



Genetic background modifies amyloidosis in a mouse model of ATTR neuropathy



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ABSTRACT

Penetrance and age of onset of ATTRV30M amyloidotic neuropathy varies significantly among different populations. This variability has been attributed to both genetic and environmental modifiers. We studied the effect of genetic background on phenotype in two lines of transgenic mice bearing the same ATTRV30M transgene. Amyloid deposition, transthyretin (TTR), megalin, clusterin and disease markers of endoplasmic reticulum stress, the ubiquitin-proteasome system, apoptosis, and complement activation were assessed with WB and immunohistochemistry in donor and recipient tissue. Our results indicate that genetic background modulates amyloid deposition by influencing TTR handling in recipient tissue and may partly account for the marked variability in penetrance observed in various world populations.

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1. Introduction

Familial amyloidotic neuropathy (FAP) Type I is an autosomal dominant sensorimotor neuropathy due to amyloid deposition in which the main polypeptide component is transthyretin (TTR). Mutation of valine to methionine at position 30 is the commonest variant hence it is named ATTRV30M neuropathy. Penetrance and age of onset of ATTRV30M neuropathy varies significantly among different populations. Penetrance in Sweden, Cyprus and Portugal is 2%, 28% and 80% while the age of onset is 52, 46 and 32 years respectively [1]. This country-specific variability in penetrance is unusual for an autosomal dominant disease caused by an identical mutation and has been attributed to genetic and environmental modifiers [2–4].

We investigated the effect of genetic background on TTR amyloidosis using two different genetic backgrounds of a transgenic mouse model of ATTRV30M amyloid neuropathy. The mice were null for the endogenous TTR and homozygous for the human ATTRV30M mutation, one on a $129 \times 1/SvJ$ background and the other on a mixed $129 \times 1/SvJ/C57BL/6J$ background. Both genetic backgrounds had the same human transgene copy number and a similar number of RNA transcripts in liver tissue, the main site of TTR synthesis. We carried out a semi-quantitative comparison of

TTR amyloid deposition and of megalin and clusterin expression, both of which are increasingly recognized for their role in tissue handling of amyloidogenic peptides [5–9]. In addition, a number of molecular markers involved in ATTRV30M disease pathogenesis were assessed including BiP (endoplasmic reticulum stress), ubiquitin (ubiquitin-proteasome system), Fas and Caspase 3 (apoptosis) and C5b9 (complement activation). We demonstrate that cross-bred $129 \times 1/SvJ/C57BL/6J$ mice exhibit significantly less amyloid deposition and secondary disease markers which are associated with significantly higher megalin and clusterin up-regulation. Thus genetic background does appear to modulate amyloidogenesis in the ATTRV30M neuropathy model and a number of players involved in misfolded protein handling may be involved.

2. Materials and methods

2.1. Animal and tissue handling

All animals were originally derived from the same transgenic mouse line [10,11]. They are both null for murine TTR and homozygous for the ATTRV30M transgene but on two different genetic backgrounds; the $129 \times 1/SvJ$ background (as of now shall be referred to as: *TM 129*) and the $129 \times 1/SvJ/C57BL/6J$ hybrid outcross background (as of now shall be referred to as: *TM 129/BL6*). Two $129 \times 1/SvJ$ and two *C57BL/6J* animals of the appropriate age group, depending on the application, were used as wild type

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control animals.

The $mTTR^{-/-}hTTR^{V30M/V30M}$ mice were generated from a single pair of $mTTR^{-/-}hTTR^{V30M}$ mice (of $129 \times 1/Svj$ background, obtained from M. J. Saraiva) that were crossed amongst them to generate $mTTR^{-/-}hTTR^{V30M/V30M}$ pups. These were then inter-crossed to generate the colony of experimental mice. The number of copies of the V30M transgene was monitored by real time PCR. These mice are referred to as *TM 129*.

To generate the *TM 129/BL6* colony, male mice $mTTR^{-/-}hTTR^{V30M/V30M}$ (in $129 \times 1/Svj$ background) were crossed to *C57BL/6j* females in order to generate F1 mice $mTTR^{+/-}hTTR^{V30M}$. These were then inter-crossed to generate F2 $mTTR^{-/-}hTTR^{V30M/V30M}$ mice which were then bred to generation 7 through successive brother-sister mating. These F7 progeny were used to produce the experimental mouse line which was used throughout the experiments. Specifically, the F7 generation was used was made up of 2 males and 6 females. Their progeny is considered to be of mixed genetic background, segregating alleles of $129 \times 1/Svj$ and *C57BL/6j* origin.

Real time PCR was used to ensure that all animals included in the experiment had the same copy of transgenes (Fig. 1A). Furthermore reverse transcription real time PCR was used to confirm that RNA expression for human TTR was similar in liver tissue of the two backgrounds used in the experiments (Fig. 1B).

