The role of fibrinogen glycation in ATTR: evidence for chaperone activity loss in disease


Transthyretin amyloidosis (ATTR) belongs to a class of disorders caused by protein misfolding and aggregation. ATTR is a disabling disorder of autosomal dominant trait, where transthyretin (TTR) forms amyloid deposits in different organs, causing dysfunction of the peripheral nervous system. We previously discovered that amyloid fibrils from ATTR patients are glycated by methylglyoxal. Even though no consensus has been reached about the actual role of methylglyoxal derived advanced glycation end products in amyloid diseases, evidence collected so far point to a role of protein glycation in conformational abnormalities, being ubiquitously found in amyloid deposits in Alzheimer’s, dialysis-related amyloidosis’ and Parkinson’s diseases. Human fibrinogen, an extracellular chaperone, was reported to specifically interact with a wide spectrum of stressed proteins and suppress their aggregation, being an interacting protein with TTR. Fibrinogen is differentially glycated in ATTR, leading to its chaperone activity loss. Here we show the existence of a proteostasis imbalance in ATTR linked to fibrinogen glycation by methylglyoxal.

Cite as Biochemical Journal (2016) DOI: 10.1042/BCJ20160290
The role of fibrinogen glycation in ATTR: evidence for chaperone activity loss in disease

Daniel Fonseca*,1,2, Samuel Gilberto*,1,2, Cristina Ribeiro-Silva1,2, Raquel Ribeiro1,2, Inês Batista Guinote5,6, Susana Saraiva1,2, Ricardo A. Gomes3, Élia Mateus4, Ana Viana2, Eduardo Barroso4, Ana Ponces Freire2, Patrick Freire6, Carlos Cordeiro1,2# and Gonçalo da Costa1,2#

1Laboratório de FTICR e Espectrometria de Massa Estrutural and Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal
2Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Edifício C8, Lisboa, Portugal
3Instituto de Tecnologia Química e Biológica/Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal
4Unidade de Transplantação Hepática, Hospital Curry Cabral, Lisboa, Portugal
5Instituto Nacional de Investigação Agrária e Veterinária - INIAV,IP, Estrada de Benfica 701, 1549-011 Lisboa, Portugal
6Laboratório de Controlo da Expressão Génica, Instituto de Tecnologia Química e Biológica, Avenida da República, Estação Agronómica Nacional, 2780-157 Oeiras, Portugal

Running Title: Fibrinogen differential glycation in ATTR

*Both authors contributed equally to this work

# – Corresponding authors:
Gonçalo da Costa

Address: Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, Edifício C8, Campo Grande, 1749-016 Lisboa, Portugal.
Tel.: +351-217500929
E-mail: gmcosta@fc.ul.pt
Fax: +351-217500088
Carlos Cordeiro

**Address:** Departamento de Química e Bioquímica, Faculdade de Ciências da
**Tel.:** +351-217500929
**E-mail:** caac@fc.ul.pt
**Fax:** +351-217500088

**Keywords:** ATTR, Fibrinogen, Chaperone, Glycation, Methylglyoxal, FTICR-MS
ABSTRACT

Transthyretin amyloidosis (ATTR) belongs to a class of disorders caused by protein misfolding and aggregation. ATTR is a disabling disorder of autosomal dominant trait, where transthyretin (TTR) forms amyloid deposits in different organs, causing dysfunction of the peripheral nervous system. We previously discovered that amyloid fibrils from ATTR patients are glycated by methylglyoxal. Even though no consensus has been reached about the actual role of methylglyoxal derived advanced glycation end products in amyloid diseases, evidence collected so far point to a role of protein glycation in conformational abnormalities, being ubiquitously found in amyloid deposits in Alzheimer’s, dialysis-related amyloidosis’ and Parkinson’s diseases. Human fibrinogen, an extracellular chaperone, was reported to specifically interact with a wide spectrum of stressed proteins and suppress their aggregation, being an interacting protein with TTR. Fibrinogen is differentially glycated in ATTR, leading to its chaperone activity loss. Here we show the existence of a proteostasis imbalance in ATTR linked to fibrinogen glycation by methylglyoxal.
INTRODUCTION

Transthyretin amyloidosis (ATTR) belongs to a class of disorders that occur as a consequence of protein misfolding and aggregation, with transthyretin (TTR) forming insoluble cross beta-fiber amyloid deposits [1-4]. ATTR is a disabling disorder of autosomal dominant trait, where protein is deposited at different organs, causing dysfunction of the peripheral nervous system (PNS) [1,4]. Partial suppression of organ function is eventually reached, resulting ultimately in full function loss, affecting the patient life quality and causing death, 10 to 20 years after disease onset [5]. ATTR is presently recognized as a worldwide spread disease, with major incidence foci in Portugal, Sweden and Japan [3,6,7].

The most widely accepted molecular model of ATTR relates variations of transthyretin (TTR) tetramer stability with TTR point mutations [8]. According to this model, point mutations promote the dissociation of the protein tetramer, followed by misfolding of the monomers into an aggregation-prone conformation. Several TTR single point mutations are associated to ATTR. The most common one is the substitution of the valine in position 30 by a methionine (V30M) [9].

Although TTR tetramer stability may be a significant factor in the initiation of amyloid fibril formation, there are a number of observations related to ATTR pathogenesis that must be considered in order to have a solid understanding of this disease. First, mutant TTR, which is present from birth does not evolve to amyloid fibril prior to late adult life [10-12]. It is unreasonable to consider that the late onset of the disease is only due to tetramer destabilization, since the mutant protein is present from birth [10-12]. Second, wild-type TTR presents intrinsic amyloidogenicity, since its aggregation in older individuals causes Systemic Senile Amyloidosis (SSA) [13]. Finally, protein misfolding and aggregation are common features to many neurodegenerative diseases referred to as “conformational disorders” [14-17]. These observations suggest that changes in the normal protein homeostasis and post-translational modifications might contribute to pathogenesis of these conditions, since the decreased ability of the proteostasis network to cope with inherited misfolded-prone proteins, aging and/or metabolic/environmental stress appears to trigger or exacerbate proteostasis diseases [15-17].
Protein glycation is an ubiquitous hallmark of neurodegenerative diseases of amyloid type [18], and the accumulation of Advanced Glycation End Products (AGEs) has also been linked to protein aggregation [19]. The most powerful glycation agent in vivo is methylglyoxal (MG), formed in all living cells as an unavoidable and non-enzymatic by-product of glycolysis [20]. MG irreversibly modifies arginine and lysine side chains, resulting in a chemically heterogeneous group of advanced glycation end-products, termed MAGE (methylglyoxal-derived advanced glycation end products) [21,22]. Argpyrimidine and hydroimidazolones are specific markers of protein glycation by MG in arginine residues, while Nε-(carboxyethyl)lysine (CEL) is derived from the specific reaction of MG with lysine residues [22,23].

