

Biomarkers in the Assessment of Therapies for Familial Amyloidotic Polyneuropathy

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The identification of specific biomarkers provides opportunities to develop early diagnostic parameters, monitor disease progression, and test drug efficiency in clinical trials. We previously demonstrated that in familial amyloidotic polyneuropathy (FAP) related to the abnormal extracellular tissue deposition of mutant transthyretin (TTR), inflammatory and apoptotic pathways are triggered in the presymptomatic stages of the disease, when nonfibrillar TTR deposits are present. In the present work, to better define biomarkers for future assessment of prophylactic and therapeutic drugs in the treatment of FAP, we extended the search for oxidative stress and apoptotic biomarkers to clinical samples and animal models presenting nonfibrillar and fibrillar TTR. We found that lipid peroxidation measured by hydroxynonenal, oxidative DNA damage measured by 8-hydroxy-2'-deoxyguanosine, and cellular redox homeostasis measured by glutaredoxin 1 were consistently increased in biopsy specimens from FAP patients and in tissues from transgenic mouse models presenting nonfibrillar TTR deposition. Death-receptor Fas, caspase-8, and Bax were also found to be increased, indicative of the involvement of death receptors in the observed apoptosis process. Removal of TTR deposition by an immunization protocol resulted in significant decreases of the selected markers we describe, corroborating the relationship between TTR deposition, oxidative stress, and apoptosis. Taken together, our results provide a robust biomarker profile for initial experimental animal studies and clinical trials to assess the application of the selected markers in therapies aimed at removal and/or inhibition of TTR polymerization.

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INTRODUCTION

Familial amyloid polyneuropathy (FAP) is an autosomal dominant hereditary disease characterized by the extracellular deposition of amyloid fibrils in the connective tissue, affecting the peripheral nervous system in particular (1,2). The onset of clinical symptoms generally occurs before age 40, with a progressive and severe sensory and autonomic neuropathy leading to death in about 10–20 years.

FAP amyloidoses are related to single transthyretin (TTR) amino acid substitutions in which the mutated protein leads to extracellular amyloid fibril deposition. Although more than 80 transthyretin mutations associated with TTR amyloidosis

have been described (3), the most common variant has a valine substituted by a methionine at position 30 (V30M) (4). TTR is a 55-kDa homotetrameric protein synthesized mainly in the liver, eye, and choroid plexus, and its main function is the transport of thyroxine (T₄) and vitamin A (retinol) associated with the retinol binding protein.

Why mutated TTR deposits in the form of amyloid is unknown, but x-ray crystallographic studies of TTR mutants related to aggressive forms of FAP show conformational changes in this protein (5). These changes may lead to tetramer dissociation into a nonnative TTR monomer with low conformational stability, which results in partially unfolded

monomeric species with a strong tendency to aggregate (6). Pathogenic events associated with TTR deposition in FAP patients have been investigated by analyses of nerve and salivary gland tissues from FAP patients and asymptomatic V30M carriers; cytotoxicity begins in a presymptomatic stage of the disease, with nonfibrillar TTR aggregates triggering oxidative damage, inflammatory responses, induction of the nuclear transcription factor κ B (NF κ B) pathway, and activation of caspase-3 before amyloid fibrillar deposition (7,8). Increased levels of the endoplasmic reticulum (ER) stress sensor BiP were found to correlate with the extracellular TTR deposition observed in salivary gland tissue from FAP patients (9).

Early detection of pathological lesions through the use of biomarkers may aid in correct clinical management of patients and possible delay of morbidity. In this regard, animal models of TTR

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deposition with defined sets of correlative biomarkers are essential tools to test and guide the application of prophylactic and therapeutic drugs before clinical testing.

Mice transgenic for human TTR V30M in a null background (hTTR Met30) serve as animal models for TTR deposition. Nonfibrillar deposition begins when the mice are three months old, and the deposits evolve to amyloid fibrils when the mice are nine months old, with particular involvement of the gastrointestinal tract and skin (10). hTTR Met 30 mice have already been used to demonstrate that TTR deposits can be removed by an immunization protocol with a TTR variant expressing a cryptic epitope, TTR Y78F (11).

By crossing hTTR Met 30 mice to mice with a heat shock transcription factor 1 (HSF1) null background, a novel transgenic mouse model was generated, hTTR Met30/HSF1-KO. This new mouse model shows TTR deposition in the peripheral [dorsal root ganglia (DRG) and nerve] and autonomic nervous systems, a deposition pattern not observed in hTTR Met30 models (12).

We sought to define new, reliable oxidative stress and apoptotic biomarkers associated with TTR deposition in human clinical samples and in transgenic mouse models before and after deposition removal by immunization.

MATERIALS AND METHODS

Human Samples

Labial minor salivary gland biopsy specimens were obtained from V30M FAP patients before they underwent liver transplantation (13), the only available treatment for this disorder. Control salivary gland tissue samples were from non-FAP volunteer individuals who had no evidence of infection. The collection of biopsy material was approved by the ethics committee of Hospital Geral de Santo António, Porto, Portugal, and all specimen donors gave informed consent.