Three groups of animals were used from each background; group **A** (3–6 months), group **B** (9–12 months) and group **C** (15–18 months). Each *TM 129* age group was comprised of 15 mice (7 M/8 F), whereas each *TM 129/BL6* group was comprised of 10 mice (5 M/5 F) each. All animals were kept in a regular 12 h light-12h dark cycle and were given free access to water and food, under SPF conditions. Animals were separated in cages depending on the age group they were assigned to and their sex. All animal involving experiments were carried out in accordance to the 86/609/EEC Directive.

Mice were anaesthetised and then euthanized using Tribromoethanol (Avertin) through IP injection at a dose of 250 mg/Kg. The animals were then exsanguinated via PBS perfusion to reduce the contribution of plasma in the measurements. Tissues were obtained at the required time points. Tissues were processed for immunohistochemistry by carrying out overnight 4% PFA fixation followed by wax embedding or were frozen and kept at $-80^{\circ}C$ for western blot analysis.

Amyloid deposition assessment was confined to stomach tissues since this organ is heavily involved in amyloid deposition at an early age in this model of ATTRV30M neuropathy.

2.2. Genotyping

Animals were routinely genotyped using the PCR method. Primers for the mouse TTR gene ($mTTR$ F 5' - CTG ACC CAT TTC ACT GAC ATT T - 3' & $mTTR$ R 5' - CAA ATG GGA ACC TGG AAC C - 3') and the human mutated transgene ($hATTRV30M$ F 5' - TGCTGATGACACCTGGGAGC - 3' & $hATTRV30M$ R 5' TCAGGTTCTGGTCACTTCC - 3') were utilized for screening with annealing temperature at $58^{\circ}C$.

2.3. Transgene zygosity assessment

For transgene zygosity assessment, the qPCR LightCycler FastStart DNA Master SYBR Green I assay (Roche Cat. No. 12 239 264 001) was used according to the manufacturer's instructions. The primers (same used for $hATTRV30M$ genotyping) were used at $0.5 \mu M$ concentration each. Wild type mice (without the transgene), heterozygote and homozygous mice were used as controls to allow for result interpretation. The GAPDH gene was used as a run control for normalization ($mGAPDH$ F 5' - CGACTTCAA-CAGCAACTCCCCTCTTCC - 3' & $mGAPDH$ R 5' TGGGTGG-TCCAGGGTTTCTACTCCTT - 3') [12] (Fig. 1A).

2.4. hTTR mRNA Expression in liver tissues

Two-step RT-qPCR was carried out to quantify the expression of $hTTR$ in the liver. Liver tissue was extracted following animal exsanguination via PBS perfusion. Approximately 25 mg of liver tissue was removed and lysed using the RNeasy Mini QIAcube Kit (74116). RNA concentration was assessed by Nanodrop2000 and maximum $100 \text{ ng}/\mu\text{l}$ was used for cDNA synthesis. The Invitrogen SuperScript™ II Reverse Transcriptase (18064-022) was used to synthesize first-strand cDNA according to the manufacturer's instructions.

TaqMan® Gene Expression Assay for human TTR was then used, containing a pair of unlabelled PCR primers and a TaqMan® probe with a FAM™ dye label on the 5' end, and minor groove binder (MGB) non-fluorescent quencher (NFQ) on the 3' end. $5 \mu\text{l}$ 2x TaqMan® Gene expression master mix was used with $0.5 \mu\text{l}$ $hTTR$ primer probe mix (Hs00174914_m1, 4331182) and $2 \mu\text{l}$ cDNA in a total reaction of $10 \mu\text{l}$. The GAPDH (4352932E) gene was used as an endogenous control in delta Ct normalization of samples. For each liver sample duplicate reactions were run (Fig. 1B).

2.5. Western blots and densitometry

The amount of each protein under investigation was expressed as a ratio of the intensity of the protein band divided by the

