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Apolipoprotein A-I: the dual face of a protein

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Conformational plasticity and flexibility are key structural features of ApoAI in lipid metabolism. Amyloidogenic single point mutations, associated with incurable familial amyloidosis with fibril deposition in peripheral organs, may have a dramatic impact on the structural and functional features of ApoAI. Here, the consistent body of data on ApoAI variants has been reviewed, with the aim of highlighting the hallmarks of the pathology. In accordance with our observations, as well as that of others, we propose a model that accounts for the alteration of the delicate balance between lipid-free/lipid-bound dynamic states which is based on monomer-to-dimer interconversion *via* domain swapping.

Keywords: aggregation; amyloidosis; Apolipoprotein A-I; conformational diseases; domain swapping; protein misfolding

The natural protein and its physiological role

Apolipoprotein A-I (ApoAI) is synthesized mainly by the liver and the intestine as a preproprotein. After cleavage of the pre- and pro-peptides, the mature protein (28 kDa, 243 amino acids) is secreted in the plasma as a lipid-poor/lipid-free protein, which is the main acceptor of cholesterol from artery wall cells [1]. It has been demonstrated that ABCA1 mediates the secretion of cellular-free cholesterol and phospholipids to ApoAI, its extracellular acceptor, to form nascent high-density lipoproteins (HDLs) [2–4]. It emerged that HDLs are dynamic entities undergoing remodeling, lipolysis, and fusion that convert small particles to large particles and *vice versa* [4]. ApoAI is the principal component (about 70% of proteins) of HDL, nanoassemblies acting as carriers for cholesterol transfer, *via* the plasma, from the periphery to steroidogenic tissues, and to the liver for catabolism in the so called reverse cholesterol transport [5,6].

A delicate balance between the lipid associated (about 95%) and not associated (about 5%) state of ApoAI accounts for its critical role as a lipid metabolism regulator [7] and as a modulator of atherosclerosis. Although the molecular mechanism of the atheroprotective action of ApoAI, as well as HDL biogenesis, is not fully understood, it is known that ApoAI plays an antiatherogenic action *in vivo*, with a protecting effect against cardiovascular diseases [8], since atherosclerosis of hypercholesterolemic mice lacking ApoAI [9,10] is counteracted by ApoAI transgenic expression [11,12].

Key structural features for ApoAI functions are conformational plasticity and flexibility, based on the presence of 10 tandem of 11/22 residue repeats (H1–H10) which form amphipathic lipid-binding α-helices. Following the first crystal structure of lipid-free human ApoAI, truncated at the N terminus (Δ1–43) [13], a high-resolution (2.2 Å) crystal structure was obtained using a C-terminally truncated (Δ185–243) form of the protein [14] to facilitate crystallization, the C-terminal region being highly unfolded in solution. The structure provided evidence that ApoAI forms a half-circle dimer, in which two elongated antiparallel helices connect two–four-helix bundles (Fig. 1A), each

Abbreviations
AApoAI, amyloidogenic variant of ApoAI; ANS, 8-anilino-1-naphthalensulfonic acid; ApoAI, Apolipoprotein A-I; CD, circular dichroism; DLS, dynamic light scattering; FAP, familial amyloidosis polyneuropathy; HDL, high-density lipoproteins; MD, molecular dynamics.

constituted by the N terminus of one chain (residues 1–121, helices I, II, III) and the C terminus of its partner (residues 143–184, helix IV). However, it has to be pointed out that the only high-resolution crystal structure so far available refers to a truncated form of the protein, and therefore could be different from the real solution form of the natural protein.

ApoAI and amyloidogenicity

Apolipoprotein A-I can be the causative agent of acquired or familial amyloidoses. In the acquired, that is, not inherited amyloidosis, the deposition of wild-type full-length ApoAI occurs in arterial plaques (atherosclerosis) [15,16]. This has to be ascribed to the lower thermodynamic stability of lipid-free ApoAI with respect to the HDL-bound protein. It has also to be highlighted that oxidation of methionine residues of lipid-free ApoAI has been identified as a primary cause of protein destabilization and amyloid deposition in atherosclerotic plaques, where high levels of oxidized lipid-free ApoAI were detected [17,18].

