Argpyrimidine, a methylglyoxal-derived advanced glycation end-product in familial amyloidotic polyneuropathy

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FAP (familial amyloidotic polyneuropathy) is a systemic amyloid disease characterized by the formation of extracellular deposits of transthyretin. More than 80 single point mutations are associated with amyloidogenic behaviour and the onset of this fatal disease. It is believed that mutant forms of transthyretin lead to a decreased stability of the tetramer, which dissociates into monomers that are prone to unfolding and aggregation, later forming β -fibrils in amyloid deposits. This theory does not explain the formation of β -fibrils nor why they are toxic to nearby cells. Age at disease onset may vary by decades for patients with the same mutation. Moreover, non-mutated transthyretin also forms the same deposits in SSA (senile systemic amyloidosis), suggesting that mutations may only accelerate this process, but are not the determinant factor in amyloid fibril formation and cell toxicity. We propose that glycation is involved in amyloidogenesis, since amyloid fibrils present several properties common to glycated proteins. It was shown recently that glycation causes the structural transition from the

folded soluble form to β -fibrils in serum albumin. We identified for the first time a methylglyoxal-derived advanced glycation end-product, argpyrimidine [*N*⁸-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-L-ornithine] in amyloid fibrils from FAP patients. Unequivocal argpyrimidine identification was achieved chromatographically by amino acid analysis using dabsyl (4-dimethylaminoazobenzene-4'-sulphonyl) chloride. Argpyrimidine was found at a concentration of 162.40 ± 9.05 pmol/mg of protein in FAP patients, and it was not detected in control subjects. The presence of argpyrimidine in amyloid deposits from FAP patients supports the view that protein glycation is an important factor in amyloid diseases.

Key words: advanced glycation end-product (AGE), amyloid, argpyrimidine, familial amyloidotic polyneuropathy (FAP), glycation, methylglyoxal.

INTRODUCTION

FAP (familial amyloidotic polyneuropathy) is a systemic amyloid disease characterized by the extracellular deposition of transthyretin in several tissues, particularly in the peripheral nervous system [1]. The main clinical symptom is a progressive polyneuropathy affecting both the peripheral and autonomic nervous systems [2]. In all amyloidoses, including FAP, fibrils are insoluble, highly stable, resistant to enzymic proteolysis and show an extensive β -sheet structure [3]. Transthyretin is a homotetrameric protein of 55 kDa found in the plasma and cerebrospinal fluid, and is responsible for the transport of thyroxine and retinol by association with the retinol-binding protein [4,5]. In certain forms of FAP, amyloid fibrils are mainly constituted by variants of transthyretin. In Portuguese-type FAP, substitution of methionine for valine at position 30 occurs [6]. Although all FAP patients have identical concentrations of variant transthyretin in the plasma and cerebrospinal fluid, age at onset varies widely between 20 and 70 years. Therefore, despite the identification of mutations in transthyretin associated with FAP, the process of fibril formation and their toxicity remain to be elucidated. The observation that non-mutated transthyretin also forms amyloid fibrils as in SSA (senile systemic amyloidosis) [7,8] implies that other factors besides genetic modifications must be considered in the pathogenesis of FAP. Since the first symptoms in FAP appear much earlier than in SSA, the point mutations in transthyretin only seem to accelerate fibril formation by increasing the amyloidogenicity of transthyretin. Moreover,

different amyloidotic proteins with no sequence homology form similar amyloid fibrils [3]. Besides amyloidogenesis, the mechanisms by which the extracellular deposits are toxic to cells are not well understood. One important observation is the presence of oxidative stress markers associated with amyloid fibrils, suggesting that interactions with specific receptors may occur [9].

At present, there is evidence that protein glycation is involved in the pathogenesis of several amyloid diseases, such as Alzheimer's disease [10–12] and dialysis-related amyloidosis [13]. Protein glycation is equivalent to a point mutation, since amino acid side chains are modified. In living cells, the effects of glycation are countered by high turnover and short half-life of most cellular proteins. In contrast, long-lived extracellular proteins accumulate glycation adducts with age [14–16]. Like amyloid fibrils, glycated proteins are resistant to proteolysis *in vivo* and are toxic to animal cells [17–19]. The cellular effects of AGEs (advanced glycation end-products) occur by interacting with specific cellular receptors, such as RAGE (receptor for AGEs), the best characterized [20,21]. The AGE–RAGE interaction *in vivo* generates a significant cellular redox stress [22].