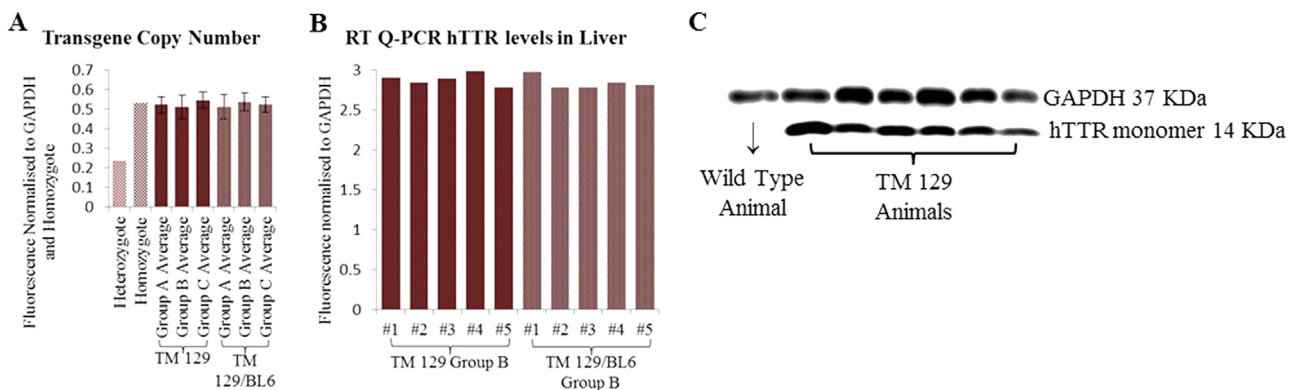


Fig. 1. (A) Real-time PCR results of the number of transgene copies found in the animals of *TM129* pure background and *TM129/BL6* mixed background, (B) Reverse transcription real-time PCR results from the liver tissue of *TM129* pure background and *TM129/BL6* mixed background animals measuring the amount of human TTR RNA expressed in liver, (C) Western blot of *TM129* stomach samples from different mice and a single wild type *129* animal for $hTTR$ and GAPDH. The strong TTR band seen consists of monomers.

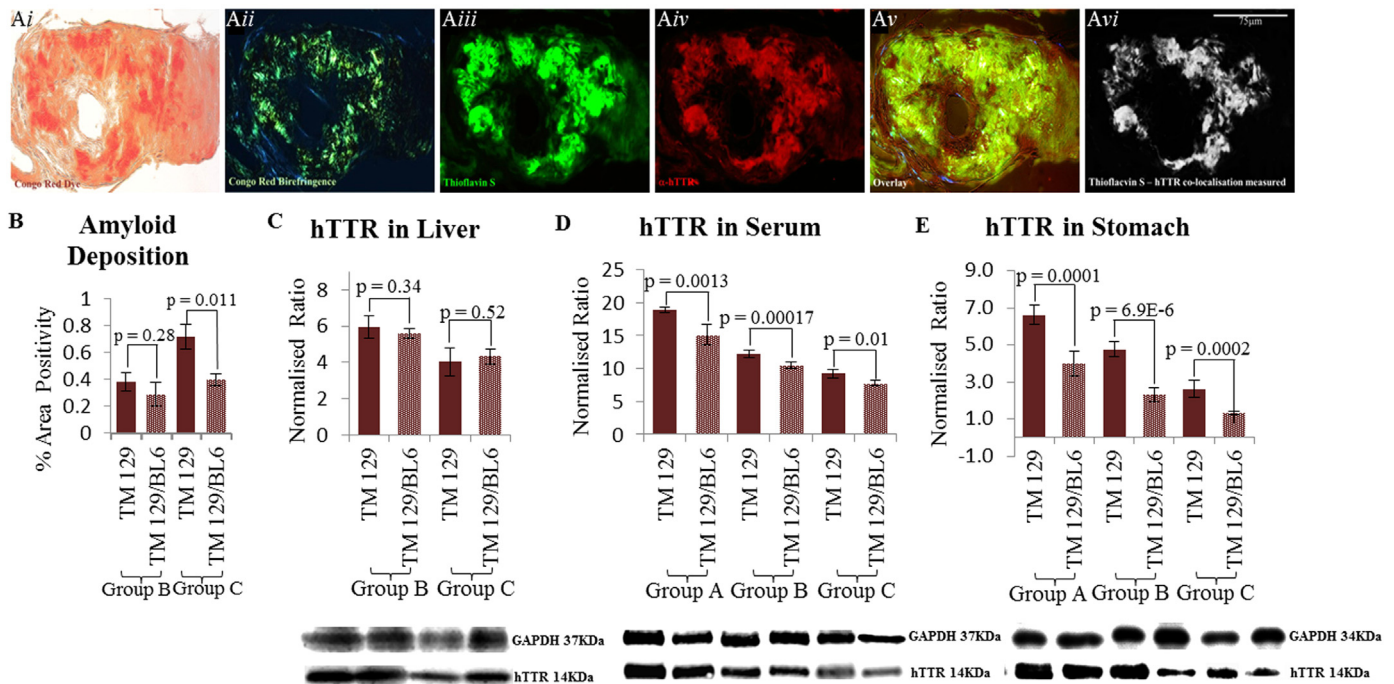


Fig. 2. (A) Amyloid plaque in the stomach that stains with Congo red and exhibits apple green birefringence (Ai&Aii). The same plaque stains Thioflavin S positive (Aiii) and is composed of human transthyretin (Aiv). The area of co-localization of Thioflavin S and TTR labelling appears yellow (Av) and morphometric measurements are carried out with the Image J software (Avi). (B) Morphometric comparison of amyloid deposition in the two backgrounds. Western blots of human non fibrillar TTR in the transgenic animals of the two backgrounds: in the liver (C), the serum (D) and stomach tissue (E).

intensity of the GAPDH band (Santa Cruz Biotechnology anti-mouse SC-32233 1/4,000 and Jackson ImmunoResearch anti-mouse 115-035-003, 1/5,000) to account for variations due to loading, even though the amount of protein loaded was previously quantified using the standard Bradford assay. This ratio was normalized by dividing it by the ratio obtained, in the same Western blot, from a standard control mouse. The same standard mouse was used to normalize all Western blots across different backgrounds and age groups. Each sample was run in triplicate and the average normalized ratio was used for comparison. Image J was used to carry out densitometry calculations.