The first analysis of amyloid deposit composition in localized forms of ApoAI-associated amyloidoses provided evidence of the presence of ApoAI-derived N-terminal fragments in aortic amyloid deposits [19]. In the knee joint menisci of patients with knee osteoarthritis, amyloid deposits were found to be mainly constituted by ApoAI produced *in situ* by chondrocytes [20].

Familial amyloidosis polyneuropathy (FAP), instead, is associated with mutations in APOA1 gene. About 20 natural mutations (Fig. 2) were mapped so far as responsible for ApoAI systemic amyloidoses. In patients carrying an amyloidogenic ApoAI variant (AApoAI), amyloid deposition occurs overtime in peripheral organs, such as heart, liver, kidneys, skin, etc. [21].

No cure is available for these pathologies, being organ transplantation the only possible treatment. In AApoAI patients, ApoAI and HDL plasma levels are generally lower than in normal subjects [22,23], although this seems not to be the cause of cardiovascular diseases [24]. ApoAI-decreased plasma levels may be related to the lower amount of secreted amyloidogenic variants with respect to wild-type ApoAI, as demonstrated for L75P-AApoAI overexpressed in stably transfected human hepatic cells [25], as well as for L75P- and L174S-AApoAI variants in transiently transfected COS-7 cells [26].

The amyloidogenic mutations described so far in patients, all heterozygous for the mutated gene, can be divided in two groups: those located within the N-terminal portion of the protein that is found in fibrils ('inside mutations'), and those located externally to this region ('outside mutations') [23] (Fig. 2). Wherever an amyloidogenic mutation is located, internally or externally to the N-terminal region, amyloid fibrils isolated *ex vivo* were found to be mainly constituted by the N-terminal fragments of AApoAI, 80–100 residue long. For instance, G26R point mutation, the first amyloidogenic mutation discovered in ApoAI [27], enhances the formation of fibrils containing ApoAI fragment 1–83, whereas amyloid fibrils extracted from patients carrying the first AApoAI outside mutation identified (L174S) [28,29] were mainly constituted by fragment 1–93. We performed a comparative analysis of the aggregation propensity of eight recombinant isoforms of ApoAI 1–93 polypeptide, each carrying a known amyloidogenic mutation of the N-terminal region [30]. The data supported the hypothesis that 'inside' amyloidogenic mutations might enhance protein

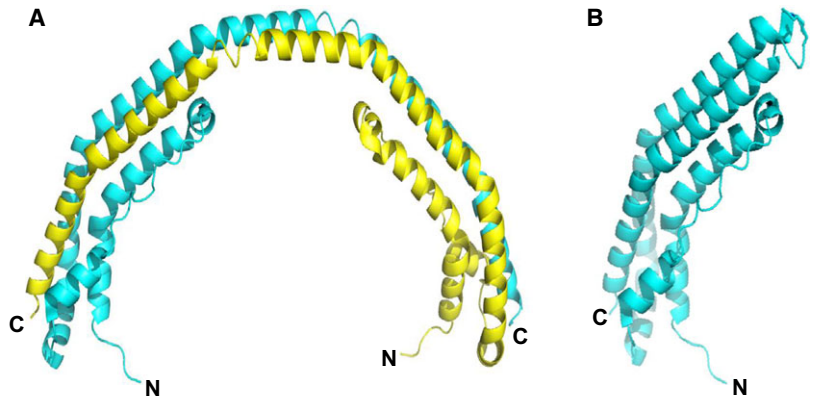


Fig. 1. (A) Ribbon representation of the crystal structure of lipid-free Δ185–243 human ApoAI dimer (PDB code 3R2P). (B) A model proposed for the monomeric structure, as suggested by Mei and Atkinson [14].