For immunohistochemical (IHC) analysis, salivary gland samples were col-

lected into 4% paraformaldehyde in PBS. The general characterization of salivary gland tissue consisted of TTR immunohistochemistry and analysis of the presence of amyloid deposits by Congo red (CR) staining. Salivary gland tissues with TTR deposition and with amyloid deposits were classified as FAP.

Transgenic Mice

Transgenic mice bearing the human TTR V30M in a TTR null background (hTTR Met300) and transgenic mice expressing the human TTR V30M crossed into an HSF1 null background (hTTR Met30/HSF1-KO) were used for the experiments (10,12). In this case, we analyzed animals heterozygous for HSF1, labeled as hTTR Met30/(+/-) HSF1-KO. Animals were killed after administration of ketamine/xylazine anesthesia. DRG and stomachs were dissected and fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5- μ m sections. The animals were housed in pathogen-free conditions in a controlled temperature room maintained with 12-hour light/dark periods. Water and food were freely available. All animal experiments were carried out in accordance with the European Communities Council Directive.

Immunohistochemistry

Sections (5- μ m thick) were deparaffinized in histoclear (National Diagnostics, Atlanta, GA, USA) and dehydrated in a descent alcohol series. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide/100% methanol, and sections were blocked in 4% fetal bovine serum and 1% bovine serum albumin in PBS. Primary antibodies used were: rabbit polyclonal anti-thioredoxin 1 (Trx1) (1:500; Chemicon, Temecula, CA, USA), rabbit polyclonal anti-glutaredoxin 1 (Grx1) (1:500; Abcam, Cambridge, UK), goat polyclonal anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) (1:1000 with proteinase K epitope retrieval; Chemicon), goat polyclonal anti-4-hydroxynonenal (HNE) (1:500; Abcam), mouse monoclonal for active caspase-8 (1:200; Cell

Signaling, Danvers, MA, USA), rabbit polyclonal for active caspase-3 (1:50; Cell Signaling), rabbit polyclonal for active caspase-9 (1:20; Abcam), rabbit polyclonal anti-Fas (antihuman CD95) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Fas L (antihuman CD95 ligand) (1:50; Santa Cruz Biotechnology), rabbit polyclonal anti-Bax (1:50; Santa Cruz), rabbit polyclonal anti-Bcl₂ (1:100; Santa Cruz Biotechnology), and rabbit polyclonal anti-Apaf-1 (apoptotic protease-activating factor 1) (1:100 with citrate buffer epitope retrieval; Abcam), which were diluted in blocking solution and incubated overnight at 4°C. Antigen visualization was performed with the biotin-extravidin-peroxidase kit (Sigma, St. Louis, MO, USA), using 3-amino-9-ethyl carbazole (Sigma) as substrate. On parallel control sections, the primary antibody was replaced by blocking buffer and by nonimmune immunoglobulins corresponding to the species used as primary antibodies, namely rabbit and goat; staining was absent in all the cases.

Semiquantitative analysis of IHC images was done with the Universal Imaging system (NIH), which performs automated particle analysis in a measured area, i.e., the area occupied by pixels corresponding to the IHC substrate color is counted and normalized relative to the total area. Each slide was analyzed in five different selected areas. Results shown represent the percentage occupied area \pm SD.

Initially, tissues of transgenic mice were selected for TTR deposition by IHC analysis using a rabbit polyclonal anti-TTR (1:1000; Dako, Carpinteria, CA, USA). TTR-positive-CR-negative tissues were classified as (+/-) and TTR-positive-CR-negative tissues as (-/-). Expression of several markers was compared in hTTR Met30 mice with TTR deposition in the stomach and age-matched mice (six months old) without TTR deposition in the stomach. The same type of evaluation was performed for stomach and DRG deposition in hTTR Met30/HSF1-KO mice.

