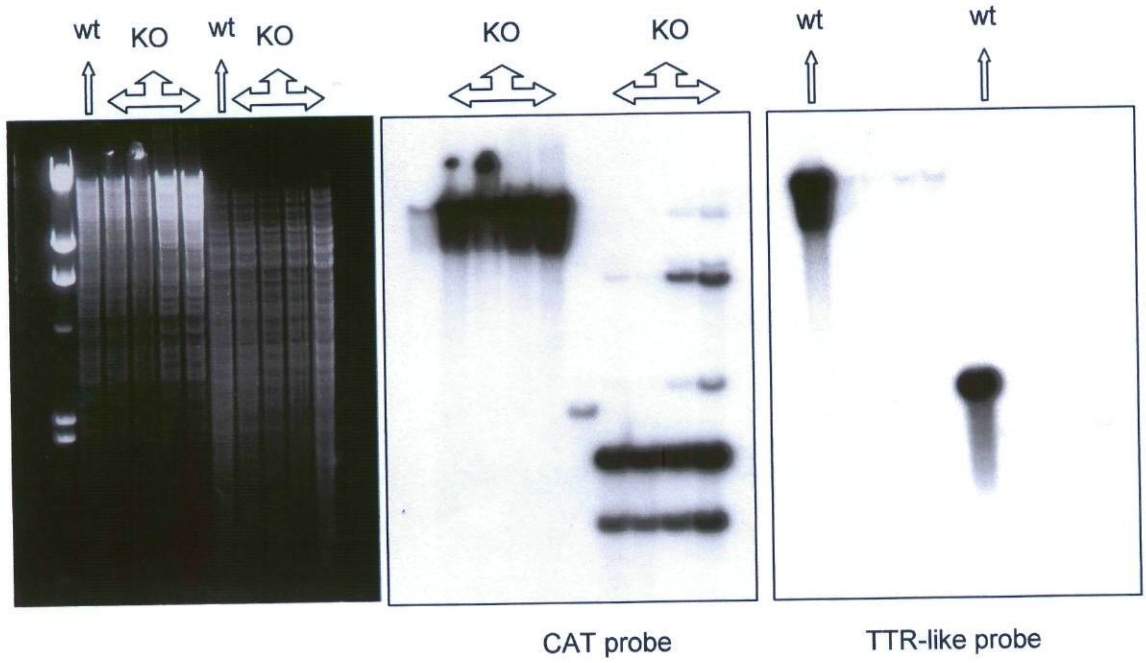


Figure 3-
Southern blot of wt *E. coli* and TTR-like KO *E. coli* DNA digested with BamH I and Pvu II restriction enzymes (left picture), using the CAT sequence as one probe (middle picture) and the TTR-like sequence as another probe (right picture).

The confirmed mutants were selected and by phage P1 transduction transferred to *E. coli* strain N99 to perform subsequent phenotype studies. The smaller sized colonies did not result in enough N99 TTR-like KO *E. coli*, contrary to big sized colonies. So, this was a hint for a possible problem with the CATTR introduced and small sized colonies were not further studied. The characterization studies were performed in a selected big sized colony.

Growth behaviour studies

The growth pattern in different media was investigated in N99 TTR-like KO mutant. The growth in LB medium was similar for N99 KO as compared to N99 wt (chart 1). In the LB medium supplemented with glucose the steady state growth phase is reached earlier than without glucose, probably by acidification of the medium but it is similar between the 2 strains.

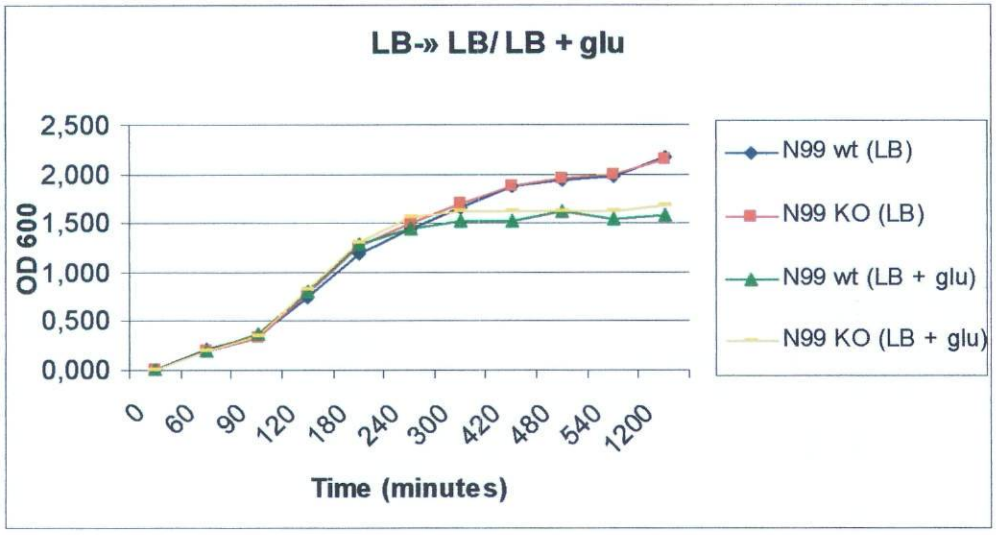


Chart 1-
E. coli growth in Luria-Bertani (LB) medium supplemented or not with glucose, measured by OD at 600 nm.

Comparing the growth in M9 medium inoculated from an overnight LB culture, no differences were detected between the N99 KO and N99 wt (chart 2). On the other hand the parental strain and the TTR-like KO grown in M9 medium originating from the same preculture medium have a distinct pattern of growth. While N99 KO is not

able to start growing initially the wt is. The growth is delayed and starts later. But cells are viable since at 1200 minutes cell density is observed and reaches the levels of the wild type. TTR-like seems to be essential for a rapid growth in minimal medium if cells come from the same minimal medium.

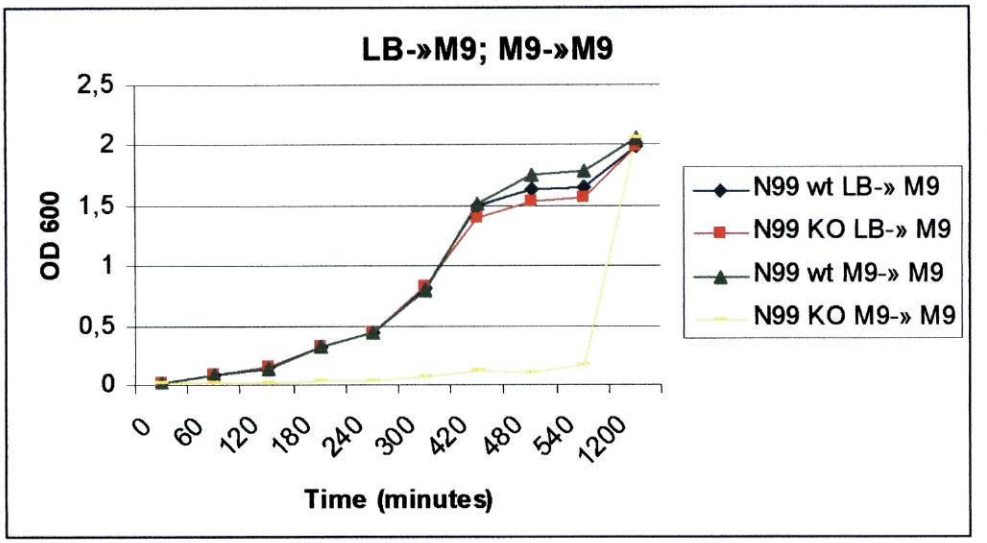


Chart 2-
E. coli growth in minimal medium (M9), from a LB or a M9 preculture, measured by OD at 600 nm.

Comparing the growth in M9 having 10 times less glucose (0,02%) than regular M9 medium, both inoculated from an overnight LB culture, the N99 KO has an initial similar growth to the wt, that is delayed afterwards (300 minutes) although not very sharply (chart 3). *E. coli* wt grows better if coming from the same medium preculture while N99 KO does not, since is not able to adapt when inoculated in M9 culture with 0.02% glucose from the M9 preculture medium. In fact, values of OD_{600} reflecting growth are only obtained at 1200 minutes, meaning also that cells are viable and although needing an extended period of time they can still grow. This result means that *E. coli* is able to an adaptive response in M9, probably activating necessary metabolic pathways that are not necessary when in a LB preculture and turning it to be better prepared to grow in a medium with low glucose levels. TTR-like seems to be involved in this response, since the KO does not show the same growth pattern.