2.6. TTR Western blot

The animals were exsanguinated after sacrifice so that blood TTR could be eliminated as much as possible. Stomach homogenate was separated via reducing SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% low fat dry milk (Régilait) for one hour at room temperature. The membranes were then incubated overnight at 4 °C with anti-human Transthyretin (TTR) (DAKO A000202) at a dilution of 1/2,000. The antibody was visualized using the Amersham ECL Western Blotting Detection Reagent (RPN2232) after incubating with anti-rabbit secondary antibody at 1/5,000 (Jackson ImmunoResearch 111-035-003) for one hour at room temperature. It should be noted that for TTR, due to the denaturing conditions, only monomers (14 kDa) were measured (Fig. 1C) so that only non-fibrillar hTTR can be visualized since plaque-bound hTTR needs to be otherwise extracted [13].

Stomach TTR was measured in all animals. Serum and liver TTR was similarly measured by western blot in 3–5 animals from each of the two transgenic backgrounds using a control for normalization.

Blood samples were collected without sacrificing any of the animals, from the orbital sinus in the absence of anticoagulant. The samples were allowed to stand overnight at 4 °C to coagulate. They were then centrifuged at 3500 rpm for 10 min and the top layer

was collected in order to obtain the serum. Samples were diluted 1/10 using injection water prior to western blot analysis as aforementioned. However the samples mixed with loading buffer were not boiled but were instead heated at 65 °C for 15 min to avoid protein coagulation.

2.7. Other Western blots

The same method was applied as with TTR for the following antibodies; Clusterin (Santa Cruz Biotechnology anti-rabbit sc-8354 1/500) GRP78 (BiP) (Santa Cruz Biotechnology anti-rabbit sc-13968 1/1000) and ubiquitin (Santa Cruz Biotechnology anti-rabbit sc-9133 1/1500).

2.8. Amyloid plaque visualisation and quantification

Thioflavin S stain combined with TTR immunofluorescence were used to identify amyloid deposits in paraffin sections obtained from stomach tissues. Paraffin sections were deparaffinised and hydrated to distilled water. Sections were then stained with Mayer's haematoxylin for 5 min, washed further with distilled water and then stained with aqueous 1% Thioflavin S solution (T1892-25G) for a further 5 min and finally differentiated in 50% ethanol before being rinsed with distilled water and then mounted using the DAKO Fluorescence Mounting Medium (S3023). Thioflavin S positive deposits were further confirmed to be amyloid by electron microscopy (data not shown). In the cases where double staining with Thioflavin S and hTTR antibody were performed, antibody immunohistochemistry was carried out first followed by staining with Thioflavin S standard protocol but omitting the Mayer's haematoxylin final staining [14]. Plaques positive for both Thioflavin S and hTTR were measured using the Image J software set to measure yellow (570–585 nm) (Fig. 2A). TTR amyloid plaques were measured over the entire area of stomach section, a percentage of the surface area occupied by plaques was calculated and an average percentage obtained over three serial sections.

2.9. Immunofluorescence for TTR and molecular markers

Paraffin sections from animals' stomachs were deparaffinised and hydrated to distilled water. Sections were then blocked with 5% BSA solution in PBS for 1 hour at room temperature and then incubated with the appropriate primary antibody overnight at 4 °C. The primary antibody used was the anti-human Transthyretin (TTR) from DAKO (A000202) at a dilution of 1/500. The slides were then incubated with Invitrogen Alexa Fluor 555 fluorescence secondary antibody for 1 hour at room temperature at 1/2,000 (anti-rabbit A-21428). Finally sections were washed with PBS and mounted using the DAKO Fluorescence Mounting Medium (S3023).

Further analysis was carried out using supplementary antibodies; Fas (Santa Cruz Biotechnology anti-rabbit sc-10231/1000) and activated Caspase-3 (Santa Cruz Biotechnology anti-goat sc-1225 1/500) to assess apoptosis, GRP78 (Santa Cruz Biotechnology anti-rabbit sc-13968 1/1000) to assess the endoplasmic reticulum stress response, and C5b-9 (EMD Millipore anti-rabbit 204903 1/4,000) to assess complement activation and Megalin (Santa Cruz Biotechnology anti-goat sc-16478 1/400). The appropriate secondary antibodies were used, anti-rabbit (Alexa Fluor A-21428 1/2000) and anti-goat (Alexa Fluor A-21432 1/2000). ECL was used to visualize the antibodies as previously explained.