MAGE are found in several conformational pathologies like Alzheimer [24,25,26,27], dialysis-related amyloidosis [28] and Parkinson’s disease [18]. ATTR is no exception to this pattern since we discovered that amyloid deposits from ATTR patients are glycated by MG [29]. Evidence collected so far point to a more intriguing contribution, as it seems they can be implicated in the progression and outcome of the senescence phenomena and related pathologies. This strengthens the hypothesis that methylglyoxal-derived protein glycation is involved in conformational disease and that glycation can be the missing link between metabolic factors proteostasis imbalance and disease.

Fibrinogen is a 340 kDa glycoprotein synthesized by the liver and assembled as a dimer of three different polypeptide chains bound to each other, termed α, β and γ [33,34]. Recent evidence from our group showed that, in the plasma of ATTR patients, fibrinogen is overexpressed and interacts with TTR [30]. Interestingly, fibrinogen has also been described as an important player in other amyloid-like pathologies (Alzheimer’s and Parkinson’s Diseases) [31,32]. In addition, human fibrinogen was reported to specifically interact with a wide spectrum of stressed proteins and suppress their aggregation [33,35-37]. Evidence suggests a potential role of fibrinogen in misfolding diseases as a molecular chaperone. Thus it is likely that, in patients with different pathologies, but sharing common molecular mechanisms, the increased levels of fibrinogen occur in response to a common increased need of extracellular chaperone activity [36,37].
In this work, aggregation and fiber formation assays were performed in the presence of fibrinogen isolated from the plasma of healthy control subjects and ATTR patients. Significant differences were observed between the chaperone activity of fibrinogen enriched fractions from healthy controls and ATTR patients. Since these individuals present different glycation profiles, mapping fibrinogen glycation by ultra-high resolution mass spectrometry (FTICR-MS) was performed. A structurally differential glycation pattern was found in fibrinogen from ATTR patients regarding healthy individuals. Moreover, glycated fibrinogen loses its capability as chaperone and to interact with TTR.

Our results support the existence of an imbalance of the proteostasis machinery in ATTR pathogenesis, as fibrinogen chaperone activity seems to be impaired in ATTR patients. Furthermore, this extracellular chaperone is differentially glycated regarding healthy individuals, suggesting that glycation by methylglyoxal causes its chaperone activity loss.
METHODS

Human samples and plasma collection

Blood samples from control healthy individuals and ATTR portuguese type TTR V30M patients (three subjects each, all males, age range 23–38 years in the two cohorts) were collected in citrate containing tubes. Samples were centrifuged at 1800 \( g \) for 5 min at 4°C. None of the individuals in the two cohorts presented any diabetic indication. The supernatant plasma was kept frozen at \(-80°C\) until further analysis. All subjects were characterized by gene typing to be heterozygous carriers for the V30M mutation except the controls that do not bear any known TTR mutation. Heterozygoty of ATTR patients was later verified in our lab, by a FTICR-MS mass spectrometry based assay [38,39]. At the time of transplantation, ATTR patients showed peripheral polyneuropathy or autonomic polyneuropathy without signs of amyloid deposition [38]. All individuals gave informed written consent and the protocol was approved according to EEC ethic rules at Curry Cabral Hospital, Lisbon.

Preparation of fibrinogen enriched fraction (FEC)

Fibrinogen enriched fractions were prepared by a modified cold ethanol fractionation procedure [40]. Human plasma from ATTR and healthy control subjects was diluted (1:10) in ultrapure water and precipitated by the slow addition of 0.22 volumes of cold 50% ethanol, lowering the temperature to \(-3°C\). After centrifugation (10 min at 10000 \( g \)), the precipitate was washed with 0.5 original volumes (O.V.) of 7% ethanol at \(-3°C\) and the solution was again centrifuged in the previous conditions. The precipitate was re-collected and dissolved in 0.25 O.V. 55 \( \mu M \) trisodium citrate buffer, pH 6.5, at 30°C for 30 min. The solution was cooled to 0°C followed by the addition of 20% cold ethanol to a final concentration of 2%. After a spin-down, a mucous-like precipitate was removed and 20% cold ethanol was added again to a final concentration of 8%, followed by centrifugation (10 min at 10000 \( g \)).

In vitro fibrinogen glycation by methylglyoxal
High purity MG was prepared by fractional distillation under reduced pressure in nitrogen atmosphere as previously described [41]. Once prepared, MG solutions were standardized by enzymatic assay with glyoxalase I and II [41]. Purity was verified by HPLC analysis. For *in vitro* glycation, human fibrinogen prepared from plasma (1 mg/ml, Calbiochem) in 0.1 M sodium phosphate buffer (pH 7.4) was incubated with 0.2, 0.5, 2.5 and 10 mM of MG for 4 days at 37ºC with stirring at 800 rpm, in the presence of NaN₃ (Merck) to prevent microbial growth. Only one batch of human fibrinogen (Calbiochem) was used for this work.

**Protein aggregation and amyloid fiber formation assay**

Human insulin (SAFC Biosciences) was prepared in sodium acetate buffer 0.1 M, pH 4.6 (with 137 mM NaCl and 2.7 mM KCl) to a final concentration ranging from 5 to 100 μM.

Each TTR variant, 0.8 mg/ml solution (10 mM phosphate, 100 mM KCl, and 1 mM EDTA, pH 7.0) of the different TTR variants was prepared in acetate buffer (200 mM NaOAc, 100 mM KCl, and 1 mM EDTA) at pH 4.6 0.002% of sterile, filtered NaN₃ (Merck) was included in the aggregation mixtures.