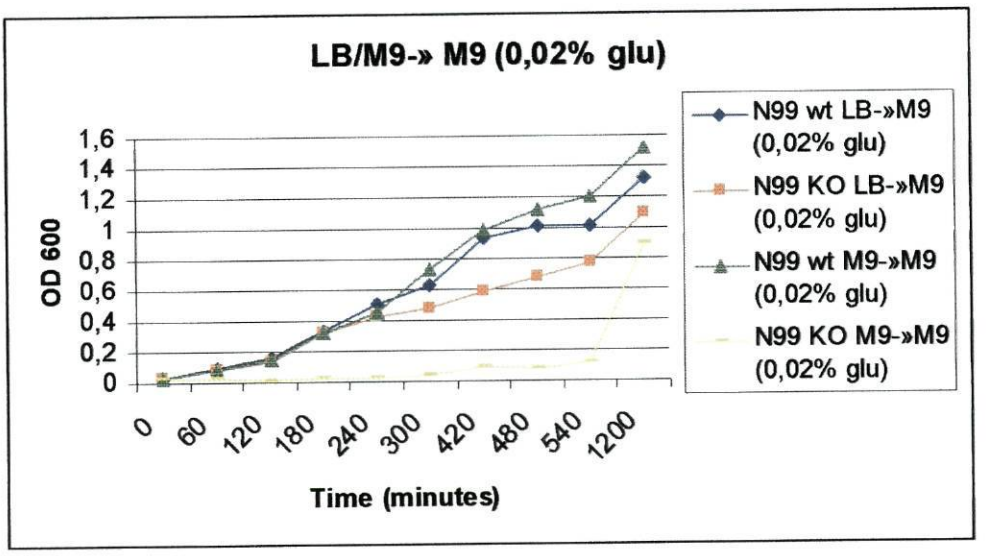


Chart 3-
E. coli growth in M9, with 0.02% glucose from a LB or a M9 preculture, measured by OD at 600 nm.

The role of TTR-like related to aminoacid availability was investigated by growing *E. coli* wt and TTR-like KO in M9 medium without casamino acids (caa). Chart 4 represents this experiment. Similar growth is obtained when N99 is inoculated from a LB preculture. But from a M9 preculture, the situation is very different. The wt is able to adapt better (grows faster) to this situation than with the previous condition (LB preculture), although at the end it reaches the same OD_{600} values. In contrast, the KO shows an increased growth as if it had turned on a metabolic pathway in M9 medium or as if it had storaged essential components from M9 medium providing a means of rapid growth in medium without available caa. The difference is seen during the initial growth: KO cells are able to start the exponential phase earlier (120 minutes) and this is reflected in all the other time points except the last measure (after an o/n, 1200 minutes) where the maximal values of OD_{600} are similar to all situations.

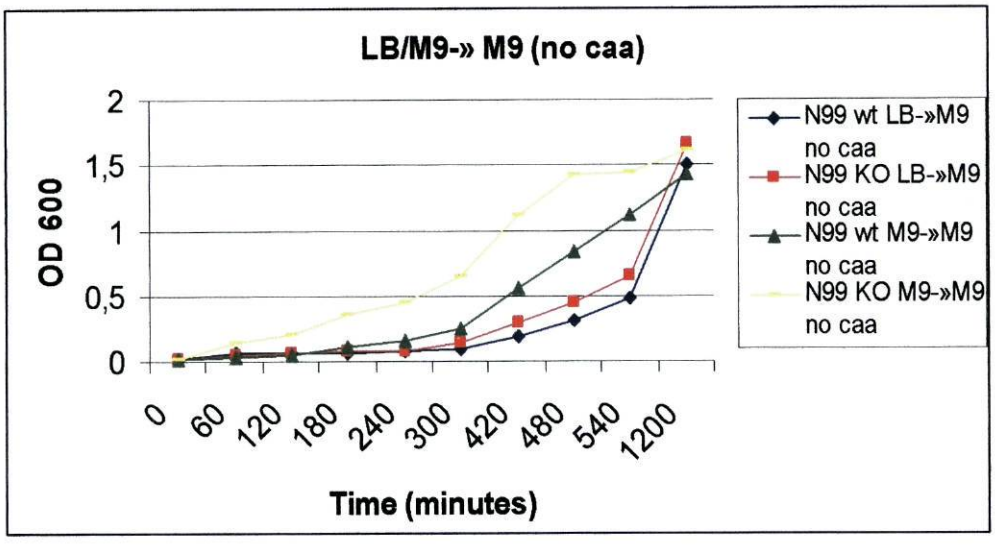


Chart 4-
E. coli growth in minimal medium (M9), without casaminoacids (caa), from a LB or a M9 preculture, measured by OD at 600 nm.

The influence of the carbon source in the growth of *E. coli* lacking TTR-like was also investigated by supplementing M9 medium with succinate which drives the aerobic metabolic pathway. In this growth condition, N99 TTR-like KO is not able to grow (chart 5). Moreover, in the case of being before in LB medium, the growth is delayed.

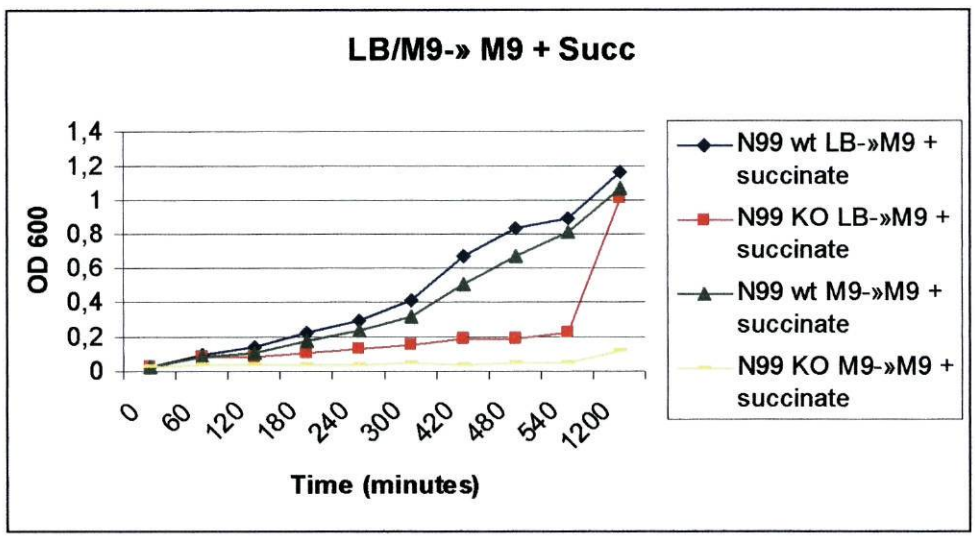


Chart 5-
E. coli growth in M9 medium supplemented with succinate (succ) from a LB or a M9 preculture, measured by OD at 600 nm

The distinct growth patterns obtained for the TTR-like KO indicates a possible intervening function in metabolic pathways in conditions of limited energy supply that is in conditional medium - M9 - that is reflected when the culture is subsequently transferred to deficient media such as M9, M9 with decreased glucose, without caa or using succinate as the carbon source.

Differential protein expression

To investigate the influence on protein expression by the absence of TTR-like in *E. coli* we analysed the protein profile of the cytoplasm and the periplasmic space (figure 4). The differential protein expression between the wt and the KO was mainly detected in the periplasmic fraction. This subcellular protein fractioning showing a different protein pattern in the periplasm of the *E. coli* TTR-like KO suggested a possible TTR-like periplasmic localization, which was addressed later.

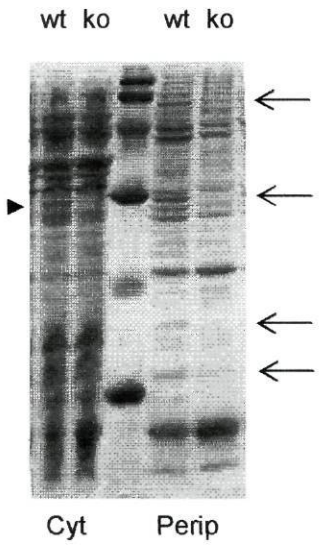


Figure 4-
Protein profile of N99 wt (wt) and N99 KO (ko) showing cytoplasmic (Cyt) and periplasmic (Perip) fractions. Middle lane shows the molecular weight markers: 97; 60; 40; 30; 20; 14 kDa from top to bottom. Arrows indicate some differentially expressed proteins in the KO. Arrow head: cytoplasmic proteins, arrow: periplasmic proteins.

We choose the proteins extracted from the periplasm (higher number of detected protein differences, as pointed by the arrows in figure 4) to be analysed by 2D (figure 5). Some evident differences were obtained which invariably involved a decreased expression in the *E. coli* TTR-like KO.

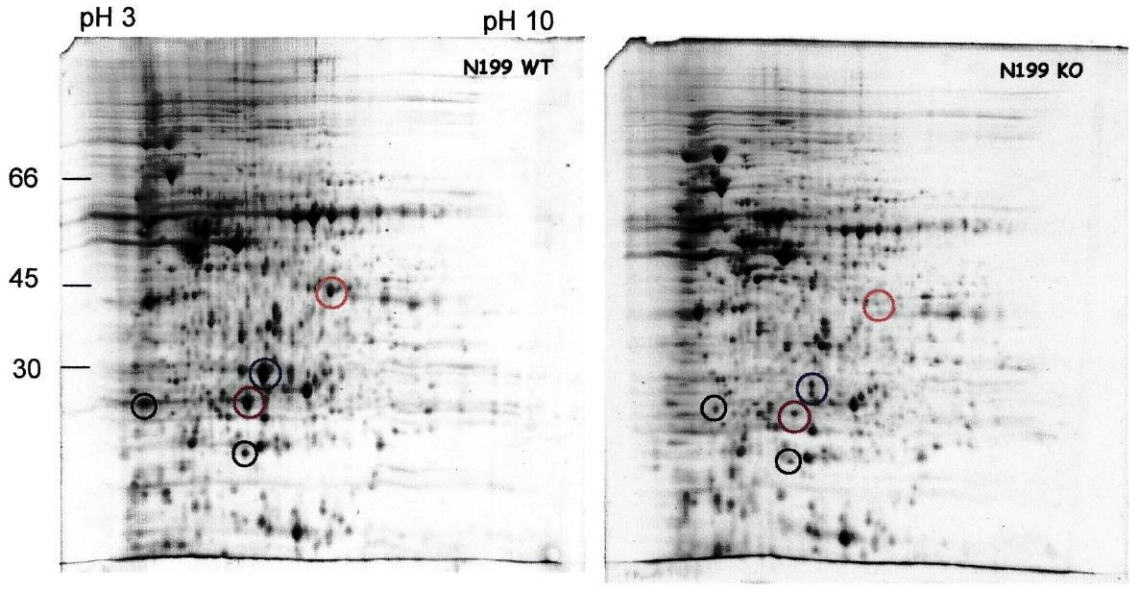


Figure 5-
Protein profile of N99 wt and N99 KO by 2D analysis. Orange circle: UDP-glucose 4 epimerase, blue circle: deoxyribose-phosphate aldolase, red circle: thymidine phosphorilase. Black circles: other differential expressed proteins not sequenced.