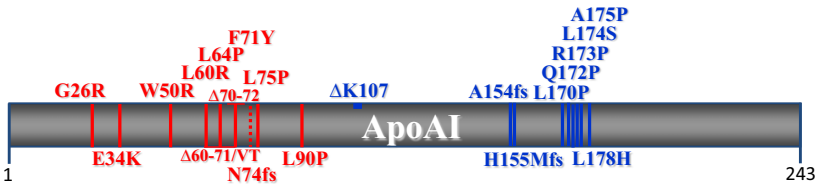


Fig. 2. ApoAI mutations associated with systemic amyloidoses. In red, the mutations located within the N-terminal fibrillogenic region ('inside mutations'). In blue, the 'outside mutations'. Δ, deletion mutations; fs, frameshift mutations.

flexibility in the proximity of the putative cleavage site releasing the N-terminal amyloidogenic domain [30], consistently with Lagerstedt *et al.* [31]. We also provided evidence that an increased aggregation propensity of isoforms of the N-terminal polypeptide is not causative *per se* of amyloidogenesis [30].

Hallmarks of a pathogenic protein: impact of mutations on protein conformation

Nothing is known about the molecular mechanism responsible for the release *in vivo* of a fibrillogenic polypeptide from a full-length AApoAI variant, nor in which context it occurs. In such an uncertain scenario, any structural or functional difference between the natural protein and a pathological version of it would represent an important clue to reveal mechanistic aspects of the disease.

Interestingly, the majority of the amyloidogenic mutations identified so far cluster in two regions: the amino-terminus (residues 1–100) and within residues 170–178, which in ApoAI 3D structure are located in the four-helix bundle domain and define a 3D hot spot mutation site [14]. Based on sequence analyses, four hot spots, corresponding to regions 14–22, 53–58, 69–72, and 227–232, have been predicted [32]. Amyloidogenic mutations occurring in these regions were proposed to perturb the protein structure prompting aggregation *via* the N-terminal region [32]. With the exception of 53–58, these regions are mainly in α-helical structure, with region 227–232 representing the primary lipid-binding site of the protein. Noticeable is that ApoAI fibrillogenic polypeptides (residues from 1 to 80–100) encompass all of the N-terminal predicted hot spots. To date, from the analysis of some of the naturally occurring full-length AApoAI variants identified so far, for example, G26R, W50R, F71Y, L75P, A164S, L170P, R173P, L174S, and L178H, interesting peculiarities of their conformational behavior have emerged.

Two hypotheses have been raised on the chronology of the events occurring during fibrillogenesis. In one case, ApoAI destabilization induced by amyloidogenic mutations allows the cleavage of the full-length protein and the release of the fibrillogenic polypeptide with consequent aggregation in fibrils [23,29–31,33,34]. Alternatively, it has been hypothesized that destabilization of AApoAI four-helix bundle promotes aggregation of the full-length variant, an event that precedes the proteolytic cleavage releasing the N-terminal region [32].

The elucidation of any structural differences between AApoAI variants and the natural protein may be fundamental to elucidate molecular determinants that make a protein amyloidogenic. From the large body of data reported on amyloidogenic variant behavior with respect to natural ApoAI, a picture emerges indicating that the amyloidogenic phenotype is associated with altered structural features, which are reported below grouped into four main categories.

Effects of mutations on protein conformation and lipid binding

Generally, amyloidogenic mutations induce protein disorder, as demonstrated by CD analyses. Variants F71Y-AApoAI and L170P-AApoAI showed a decreased CD signal at 185–200 nm [32]; similar effects, although much less evident, have been described for G26R and W50R mutations [32,35]; in the case of L75P-AApoAI and L174S-AApo-I, an increased percentage of β-sheet structure has been detected [36]; G26R, W50R, and F71Y mutations cause marked changes in near-UV CD, while L170P substitution is associated with the largest change with more negative CD signals at 255–280 nm. Moreover, studies by amide hydrogen–deuterium exchange demonstrated that the G26R mutation destabilizes the protein through the large region spanning residues 10–114, causing changes in protein structure and dynamics [37]. As a consequence, the N-terminal helix unfolds, inhibiting interactions within the N-terminal helix bundle, which is destabilized.