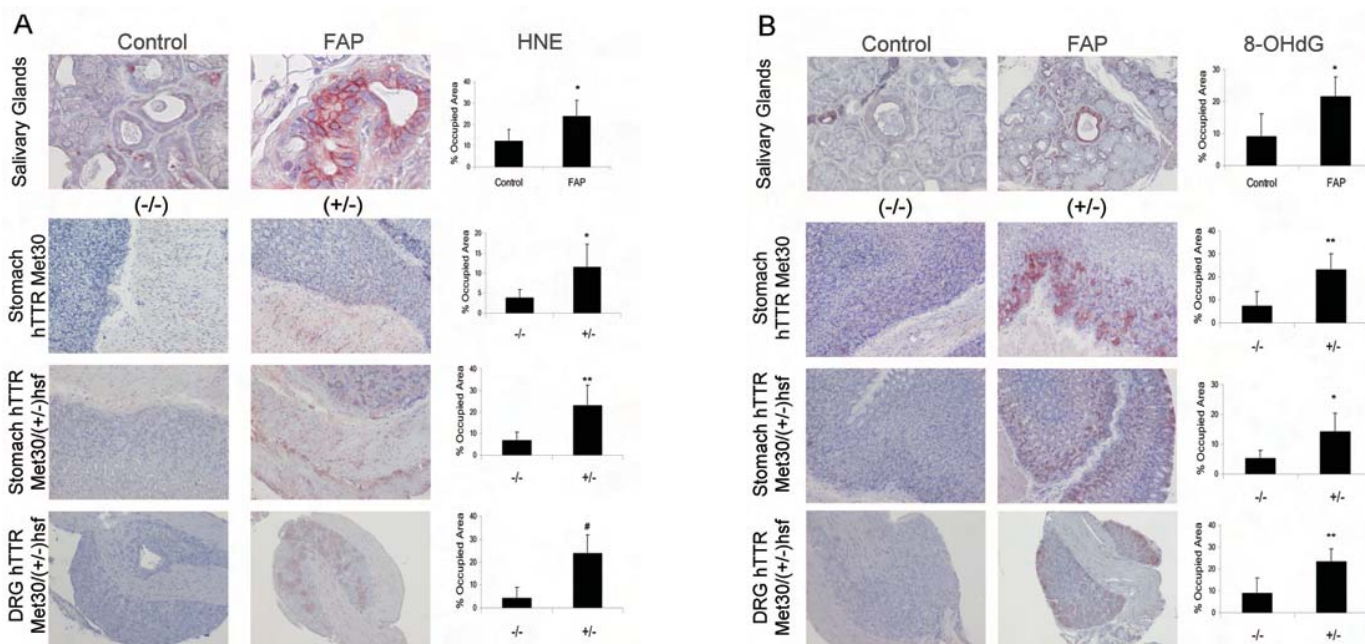


Figure 1. (A) *Top panels:* Representative 4-hydroxy-2-nonenal (HNE) staining of salivary glands from healthy individuals (left, n = 6) and FAP patients (right, n = 6). *Middle panels:* Representative HNE staining in stomach tissue of hTTR Met30 mice without TTR deposition (-/-, left, n = 5) and with TTR deposition (+/-, right, n = 5) and in stomach tissue of hTTR Met30/(+/-)HSF1-KO mice, without TTR deposition (-/-, left, n = 5) and with TTR deposition (+/-, right, n = 5); *Lower panel:* Representative HNE staining in DRG of hTTR Met30/(+/-)HSF1-KO mice without TTR deposition (-/-, left, n = 4) and with TTR deposition (+/-, right; n = 4). 20x magnification. Quantitation of IHC images of HNE staining are represented as percentage of occupied area ± SD (*P < 0.05; **P < 0.01, and # P < 0.005). (B) *Top panels:* Representative 8-OHdG staining of salivary glands from healthy individuals (left, n = 6) and FAP patients (right, n = 6). *Middle panels:* Representative 8-OHdG staining in stomach tissue of hTTR Met30 mice without TTR deposition (-/-, left, n = 5) and with TTR deposition (+/-, right, n = 5) and in stomach tissue of hTTR Met30/(+/-)HSF1-KO, without TTR deposition (-/-, left, n = 6) and with TTR deposition (+/-, right, n = 6); *Lower panel:* Representative 8-OHdG staining in DRG tissue of hTTR Met30/(+/-)HSF1-KO mice without TTR deposition (-/-, left, n = 4) and with TTR deposition (+/-, right; n = 5). 20x magnification. Quantitation of IHC images of 8-OHdG staining are represented as percentage of occupied area ± SD (*P < 0.05 and #P < 0.005).

Statistical Analysis

Group values, expressed as the mean ± SD, were compared by Student *t* test, and *P* values <0.05 were considered significant.

RESULTS

Tissue Markers for Oxidative Stress

Several markers of oxidative damage have been widely studied in different pathologies, such as Alzheimer disease (14), and include 8-OHdG, HNE, glutaredoxins such as Grx1, and thioredoxins such as Trx1. We investigated the expression of HNE, 8-OHdG, Grx1, and Trx1 in biopsy specimens from human salivary glands and in tissues from two FAP animal model strains.

IHC analyses for the lipid peroxidation biomarker HNE were performed in salivary gland tissue biopsies from V30M FAP individuals (n = 5) and non-FAP controls (n = 4). In FAP-patient salivary gland tissue, HNE was increased by approximately 50% compared with controls (Figure 1A); the immunostaining was exclusively cytoplasmic and was present in epithelial cells in salivary gland excretory ducts, at the same location of TTR fibrillar amyloid deposits (13). This result is in accordance with a previous report of the involvement of lipid peroxidation in colon biopsies of FAP patients (15). In addition, we investigated a possible correlation between nonfibrillar TTR deposition and HNE levels in the transgenic

mice strains under study. In the stomach and DRG in both hTTR Met30 and hTTR Met30/(+/-)HSF1-KO transgenic mice, HNE were significantly increased in mice with TTR deposition compared to mice without TTR deposition (Figure 1A). Quantification of IHC images of HNE staining revealed that HNE was increased approximately 70% in the stomach and 80% in the DRG (Figure 1A), correlating with nonfibrillar TTR deposition. These results validate the use of hTTR Met30 and hTTR Met30/(+/-)HSF1-KO mice models as tools for lipid peroxidation studies in FAP.