2.10. Morphometric image analysis

This analysis was carried out to measure areas of positivity by immunofluorescence among the various animals. Pictures were taken using a Zeiss fluorescence microscope using the 20 × objective lens (for measurement purposes). Four (4) pictures were taken of each stomach in order to visualize the entire stomach cross section. Percentage area of antibody positivity was measured using the Image J software set to measure red (665–700 nm) and averaged from three serial cross sections for each animal. A control (wild animal) was similarly measured to obtain an indication of background positivity.

2.11. Statistical analyses

Using the Microsoft Office Excel 2010 suite, mean, standard deviation and p-values were calculated. Using this information, graphical charts representing the data were prepared. The error bars depicted represent the standard deviation (1 SD) for each group of data.

3. Results

The main hallmark of FAP Type I neuropathy is the extracellular deposition of amyloid fibrils composed of TTR protein. Detectable amyloid deposits (by Thioflavin S/TTR immunofluorescence) in the stomach appear after the age of six months. Our results indicate that TTR amyloid deposition was not statistically different between the two genetic backgrounds between ages 9–12 months but by the age of 15–18 months amyloid deposition was significantly higher in the *TM129* mice by about 30% compared to the *TM129/BL6* (Fig. 2B).

We next asked if this difference was due to differences in the production of TTR in the liver, the major organ of TTR production and subsequent release in the serum. Western Blot analysis indicated no significant difference in liver TTR among the two transgenic backgrounds (Fig. 2C). However, TTR measured in the serum was significantly lower in the *TM 129/BL6* compared to the *TM 129* background (Fig. 2D) at all ages. As the animals aged there was reduction in circulating TTR in both backgrounds, which

coincided with appearance of amyloid deposits. Serial body weights did not differ significantly between the two backgrounds for any of the age groups (data not shown). Non-fibrillar TTR, was also significantly lower in the *TM 129/BL6* as compared to the *TM129* background between the ages of 3–6 months (Fig. 2E). There was also a great reduction in the tissue levels of non-fibrillar TTR in Group B of both lines, which coincided with the appearance of amyloid deposits. Significant difference was also observed during the third group.

Previous studies demonstrated that BiP, a major regulator of endoplasmic reticulum (ER) function involved in both protein folding and assembly, has been found to be over-expressed in tissues of FAP patients and transgenic mice. We evaluated the expression of this marker in donor tissue (liver), as a means of evaluating the stress imposed on the ER by misfolded intracellular TTR, and recipient tissue (stomach) as a means of evaluation extracellular stress due to extracellular TTR. In the liver, BiP levels were not statistically different between the two genetic backgrounds but was overexpressed compared to wild type animals (Fig. 3A). In the stomach, BiP appeared to be overexpressed in both backgrounds compared to wild type animals, before the appearance of amyloid deposits, but less so in the *TM 129/BL6* mice (Fig. 3C).

We then analysed ubiquitin which is expressed in all cells and is central in the degradation of aberrant/misfolded proteins. In TTR amyloidosis ubiquitin has been shown to be up-regulated by extracellular amyloid. We found that its level was significantly higher in the *TM129/BL6* background in both stomach (despite the lower amyloid deposits) and in liver. In both backgrounds there was up-regulation of ubiquitin compared to control in the liver tissue (Fig. 3B), whereas in the stomach a significant difference was recorded between the transgenic backgrounds wild type animals in the third age group (Fig. 3D).

Increased amyloid deposition leads to complement activation and increased apoptotic events in TTR amyloidosis. We thus tested markers for apoptosis (Fas and Caspase-3) and complement activation (C5b-9) and found them all up-regulated in both transgenic backgrounds in an age dependent manner with the *TM129* mice exhibiting higher expression than the *TM 129/BL6* background (Fig. 4). The up-regulation predated the appearance of amyloid deposits.

Finally, Megalin, a protein that acts as a multi-ligand membrane receptor, and clusterin, that acts as an extracellular chaperone of amyloidogenic peptides but also exists within the cell, both recently recognized for their role in amyloidogenesis were assessed. Our results show that both were up-regulated in the two backgrounds compared to wild type animals but tended to be more overexpressed in the *TM129/BL6* mice (Fig. 5A–B). Both clusterin and megalin co-localize with TTR intracellularly (Fig. 5C).

4. Discussion

In the present study we examined the effects of different genetic backgrounds on amyloid deposition and various related molecular pathogenic markers in a transgenic model of ATTRV30M. Genetic background has previously been shown to affect the phenotype and severity of other neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) [15–17]. Considering that the two lines of mice used in the current study bear the same mutation with regards to the disease-causing locus, our results suggest that genetic modifiers affect disease progression, or in this case, amyloid deposition.