One of the most powerful glycation agents *in vivo* is methylglyoxal (2-oxopropanal) formed mainly in all living cells from dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate in glycolysis. Methylglyoxal irreversibly modifies lysine and arginine residues in proteins. However, methylglyoxal arginine AGEs appear to be more relevant, considering the existence of specific receptors for hydro-imidazolones [23]. Methylglyoxal

Abbreviations used: AGE, advanced glycation end-product; ALS, amyotrophic lateral sclerosis; FAP, familial amyloidotic polyneuropathy; RAGE, receptor for AGEs; SOD-1, superoxide dismutase 1; SSA, senile systemic amyloidosis; TFA, trifluoroacetic acid.

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also forms argpyrimidine [*N*^δ-(5-hydroxy-4,6-dimethylpyrimidin-2-yl)-L-ornithine], a fluorescent product [24]. Recently, argpyrimidine was found in spinal cord of familial sporadic ALS (amyotrophic lateral sclerosis) patients and mutant SOD-1 (superoxide dismutase 1) mice [25]. Methylglyoxal modification of arginine may contribute to the pathophysiology associated with aging and other diseases [25,26].

In the present study, we unequivocally identified argpyrimidine, by chromatographic methods, in amyloid fibrils from Portuguese-type FAP patients. The formation of AGEs in FAP may play an important role in the molecular mechanism of amyloidogenesis either by promoting the pathway of amyloid fibril formation or by increasing its toxicity to nerve cells.

MATERIALS AND METHODS

Chemicals and materials

L-Amino acids, *N*^α-acetylarginine, thymol, pepsin, Pronase E (protease type XIV), aminopeptidase and dabsyl (4-dimethylaminoazobenzene-4'-sulphonyl) chloride were purchased from Sigma Chemical Co. Solid-phase extraction columns (LiChrolut RP-18, 500 mg) were from Merck. All solvents were HPLC grade. HPLC analysis was performed in a Beckman-Coulter column with a high-pressure binary gradient pump 126, a Beckman-Coulter 168-diode-array detector (1 nm resolution) and a fluorescence detector FP-2020 Plus (Jasco). In all assays, a Merck LichroCART 250-4 (250 mm × 4 mm) column with stationary-phase Lichrospher 100 RP-18, 5 µm, was used at a flow rate of 1 ml/min. MS analysis of *N*^α-acetyl-argpyrimidine was performed in an ESI (electrospray ionization)-MS Thermo-Finnigan LQC Duo, and NMR analysis was performed in a Bruker Advance 400 [using DQF (double-quantum-filtered)-COSY, HMQC (heteronuclear single quantum correlation) and HMBC (heteronuclear multiple bond correlation) NMR sequences].

Human samples

Adipose tissue samples from FAP patients (two males and one female; mean age 30, range 26–33 years) were collected during the initial phase of liver transplantation. Non-FAP controls (three males and two females; mean age 51, range 28–69 years) comprised patients receiving transplants following liver cirrhosis or autoimmune liver disease. One of the control subjects, suffering from a pancreatic tumour, did not undergo liver transplantation. Adipose tissue samples were obtained during surgery. For all FAP and non-FAP subjects, blood D-glucose concentration was determined, in fasting and post-prandial conditions. Neither FAP patients nor control subjects were diabetic or had any carbohydrate-related disorders. There were no other exclusion criteria. Moreover, blood methylglyoxal concentration was determined in the same conditions, and no significant differences between FAP and control subjects were found (results not shown). All patients gave informed written consent, and the protocol was approved by the Curry Cabral Ethical Commission according to EEC approved protocols.

Extraction of amyloid proteins from adipose tissue

Amyloid proteins were extracted from samples of FAP patients and non-FAP subjects following a modified procedure based on the method developed by Kaplan et al. [27]. Briefly, adipose tissue was dispersed in 5 ml of 0.154 M KCl and incubated for 1 h at room temperature (20 °C). Samples were centrifuged for 10 min

at 19000 *g*, and the sediment was washed twice in the same solution. Lipid extraction was then performed with the addition of 5 ml of chloroform/methanol (2:1, v/v) and incubation for 5 min at room temperature. Samples were centrifuged for 10 min at 19000 *g*, and the sediment was suspended with aqueous 20 % acetonitrile containing 0.1 % TFA (trifluoroacetic acid). Samples were incubated for 1 h at room temperature, and the amyloid proteins extracted were collected in the supernatant after centrifugation for 10 min at 19000 *g*. Amyloid protein extraction was repeated once more. The extracted material was evaporated to dryness under a stream of N₂ at 40 °C and suspended in 500 µl of aqueous 20 % acetonitrile with 0.1 % TFA.