Although 8-OHdG DNA oxidation has been extensively investigated in other neurodegenerative pathologies, this

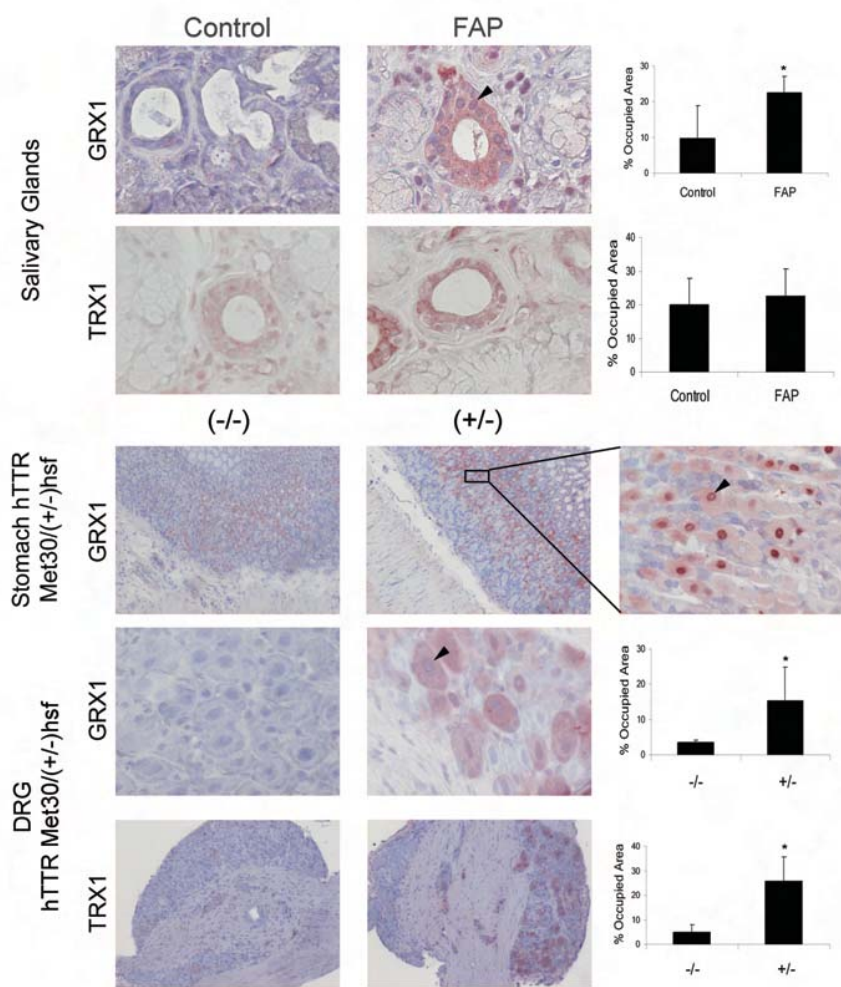


Figure 2. Top 2 panels: Representative Grx1 and Trx1 staining of salivary glands from healthy individuals (left, $n = 6$) and FAP patients (right, $n = 6$); nuclear staining is indicated by the arrow. Middle panel: Representative Grx1 staining in stomach tissue of hTTR Met30/(+/-)hsf mice without TTR deposition (-/-, left, $n = 5$) and with TTR deposition (+/-, right, $n = 5$). Lower 2 panels: Representative Grx1 and Trx1 staining in DRG tissue of hTTR Met30/(+/-)HSF1-KO mice without TTR deposition (-/-, left, $n = 4$) and with TTR deposition (+/-, right; $n = 5$); nuclear staining is indicated by the arrow. 20x and 40x magnification (inset). Quantitation of IHC images of Grx1 and Trx1 staining are represented as percentage of occupied area \pm SD (* $P < 0.05$).

study is the first to assess 8-OHdG DNA oxidation in FAP patients. Immunostaining of FAP salivary gland tissue samples revealed 50% more oxidized DNA than in control samples (Figure 1B). The increase in DNA oxidation was even more dramatic in the transgenic mice strains under study. Stomach tissue from transgenic hTTR Met30 mice with TTR aggregates showed approximately 75% more

8-OHdG than tissues without TTR deposition (Figure 1B), and in hTTR Met30/(+/-)HSF1-KO mice we confirmed approximately the same level of increase in DRG (Figure 1B). Thus, we validated the use of these animal models as tools to investigate oxidative stress involving DNA damage.