The two lines of mice used here were the *TM 129* mice, which were on a pure *129 × 1/SvJ* background and the *TM129/BL6* mice were on a mixture of *129 × 1/SvJ* and *C57BL/6J* backgrounds. The

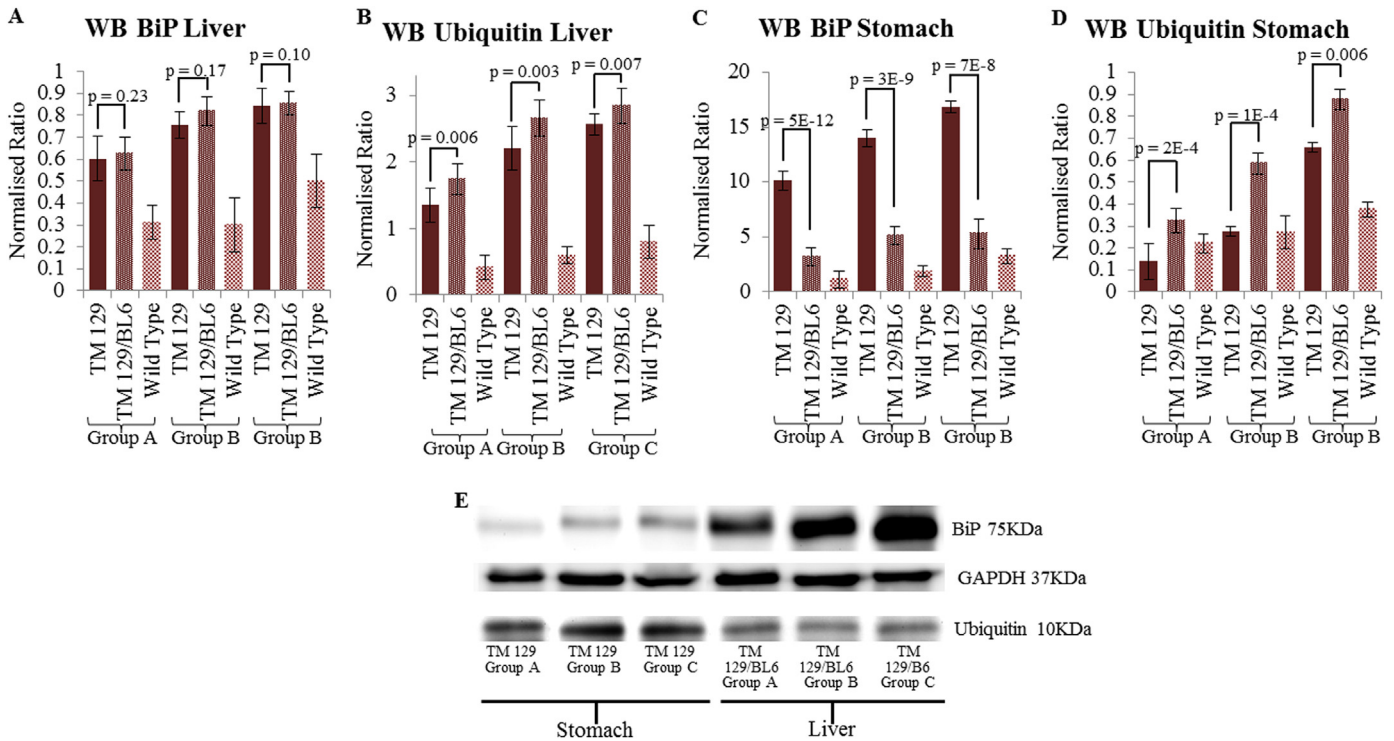


Fig. 3. Western blot (A–E) comparisons of BiP in liver (A), Ubiquitin in liver (B) BiP in stomach (C) and Ubiquitin in stomach (D) in the two backgrounds.

latter group was considered genetically quite distinct from the reference group, bearing in mind the number of generations used for interbreeding. We have confirmed, through real-time PCR, that all mice used in this study carried the same number of disease causing mutated human TTR transgenes (V30M) (Fig. 1A). Furthermore, employing reverse transcription real-time PCR, we were able to quantify the expression of hTTR in the liver, the main site of TTR production (Fig. 1B). Our results indicate that TTR production in liver cells was indeed equivalent in both lines of animals (Fig. 2C). Secretion efficiency is likely similar in both lines since the TTR mutation is the same and hepatic levels of BiP are also the same (Fig. 3A). Therefore, any differences in amyloid deposition in stomach are likely due to differences in recipient tissue handling

of the amyloidogenic protein.

Thioflavin S and TTR immunofluorescence were used to provide quantification of the TTR specific amyloid deposits (Fig. 2A, B). Our results indicate that as amyloid deposition progresses, the mixed background mice appear to have less amyloid in the stomach (the main site of deposition in this mouse model) than their 129 background counterparts. Blood and stomach non-fibrillar TTR are similarly lower in the mixed background mice. The lower levels of serum and tissue non-fibrillar TTR found in the mixed background animals may be a result of enhanced peripheral tissue “clearance” (Fig. 2D, E).