Three protein spots from wt *E. coli* that were decreased in the TTR-like KO *E. coli* were chosen for identification by mass spectrometry. The results were: thymidine phosphorylase (TP) a homodimer of 43 kDa and a pI of 5.46; deoxyribose-phosphate aldolase (DA) that is 27 kDa and has a pI of 5.78 and UDP-glucose 4 epimerase which is 37 kDa and has a pI of 6.28. These differences were confirmed by RT-PCR. A decreased expression was detected in the N99 KO as depicted in figure 6 (picture and chart). TP and DA are proteins involved in metabolism of pyrimidines. UDP-glucose 4 epimerase is important for galactose degradation. Such alterations point toward an influence of TTR-like in metabolism and catabolic functions.

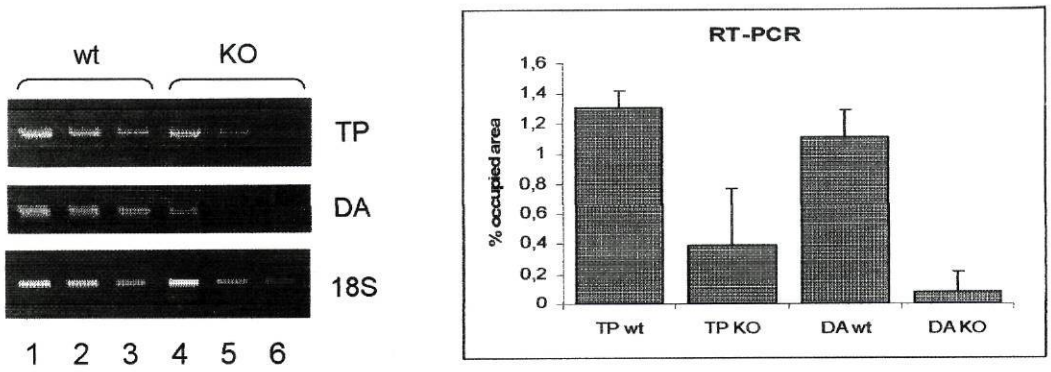


Figure 6-
RT-PCR of N99 wt and N99 KO for thymidine phosphorylase (TP) and deoxyribose-phosphate aldolase (DA). 18S RT-PCR is shown for normalization of cDNA added. Lane 1: 4 fold dilution of cDNA wt template; Lane 2: 8 fold dilution of cDNA wt template; Lane 3: 16 fold dilution of cDNA wt template; Lane 4: 1 µl of cDNA KO template; Lane 5: 4 fold dilution of cDNA KO template and Lane 6: 8 fold dilution of cDNA KO template.

TTR-like localization

To investigate the cellular localization of TTR-like, we cloned TTR-like with and without the signal sequence, named ss TTR-like and mc TTR-like respectively. Both proteins had a his-tag at the C-terminus. These proteins were overexpressed in BL21 *E. coli* and extracted from both total (cytoplasmic + periplasmic) and periplasmic fractions. In the SDS-PAGE of total extracts (figure 7) two bands between 14 and 20 kDa molecular weight markers were detected in ss TTR-like that we assumed to be the protein with the signal sequence and the processed protein without the signal sequence: the mature chain, with approximately 15 kDa. In the cytoplasmic fraction of the mc TTR-like only one band of 15 kDa was detected - the mature TTR-like since there was no signal sequence. Analysing the periplasmic fractions some intriguing results were obtained since ss TTR-like is detected only in the processed form, as one would expect, but the mc TTR-like is also present. This protein does not possess the signal sequence that directs it to the periplasm, so two explanations might be formulated. One is that TTR-like is a membrane associated protein extracted by osmotic shock and the signal peptide

would not be important in this situation and the other is that the technique of protein extraction by osmotic shock also extracts some cytoplasmic proteins as it has been reported for cytidine deaminase, deoxyribomutase and uridine phospholylase for example.

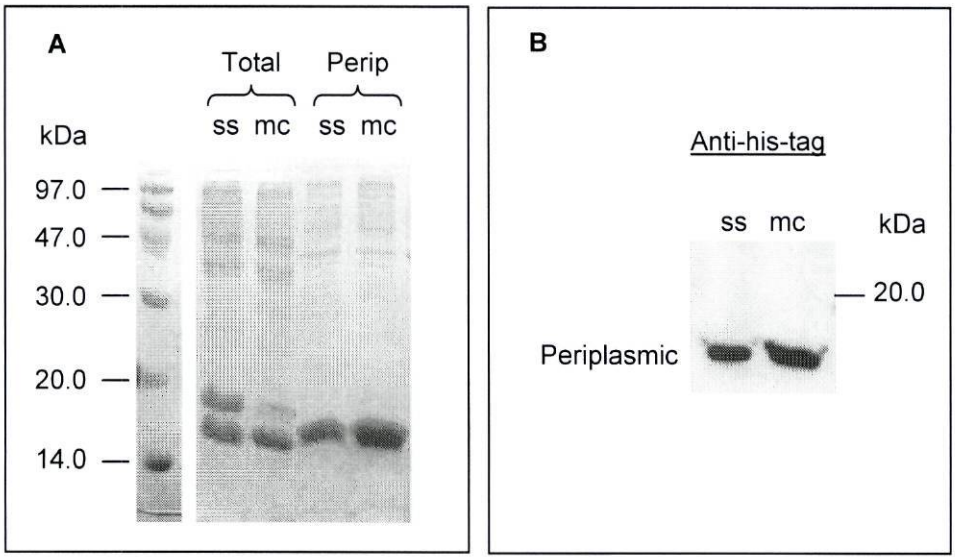


Figure 7-
TTR-like production tagged to his-tag, with (ss) and without (mc) the signal sequence. **A**: Coomassie staining of total protein and periplasmic extracts. **B**: Western blot anti-his-tag of periplasmic extracts.

Studies by electron microscopy were performed to analyse in detail the cellular localization of the TTR-like with and without the signal peptide. Ss TTR-like shows accumulation of a large mass of protein in the periplasmic space related to TTR-like overexpression, resulting in an enlargement of the periplasmic space that is not observed neither in mc TTR-like nor in the BL21 *E. coli* control (figure 8). Thus, TTR-like is localized in the periplasm and it is the signal peptide that directs this protein there.

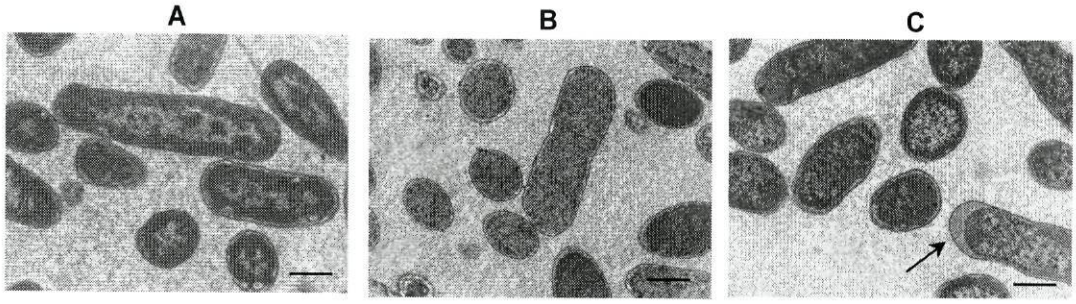


Figure 8-
Images of *E. coli* BL21 obtained by TEM. A: BL21 control, C: BL21 expressing mc TTR-like, C: BL21 expressing ss TTR-like. An accumulation in the periplasm of the TTR-like is observed (arrow). Scale bar: 600 nm

Physical-chemical characterization

E. coli TTR-like protein tagged with flag peptide was cloned, produced and isolated to study its biochemical and physical properties. TTR-like is a homotetrameric protein of approximately 55 kDa, as determined by HPLC analysis (figure 9).