Finally, IHC studies on salivary glands revealed that Grx1 is up-regulated in

FAP individuals with TTR deposition compared with normal individuals (Figure 2); in contrast, Trx1 levels did not differ in FAP and control individuals (Figure 2). Grx1 resides mainly in the cytosol, although it may translocate into the nucleus in response to certain stimuli. For example, in the brains of patients with Alzheimer disease, control samples revealed Grx1 was located mainly in the cytosolic compartment, contrary to the nuclear staining in Alzheimer disease neurons (16). In our study nuclear translocation of Grx1 was evident in FAP salivary glands (indicated by arrows in Figure 2).

We then investigated Trx1 and Grx1 endogenous antioxidant systems in hTTR transgenic mice. In both hTTR Met30 and hTTR Met30/(+/-)HSF1-KO mice, increased Grx1 was associated with TTR deposition in the stomach (Figure 2) but Trx1 was not (not shown). In the peripheral nervous system of hTTR Met30/(+/-)HSF1-KO mice, represented by DRG tissue samples, Grx1 and Trx1 were both up-regulated in samples with TTR deposition (Figure 2).

Tissue Markers for Apoptosis

We investigated the Fas death receptor in FAP salivary glands and in FAP transgenic mouse models and observed that although there were no differences in FasL expression (Figure 3), FAP salivary glands expressed higher amounts of the receptor protein, probably related to increased activation of this apoptosis-initiating cascade (Figure 3). We next evaluated the cleavage of caspase substrates in FAP salivary glands. In FAP salivary glands, both the initiator caspase-8 as well as the effector caspase-3 were cleaved more extensively compared with controls (Figure 3), presenting as much as 70% increase in activation. However, no differences were found for caspase-9 activation (not shown).

We then investigated the role of Bcl-2 family proteins in FAP biopsies. We did not find differences in Bcl-2 or Apaf-1 levels between FAP and control salivary gland tissue (not shown), concordant

with the results on caspase-9, which did not show evidence for activation. Because Bax and Bak are proapoptotic proteins that initiate mitochondrial dysfunction but also localize to the ER (17) we next assessed Bax levels in salivary gland tissue samples. A slight, but not significant, increase was detected in FAP glands compared with controls (Figure 3).

We further investigated a possible correlation between nonfibrillar TTR deposition and the above-defined markers of apoptosis in the mice transgenic for human TTR V30M and the novel hTTR Met30/(+/-)HSF1-KO mice. A dramatic increase in the Fas death receptor signal was observed in tissues with TTR deposition in the transgenic mouse strains under study. Transgenic hTTR Met30 mice presented an increase of approximately 75% in Fas in the stomach of TTR-positive animals (Figure 4), and the same differences were observed in the stomach and DRG of hTTR Met30/(+/-)HSF1-KO mice (Figure 4) in TTR-positive compared with deposit-free tissues. As in human salivary gland tissue, FasL was expressed at the same levels in mouse tissues with or without TTR deposition (data not shown).

We found increased levels of Bax (Figure 4) associated with TTR deposition in the stomach of both transgenic mice strains as well as in DRG from hTTR Met30/(+/-)HSF1-KO mice (Figure 4).

Based on these results, we believe that the apoptotic pathway mediated by the Fas receptor is definitely involved in neurotoxicity observed in FAP salivary gland tissue and in the animal models under study that present TTR deposition, and in FAP in general.

Oxidative Stress and Apoptotic Markers after TTR Removal

IHC analyses of tissues from transgenic hTTR Met30 mice immunized with Y78F and presenting with TTR removal revealed a decrease in some of the markers described above. We tested one marker of oxidative stress, 8-OHdG, a radical-damaged guanine nucleotide representative of oxidation at the DNA