Which are the consequences of an altered conformation? One should consider that all the above mentioned residues contribute in the native protein to the

four-helix bundle stability. In particular, residue L75 is located in the middle of the four-helix bundle, within the short helix 70–76 that contributes to define the relative helical orientation in the bundle; G26 and W50 are also located in the middle of the bundle [38], whereas F71, L170, and L174 are located at the 'bottom' of it [32]. Therefore, it is conceivable that, associated with these mutations, and as a consequence of the altered conformation, hydrophobic regions are more exposed to the solvent than in the native protein, as it has been described for L75P-AApoAI and L174S-AApoAI, with a consequent increase in tryptophan and 8-anilino-1-naphthalensulfonic acid (ANS) fluorescence emission [36]. A slight increase in ANS emission was detected also for G26R-, W50R-, and F71Y-AApoAI [35]. On the other hand, for L170P-AApoAI a decrease in ANS emission was described, possibly due to an increased order in the C terminus, a potential ANS-binding site. This is in line with a significantly decreased deuteration of L170P-AApoAI in its C-terminal tail (see below).

Generally, mutations do not promote dissociation of the free protein from HDL [32], nor do they impair the protein ability to bind to model phospholipids generating HDL-like complexes of unaltered size. However, mutations may decrease the lipoprotein stability [35]. To this regard, G26R and W50R mutations slightly reduce protein ability to recruit lipids to form HDL [35], whereas larger destabilization occurs in the case of the 'outside' mutation L170P. Mutation L178H is responsible for an altered lipid-binding profile [39]; similarly, R173P-AApoAI shows lower efficiency to bind to phospholipid vesicles [40] and A164S-AApoAI has lower binding affinity to lipids [41].

Effects of mutations on protein flexibility and stability

A generally decreased compactness is described for AApoAI variants. This is at least in part related to an increased flexibility of the protein chain, accompanied by a reduced stability and loss of unfolding cooperativity, as it has been observed for several AApoAI variants [32,35–37]. Importantly, it has been pointed out [35] that destabilization is not necessarily associated with misfolding, as demonstrated by some destabilized, but not amyloidogenic, variants (e.g., Milano variant) [42].

The DLS measurements of the hydrodynamic diameter indicated that L75P-AApoAI is much less compact than the wild-type protein and L174S-AApoAI. Upon 7 days of incubation at 37 °C, L75P-AApoAI and L174S-AApoAI diameter increased by about 50% and 44%, respectively [14]. Mutation-driven protein destabilization occurs at different degrees, being less evident in case of G26R-AApoAI and W50R-AApoAI variants (−5 °C Tm), and more evident for L170P-AApoAI (−11 °C Tm), L174S-AApoAI (−12 °C Tm), and L75P-AApoAI (−14 °C Tm) [31,32,35,36].

Again, a direct relationship between destabilization and aggregation propensity cannot be envisaged. Hydrogen–deuterium exchange studies revealed that G26R mutation greatly increases solvent accessibility and dynamics at the N terminus (first 90 residues), as reported above [37]; L170P-AApoAI is less protected from exchange at the major hot spot 14–22 among the variants tested [35]. Mutations F71Y and W50R have little effects on the C-terminal deuteration, while mutation L170P induces a significant decrease in deuteration, probably due to a more extensive packing of the protein tail against the helix bundle, as reported above.

Effects of mutations on susceptibility to proteases

It is conceivable that, as a direct consequence of the decreased compactness and increased flexibility, susceptibility to proteases increases.

Enhanced susceptibility to protease cleavage has been demonstrated in the N-terminal region of L75P-AApoAI and L174S-AApoAI [36], to be related to N-terminal destructuration and disorder. Exposure of a proteolytic site at Y18, belonging to the major hot spot 14–22, was revealed in variants G26R-, L75P-, L174S-, and L178H-AApoAI [31,36,39], while, in the case of L174S-AApoAI, cleavages at R153 and Y166 occur, as expected from MD simulations experiments which indicated that region 153–166 is unstructured [36]. Similarly, in L75P-AApoAI the accessibility of proteolytic sites at E34 and F57 (the latter belonging to the minor hot spot 53–58), both hindered in the native protein, is the consequence of the propagation of a local perturbation (helix 70–76) to the other predicted hot spots and the cause of the decreased protein compactness.