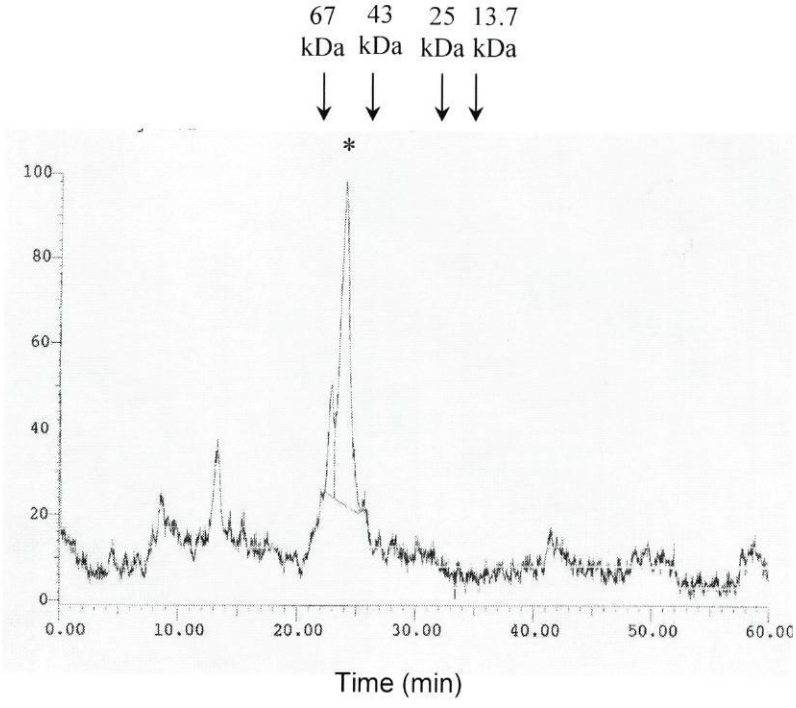


Figure 9-
HPLC elution pattern of TTR-like protein. A peak of TTR-like protein is eluted from fractions 23 to 25. Protein standards to calibrate the column are indicated by arrows: 67 kDa- BSA; 43 kDa- ovalbumin; 25 kDa chymotrypsinogen; 13.7- ribonuclease A.

In a SDS-PAGE, it runs as a monomer of approximately 15 kDa as shown in the expression experiments. Human TTR is also a homotetrameric protein and in a SDS-PAGE gel dissociates mainly to a monomer and to a much less extent to a dimer.

We tested the effect of heating and β–mercaptoethanol on TTR-like tetramer dissociation observed during electrophoresis (figure 10). Heating treatment by boiling destroys the tetrameric TTR-like structure totally into a monomer in the presence or absence of β–mercaptoethanol. In contrast, under the same conditions, human TTR partially dissociates into a dimer and mostly to a monomer.

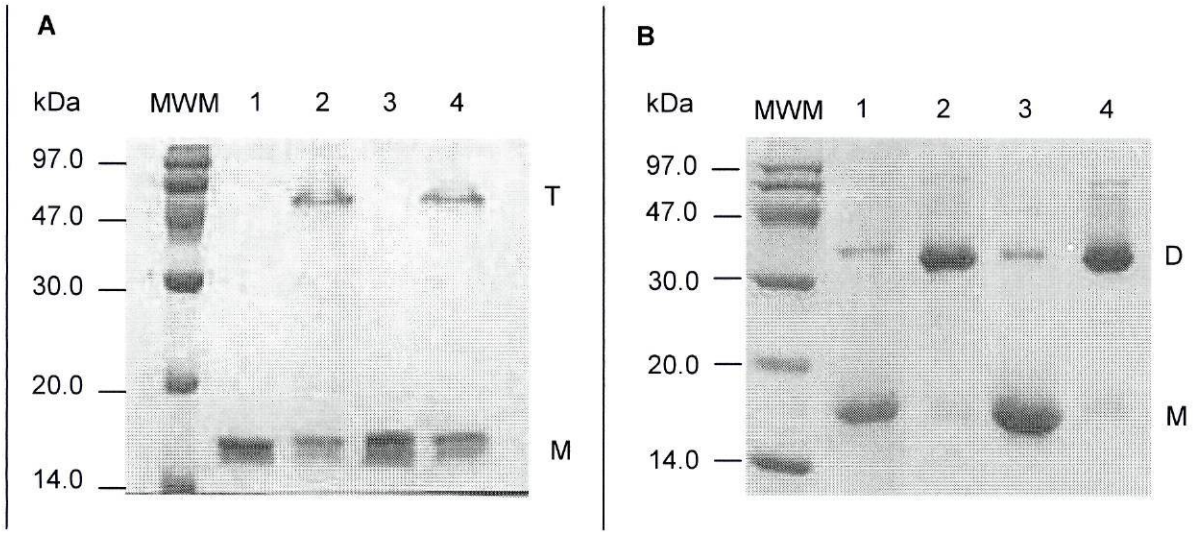


Figure 10-
SDS-PAGE of TTR-like protein (A) and human TTR (B). Lane 1: Boiled, with β–mercaptoethanol; Lane 2: Not boiled, with β–mercaptoethanol; Lane 3: Boiled, without β–mercaptoethanol; Lane 4: Not boiled, without β–mercaptoethanol. T- tetramer; D- dimer, M- monomer.

The search for an interaction between TTR-like and TTR was formulated. We mixed human TTR with TTR-like from *E. coli* and run a native PAGE (figure 11 A). When the two proteins were mixed for 3 days at 4°C, two extra bands were observed in the mix sample (figure 11 A, lane 4) that are not present when TTR or TTR-like are tested alone which indicates a possible binding between the two proteins or heterotetramer formation. Incubation o/n at the same temperature did not show this extra band; probably the period of time is not enough to detect

binding/heterotetramer formation. To confirm that this band is composed of both proteins a western blot against the flag peptide was performed but no extra band was detected (not shown). This result could be due to the relative amount of TTR-like in a complex with TTR being lower or due to a lower limit of detection by the Flag antibody, or instead, the extra band is mainly TTR, presenting a different mobility in the presence of TTR-like. We also run a gel filtration column with both proteins incubated for 3 days at 4ºC and analysed different fractions (figure 11 B). TTR-like (fractions 23 and 24) is eluted after TTR (fractions 21 and 22) reflecting its lower molecular weight. Fraction 12 analysed by dot blot against Flag and TTR has positive signal for both, as it is a high molecular weight fraction there is probably a binding between both proteins. Note that the amount of TTR-like detected is very low by comparative analysis to TTR immunoreactive signal, which confirm the hypothesis stated above: that the Flag antibody shows a weak detection signal, as for the same amount of proteins (TTR-like and TTR) it gives a lower signal than for TTR.

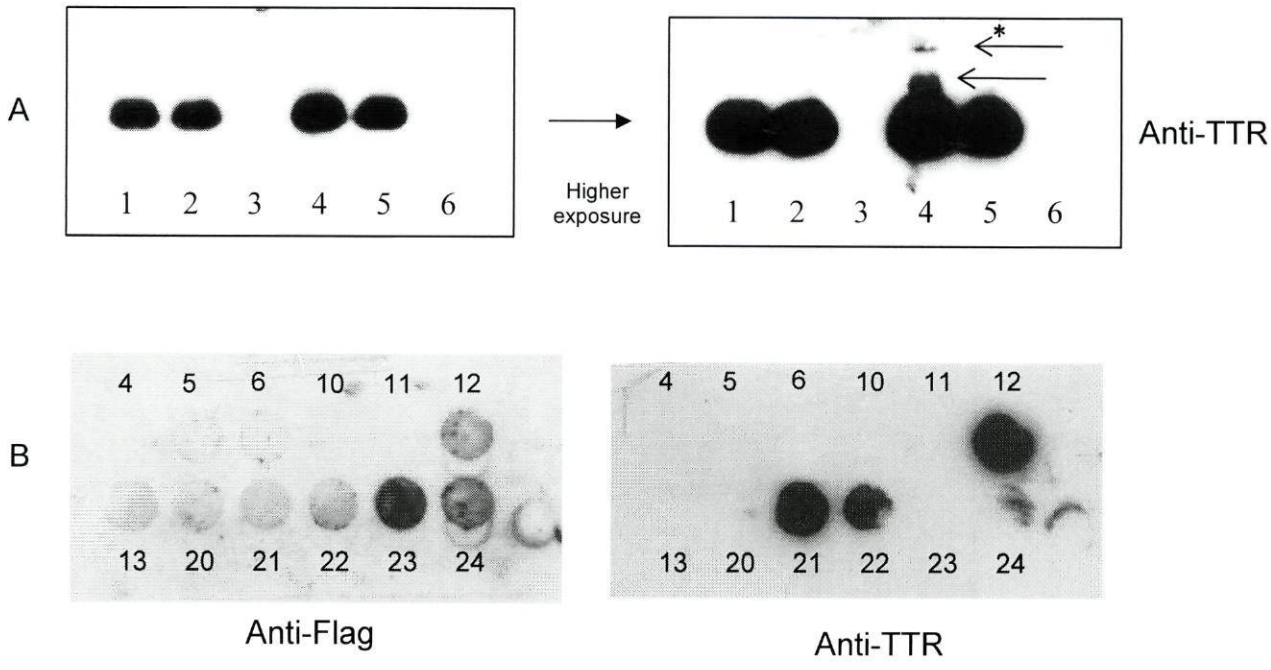


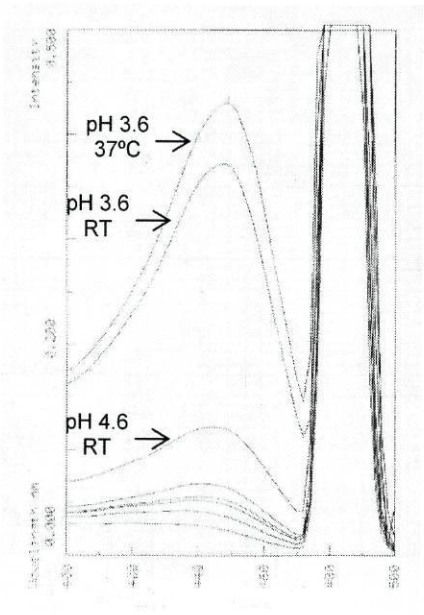
Figure 11-
TTR-like and human TTR. **A**: Western blot of a native PAGE. Lanes 1,4: TTR + TTR-like; Lanes 2,5: TTR; Lanes 3,6: TTR-like. Lanes 1, 2 and 3: proteins incubated at 4ºC o/n. Lane 4,5 and 6: proteins incubated at 4ºC for 3 days. Right is the same as left picture, but overexposed. **B**: HPLC fractions of human TTR + TTR-like mix after incubating at 4ºC for 3 days and blotted against Flag-tag (left pictures) and TTR (right pictures).