Protein aggregation was followed by absorbance at 330 nm increase due increased light scattering in time. To monitor fibril formation, aggregation mixtures were added to a solution of glycine-NaOH 50 mM pH 8.5 buffer with 0.5 μM of Thioflavin T. Fibril formation was revealed by the appearance of new emission maxima of the Thioflavin T fluorophore, at 450 nm and 482 nm, respectively, corresponding to the described maxima in its fluorescence spectra after binding to amyloid fibrils of different nature. Fluorescence measurements were made on a Fluorolog-3 (Horiba Jobin Yvon) in a quartz cuvette with 1 cm optical path in Instrument Control Center v2.2.13 software. Both excitation and emission bandwidths were 2.5 nm and fluorescence measurements correspond to an average of 10 readings. Fluorescence intensity time courses were recorded at 482 nm (excitation at 450 nm).

To monitor fibrinogen chaperone activity, the same reaction mixtures described previously were incubated with 0.5 μM and 1 μM fibrinogen (enriched fraction and Calbiochem® fibrinogen, respectively), for 12 to 72 hours at 37ºC with constant stirring (900 rpm). Aggregates and fiber formation were detected as described before.
Polyacrylamide gel electrophoresis

Fibrinogen chains were analyzed by Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis (SDS-PAGE) in mini-gel format (7x7 cm Tetra system from Bio-Rad, 10%). For western blot, 2 micrograms were used per lane. For peptide mass fingerprint (PMF) and glycation site mapping, 8 micrograms were used per lane. Prior to electrophoresis, samples were added of reduction buffer (6.25 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol) and heated at 100°C for 2 min. Protein bands were stained with Coomassie brilliant blue G-250 (BioRad).

Western blotting

Proteins were transferred from the polyacrylamide gel to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were blocked for 1 h at room temperature with TBS-T (10 mM Tris–HCl, 150 mM NaCl, pH 7.5 with 0.1% (v/v) Tween 20) containing 5% (w/v) skimmed milk. Membranes were then incubated overnight at 4°C in TBS-T containing 1% (w/v) skimmed milk with the primary antibody anti-argpyrimidine (JaICA- monoclonal antibody 1/10000), anti-fibrinogen (Calbiochem - polyclonal antibody 1/10000) and anti-transthyretin (DAKO 1/2000). Membranes were washed three times, 15 min each, with TBS-T and incubated for 3 h at room temperature with the secondary antibodies: anti-mouse IgG (Sigma Aldrich- 1/4000) and anti-rabbit IgG (Sigma Aldrich - 1/4000). Immunoreactivity was detected by chemiluminescence following the manufacturer’s instructions (Pierce ECL Western Blotting Substrate).

In gel protein digestion

In gel protein digestion was performed as previously described [42-44]. Briefly, protein bands were excised, washed in ultrapure water, destained in 50% acetonitrile (ACN) and subsequently dehydrated with 100% ACN. Cysteine residues were reduced with
Differential glycation in ATTR - imbalance of proteostasis

10 mM DTT and alkylated with 50 mM iodoacetamide. Gel pieces were dehydrated using 100% ACN and rehydrated at 4°C in digestion buffer containing either 50 mM NH₄HCO₃ with 6.7 ng/µl of trypsin (modified porcine trypsin, proteomics grade, Promega) or 25 mM sodium phosphate buffer, pH 7.8 with 10 ng/ml of endoproteinase Glu-C (sequencing grade, Roche). After 45 min, excess supernatant was removed, discarded and 50 µl of 50 mM NH₄HCO₃ (for trypsin digestion) or 25 mM sodium phosphate buffer pH 7.8 (for Glu-C digestion) were added. Digestions were allowed to proceed at 37°C overnight (~16 hours). After digestion, the remaining supernatant was removed and stored at -20°C until further analysis.

Mass spectrometry

Samples were desalted and concentrated in homemade micro columns containing reverse phase media POROS R2 or OLIGO R3 (Applied Biosystems) and eluted sequentially to the MALDI target AnchorChip (BrukerDaltonics, Bremen, Germany) with the appropriated matrix. For α-Cyano-4-hydroxycinnamic acid (CHCA, Fluka), 10 µg/µl matrix solutions were prepared in 0.1% trifluoroacetic acid (TFA) with 20%, 50% or 80% ACN. The 10 µg/µl 2,5-dihydroxybenzoic acid matrix (DHB, Fluka) was prepared in 0.1% trifluoroacetic acid (TFA) with 10%, 50% or 80% ACN. Peptide mixtures were analyzed by MALDI-FTICR-MS in a Bruker Apex UltraQe, Apollo II ESI-MALDI combi-source (BrukerDaltonics, Bremen, Germany), with a 7 Tesla magnet (Magnex corporation, Oxford UK). Monoisotopic peptide masses were determined using the SNAP 2 algorithm in Data Analysis software version 3.4 (BrukerDaltonics). External calibration was performed by using BSA tryptic digest spectrum, processed and analyzed with Biotools 3.1 (BrukerDaltonics, Bremen, Germany). For protein identification purposes, monoisotopic peptide masses from non glycated fibrinogen peptides were used for database search using Mascot (Matrix Science, London, UK; http://www.matrixscience.com). Data was submitted and analyzed with BioTools 3.1 (BrukerDaltonics). Database searches were performed against Swiss-Prot, a non-identical protein sequence database (http://csc-fserve.hh.med.ic.ac.uk/msdb.html). The following criteria were used to perform the search: 1) mass accuracy better than 5 ppm; 2) one missed cleavage in peptide masses; and 3) carboxamidomethylation of Cysteine and oxidation of Methionine as fixed and
Differential glycation in ATTR - imbalance of proteostasis

variable amino acid modifications, respectively. Criteria used for protein identification in the Mascot software were (1) significant homology scores achieved in Mascot; (2) at least 20% sequence coverage and 4 peptide matches; (3) similarity between the protein molecular mass calculated from the gel and from the identified chain. For the analysis of glycated peptides, BioTools was used to compare the obtained monoisotopic peptide masses with predicted monoisotopic mass values considering optional modifications by methylglyoxal. In the case of tryptic digests, hydrolysis was considered not to occur at modified sites and 2 missed cleavages were allowed. For MS/MS analysis, a MALDI-TOF/TOF 4800 Plus mass spectrometer (Applied Biosystems) was used. Glycated peptides identified with BioTools were used to create an inclusion list for tandem experiments. MS/MS analyses were performed using CID (Collision Induced Dissociation) with 1 kV collision energy at a pressure of 1x10^6 torr. 2000 Laser shots were collected for each MS/MS spectrum using a fixed laser intensity of 4000 V. Raw data was generated by the 4000 Series Explorer Software v3.0 RC1 (Applied Biosystems). MS/MS data was analyzed in Data Explorer 4.5 (Applied Biosystems).