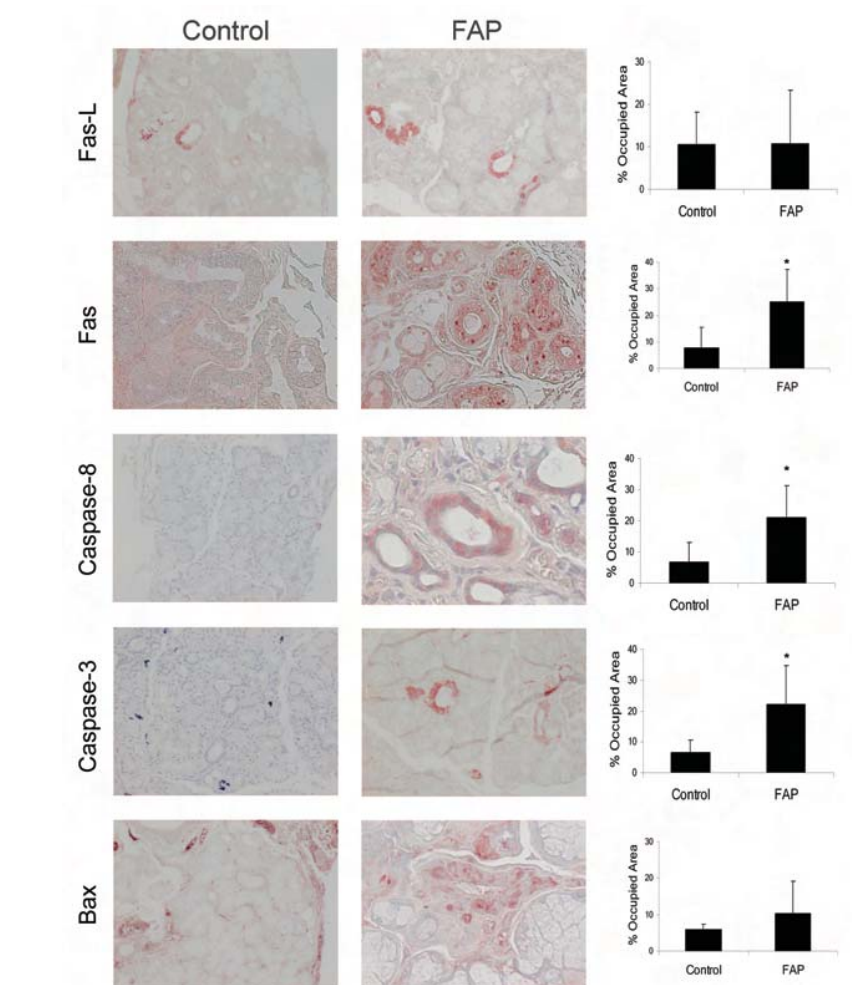


Figure 3. Representative FasL, Fas, active caspase-8, active caspase-3, and Bax staining of salivary glands from healthy individuals (left, n = 6) and FAP patients (right, n = 6). 20x magnification. Quantitation of IHC images of FasL, Fas, active caspase-8, active caspase-3, and Bax staining are represented as percentage of occupied area \pm SD (* $P < 0.05$).

level; two markers of apoptosis, the death receptor Fas and the pro-apoptotic Bax; the ER chaperone BiP; and the endogenous antioxidant protein Grx1.

IHC analysis of the gastrointestinal tract of TTR Y78F immunized mice (n = 6) revealed decreased levels of these markers in the stomach compared with nonimmunized age-matched controls (n = 6). The decrease was approximately 50% for Fas and BiP and was even more dramatic, approximately 70%, for the oxidative stress markers 8-OHdG and Grx1. Although Bax levels were lower in

the tissues of immunized mice, the difference was not significant (Figure 5).

These results correlate with the decrease of TTR in the stomach of TTR Y78F immunized animals reported by Terazaki et al. (11), further validating the use of 8-OHdG, Grx1, Fas, and BiP for follow-up of therapeutic approaches in FAP patients.

DISCUSSION

We generated a set of biomarkers associated with FAP pathology by the use of salivary gland tissue from FAP pa-