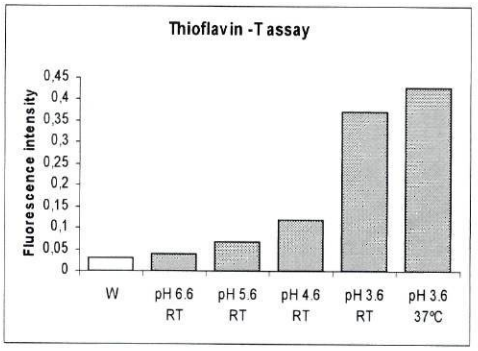
Amyloid formation

Human TTR is able to form fibrils *in vitro* by acidification (Cardoso et al, 2002). At low pH the TTR tetramer dissociates into monomers favouring aggregation and fibril formation. Human TTR fibrils are 8 nm wide mostly, although 4-5 nm and 9-10 nm wide fibrils can be observed. With time, TTR fibrils length can be up to 300 nm after an extended period of time. TTR-like susceptibility to form fibrils was investigated at acidic pH and at neutral pH. Upon acidification of TTR-like, Th-T binding was observed. The characteristic peak for amyloid was identified after incubation at pH 3.6, during 15 days at room temperature (figure 12 A). TTR-like fibrils were increased if the incubation was done at 37ºC. At pH 4.6 some fluorescence is obtained, that is decreased at pH 5.6 and negligible at pH 6.6. If TTR-like is incubated at neutral pH, no fibrils are formed and if pre-treated with acidic buffer for 3 days before incubating at neutral pH, no fibrils are formed as well (not shown), contrary to what happens to human recombinant TTR wt, which is able to form fibrils by this procedure. So, TTR-like is able to form fibrils although not so prone as human TTR. To confirm the aggregate state of the Th-T positive preparation, a dot blot filter assay was performed. This technique allows the retention of aggregated and fibrillar protein in a membrane, while soluble protein passes through the filter membrane. By western blot TTR-like was identified only in the dot were fibrillar TTR-like was added (figure 12 B) while added soluble TTR-like was not detected.

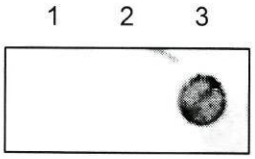
TTR-like fibrils were observed by TEM (figure 12 C). Initially TTR-like is soluble (figure 12 C) but with acidification fibrils are formed. After 5 days incubation at 37ºC, aggregates and fibrils are seen and at 10 and 15 days defined fibrils can be observed. The measurements of the TTR-like fibrils indicated a width of 6 nm. Alongside, some annular structures with a diameter of 2.5 nm were also observed, that might represent oligomers.

A





B



Anti-Flag
western blot

C

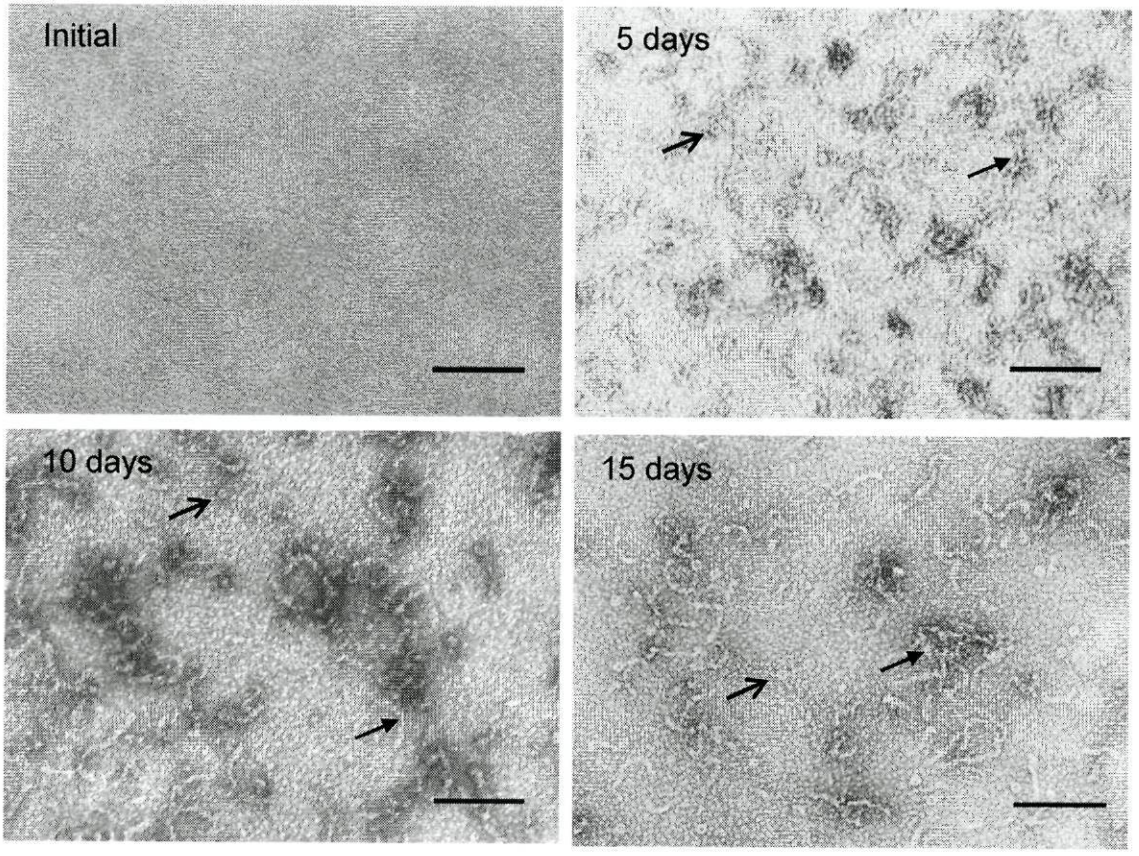


Figure 12-
TTR-like fibril formation. **A**: Thioflavin-T assay to measure TTR like fibrils induced at different pHs. Chart: Fluorescence intensity displayed by each condition due to Thioflavin-T binding. B: Dot blot filter assay to detect TTR-like fibrils with anti-flag antibody. Dot 1: No protein; dot 2: Soluble TTR-like; dot 3: TTR-like fibrils. **C**: Negative staining of TTR-like fibrils induced at pH 3.6, visualized by TEM, showing different time points of incubation. Filled arrows indicate a TTR-like fibril of 6 nm wide and open arrow a TTR-like anular structure with 2.5 nm of diameter. Scale bar: 100 nm.

TTR-like fibrils were tested for their ability to function as seed for human TTR fibril formation. We observed, as a function of time, that TTR-like is able to induce human TTR fibril formation at neutral pH (figure 13). If this effect was tested for soluble TTR-like, no induction could be seen (not shown). It is known that human TTR fibrils do not have a seed effect on soluble human TTR which apparently is extended for other proteins, such as TTR-like.

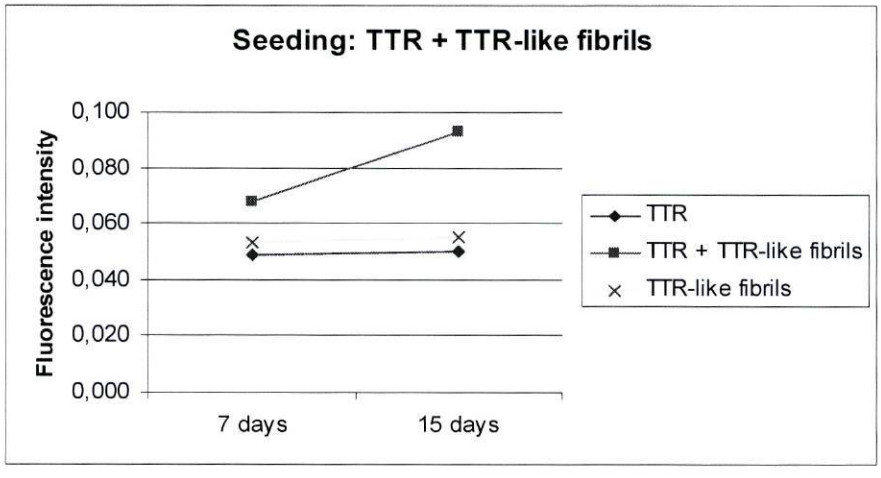


Figure 13-
hTTR wt fibril formation induced by seeding with TTR-like fibrils at pH 7.0 and at 37°C.

T4 binding

T4 binding to TTR-like was tested. Figure 14 A, lane 1, shows very low binding of labelled T4 to TTR-like and most of the labelled T4 in the free form. Next lane shows recombinant human TTR that completely binds all labelled T4.