Plasma TTR co-purification

Fibrinogen and glycated fibrinogen (1 mg) were coupled on two Aminolink columns as described in the Aminolink Plus immobilization kit manufacture’s protocol. Columns were washed with phosphate buffered saline (PBS) and were incubated with human plasma samples (1:10 dilution in PBS) for 1 h at room temperature, with continuous rocking. After washing the columns 4 times with PBS, proteins were eluted by gravity-flow with 2 ml 0.1 M glycine-HCl, pH 3.0, following neutralization with 0.1 M NaOH. Western blot was performed after resolving the eluted protein samples by SDS-PAGE blotted into a PVDF membrane. Membranes were blocked in 5% skimmed milk, and the proteins were probed against 1:2000 polyclonal rabbit anti-TTR antibody (DAKO) overnight, and then incubated with 1:4000 peroxidase-labeled.

TTR expression and purification
Differential glycation in ATTR - imbalance of proteostasis

TTR human genes (for wild-type and V30M variant) were amplified by polymerase chain reaction (PCR). PCR was carried out using primers with recombination sequences ("Gateway att" sites) added to the 5’, and 3’ end of the gene (forward primer 5’GGGGACAAGTTTGTACAAAAAAGCAGGCTTGGAGGAG ATAGAACCATGGGCCCTACGGGCACCG 3’ and reverse primer 5’GGGGACCACTTTGTACAAGAAAGCTGGGTATTCCTTGGGATTGATGCAG 3’). PCR amplification products were inserted into the pDONRTM221 plasmid from the Gateway System (Invitrogen) and sequenced. The gene cassette in the Gateway Entry clone was then transferred to a Gateway Destination vector Pet23-b with His tag at C-terminal using the proprietary enzyme mix, “LR Clonase” (Invitrogen).

Recombinant human TTR variants were expressed in E.coli strain BL21 (DE3). A preculture (10 ml) was grown overnight at 37ºC in Luria Bertani broth (LB) medium with ampicilin (100 µg/ml) and inoculated to 2.5 L of fresh LB medium. Protein expression was induced with 1 mM IPTG when absorbance at 600 nm reached 0.5. After growth for 4 h at 37ºC with shaking, cells were harvested by centrifugation at 8,000 rpm for 10 min at 4ºC.

Cell pellets were suspended in 50 ml of Lysis Buffer (50 mM Tris, 400 mM NaCl, 10 mM Imidazole, 0.5 mM PMSF, pH 7.5) and lysed by sonication at 4ºC. After centrifugation at 11,000 rpm for 30 min at 4ºC, supernatant was added to 1.5 ml of nickel-nitrilotriacetic acid (Ni-NTA) beads (QIAGEN). The lysate-Ni-NTA mixture was mixed gently by shaking at room temperature for 2 hours. Beads were washed 3 times for 5 min with 10 ml of washing buffer (50 mM Tris, 400 mM NaCl, 10 mM Imidazole, 0.5 mM PMSF, 10% glycerol, pH 7.5). Elution of the fusion proteins was carried with 5 ml of Elution Buffer (50 mM Tris, 400 mM NaCl, 250 mM Imidazole, 0.5 mM PMSF, pH 7.5). Solutions of recombinant TTR variants were dialyzed against 20 mM sodium acetate, 6 mM HEPES, 0.8 mM PMSF, pH 7.5 and then lyophilized.

**Gel filtration chromatography**

Purified TTR protein variants were subjected to gel filtration on a Sephacryl S-200 HR column (Amersham Biosciences) pre-equilibrated with 30 mM HEPES 10 mM NaOAc, pH 7.4. Elution was performed at a flow rate of 0.5 ml/min in the same buffer. Apparent
molecular masses were assessed based on elution volumes of suitable markers (bovine serum albumin - 66.5 kDa, and SmpB - 18 kDa).

**Atomic Force Microscopy**

Tapping mode atomic force microscopy (AFM) was performed in air, on a Multimode AFM instrument with Nanoscope IIIa controller from Digital Instruments, Bruker. Etched Si probes with a spring constant of 42 N. m\(^{-1}\) and a resonance frequency of about 300 kHz (TESP, Bruker), and a scan rate of ca. 1.6 Hz were employed for imaging. Approximately 50 µl of sample was placed onto a freshly cleaved atomically flat mica surface for 20 minutes and dried under nitrogen.

**RESULTS**

**Fibrinogen chaperone activity is decreased in ATTR patients**

The description of fibrinogen as a molecular chaperone [35] highlights its potential role in misfolding diseases, in particular in ATTR, since it was found to be an interacting partner of Transthyretin [30]. Hence, we investigated whether fibrinogen from ATTR patients and control subjects displayed a differential chaperone activity.

Fibrinogen from ATTR patients and healthy individuals was purified using a modified fibrinogen enrichment protocol. By Western blot analysis we observed that fibrinogen is abundant in the enriched fraction, whereas transthyretin or albumin were not detected (Figure 1- B and C). Indeed, the most abundant protein in all plasma electrophoretic profiles corresponds to human serum albumin (HSA) that is absent from the enriched fraction (Figure 1- A).

SDS-PAGE analysis of control and ATTR fibrinogen enriched samples shows homogenous protein profiles between the two sets of individuals and within subjects in the same group (Figure 1- D). In the SDS-PAGE of the enriched fraction the most abundant proteins present are the α, β and γ chains of fibrinogen and whose identities
were confirmed by peptide mass fingerprint (Figure 1- D and E). Furthermore, using gel-densitometry analysis, we observed that fibrinogen represents 50% of the whole protein content in the enriched fraction (Figure S1).