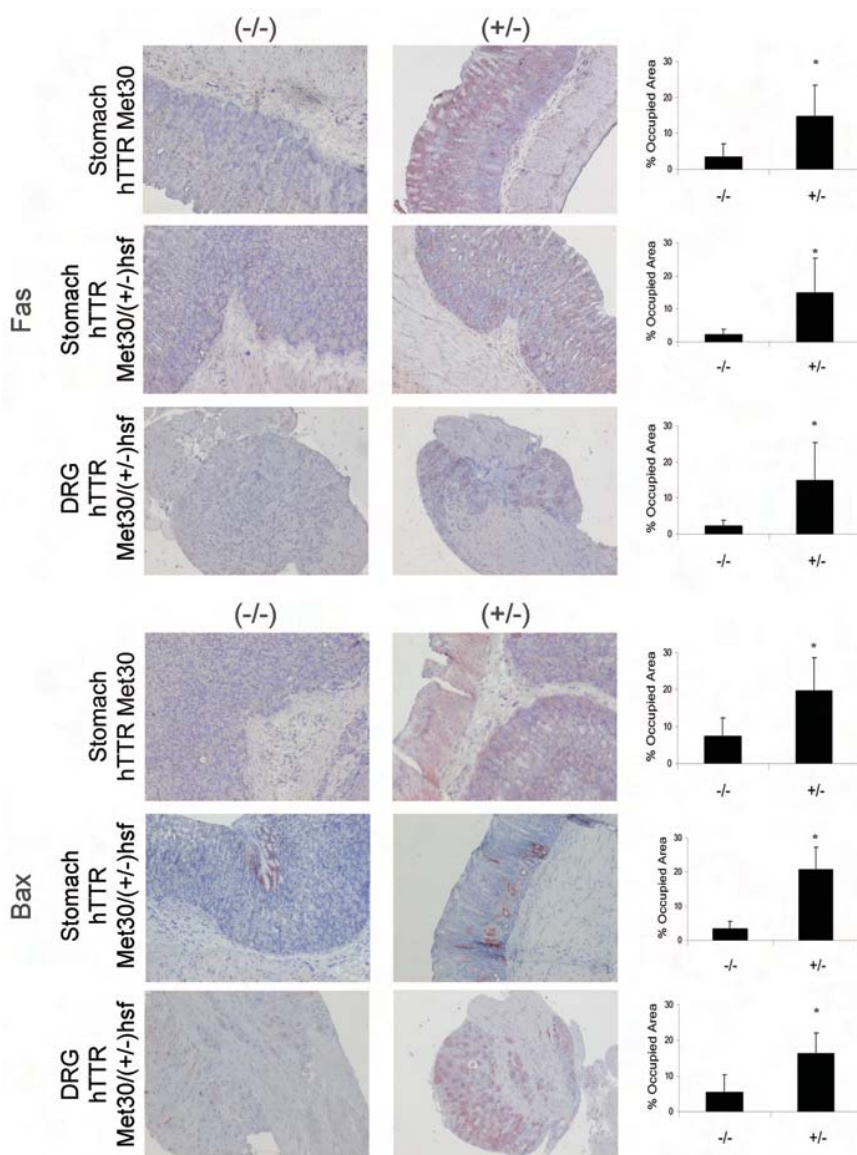


Figure 4. Representative Fas (upper panels) and Bax (bottom panels) staining in stomach and DRG tissue of TTR transgenic mice. Upper panels: Stomach tissue of hTTR Met30 mice without TTR deposition (-/-, left, n = 5) and with TTR deposition (+/-, right, n = 5), of hTTR Met30/(+/-)HSF1-KO, without TTR deposition (-/-, left, n = 6) and with TTR deposition (+/-, right, n = 6) and DRG tissue of hTTR Met30/(+/-)HSF1-KO mice without TTR deposition (-/-, left, n = 6) and with TTR deposition (+/-, right; n = 6). Bottom panels: the same organization as above. 20x magnification. Quantitation of IHC images of Fas and Bax staining are represented as percentage of occupied area \pm SD (* $P < 0.05$).

tients with amyloid deposition and unique tissues, stomach and DRG from 2 different animal models with nonfibrillar TTR deposition, that resemble the asymptomatic phase of the disease. A

major novel finding from our study was the detection of specific oxidative and apoptotic markers not only in amyloid-laden tissues, but also before amyloid deposition, in tissues presenting nonfibrillar

deposition. The biomarkers we found to be associated with deposition did not deviate considerably from those found in other neurodegenerative disorders; for instance, in Alzheimer disease abnormal oxidative metabolites, including HNE and cholesterol-derived aldehydes, have been reported to modify A β and accelerate the early stages of amyloidogenesis (18). The biomarker 8-OHdG, a modified base that occurs in DNA due to attack by hydroxyl radicals formed as byproducts and intermediates of aerobic metabolism and during oxidative stress, has been increasingly used as a sensitive, stable, and integral marker of oxidative damage in cellular DNA (19). In a neuroblastoma cell line, A β fibrils caused transient oxidation of both Grx1 and Trx1 (16). Cytosolic mammalian Trx1 has numerous functions in defense against oxidative stress, control of growth, and apoptosis. Grx1 catalyses glutathione disulfide oxidations, overlapping the functions of thioredoxins and using electrons from NADPH via glutathione reductases (20). Despite the importance of Grx1 and Trx1 as regulators of oxidative stress and apoptosis, their role in FAP is completely unknown. We believe that overexpression of Grx1 may have a protective role, acting to detoxify FAP tissues as it does the frontal cortex and hippocampal neurons of the brain in Alzheimer disease (16). Trx1 was not up-regulated in the analyzed tissues with TTR deposition, except for DRG, probably because salivary glands and stomach mucosa are in contact with multiple stimuli and irritations. The Trx1 system is probably activated in a constant way by contact with external stimuli, whereas Grx1 better protects against cellular injury. In addition to measurement of the levels of these proteins, their reduced and oxidized states should also be investigated, because understanding of the downstream cascades that Grx1 and Trx1 trigger will elucidate the roles of Trx1 and Grx1 in TTR amyloidosis and may lead to the development of therapeutic interventions to modulate these markers.

Apoptotic features are present extensively in the most common neurodegenerative diseases. Previous studies have evaluated A β -induced apoptotic cell death in cerebral endothelial cells by investigation of increased oxidative stress, caspase activation, mitochondrial dysfunction, and DNA damage (21).

The evidence we obtained for Fas and caspase-8 activation in tissues with TTR deposition suggest activation of death-receptor pathways not involving the mitochondria (22,23); a possible explanation for caspase-8 activation is the induction of Fas-associated death domain-independent pathways deriving from prolonged activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) (24) and activation of ERK1/2, which was shown in FAP salivary gland tissue and nerves and in tissues from TTR transgenic mice presenting TTR deposition (25). Further suggestion for a secondary role of mitochondrial involvement was the lack of evidence in the present study for changes in Bcl2 and Apaf-1 in tissues with TTR deposition (22,26). Additional studies regarding morphological and functional alterations in mitochondria, namely the measurement of mitochondrial membrane potential, should clarify this issue as tested in another model system (27). Moreover, the trophic signals relayed from TTR to mitochondria remain elusive, with a possible role of intracellular Ca²⁺ remaining to be investigated. The ER contributes to pathways in FAP, a characteristic that differs from other neurodegenerative protein misfolding disorders and is suggestive of different pathways involved in apoptosis, possibly related to the fact that in FAP protein aggregation occurs extracellularly rather than intracellularly. Recent data showed activation of the classical unfolded-protein response pathways in tissues not specialized in TTR synthesis but presenting extracellular TTR aggregate and fibril deposition. We have previously demonstrated cytotoxicity by Ca²⁺ efflux from the ER in cell cultures incubated with TTR oligomers, evidence for ER