Moreover, some mutations (e.g., L170P and G26R) cause a disorder in the α-helical segment 83–93 by possibly exposing a proteolytic site that might be involved in the release of the N-terminal region, while other mutations (e.g., F71Y and W50R) do not [32]. Similarly, in L174S-AApoAI, the C terminus seems to be protected, as shown by the disappearance of the E223 cleavage site (exposed in the native protein and in L75P-AApoAI), as well as by slower hydrolysis kinetic at R188 and Y192 residues [36], thus suggesting that conformational changes occurred in this region.

Effects of mutations on aggregation propensity

A time-dependent increase of the β-sheet structure in L75P-AApoAI and L174S-AApoAI has been reported [36], similarly to G26R-AApoAI [39], whereas for L178H-AApoAI an increase in the α-helical content has been described over time, accompanied by the formation of very short fibrillar structures [39]. Beaded morphology of L75P-AApoAI fibrils well correlates with the lower protein compactness, whereas the more compact structure of L174S-AApoAI, combined with its high flexibility, is in agreement with the formation of thinner and longer fibrils.

Common features of amyloidogenic mutations effects

A key feature of ApoAI is its conformational plasticity and flexibility that make possible a prompt adaptation of the protein to different environmental conditions. ApoAI is, therefore, an optimal flexible scaffold for lipidation/delipidation and cholesterol circulation, since, due to its molten globule state, the protein is able to easily switch from the presumed monomeric state in the absence of lipids (about 5% of total ApoAI) to an extended double belt-like conformation in the lipid-bound state, with antiparallel α-helices wrapped around the edge of nascent HDL. On the other hand, the high protein flexibility is a key element involved in pathological conditions, when an amyloidogenic mutation alters the protein architecture, switching the dynamic state of the native protein toward a 'pathology-competent state'.

A wide range of effects can be ascribed to point mutations. From a general point of view, the effects of mutations can be summarized here as follows: (a) induction of protein disorder with loss of protein compactness; (b) destabilization of the N-terminal helix bundle; (c) exposure of hydrophobic regions to the solvent; (d) increase in protein flexibility and consequent susceptibility to proteases; (f) altered lipid-binding profile.

The model recently proposed by Das *et al.* [32] prompts that AApoAI amyloidogenic mutations perturb one of the four predicted hot spots of the protein (14–22, 53–58, 69–72, and 227–232), with a consequent full-length protein aggregation *via* the N-terminal region. In particular, mutation-driven perturbations of the native structure would allow the exposure of the major amyloid hot spot 14–22, normally buried in the middle of the four-helix bundle, triggering protein misfolding and aggregation. Thus, the paradigm proposed is the following: conformational perturbation—exposure of hot spots—protein aggregation.

However, some further considerations may be added. First, a question can be raised: from the data available so far on AApoAI variants can a more general rule be drawn to classify the effects of mutations on protein misfolding? To this regard, on the basis of experimental evidence, it is tempting to propose that 'inside' and 'outside' mutations have a different impact on the protein, with internal mutations destabilizing preferentially the N-terminal region, and significantly altering protein conformation and compactness, while external mutations, besides determining N-terminal destabilization, increase the overall protein flexibility and induce a more pronounced aggregation-competent state. As prototypes of internal and external mutations, respectively, L75P-AApoAI is less compact, while L174S-AApoAI is more flexible. In the natural protein, the flexible C-terminal tail has low protection as it interacts weakly with the helix bundle. In the presence of external mutations (mainly clustered in the region 170–178), the C-terminal tail is more protected and less exposed, as demonstrated by L170P-AApoAI lower deuteration kinetics, as well as by L174S-AApoAI and L178H-AApoAI lower susceptibility of this region to proteases with respect to the natural protein. Based on the observation that L170P mutation greatly reduces protection from hydrogen–deuterium exchange in the 83–93 region [32], it has been hypothesized that an increased disorder in the well-ordered α-helical segment 83–93 may facilitate proteolytic cleavage(s) in AApoAI variants.