“Clearance” refers to the amount of non-fibrillar TTR that is removed either from the circulation and/or from the extracellular space and is either sequestered intracellularly and degraded in the

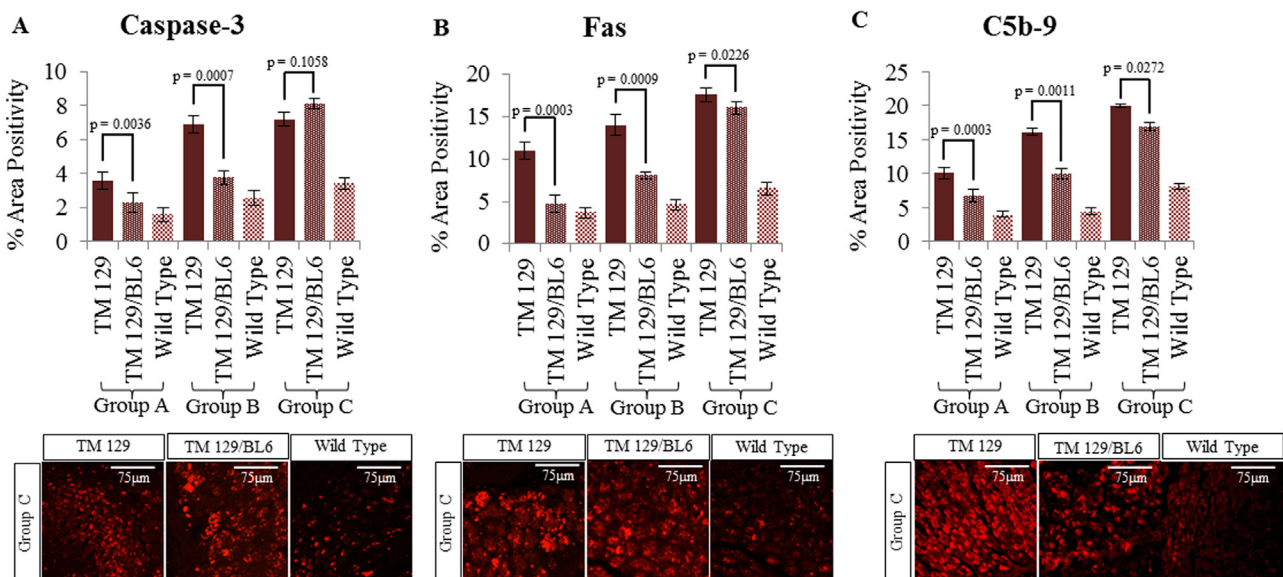


Fig. 4. (A–C) Morphometric comparison of immunofluorescence for Caspase-3, Fas and C5b9 in the two backgrounds along with representative images for Group C animals.