To evaluate the chaperone activity of fibrinogen, a well-established amyloid model protein, insulin, was used. Aggregation and fiber formation were induced under acidic conditions and detected by light scattering and Thioflavin T fluorescence, respectively [42,43] (Figure S2). Human fibrinogen (Calbiochem) was able to suppress both aggregation and fiber formation of insulin in vitro in a dose-dependent manner (Figure 1- F,G). Aggregation assays were repeated in the presence of fibrinogen enriched fractions from ATTR and from healthy individuals. Our results show that the enriched fraction from healthy individuals decreased insulin aggregation by 50% (Figure 1- H) (p = 0.0280), and decreases fiber formation by 40% (Figure 1- I) (p = 0.0018). Fibrinogen from ATTR patients did not show statistical significant effects on the suppression of insulin aggregation neither on fiber accumulation. To rule out any consequence of a molecular crowding effect on the observed chaperone activity caused by other proteins present in the fibrinogen enriched fractions [47], we conducted the same assay using bovine serum albumin (BSA) at the same respective molar protein concentration of the enriched fraction. We observed that BSA does not influence the observed chaperone effects (Figure 1- H, I). To confirm that fibrinogen was indeed responsible for the suppressive effect on insulin aggregation, polyclonal antibody anti-fibrinogen was added to the reaction mixture containing fibrinogen enriched fraction from healthy individuals. This antibody binds selectively to fibrinogen preventing its interaction with natural target proteins, thus inhibiting fibrinogen chaperone activity [44]. Our results show that the fibrinogen enriched samples from healthy subjects, incubated with this antibody exhibit an aggregation and fiber formation pattern similar to insulin alone (Figure S3). This confirms that, despite the heterogeneous composition of the enriched fraction, fibrinogen is the major component involved in the observed reduction of aggregation and fiber formation. These results strongly support the hypothesis that fibrinogen chaperone-like activity is decreased in ATTR patients compared to healthy individuals.
Fibrinogen from ATTR patients displays an increased glycation pattern

We have previously found that TTR fibers from ATTR individuals are glycated by methylglyoxal [29] and that ATTR patients show increased glycation levels in some plasma proteins [30]. Considering the fact that protein glycation by MG is known to exert effects on the activity of chaperone proteins [48] we speculated that this could also be the case of fibrinogen in the context of ATTR pathogenesis.

To evaluate the effects of glycation on chaperone activity, fibrinogen (Calbiochem) was glycated in vitro by incubation with increasing concentrations of MG from 0 to 10 mM. Glycation by MG was probed by western blot [49], confirming an increase of the MG advanced glycation end products (MAGE) the signal being proportional to the molecular mass increment of the fibrinogen chains. Fibrinogen incubated with different concentrations of MG was later incubated with insulin to determine the impact on its chaperone's activity (Figure 2 – A,B).

Since fibrinogen glycation by methylglyoxal in vitro decreases its chaperone activity, we investigated if the differential fibrinogen glycation in ATTR patients could explain the observed absence of reduction of insulin aggregation. Therefore, both fibrinogen enriched fractions collected from healthy subjects and from ATTR patients were analyzed by SDS-PAGE and argpyrimidine-modified proteins detected by western blot using a monoclonal antibody specific to this MAGE (Figure 2- C, D) [49]. Higher levels of argpyrimidine modified proteins were found to be present in ATTR subjects by comparison with healthy individuals (Figure 2- C). This was particularly noticeable in a protein band with a molecular mass slightly under 75 kDa that shows a stronger signal for argpyrimidine only in ATTR individuals (Figure 2- C). This protein was identified as being the α-chain of fibrinogen.

Mapping fibrinogen glycation in ATTR

To investigate the molecular effects of fibrinogen glycation on its chaperone activity, we identified the location of the glycated residues in fibrinogen from ATTR patients
and from healthy subjects. Since fibrinogen is a large, multidomain glycoprotein composed of two pairs of three non-identical polypeptide chains (Aα, Bβ and γγ) [33,34] it is quite difficult to map its post-translational modification either by top-down or conventional bottom-up strategies. An added problem is the low abundance of glycation [50]. Hence, the first step was to optimize MS detection and characterization of MAGE modified peptides using in vitro glycated fibrinogen. To increase protein sequence coverage and the likelihood of detecting methylglyoxal modified residues, we combined three methods: 1) different micro-chromatography conditions, by using different resins and sequential peptide elution; 2) parallel use of more than one protease; and 3) different MALDI matrices. Combining peptide matches obtained with all of these methods allowed us to increase the average sequence coverage from 31% (classic approach based on trypsin digestion, R2 sample clean up and single matrix elution) to an outstanding sequence coverage of 82%, more adequate for post-translational modification search. The ultr-high resolving power of FTICR-MS was critical to resolve closely adjacent isotopic series, thus enhancing peptide ion detection and contributing to increased protein sequence coverage.

In vitro glycated fibrinogen polypeptide chains were separated by SDS-PAGE, followed by in-gel digestion, sample cleanup and spectra acquisition. Given that MG-HI, argpyrimidine and CEL are the most frequent MAGE [51], we considered the respective mass increments for arginine and lysine residues [52] (Figure S5).

We found a total of 56 different peptide ions exclusively for the glycated fibrinogen mass spectra whose mass values corresponded to fibrinogen peptides containing MG adducts: 24, 21 and 11 for fibrinogen’s α, β and γ chains, respectively. In Figure 3- A we show two spectra for the fibrinogen β chain of non-glycated (top) and glycated fibrinogen (bottom) peptides. We confirmed the sequence of the non-glycated peptides by tandem MS (Figure 3- B, F) and assigned the observed mass shifts (Figure 3- C) corresponding to one, two MG-HI adducts (Figure 3- D) and one argpyrimidine (Figure 3- G). We detected a total of 36 different ions corresponding to peptides with 10 MG-H1, 16 with argpyrimidine and 16 with CEL, for all fibrinogen chains. Interestingly, peptides containing more than one MG adduct were also found, for example two peptides, not present in the non-glycated fibrinogen β chain, shown in figure 3- A and B, corresponding to different MAGE of the same peptide (TVNSNIPTNLRVLRILE, TVNSNIPTNLRVLRILE).
Differential glycation in ATTR - imbalance of proteostasis

The peak with m/z 2147.19 matches this peptide with two MG-HI adducts in both arginine residues 166 and 169, determination further confirmed by MS/MS (Figure 3- D), while the peak with m/z 2093.17 corresponds to the same peptide, but with only one mass adduct. We found by MS/MS that this last peptide carrying a MG-HI adduct, was in fact a mixture of two peptides with one MG-HI adduct in each arginine residue. The parent ion is a contribution of peptides glycated exclusively in arginine 166 or in arginine 169 (Figure 3- C). Another peptide (ALLQQERPIRNSVDE, m/z 1767.94) was detected containing either MG-HI or argpyrimidine adducts (Figure 3- E, F). These data confirm the inherent complexity and heterogeneity of glycation [52].