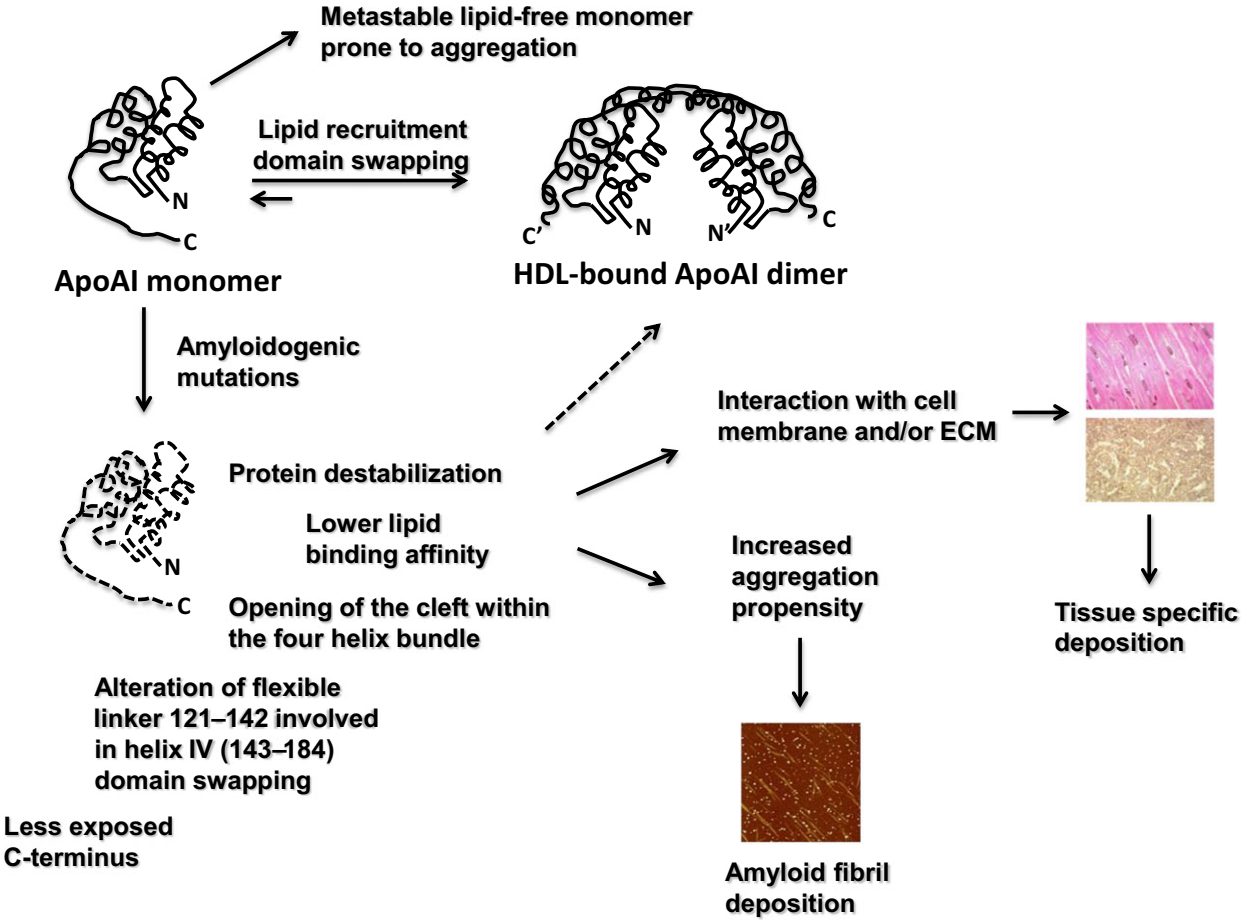
A second consideration emerges from the model proposed by Das *et al.* [32], suggesting that a mutated protein is *per se* endowed with special molecular features that make it amyloidogenic. At the same time, intriguing but fully unexplained so far is the observation that for some AApoAI variants a preferential fibril deposition in certain tissues occurs. As an example, variant L75P-AApoAI is associated with a hereditary systemic amyloidosis characterized by preferential accumulation of fibrils in kidneys and liver [43]. Instead, in the case of L174S-AApoAI, a predominant accumulation of fibrils in heart, skin, testes, and larynx was observed [21]. The molecular bases of the differential localization of amyloid fibrils are is still unknown. Different environments (e.g., composition of cell membrane, extracellular matrix, soluble molecules) might promote the unfolding/aggregation pathway of a particular variant. If so, the physiological context would be crucial and might play a key role to determine the still unclear phenomenon of tissue specificity of fibril deposition described for some variants.