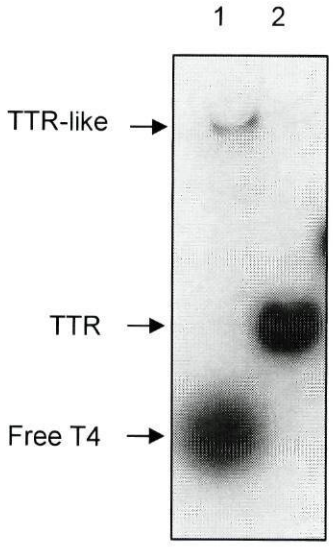


Figure 14-
Autoradiography of Thyroxine (T4) binding gel electrophoresis. Lane 1: TTR-like; Lane 2 Recombinant TTR.

A competition assay was accomplished to test the specificity of TTR-like binding to T4. We found a maximum binding of 3% by this technique (figure 15, chart A) that is negligible and too low to be considered as specific binding to T4. For human TTR, the maximum value obtained was close to 70% of T4 binding (figure 15, chart B). Probably, the previous result (figure 14 A) corresponds to a non-significant binding of T4 to TTR-like.

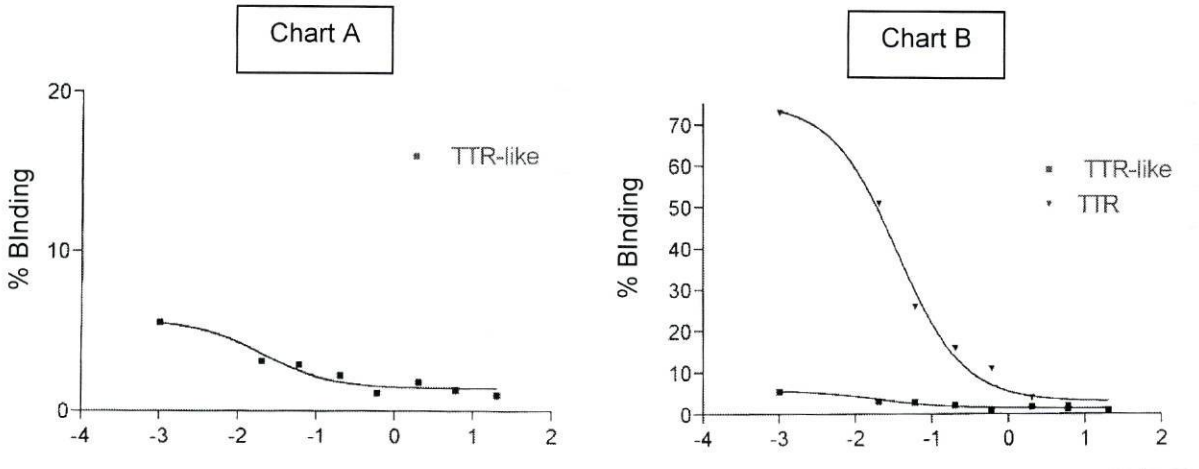


Figure 15-
T4 competition assay. Chart A: percentage of binding of TTR-like to T4. Chart B: comparison between TTR-like and human TTR wt binding to T4.

Discussion

Human TTR is well characterized in terms of biology, biochemistry and biophysics. On the other hand, homologous sequences to TTR found in prokaryotes – TTR-like proteins are only now emerging and studies on their physiological importance are starting to be explored. Within the prokaryotic TTR-like family, the considerable sequence similarity to human sequences, involved in ligand binding sites suggests the existence of common ligands. Until today, there is no characterized common ligand, neither a unifying role for this protein family. As an approach to understanding the function of TTR-like sequences, an *E. coli* TTR-like knock-out mutant was generated. The KO is not lethal and presents phenotypic differences when compared to the parental strain in terms of growth dynamics.

When different kinds of nutrients are available, bacteria grow at different rates. The richer the medium is, the faster they grow and spare themselves of the task to synthesize a surplus of compounds already available, thus during the exponential phase, only required molecules for growth are synthesized (Siegele and Kolter, 1992). In the stationary phase and under conditions of starvation, bacteria accumulate components that are not used immediately, as a strategy for rapid growth for the exponential phase. TTR-like KO does not exhibit this behaviour in every situation. Thus, in conditions of low supply of nutrients (M9 medium) TTR-like KO *E. coli* growth is delayed in comparison to the parental wild type strain. Interestingly, TTR-like KO *E. coli* shows a growth advantage in medium without aminoacids as the nitrogen source if earlier is adapted in minimal medium. We hypothesised that TTR-like is necessary for the utilization of nitrogen as an energy source. In the TTR-like KO a deficiency in oxidation of inorganic elements explains the absence of any effect in a limited nitrogen supply.

TTR-like KO *E. coli* is not able to grow aerobically with succinate, therefore we propose that TTR-like is part of the complex regulatory network involved in aerobic metabolic pathways, probably in reactions occurring after succinate oxidation in the Tricarboxylic Acid Cycle (TCA) or in the respiratory chain.

The protein profile of the *E. coli* TTR-like KO is very different in comparison to the parental strain. The periplasm shows a higher number of differentially expressed

proteins than the cytoplasm. Normally, the periplasm contains diverse proteins: i) hydrolytic enzymes required for metabolism of large substrates; ii) binding proteins which function in the transport of small molecules, in nutrient processing and uptake; iii) enzymes required for chemotaxis; iv) detoxifying enzymes that inactivate toxic molecules. It is a dynamic and important space for reactions of a large and varied pool of substrates that enter and leave the cell. The identification of some proteins whose expression is affected in *E. coli* TTR-like KO resulted in enzymes involved in metabolic reactions. The change in expression of thymidine phosphorylase (TP), deoxiribose-phosphate aldolase (DA) and UDP-glucose 4 epimerase in *E. coli* lacking TTR-like can reflect the compromised alteration in the metabolic pathways related to the TCA. It is significant to correlate these results with studies performed with TTR-like from *B. subtillis* which implies this protein in the metabolism of purines as a nitrogen source. TP and DA are both involved in salvage pathway of nucleotides or, instead, degradation and utilization as carbon and energy sources. More specifically, TP catalyzes the reaction of thymidine into thymine and 2-deoxy-D-ribose 1-phosphate that is converted into intermediates of the pentose phosphate shunt and glycolysis. DA catalyzes the conversion of 2-deoxy-D-ribose 5-phosphate into D-glyceraldehyde 3-phosphate and acethaldehyde. UDP-glucose 4 epimerase catalyzes the reaction of UDP-galactose into UDP-D-glucose as part of galactose metabolism. Therefore, these enzymes have in common the participation in attaining metabolic energy.

The cellular localization of TTR-like was shown to be the periplasmic space. It is interesting to note that this was the cell area where higher differences were obtained concerning protein expression.

In biochemical terms many characteristics of TTR-like resemble human TTR: i) its homotetrameric structure, ii) its susceptibility to heat treatment, resulting in monomeric species, iii) the ability to form fibrils at acidic pH with similar appearance and width to human TTR. Concerning binding to thyroid hormones we were not able to detect a significant binding of TTR-like to T4. Of interest to know is the possible connection between bacteria and thyroid hormones from studies published a long time ago. It was implied a role in degradation of conjugated thyroid hormones in the intestine by Briggs et al., 1953. Cultures of *E. coli* were shown to bind radioiodinated T3 and T4, and release radioiodide (Roche et al., 1960).

Another study with cultures of *E. coli* demonstrated a rapid uptake of thyroid hormones, and binding of thyroid hormones, with higher affinity for T3 than T4 (Salvatore et al., 1963). Not so distant, DiStefano and co-workers (DiStefano et al., 1993) showed a greater binding of radioiodinated T3 and T4 by fecal and cecum samples from control rats, than from rats which had bee treated with antibiotics, and so without bacteria. The only degradation products detected were radioiodide, whose production was not effected by the presence of inhibitors of deiodinases. It was not clarified which *E. coli* protein was responsible for T4 and T3 results; and if in fact the bacteria TTR-like protein could then play a role in the thyroid hormones binding and oxidative degradation. If so, the significance and implications of this binding remains to be investigated. In the absence of T3 binding studies, a correlation for a functional role of TTR-like in the metabolism of hormones cannot be drawn.

Other biochemical characteristics of TTR-like in comparison to human TTR are however distinct: its high pI, the absence of dimeric species in SDS-PAGE that are observed in human TTR, and absence of fibril formation at neutral pH after inducing aggregate formation. Thus, the properties of TTR-like related to amyloid in bacteria differ from human. Human TTR fibrils are induced by lowering the pH but seeding by TTR fibrils does not initiate fibril formation (Bonifácio et al., 1996). In contrast, TTR-like seeds facilitate human TTR fibril formation which might be associated with an interaction between TTR and TTR-like. The interaction between these two proteins might relate to their homology and probable similar structure. Future studies are necessary to better understand TTR evolution relating its structure and function.

If TTR-like is in fact an ancestor of TTR from vertebrates, we can observe that its amyloidogenic potential was kept. During evolution, TTR was conserved in a selective process, though its function has changed.

ACKNOWLEDGEMENTS

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CONCLUSIONS AND PERSPECTIVES

Conclusions and future perspectives

The knowledge of TTR pathological involvement in FAP is growing and becoming more extensive over the years, through the effort of many scientific groups. Such wide comprehension has allowed following up patients during the asymptomatic phase and at a later symptomatic phase when neurodegeneration occurs. More important, is the possibility of implementing therapeutic and pharmacological methods that can halt the disease. In order to do so, it is compulsory to understand better the complex interplay between TTR aggregation, protective mechanisms and neuropathogenesis in FAP so that the best strategy is chosen. In parallel, it is crucial that animal models that mimic the human disease are available.