Protein concentration was determined using Coomassie Brilliant Blue dye as described in [28]. Extracted proteins were analysed directly by HPLC using a binary gradient made of solvent A (water with 0.1 % TFA) and solvent B (acetonitrile with 0.1 % TFA). The gradient program was 0–20 min, 20–90 % solvent B; 5 min isocratic at 90 % solvent B; and 25–30 min, 90–20 % solvent B. The eluate was monitored at 220 and 260 nm (diode array detector) and by the fluorescence signal at $\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$ of 320/385 nm (double-monochromator fluorescence detector).

Preparation of argpyrimidine standard

Argpyrimidine was prepared by the reaction of *N*^α-acetyl-L-arginine with methylglyoxal. *N*^α-Acetyl-L-arginine (100 mM) was incubated with 100 mM methylglyoxal in 100 mM sodium phosphate buffer (pH 7.4) at 70 °C for 72 h. Methylglyoxal was prepared by acid hydrolysis of 1,1-dimethoxypropanone, as described previously [29]. The products obtained were analysed by HPLC using a binary gradient, solvent A (water with 0.1 % TFA) and solvent B (acetonitrile with 0.1 % TFA). The gradient program was: 0–8 min, 2–10 % solvent B; 8–13 min, 10 % solvent B isocratic; 13–23 min, 10–20 % solvent B; 20 % solvent B isocratic for 2 min; and 25–28 min, 20–2 % solvent B. *N*^α-Acetyl-argpyrimidine was identified by its characteristic absorption spectra and characteristic fluorescence at excitation wavelength of 320 nm and emission wavelength of 385 nm. The resulting *N*^α-acetyl-argpyrimidine was purified in a RP-18 column (LiChrolut, 500 mg). Purification was followed by HPLC analysis as described above; the fractions containing pure *N*^α-acetyl-argpyrimidine were combined and freeze-dried. The identity of *N*^α-acetyl-argpyrimidine was confirmed by MS (molecular mass of 298.11 Da) and NMR spectroscopy analysis. To prepare the chromatographic argpyrimidine standard, the acetyl group was removed by enzymic hydrolysis using leucine aminopeptidase for 2 days at 37 °C.

Argpyrimidine was quantified by HPLC using a calibration curve relating argpyrimidine concentration to the area of the corresponding peak (fluorescence detection).

Amino acid analysis: chromatographic detection of argpyrimidine

Before amino acid analysis, samples containing amyloid proteins extracted from FAP patients and samples containing proteins extracted from non-FAP patients were washed by ultrafiltration (5 kDa cut-off membrane) and subjected to enzymic hydrolysis as described previously [30]. Briefly, to 20 µl of sample (approx. 10–20 µg) 25 µl of 40 mM HCl, 5 µl of 2 mg/ml thymol and 5 µl of 2 mg/ml of pepsin (both prepared in 20 mM HCl) were added, and the samples were incubated at 37 °C for 24 h. Samples were neutralized and buffered at pH 7.4 with the addition of 25 µl of 0.5 M potassium phosphate buffer, pH 7.4, and 5 µl of 260 mM

Figure 1 Direct HPLC analysis of proteins extracted from a FAP patient using two different methods

Absorbance was monitored at 220 nm. Samples underwent ultrafiltration before analysis (5 kDa cut-off). The inset shows a magnification of the chromatogram between 5 and 25 min. Solid line, extraction by the modified procedure; dotted line, extraction by the method of Kaplan et al. [27]; mAU, milli-absorbance units.

KOH. Then 5 μ l of Pronase E solution (2 mg/ml in 10 mM phosphate buffer, pH 7.4) was added, and the samples were incubated for 24 h at 37°C. Finally, 5 μ l of leucine aminopeptidase and protease solutions (both prepared in 10 mM phosphate buffer, pH 7.4) were added and the mixture was incubated for 48 h at 37°C. This enzymic hydrolysate (100 μ l) was used for the argpyrimidine assay using dabsyl chloride as the derivatization reagent.

An aliquot of the enzymic hydrolysate (50 μ l) was mixed with the same volume of 2 mM dabsyl chloride in acetonitrile and incubated at 60°C for 10 min. The resulting sample was filtered and analysed by HPLC. A binary system of 25 mM sodium acetate buffer, pH 6.5 (solvent A), and acetonitrile (solvent B) was used. The gradient program was 0–30 min, 20–40% solvent B; 30–55 min, 40–90% solvent B; 5 min isocratic with 90% solvent B; and 60–65 min, 90–20% solvent B. The eluate was monitored at 452 nm and by the fluorescence signal at $\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$ of 320/385 nm. The argpyrimidine standard solution was submitted to the same treatment.