To compare the glycation landscape of fibrinogen in ATTR patients the one from healthy subjects, we applied the MS method used to characterize the in vitro glycation pattern of fibrinogen.

The data acquired shows greater spectral complexity, less intense signals and lower signal to noise ratio relatively to the data obtained for in vitro studies, as expected when comparing PTMs from in vitro and in vivo samples. Considering only non-glycated peptides and combining the data from all individuals in the same group, we obtained similar sequence coverage values for fibrinogen from both ATTR and control individuals, 85% and 83.6% respectively and a lower inter-individual variation.

Figure 3 - A shows a zoom in of one spectrum from a healthy subject’s fibrinogen α chain. The peak in red, absent in the ATTR fibrinogen α chain spectra (Figure 3 - B), corresponds to one MG-HI adduct of the peptide WKALTDMPQMRME (m/z 1636.75). As shown in figure 4 the peak with m/z 1779.87, matching the peptide MKGLIDEVNQDFTNR with one CEL adduct in lysine residue 52 (Figure 4- D) is present in the ATTR spectra, not being detected in the control’s fibrinogen α chain.

We detected a total of 46 MAGE for ATTR subjects (an average of 15 per individual), corresponding to 15 MG-HI, 15 argpyrimidine and 16 CEL adducts, for all three chains. In healthy control individuals the total number of MAGE found was 18 (making an average of 6 per individual) and from those 8 MG-HI, 5 argpyrimidine and 5 CEL adducts.
Analysis of the distribution of fibrinogen glycation sites from ATTR patients show that glycated residues are mainly found on its globular domains (Figure 4– C). In contrast, the fibrinogen glycated residues from control individuals show a wider and seemingly random distribution (Figure 4– E). Moreover, glycation by methylglyoxal is more extensive in fibrinogen from ATTR patients, and the in vivo results reproduce the inherent complexity and heterogeneity of glycation reactions observed for in vitro glycation analysis, particularly for such a large protein. Moreover, glycation seems to have a specific distribution in fibrinogen’s globular domains suggesting that this metabolic alteration can be related to fibrinogen’s reduced chaperone capacity in these individuals.

**Fibrinogen chaperone activity prevents TTR fibril formation**

Glycation causes protein structural changes thus affecting protein-protein interactions. TTR interacts in a very stable way with fibrinogen (Figure 5– A). However the eluted TTR amount is reduced upon binding to glycated compared to non-glycated fibrinogen. This indicates that glycation decreases with the interaction between these two proteins.

The ability of fibrinogen to inhibit TTR aggregation was investigated using recombinant TTR. As human TTR is mainly tetrameric, it was important to confirm whether the recombinant TTR variant could form a tetramer under physiological conditions. As presented in Figure 5– B, the elution profile obtained by size exclusion chromatography, for both TTR WT and TTR V30M, showed in each case a single peak, with an estimated molecular mass of 60 kDa, the approximate mass of the tetramer. Knowing that TTR partially unfolds under acidic conditions, forming amyloid fibril, , the hallmark of pathological conditions such as ATTR and SSA.

To evaluate the effects of glycation on fibrinogen’s chaperone activity over TTR, we compared the ability of glycated and non-glycated fibrinogen to prevent TTR amyloidogenesis at pH 4.6 using light scattering and ThioflavinT binding (Figure 5– C). TTR WT Amyloid fibril formation is lower than TTR V30M, as expected. Atomic force microscopy (AFM) was used to confirm the presence of TTR amyloid fibers. After 12 hours incubation, WT TTR predominantly aggregates into fibrils with a length of of 2–3 nm, although longer fibers were also present (Figure 5 F, H).
As shown in Figure 5–C and D, TTR WT and TTR V30M aggregation and amyloid fiber formation were significantly suppressed by fibrinogen in a dose dependent manner (p <0.05). This observation is in agreement with the effect of fibrinogen on insulin aggregation. Using AFM in the presence of Fibrinogen WT TTR fibrils were not detected after 12 hours (Figure 5 G).

Upon TTR fibril formation, in the absence of fibrinogen, no detectable TTR was found in the supernatant (Fig 5E lane S1), most TTR being found in the insoluble fraction (Fig 5E lane P1). By contrast, in the presence of fibrinogen, almost all TTR was recovered in the supernatant (Fig 5E lane S2) and virtually no detectable TTR was found in the insoluble fraction (Fig 5E lane P2). These results show the ability of fibrinogen to maintain TTR soluble and properly folded.

**DISCUSSION**

The extracellular space imposes an additional challenge to protein stability, due to its higher oxidizing environment and mechanical stress, due to the continuous blood pumping, enhancing protein unfolding and aggregation. Molecular chaperones are essential to intra- and extracellular proteostasis. Extracellular protein misfolding and aggregation underlie many of the most serious amyloid diseases, including ATTR, Alzheimer's, Huntington's and Parkinson's [53].