The following section refers to the aims proposed for the research project, the main conclusions drawn and future perspectives.

1) *Investigation of stress as an environmental factor affecting FAP, either as a factor accelerating or anticipating the disease or aggravating its development.*

Transgenic mice, expressing TTR variants are the first tool to study FAP. Previous characterizations of these mice demonstrated that TTR had a systemic pathological deposition occurring as a function of time. Both aggregated and fibrillar deposition was observed. There were however, reports that suggested differential expressivity of the disease. Specifically, the same animal strain proceeding from another laboratory, did not behave in the same manner in our facilities: they showed later development of amyloid. The difference was the housing conditions, while our facilities were SPF, in the previous laboratory they were not, and animals were maintained in crowded cages. Moreover, a paper reported this influence of accelerated pathogenesis due to bad housing conditions and suggested an influence of the gut flora. Our studies included the same transgenics: mice expressing the Val30Met human TTR. A schedule of unpredictable chronic mild stress was imposed over one year. Afterwards, mice tissues were analysed in

terms of TTR deposition and amyloid formation. Additionally, TTR and corticosterone serum levels were measured. Stress stimulus did not increase TTR deposition in the transgenic, but amyloid formation was anticipated. Corticosterone levels did not reflect the stress situation, except for one group of animals. We believe the stress stimulus was below the threshold necessary to cause aggravated deposition onset in the mice. To date, there are no experimental results relating stress in humans and FAP and we cannot exclude this possibility. Nevertheless, the treatments imposed to the mice were not sufficient to accelerate the disease, nor to extend it to the peripheral nervous system.

In the future, other acute stressful conditions might be applied to this or other transgenic mice model for FAP in order to anticipate TTR deposition and possibly extend it to the nervous tissue.

Stress influence in humans would be very interesting to analyse, though more difficult to address. This would require a wider and closer descriptive clinical history, associated to measurements of cortisol levels, to correlate with age of onset and FAP progression.

2) *Generation and characterization of a transgenic mouse model for Val30Met, compromised in the heat shock response.*

Several transgenic mice expressing TTR Val30Met were generated by different groups. Improvements were made in successive attempts, but the mice never recapitulated the whole pathological features observed for the FAP patients since TTR was not deposited in the peripheral nervous system. Afterwards, transgenic mice expressing more amyloidogenic TTR were characterized, but with no overall improvements. Also, double transgenics were created bearing the human SAP which is known to be involved in the amyloidogenesis cascade. Again, the peripheral nervous system was spared. The reason for these results might be related to species differences, higher protective mechanism in mice towards TTR deposition, or tissue related factors preventing TTR from being deposited in the nervous tissue.

Of great importance is the generation of an animal model that resembles FAP pathology in its entire dimension within a reasonable time period, so that suitable studies can be performed to understand the complex pathways of FAP pathogenesis or to use drugs that interfere with the process, preventing TTR deposition or eliminating tissue TTR.

Our approach was to cross the transgenic for the TTR Val30Met with knock-out mice for the transcription factor HSF1. These mice had an impaired stress response, since heat shock proteins (hsps) were not upregulated when needed. And, if this important response was involved in FAP as a protective mechanism, then its impairment would have a detrimental influence. HSF1-KO mice were characterized by others and us. We detected major alterations in the central nervous system with neurodegeneration, gliosis and disregulation of hsps. The lateral ventricles were enlarged with concomitantly increase in the CSF volume. These findings were also present after crossing with transgenic mice expressing human TTR Val30Met. The resulting transgenic mice - TTR/HSF1-KO - were shown to be very promising. TTR deposition was in fact aggravated in extent and in time, and also the peripheral nervous system was affected. Analysis of markers for FAP, such as pro-inflammatory cytokines, RAGE and activation of the NF-kB, known to be upregulated in human nervous system of FAP patients, were investigated in these new transgenic mice. Concordant to the human situation, these mice presented an increase in their expression, correlated with the disease.

The obtained data suggested that the heat shock response was involved in FAP, which prompted us to evaluate hsps in tissues affected with deposition. Interestingly, transgenic mice for TTR Val30Met in a wt background for HSF1, with tissue TTR deposition showed a correlated increase in hsp70 while mice TTR/HSF1-KO, with TTR deposited were not able to show. These results added the reasoning for the improved mouse model obtained: disruption of the heat shock response prevents cells from activating a mechanism of protection against TTR deposition, which in turn leads to a broad and aggravated disorder. Next, we searched for the mechanism occurring in human patients. There was an upregulation of hsps either in symptomatic and asymptomatic cases. This was observed in nervous and non-nervous tissues. So, early in FAP, cells respond to TTR deposition by increasing hsps which function as protective entities. Such

findings will open a new era in FAP. In one hand the availability of transgenic mice with closer features to FAP patients opens new possibilities in the study of mechanisms occurring in the peripheral nervous system due to TTR deposition; in the other hand it includes the heat shock response as a pharmacological target to treat FAP.

Future perspectives aim to understand the mechanism of protection elicited by HSF1 and hsps. This will be addressed by functional studies of HSF1 activation in conditions of pathology. Moreover, specific activity of hsps against neurodegeneration and apoptosis are of utmost importance.

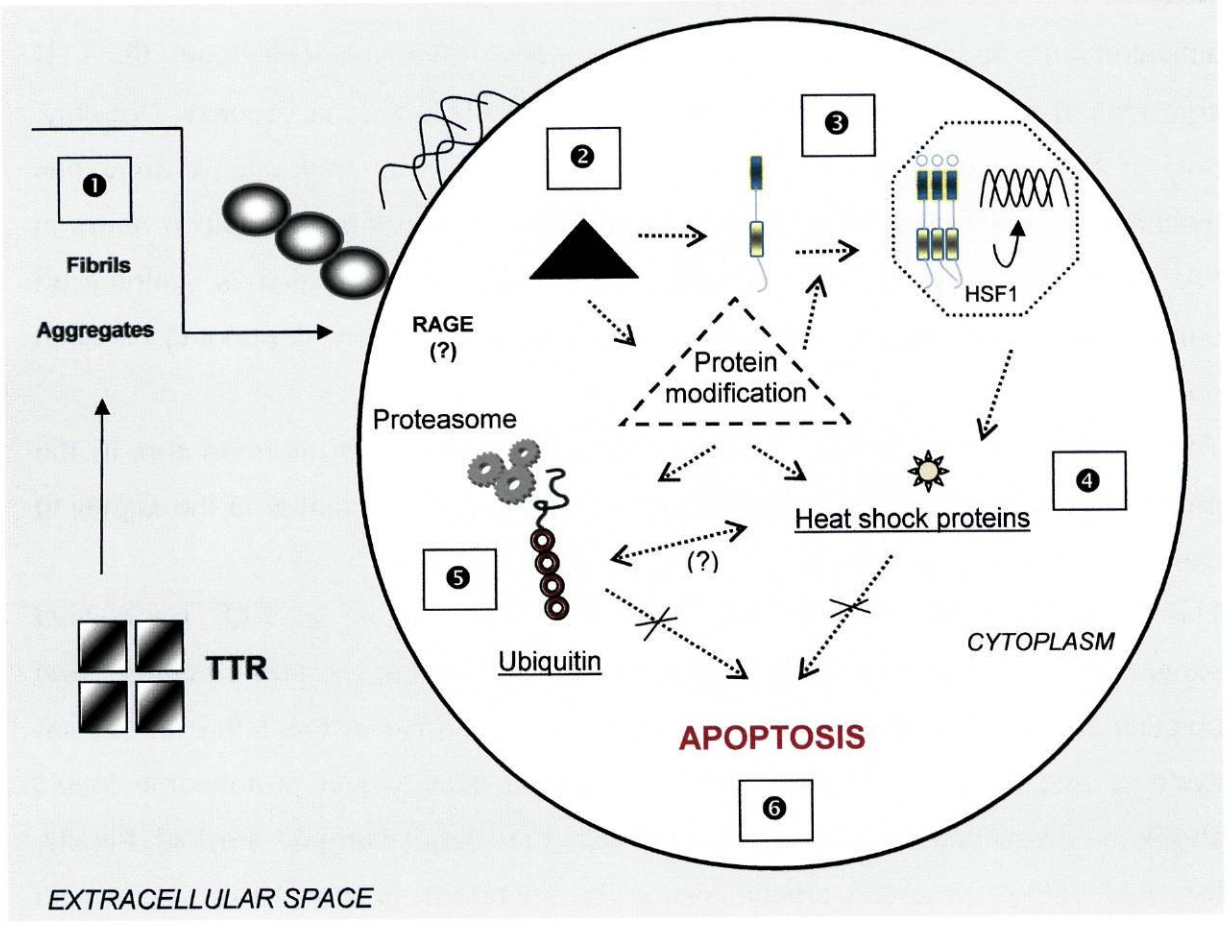
3) *Investigation of ubiquitin involvement in FAP.*

Similar to FAP, other neurodegenerative disorders are characterized by the presence of protein aggregates. The protein conformational disturbance leads to aggregation and eventually fibril formation. Among factors that distinguish this class of pathologies is the space where aggregation occurs. In FAP, TTR is found aggregated in the extracellular space, but in other diseases the altered protein can be detected inside the cell. Extensive studies in the latter situation were accomplished by diverse groups. Associated to these protein conformational disorders is ubiquitin. This protein is important in the targeting of misfolded proteins to be degraded by the proteasome. The impairment of the ubiquitin proteasome function was shown to be affected by neurodegenerative diseases.