RESULTS AND DISCUSSION

Extraction and characterization of amyloid deposits from FAP patients

Amyloid proteins were extracted using a modified procedure based on the method developed by Kaplan et al. [27]. In the present study, an additional step was added to extract lipids and phospholipids with a chloroform/methanol mixture before amyloid protein extraction. Since proteins are the main components of amyloid fibrils, protein concentration is a measure of the efficiency of both methods. With the modified procedure we obtained a higher ratio of protein amount per initial amount of tissue $[(1.32 \pm 0.198) \times 10^{-3}; n=3]$ in comparison with the Kaplan method $[(6.9 \pm 0.613) \times 10^{-4}; n=4]$ [27]. Therefore the new lipid extraction step improves the efficiency of protein extraction from adipose tissue samples. Comparing the chromatograms obtained after an HPLC analysis of the extracted material with both methods for a

representative FAP patient (Figure 1), it is clear that more protein material is obtained with the modified method. This improvement in the extraction method is of extreme importance, since AGEs are present in vestigial quantities. Therefore it is of utmost importance to extract the highest amount of amyloid fibrils with the least amount of contaminants. Amyloid fibrils in FAP are associated with lipids, collagen and amyloid P, all of which may interfere with X-ray diffraction studies. The removal of contaminants does not affect the chemical and structural integrity of the amyloid fibrils [31].

AGEs in amyloid deposits from FAP patients

One of the major consequences of protein glycation is the formation of fluorescent products with characteristic excitation and emission wavelengths [18,19]. The appearance of new fluorescence properties is strong evidence for protein glycation. Methylglyoxal, a potent and ubiquitous glycation agent, forms stable adducts with arginine residues leading to non-tryptophan fluorescence (excitation, 320 nm; emission, 340–500 nm) [16]. Shipanova et al. [24] identified a methylglyoxal-derived AGE (argpyrimidine) as the major fluorescent product ($\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$, 320/385 nm) present *in vivo*. Recently, argpyrimidine was found in spinal cord of familial sporadic ALS patients and mutant SOD-1 mice [25].

Direct HPLC analysis with fluorescence detection at $\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$ 320/385 nm of the extracted material derived from Portuguese-type FAP patients and control subjects (Figure 2) revealed that only the extracted amyloid fibrils from the FAP patients presented a significant fluorescence peak. Despite intensity differences of the fluorescence peak among different patients, it should be noted that protein concentration after extraction differs among samples, so fluorescence intensities are not directly comparable. In contrast, proteins extracted from non-FAP subjects have none or are barely modified. Comparing the chromatographic analysis with fluorescence detection of the controls, there were no significant differences between them. Only one control sample showed a minor fluorescence peak in the same region of the

Figure 2 Comparison of HPLC chromatograms with fluorescence detection $\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$ 320/385 nm, of the proteins extracted from FAP patients (solid line, FAP patient 1; broken line, FAP patient 2) and non-FAP subjects (dotted line)

All samples were submitted to the modified method of extraction. To simplify, just one non-FAP sample and two FAP samples were shown.

chromatogram. All samples were purified by ultrafiltration (5 kDa cut-off membranes) before HPLC analysis, which means that these samples are free from low-molecular-mass contaminants that could interfere with fluorescence analysis.

The differences between fluorescent properties of the extracted material from the control subjects and FAP patients show clearly that amyloid proteins from FAP patients are glycosylated. Considering the excitation and emission wavelengths that produce a measurable signal in the fluorescence detector, argpyrimidine is likely to be present in the proteins extracted from FAP patients.