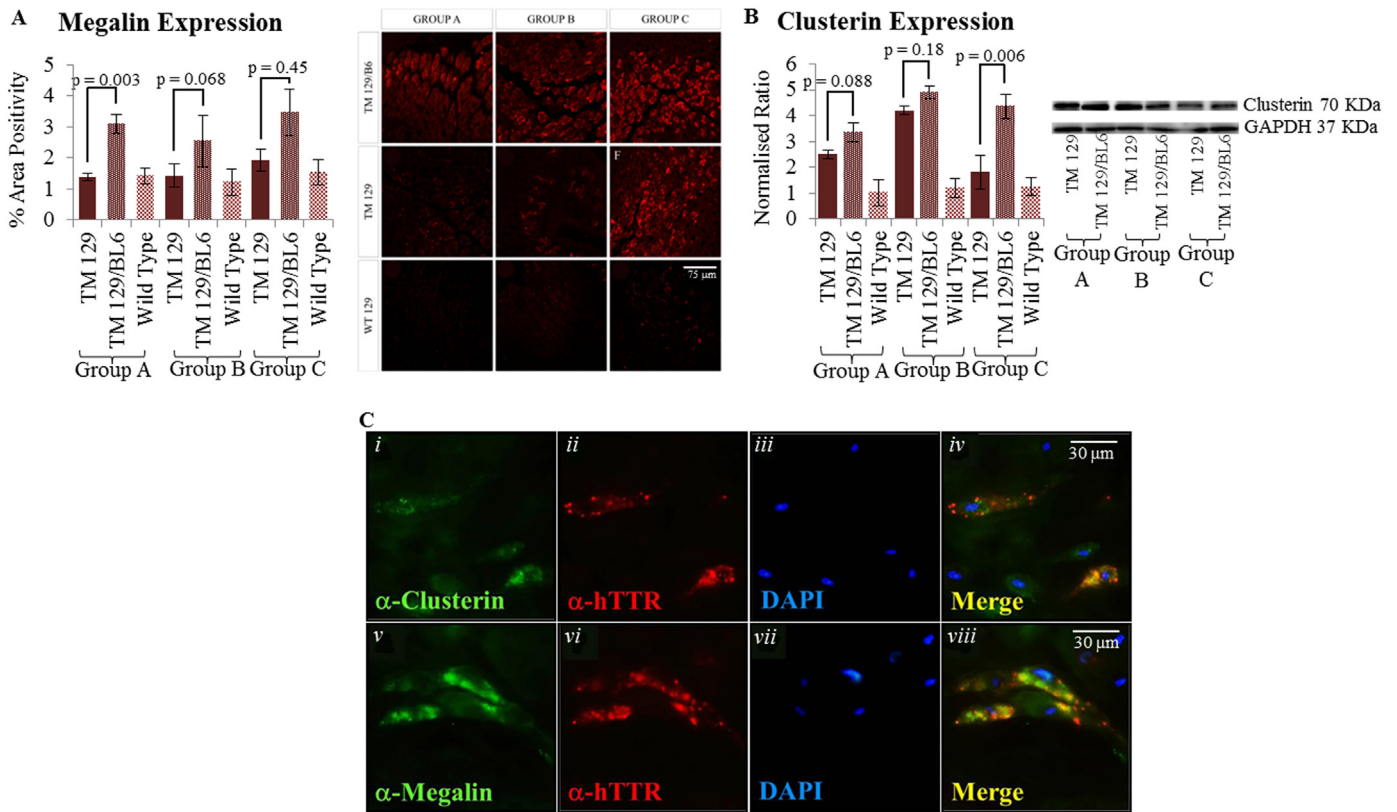


Fig. 5. (A) Morphometric comparison for megalin and Western blot comparison for clusterin (B) in the two backgrounds, (C) Intracellular co-localization of TTR with clusterin (Ai-Aiv) and megalin (Av-Aviii) in stroma stomach cells.

tissue bed or deposited as amyloid. Data obtained from both human and animal studies indicate that low levels of serum TTR have been associated with more unstable and more amyloidogenic TTR variants [18]. Furthermore human ATTRV30M carriers have a lower level of TTR compared to non-carriers; while symptomatic carriers have lower levels than asymptomatic carriers, presumably as a result of mutated TTR penetrating and forming amyloid in tissues in increasing amounts [19]. Interestingly a study of serum TTR in a group of normal human controls showed that TTR concentration was statistically related to age, gender and ethnicity [20]. The explanation is currently poorly understood but could relate to genetic/age related variability in peripheral tissue “clearance”.

In the stomach BiP appears significantly higher in the *TM129* mice which also exhibited higher amyloid deposition. BiP has previously been associated with extracellular TTR plaques in tissue obtained from FAP patients and been reported as an important biomarker that can be used to assess progression of the disease [21].

Ubiquitin on the other hand was expressed at higher levels in the stomach of *TM129/BL6* mice at all ages despite lower levels of extracellular TTR monomers and amyloid deposits (Fig. 3D). Whether this relates to a higher internalization of TTR and ubiquitination by the resident cells is unclear however, it should be noted that intracellular TTR have been reported in FAP murine models. Intracellular localization of TTR has been previously demonstrated intracellularly in fibroblasts, macrophages and Schwann cells. Endocytosis of extracellular TTR as well as in-situ synthesis are two possible explanations that been proposed [22,23]. Another possibility may be that the higher ubiquitin levels may be accounted for by the higher expression of clusterin which is a highly poly-ubiquitinated protein in the *TM129/BL6* mice [20].

Higher ubiquitin levels also expressed in the liver of *TM129/BL6* mice perhaps suggesting that the two backgrounds have different

capacities “to chaperoning at a distance” (Fig. 3B) [21].

Clusterin and megalin, tend to be more overexpressed in the *TM129/BL6* than the *TM129* mice. Clusterin, an extracellular chaperone is overexpressed in both TTR amyloidotic neuropathy and TTR amyloidotic cardiomyopathy and is thought to play a protective role in TTR amyloidosis [5,6,24]. Megalin, a multi-ligand membrane receptor, binds clusterin and has been shown to mediate internalization of TTR [7] (Fig. 5C). Thus, we hypothesize that the overexpression of these two molecules results in higher rates of sequestration and degradation of TTR in *TM129/BL6* mice resulting in less amyloid formation. A generic modulating role for clusterin and megalin in the extracellular amyloidosis probably operates in the light of data pertaining to Alzheimer disease [8,9,25].

In keeping with previously published work, outlining the toxicity of both nonfibrillar TTR as well as fibrillar TTR, molecular markers of apoptosis (Fas and Caspase-3) and complement activation (C5b-9) were found to be significantly up-regulated in both backgrounds [21,26–28]. As expected these markers were more up-regulated in the *TM129* background animals which exhibited greater amounts of non-fibrillar and fibrillar TTR (Fig. 4).

All currently available therapies for ATTRV30M amyloidosis aim to drastically reduce blood TTR (liver transplantation, silencing RNAs) or reduce the dissociation of TTR tetramers (Tafamidis, Diflunisal) [29]. Other therapeutic approaches that perhaps should be intensely considered are those that target molecular players involved in modulation of amyloid deposition [25].

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Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.08.005>.

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