Concerning ATTR, extracellular chaperones like clusterin, have been implicated in the pathology of ATTR and SSA [54, 55]. Another extracellular protein, fibrinogen, was found by us to be an interacting partner of TTR, being noteworthy that higher levels of fibrinogen were found in V30M ATTR individuals than in healthy control subjects [30, 56]. Increased levels of fibrinogen were also found in both Alzheimer’s disease and vascular dementia [57]. Moreover, fibrinogen specifically interacts and suppresses aggregation of a wide spectrum of stressed proteins [35]. In this work we demonstrated that fibrinogen from ATTR patients displays an impaired chaperone capacity, most likely due to differential glycation by methylglyoxal, when compared with fibrinogen from control subjects.
ATTR is an amyloid disease that is highly heterogeneous at the phenotypic level, regarding penetrance, incidence and also symptomatology. Considering TTR tetramer stability as a significant factor in the initiation of amyloid fibril formation, and that variant forms of TTR accelerate tetramer dissociation, one should expect that fibers would be essentially composed of TTR mutant forms, which is not the case. Furthermore, amyloidogenic TTR variant, present since birth, does not cause amyloid fibril formation prior to adult life. This evidence strongly supports the hypothesis that there are non genetic factors involved in the pathogenesis of amyloid fibril formation [58] that enhance disease penetrance and incidence. Several markers have been related to organism and cell senescence. In particular, some of these markers are related to stress phenomena and to the action of by-products resulting from metabolic processes. The formation and accumulation of AGEs has been linked to the progression of age-related diseases such as diabetes, Parkinson’s disease and SSA [59-61].

Research concerning the role of protein glycation in disease progression is a field of study in great expansion [22]. As any post-translational modification, glycation has been described as having a significant effect on protein folding, conformation, stability, turnover and function [62] which may be associated with the cell and tissue damage observed in aging and several related pathological conditions [63], among which ATTR [30]. Our results show that fibrinogen from ATTR patients displays an increased glycation profile compared with healthy control subjects as well as a differential structural pattern. In ATTR, fibrinogen presents MAGE mainly on the globular domains. Indeed FTICR-MS analysis allowed us to map the extension of this modifications in both ATTR and control individuals. It was interesting to observe that, although the majority of the sequence pattern for glycation distribution was not conserved within individuals, tri-dimensional motifs were preserved for AGEs in ATTR patients: MG derived modifications in ATTR individuals have a specific spatial distribution preferentially localizing at the fibrinogen’s globular domains. These observations reveal the inherent complexity and heterogeneity of glycation and suggest that this post-translational modification reduces fibrinogen chaperone activity in ATTR individuals. Fibrinogen’s globular domains are rich in basic amino acid residues and do not present a hydrophobic core, suggesting that this region is probably not directly involved in the chaperone activity. In fact, structural data on human fibrinogen indicate
that this molecule must be flexible, mainly in the coiled-coil regions. We propose that glycation of target residues is sufficient to destabilize fibrinogen quaternary structure and thus affect its ability to suppress protein aggregation, since it is already established that glycation is capable of affecting enzymatic activity [20, 64]. In agreement with our observations, we speculate that the differential glycation profile observed in ATTR patients can explain the loss of chaperone activity observed in these individuals.

ATTR patients have an abnormal metabolism of glucose [66], as they show a high hypoglycemic and hyperinsulinemic profile after being administrated with glucose. In fact, a modest increase in cellular glucose metabolism results in a substantial increase in AGEs accumulation [67]. This suggests that an elevated cellular uptake of this molecule may contribute to the formation glycated proteins.
FOOTNOTES

The authors acknowledge Nurse Margarida, from Hospital de Curry Cabral, Lisboa, Portugal, for her outstanding cooperation in this work regarding sample collection. The authors report no conflicts of interest. Work was supported by grants SFRH/BPD/74711/2010 (I.B.G.), SFRH/BPD/41037/2007 (R.A.G.), IF/00808/2013 (A.V) and IF/00359/2014 (G.C), PEst-OE/QUI/UI0612/2011, UID/MULTI/00612/2013, PTDC/QUI/123060/2010 and REDE/1501/REM/2005 from the Fundação para a Ciência e a Tecnologia, and also by the 2011 Junior Research grant from the amyloidosis foundation.
REFERENCES


Differential glycation in ATTR - imbalance of proteostasis

Val30Met familial amyloid polyneuropathy. Journal of the neurological sciences. 287, 178–84


methylglyoxal-derived advanced glycation end-product in familial amyloidotic polyneuropathy. Biochem J. 385, 339-345


Differential glycation in ATTR - imbalance of proteostasis


Differential glycation in ATTR - imbalance of proteostasis


Differential glycation in ATTR - imbalance of proteostasis

polyneuropathy: hypersensitivities of the autonomic nervous system and therapeutic prevention. J Auton Nerv Syst. 35, 63–70

FIGURES LEGENDS

Figure 1 – Fibrinogen Chaperone activity is altered in ATTR patients.

**Fibrinogen characterization (A-E)** – A) SDS-PAGE profile comparison between fibrinogen enriched fraction from human plasma (FP) and total plasma. B) and C) Western blot analysis of FP fraction and total plasma using anti-Fibrinogen and anti-Transthyretin antibodies. D) SDS-PAGE protein profile of the FP fractions from ATTR and Healthy subjects relatively to fibrinogen (CF) and in vitro glycated fibrinogen (GF) and E) Western blot analysis of the FP fractions from ATTR and Healthy subjects relatively to Calbiochem® fibrinogen (CF) and in vitro glycated fibrinogen (GF) using anti-Fibrinogen antibody.

**Fibrinogen effect on insulin aggregation (F-I)** – Increasing concentrations of Calbiochem® Fibrinogen samples (n=3) were incubated with insulin for 3 days at pH 4.6 - 37ºC and insulin aggregation was measured. (F, G) Fibrinogen enriched fraction. (H, I) samples (n=3) from Healthy and ATTR individuals were incubated with insulin for 3 days at pH 4.6 - 37ºC and insulin aggregation was measured. BSA was used for control purposes. (F-H) Light Scattering (Abs. 600 nm), (G-I) Fluorescence intensity (IF) of Thioflavin T probe. (□) Insulin with fibrinogen; (●) fibrinogen alone; Normalized data using non incubated insulin (*p-value <0.05; **p-value <0.01).

Figure 2 – Effect of glycated fibrinogen on insulin aggregation (A, B) – *In vitro* glycated fibrinogen samples (n=3) added of increasing concentrations of methylglyoxal were incubated with insulin for 3 days at pH 4.6 - 37ºC and the resulting insulin aggregation measured. A) Light Scattering (Abs. 600 nm). B) Fluorescence intensity, (IF) of Thioflavin T probe. (●) Insulin with fibrinogen, non-glycated or glycated with methylglyoxal 0.2, 0.5, 2, 5 and 10 mM; (○) fibrinogen alone; (--) insulin alone. Normalized data using non incubated insulin. MG, methylglyoxal.