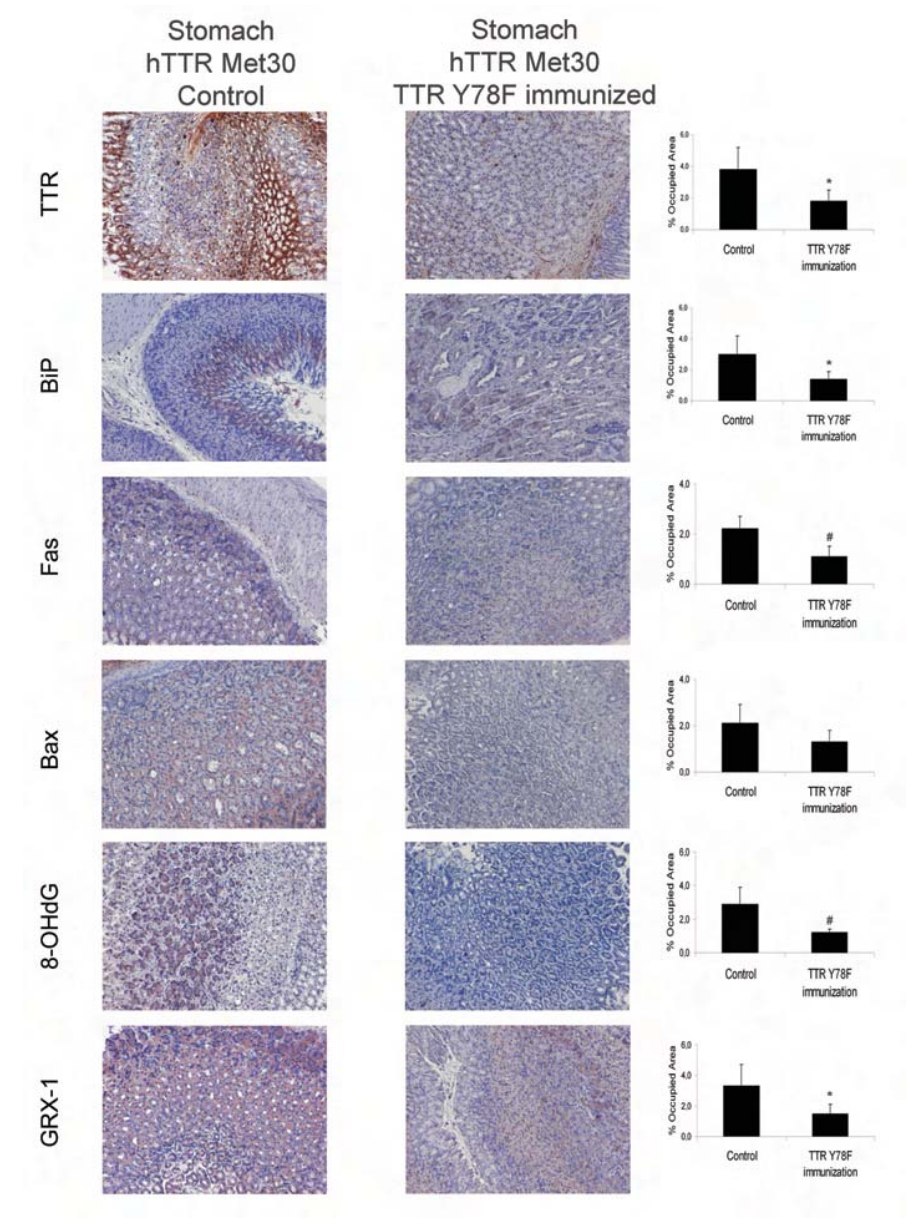


Figure 5. Representative immunohistochemistry of TTR, BiP, Fas, Bax, 8-OHdG and Grx1 in stomach tissue of TTR Y78F immunized transgenic mice 9-10 months old (right; n = 6) and nonimmunized age-matched controls (left; n = 6). 20 \times magnification; Quantification of IHC images of TTR, BiP, Fas, Bax, 8-OHdG, and Grx1 staining are represented as percentage of occupied area \pm SD (* P < 0.05, # P < 0.005).

stress associated with extracellular aggregates (9).

We have demonstrated that the biomarkers of oxidative stress and apoptosis we investigated are correlated with TTR deposition, as corroborated by the results in tissues from which deposition

was removed by immunization. These results confirm the usefulness of this set of biomarkers in the follow-up of therapeutic approaches and highlight the importance of pursuing FAP treatments based on antioxidative and antiapoptotic therapies.

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