We searched for a possible influence in the ubiquitin/proteasome system of FAP patients and animal models. It is clear that TTR does not form detectable fibrils intracellularly in the liver at the time of its synthesis, nor close to the cell of origin. After secretion, TTR is able to circulate in a soluble conformation until, due to unknown factors, it begins to unfold culminating in aggregated protein with propensity to form fibrils. Analysis of ubiquitin in the liver did not reveal increase in ubiquitin conjugates. Therefore, the protein quality control during synthesis cannot discriminate between TTR wt and TTR amyloidogenic variants. The difference in ubiquitin occurs in tissues where aggregated and amyloid TTR is found. Cellular accumulation of free and conjugated ubiquitin may be interpreted as a cell attempt

to deal with the extracellular insult, consequence of TTR deposition. The existence of a certain degree of blockage of the degradative cell activity due to ubiquitin accumulation can result from the general decreased homeostasis of the cells affected with TTR deposition. The fact that TTR is inappropriately interacting with cellular membranes perturbing the cellular physiology is logically reflected inside the cell in various points. The known activated mechanism involved is apoptosis. Mechanisms may be induced to prevent or delay apoptosis. In this context, ubiquitin may be one way of the cell to interfere with the fatal process of activated programmed cell death.

If a crosstalk between chaperones and the ubiquitin proteasome system exists, in FAP, the mechanism may start with TTR deposition extracellularly (See figure below, ❶).



Schematic representation of mechanisms, possibly elicited by TTR deposition alone or together with other factors.

TTR deposits signals to the interior of the cell via the RAGE receptor, though others yet unknown may exist. Intracellularly, damage to proteins might occur or pathways may be impaired perturbing cellular homeostasis ❷. Factors that lead directly to this disregulation (shown in the above figure) remain to be fully explored. Unknown signals, deriving from disregulated homeostasis (represented by a black box) activates HSF1 ❸. HSF1 is translocated to the nucleus, trimerizes, becomes phosphorylated and binds to heat shock elements inducing hsps synthesis ❹.

HSPs help to target damaged and oxidized proteins to the UPS ❺ and ubiquitin in turn signals those proteins to the proteasome. Ubiquitin would be important in the process of prevention of toxic proteins and intermediates to accumulate intracellularly. If damaged proteins and impaired pathways continue to persist, because of hsps and ubiquitin compromised activity, Jnk kinases, for example, are activated and apoptosis occurs, culminating with irreversible cell death ❻. TTR aggregation in tissues may not be the sole factor involved in this process. Possibly, only in conjunction with oxygen-free radicals, aging, or other stimuli does this mechanistic hypothesis occur. This is in accordance with the observation made in FAP patients: the symptoms appear late in life, although the protein is synthesized early in embryonic life and the clinical symptoms are different depending on each patient.

The complete puzzle in FAP must be clarified; therefore all intermediates in the pathogenesis cascade need to be identified such as intermediates in the signaling cascade leading to apoptosis.

The involvement of ubiquitin and hsps has been shown in FAP by distinct experiments. The proposed crosstalk between HSF1/hsps and ubiquitin/proteasome should be addressed and confirmed in the future in cellular systems and tissues. Moreover, the proteasome activity and proteasome levels should be investigated in tissues affected with TTR deposition and amyloid. Finally, the FAP stage where ubiquitin becomes important is not known. Ubiquitin involvement may be needed during the whole course of the disease or it may become more important in some stage of FAP progression.

4) Characterization of E. coli TTR-like protein

TTR is a conserved protein through evolution. However, it is a potential dangerous protein: it has an intrinsic propensity to acquire a pathological conformation which becomes evident with age and this feature is increased with amyloidogenic mutations. Its function was maintained in vertebrates as well as its structural features.

TTR-like protein was investigated in the prokaryote *E. coli*. It was expressed as a functional protein. Its absence was determined to be important for the cell, in conditions of limited nutrient supply. Its growth behaviour suggested that TTR-like was involved in the utilization of nitrogen as an energy source and its role may be required in the network of aerobic metabolic pathways.

TTR-like was localized in the periplasm due to the existence of a signal peptide.

We also evaluated TTR-like biochemical characteristics and its amyloidogenic potential. This protein is synthesized as a homotetramer similar to TTR from vertebrates. Its stability as a tetramer may be decreased in comparison to human TTR. Interestingly, a binding between TTR-like to TTR was observed which could be related to its homology.

TTR-like was able to form amyloid in acidic conditions. The width of the fibrils is similar in comparison to human TTR fibrils, albeit slightly smaller. Annular structures were also observed to form alongside with fibrils. TTR-like fibrils can seed human TTR amyloidogenesis. We also investigated the binding of TTR-like to T4, one of the TTR ligands, which was not observed. It seems that TTR function has changed during evolution, though its amyloidogenic potential was maintained.

Studies of comparative structure by site-directed mutagenesis should help in designing strategies to modulate amyloidogenesis. Additionally, further studies should address seeding in inducible models of the disease.

Concerning further TTR-like characterization, the search of other ligands is required. The toxicity of TTR-like aggregates and fibrils should also be addressed in the future, similar to what has been described for human TTR. Moreover, the

understanding of TTR-like participation in metabolic pathways is important and could be determined by 1) identification of other differentially expressed proteins of the knock-out grown in different media; 2) growth dynamics of the mutant in other conditional media and 3) rescue the phenotype by adding external intermediates suspected to be part of the metabolic pathway.

PART III

Apendix

Abbreviations

aa: Aminoacids

Aβ: amyloid β-peptide

AβPP: Amyloid β-protein precursor

AD: Alzheimer's disease

AEC: 3-amino-9-ethyl carbaxole

ALS: Amyotrophic lateral sclerosis

Apo AI: Apoliprotein AI

Apo E: Apoliprotein E

BSA: Bovine serum albumin

BSE: bovine spongiform encephalopathy

CJD: Creutzfeldt-Jakob disease

CNS: Central nervous system

CSF: Cerebrospinal fluid

DA: Deoxyribose-phosphate aldolase

DAB: 3,3´diaminobenzidine

DMEM: Dulbeco's minimal essential medium

DMSO: Dimethylsulphoxide

DRG: Dorsal root ganglion

DTSP: Dithiobis(succinimidyl propionate)

DTT: Dithiothreitol

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EIA: Enzyme immunoassay

ELISA: Enzyme linked immunoreactive assay

EM: Electron microscopy

ER: Endoplasmic reticulum

FAC: Familial amyloidotic cardiomyopathy

FAP: Familial amyloidotic polyneuropathy

FBS: Fetal bovine serum

GAG: Glycosaminoglycan

GFAP: Glial fibrillar acidic protein

GI: Gastrointestinal tract

HD: Huntigton's disease

HDL: High density lipoproteins

HPLC: High performance liquid chromatography

HSF: Heat shock transcription factor

Hsp: Heat shock protein

hmTTR/HSF1-KO: Transgenic mice expressing the human TTR Val30Met and knock-out for the HSF1 gene

hTTR/HSF1-KO: Transgenic mice expressing the human TTR Val30Met and knock-out for the endogenous mouse TTR gene and HSF1 gene

IDOX: 4'-iodo-4'-deoxydoxorubicin

IEF: Isoelectric focusing

IL1-β: Interleukin1-beta

IL-6: Interleukin-6

LB medium: Luria.Bertani medium

LDL: Low density lipoprotein

LDLr: Low-density lipoprotein receptor family

LPS: Lipopolysaccharide

M9 medium: Minimal medium

MC: Main chain

MCP-1: Monocyte chemoattractant protein-1

MMP-9: Matrix metalloproteinase-9

MT: Metallothionein promoter

mTTR-KO: Mice knock out for the endogenous TTR gene

NF-kB: Nuclear transcription factor kB

NGAL: Neutrophil gelatinase-associated lipocalin

OD: Optical density

O/N: Overnight

ORF: Open reading frame

PBS: Phosphate-buffered saline

PBS-T: Phosphate-buffered saline-0.05% tween20

PD: Parkinson's disease

PDB: Protein data bank

PG: Proteoglycan

pI: Isoelectric point

PMSF: Phenylmethylsulfonyl fluoride

PNS: Peripheral nervous system

RAGE: Receptor for advanced glycation end products

RAP: Receptor-associated protein

RBP: Retinol binding protein

RIA: Radioimmunoassay

ROS: Reactive oxygen species

RT: Room temperature

SAP: Serum amyloid P component

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SOD: Superoxide dismutase

SS: Signal sequence

SSA: Systemic senile amyloidosis

T3: Triiodothyronine

T4: Thyroxine

TA: Thymidine phosphorylase

TBG: Thyroxine-binding globulin

TCA: Tricarboxylic acid cycle

TEM: Transmission electron microscopy

Th-T: Thioflavin T

TNF- α: Tumor necrosis alpha

TTR: Transthyretin

TTR Leu55Pro (L55P): Leucine for proline exchange at position 55 of transthyretin.

TTR Val30Met (V30M): Valine for methionine exchange at residue 30 of transthyretin

UPR: Unfolded protein response

UPS: Ubiquitin/proteasome system

WT: Wild-type

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