**Glycation pattern of Fibrinogen from ATTR patients.**

**Glycated Fibrinogen Characterization (C, D) –** Western blot analysis of Fibrinogen enriched fraction from ATTR and Healthy control subjects’ in comparison to Calbiochem® Fibrinogen (CF) and *in vitro* glycated fibrinogen (GF) using anti-Argpyrimidine antibody. D) – SDS-PAGE protein profile of fibrinogen enriched
Differential glycation in ATTR - imbalance of proteostasis

fraction from ATTR and Healthy control subjects’ in comparison to Calbiochem® Fibrinogen (CF) and in vitro glycate fibrinogen (GF).

Figure 3 - In vitro Glycation sites
A) Zoom in of the spectra of fibrinogen β chain after Glu-C digestion, using R3 resin and elution with CHCA in 50% ACN, 0.1% TFA - non glycated (top) and glycated fibrinogen (bottom). (B-D) Tandem MS of the glycated and non glycated peptide TVNSNIPTNLRLRSILE (m/z 2039.16) - B) non glycated; C) peptide with one MG-HI adduct; D) peptide with two MG-HI adducts. E) Zoom in of the spectra of fibrinogen β chain after Glu-C digestion, using R2 resin and elution with CHCA in 20% ACN, 0.1% TFA - non glycated (top) and glycated fibrinogen (bottom). (F, G) Tandem MS of the glycated and non glycated peptide ALLQQERPIRNSVDE (m/z 1767.94): F) non glycated; G) peptide with one argpyrimidine in the arginine 91.

Figure 4 - In vivo Glycation sites
Zoom in of the spectra of fibrinogen α chain for peptide WKALTDMPQMRME (m/z 1636.75). A) – ATTR individual and B) – Healthy subject. B) – Peptide with one MG-HI adduct (in red).

Zoom in of the spectra of fibrinogen α chain for peptide MKGLIDEVNQDFTNR (m/z 1779.87). A) – Healthy individual and B) – ATTR subject. B) – Peptide with one CEL adduct (in red).

Mapping Fibrinogen Glycation profile. E) – Fibrinogen 3D structure - residues found as differentially glycated in control individuals are assigned as green and residues found as differentially glycated in ATTR indivisuals are assigned as red. Structure PDB ID: 3GHG.

Figure 5 - Fibrinogen Glycation promotes TTR fibril formation
A) Endogenous TTR interacts stronger with Fibrinogen (FIB) than with in vitro glycated Fibrinogen (FIBG). Plasma TTR was purified using Fibrinogen and glycated fibrinogen affinity collumn, run on SDS-PAGE, and then Western blotted with anti-TTR antibody. B) Analytical Size exclusion of WT TTR and V30M TTR - Molecular weights are assigned on top of the chromatogram. C-D) TTR purified protein (n=3) incubated with for 3 days at pH 4.6 - 37°C in the presence of Fibrinogen (0.5 μM
and 1 µM) and Glycated fibrinogen (1 µM) – C) Light Scattering (Abs. 600 nm); D - Fluorescence intensity (IF) by Thioflavin T probe; Normalized data using TTR V30M alone (*p-value<0.05). E) Western blot analysis of precipitated TTR in the presence of Fibrinogen. T1, S1, and P1, total sample, soluble fraction, and insoluble/pellet fraction of TTR V30M formed in the absence of fibrinogen; T2, S2, and P2, total sample, soluble fraction, and insoluble/pellet fraction of TTR formed in the presence of fibrinogen. F) Tridimensional AFM topographic images of TTR WT after 12 h, deposited onto mica, in the absence a) and presence b) of 1 µM of Fibrinogen. Figure c) shows a cross section of the fibrils detected in figure a).
Figure 1 – Fibrinogen Chaperone activity is altered in ATTR patients

A. FP and Plasm fibrinogen bands

B. Fibrinogen bands

C. TTR bands

D. Healthy and ATTR fibrinogen bands

E. Fibrinogen bands

F. Light scattering vs. fibrinogen concentration

G. Light scattering vs. fibrinogen concentration

H. Light scattering comparison among different samples

I. Light scattering and turbidity comparison among different samples
Figure 2 – Fibrinogen from ATTR patients display an increased glycation pattern

A

B

C

D

[Graphs and images related to Figure 2 are shown, illustrating data on light scattering and fibrinogen glycation.]
Figure 3 - *In vitro* Glycation sites

**Intensity**

**A**

\[ \beta_{[156-173]}^{2039.161} \]

\[ + 2\times MG-H1 \]

\[ + MG-H1 \]

\[ \beta_{[156-173]}^{2093.173} + 54 \text{ Da} \]

**B**

\[ \text{TVN, SN, PTN, LRV, RSILE} \]

\[ b_3, b_5, b_6, y_5, y_6, y_8, y_9, b_{10}, b_{14} \]

**C**

\[ \text{TVN, SN, PTN, LRV, RSILE} \]

\[ b_3, b_5, b_6, y_5, y_6, y_8, y_9, b_{10}, y_{12}, b_{13}, b_{14}, b_{15}, y_{13} \]

**D**

\[ \text{TVN, SN, PTN, LRV, RSILE} \]

\[ b_3, b_5, b_6, y_5, y_6, y_8, y_9, b_{10}, y_{12}, b_{13}, b_{14}, y_{15} \]

**E**

\[ \beta_{[85-96]}^{1767.935} \]

\[ + \text{Arggirmidina} \]

\[ + MG-H1 \]

\[ \beta_{[85-96]}^{1821.943} + 54 \text{ Da} \]

\[ \beta_{[85-96]}^{1847.961} + 60 \text{ Da} \]

**F**

\[ \text{ALLQGERPJRNSVDE} \]

\[ y_6, y_8, y_{10}, b_3, b_4, b_5, b_{10}, b_{14} \]

**G**

\[ \text{ALLQGERPJRNSVDE} \]

\[ y_6, y_{10}, b_3, b_{14} \]
Figure 4 - *In vivo* Glycation sites

A

![Graph showing glycation sites](image)

B

![Graph showing glycation sites](image)

C

![Graph showing glycation sites](image)

D

![Graph showing glycation sites](image)

E
Figure 5 - Fibrinogen Glycation promotes TTR fibril formation