Amino acid analysis may be used to identify AGEs, since these compounds are actually modified amino acids and therefore additional peaks (from these modified amino acids) will appear in a chromatogram [30]. Dabsyl chloride was chosen as the derivatizing reagent, since it does not interfere with the fluorescence detection. Enzymic hydrolysis of proteins is also essential, instead of the commonly used acid hydrolysis with 6 M HCl, since argpyrimidine is acid-labile. A chromatogram of a standard amino acid mixture and a chromatogram from a representative FAP patient are presented in Figure 3. The chromatogram of the amino acid analysis of the extracted material is substantially more complex with several new peaks appearing. These new peaks could be due to several AGEs, or caused by peptides resulting from incomplete protein hydrolysis. Using fluorescence detection, two major peaks were detected with retention times of 10 and 49 min (Figure 4). The 10 min retention time peak, found in all samples, is due to the derivatization reagent, also observed in a control derivatization assay without the addition of enzymic hydrolysates (Figure 5). In contrast, the fluorescence peak with a retention time of 49 min only appears in the amino acid analysis of the extracted amyloid fibrils from FAP patients. A comparison between this chromatogram and the argpyrimidine chromatographic standard (Figure 6) clearly indicates that the fluorescence peak with a retention time of 49 min is argpyrimidine. Retention time and spectroscopic properties are identical. Moreover, possible interferents, such as pentosidine or oxidation products of aromatic amino acids, have distinct retention times [30]. Chromatographic peaks were also analysed for symmetry and purity with spectral data with 1 nm resolution using the Beckman-Coulter 32Karat software (version 5.0). No shoulders are apparent, and the peaks were judged to be pure.

Figure 3 HPLC amino acid analysis using dabsyl chloride of a standard amino acid mixture (a) and hydrolysed amyloid proteins extracted from a FAP patient by the modified extraction method (b)

Absorbance was monitored at 452 nm. mAU, milli-absorbance units.

Our data clearly demonstrate the presence of AGEs in amyloid deposits characteristic of FAP patients, and the chromatographic methods allowed us to identify argpyrimidine in these deposits. In FAP patients, 152 pmol, 166.7 pmol and 168.5 pmol of argpyrimidine/mg of protein were detected (162.40 ± 9.05 pmol/mg of protein; $n = 3$). In contrast, only 1 pmol of argpyrimidine/mg of protein in just one of the five non-FAP patients analysed was measured. This subject, undergoing surgery for a pancreatic tumour, was the oldest individual analysed. The amount of argpyrimidine quantified in the present study is similar to recent results obtained by others: using an LC (liquid chromatography)-MS based method, Ahmed et al. [32] obtained 205 ± 19 pmol of argpyrimidine/mg of protein in human lens proteins, which are known to accumulate high levels of AGEs.

The Maillard reaction in FAP

Increased AGE formation has been linked to the development of cataracts [33], clinical complications of diabetes mellitus [34], uraemia [35], atherosclerosis [36] and age-related disorders. In amyloid diseases, the formation of AGEs is relevant in Alzheimer's disease [10–12], Parkinson's disease [37] and dialysis-related amyloidosis [13]. In Alzheimer's disease, glycation of β -amyloid peptide promotes the nucleation and precipitation of this peptide, suggesting an additional mechanism by which the Maillard reaction may accelerate the progression of Alzheimer's disease [11].

Figure 4 HPLC amino acid analysis with fluorescence detection at $\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$ 320/385 nm of the material extracted from FAP and non-FAP subjects

Solid line, FAP patient; broken and dotted lines, non-FAP individuals. Inset, magnification of the chromatogram between 46 and 56 min, showing marked differences between the FAP and non-FAP samples. A significant fluorescence peak with the retention time of 49 min appeared only on the FAP sample. A minor fluorescence peak, later quantified as 1 pmol of argpyrimidine/mg of protein (less than 1% of the FAP patient's values), was noted in a sample from just one control subject, who was undergoing surgery for a pancreatic tumour.

Figure 5 HPLC analysis of a control derivatization assay, with no sample added (solid line), amino acid analysis of a FAP sample (broken line) and non-FAP sample (dotted line)

A fluorescence peak with a retention time of 10 min appears in all three assays. Fluorescence detection at $\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$ 320/385 nm.

In FAP, the mechanisms involved in amyloid deposit formation and toxicity are largely unknown. Concerning amyloidogenesis, it has been showed that transthyretin tetramer dissociates to non-native monomeric species under physiological conditions [38]. These non-native monomeric species lead to the formation of partially unfolded monomeric species and high-molecular-mass soluble aggregates (the so-called proto-fibrils). These soluble aggregates will lead to amyloid fibrils by an unknown mechanism. Based on our data and the FAP amyloidogenic model discussed below, we propose that protein glycation could be directly involved in amyloid fibril formation from these soluble aggregates. Indeed, the formation of high-molecular-mass soluble aggregates precedes amyloid fibril formation by several years in FAP

Figure 6 Argpyrimidine in amyloid proteins extracted from adipose tissue samples of FAP patients

Argpyrimidine was detected by HPLC analysis with fluorescence detection at $\lambda_{\text{emission, max}}/\lambda_{\text{excitation, max}}$ 320/385 nm. The argpyrimidine chromatographic standard was treated as the samples from FAP and non-FAP subjects. The amino acid analysis of the FAP sample reveals a fluorescence peak at 49 min (broken and dotted lines) coincident with the argpyrimidine standard (solid line). To simplify, just two of the three FAP samples are shown.

patients. Transthyretin can be detected by immunohistochemical methods in different tissues, years before amyloid fibrils are formed [39]. Similar results, on a different time scale, were obtained with a transgenic rat model overexpressing a particularly aggressive transthyretin mutation [40]. The missing link in amyloidosis models lies in the mechanisms that cause the structural transition from soluble aggregates of partially unfolded proteins to insoluble fibrils. Recent research suggests that glycation of albumin is involved in conformational transitions, inducing partially unfolded intermediary formation, which in turn leads to the aggregation and fibril formation of albumin. A native all- α -motif

protein turns into a cross- β structure characteristic of amyloid fibrils, as shown by Congo Red, Thioflavin T assays and by transmission electron microscopy [41].

Moreover, glycation of soluble protein aggregates can be seen as a fixative process, since, after glycation, it is impossible to reverse the process of amyloid fibril formation. In contrast, the formation of soluble aggregates can be reversed [42]. Therefore extensive glycation could mark the difference between soluble aggregates and toxic amyloid deposits. The current understanding of amyloid diseases indicates that all forms of amyloid share biophysical and biochemical features (most important is the similar structure with an extensive β -sheet), despite the lack of existence of any relevant homology between the different amyloid precursor proteins. This observation suggests that the same process may be involved in amyloidogenesis of different types of amyloid and that this process is common to all forms of amyloidosis. The involvement of protein glycation also explains the formation of amyloid deposits derived from non-mutated transthyretin, as present in SSA.

Amyloid deposits are thought to be the direct cause of cell toxicity in different forms of amyloidosis, as they are present in areas of neurodegeneration. An important observation is the presence of oxidative stress markers co-localizing with amyloid deposits [9,36,43]. In Alzheimer's disease and in FAP, lesions have been correlated with the expression of lipid membrane peroxidation indicative of oxidative stress. Moreover, toxicity could be blocked *in vitro* by catalase, indicating an oxygen-containing free-radical-dependent mechanism [44]. Studies concerning Alzheimer's disease reveal an apoptotic induction by the amyloid deposits *in vivo* and *in vitro* through a mechanism involving the generation of free radicals [45]. The formation of AGE in FAP amyloid deposits can also contribute to cell toxicity and neurodegeneration via oxidative stress. In agreement with this hypothesis, glycated proteins may generate oxidative stress themselves [46] or by the interaction with RAGE [12,47]. Moreover, AGE-modified proteins are toxic to animal cells [17]. So, protein glycation induces cell toxicity due to the production of local oxidative stress. This local oxidative stress formed upon the interaction between AGE and RAGE is responsible for the activation of important transcriptional factors, such as NF- κ B (nuclear factor κ B) and TNF α (tumour necrosis factor α), that could trigger a neuronal inflammatory and apoptotic pathway that is important in FAP neurodegeneration [9,47].

Several studies demonstrated that the tissue targeting of amyloid-induced toxicity is not cell-type-specific, and it has been proposed that local factors might facilitate conformational change of mutated transthyretin, leading to toxic aggregates [44]. We propose that these 'local factors' might modulate the glycation of the proteins in amyloid fibrils or pre-fibrils.

The present work provides new insights into the role of glycation in amyloid deposit formation, since argpyrimidine was identified unequivocally and quantified for the first time in samples from FAP patients, using chromatographic methods. Since argpyrimidine is not one of the major AGEs found *in vivo*, we expect that other AGEs might be present in higher quantities in these deposits. Nyhlin et al. [48] found AGE immunoreactivity in tissue samples rich in amyloid deposits from FAP patients [35]. No particular AGEs were identified or quantified, but these results show that AGEs are indeed present in amyloid deposits in FAP.

So far, the major research in this area was directed to *in vitro* studies, with the production of the fibrils *in vitro*, and mainly concerned structural changes in transthyretin induced by amyloidogenic mutations [49–51]. In contrast, in the present study, we used samples from FAP patients and studied amyloid deposits found *in vivo* directly.

Despite the genetic origin of the disease, glycation should be considered seriously as an additional factor in FAP.

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