A possible model

It has been proposed that, following binding to lipids, ApoAI dimerization occurs *via* the opening of the N-terminal four-helix bundle and the displacement of the C-terminal region toward a second monomer and *vice versa* to form an antiparallel helical 'double belt' on the HDL surface [14,44–46].

As monomers are metastable, they might switch from a nonswapped to a swapped conformation, with consequent dimerization, so that domain swapping could be a natural consequence of the metastability of the interconverting species. It is tempting to speculate that such a flexible, aggregation prone protein, as it is wild-type ApoAI, may escape aggregation *via* lipid binding, which would promote dimer formation through domain swapping.

In the model we propose (Scheme 1), lipids facilitate monomer-to-dimer conversion, while AApoAI mutations, perturbing the natural protein conformation (dotted line structure in Scheme 1), alter its propensity to dimerize, modifying consequently the physiologic dynamic equilibrium between the interconverting species. In case of an altered equilibrium between lipid-bound and lipid-free ApoAI, not efficiently balanced by ApoAI lipid-free clearance, the risk of aggregation would dramatically increase. Indeed, studies on a recombinant N-terminal fibrillogenic polypeptide (residues 1–93) demonstrated the effects of cholesterol, as well as of zwitterionic, positively and negatively charged liposomes in trapping the polypeptide in a helical state, preserving it from aggregation [47]. A similar effect of the lipid environment has also been described for other amyloidogenic proteins, for



Scheme 1. Schematic representation of the monomeric and dimeric state of ApoAI. The conversion from monomer to dimer is facilitated by lipid binding and achieved by domain swapping of the C-terminal tails between two ApoAI chains. Amyloidogenic mutations alter structural and functional parameters of the protein, deviating the monomer-to-dimer balance toward unstable forms of the monomer prone to aggregation. We hypothesize that a key factor responsible for tissue-specific deposition of ApoAI amyloidogenic variants is their interaction with cell membrane and/or extracellular (ECM) matrix components.

example, for α-synuclein, a protein with an apolipoprotein lipid-binding motif [48].

To accomplish domain swapping, the C-terminal region of a monomer has to displace from its initial position and to shift toward the partner molecule by breaking the intramolecular interactions within the four-helix bundle and the reestablishing of similar contacts with the N-terminal α-helices belonging to the partner molecule. A lower exposition of the C-terminal region to the solvent, as it has been found for L170P-, L174S-, and L178H-AApoAI, could impair protein interaction with lipids, and/or the C-terminal displacement required for domain swapping, that is, it could impair monomer-to-dimer conversion.

The alteration of protein conformation due to amyloidogenic mutations is able to propagate to the far sites of the protein *via* the central hinge region (repeat H5) that allows the interconversion between the monomer and dimer. Therefore, a potentially key element is expected to be the structural organization of the central linker 121–142, and possibly its flanking regions, which is highly flexible and probably facilitates the domain swapping of helix IV (residues143–184) of the four-helix bundle during monomer-to-dimer conversion. In this regard, partial exposure to proteases of L75P-AApoAI at residue K96, and of L174S-AApoAI at residue Y115 [36], as well as the destabilization of region 81–115 in AApoAI-G26R, which allows proteolysis at residue 83 *in vivo* and consequent release of a fibrillogenic polypeptide [34,37], could be clues of the altered propensity to undergo monomer-to-dimer conversion. Dynamic simulation studies on the nonamyloidogenic ApoAI variants, Milano and Paris [49], in which an arginine residue at position 173 and 151, respectively, is mutated into a cysteine, suggest that in the lipid-bound protein the two antiparallel strands of the central linker undergo reorganization by a rotationally circular motion of the two monomers to find proper registration, thus supporting the intrinsic protein flexibility.

We hypothesize that in a cellular environment the interactions of the protein with lipids may play a critical role in impeding, or in slowing down, the fibrillogenic process by entrapping the protein in a stable helical structure unable to aggregate. Therefore, the occurrence of 3D-domain swapping seems to have a physiological significance. Membrane and extracellular matrix components may regulate this interconversion, altering the balance between aggregated and unaggregated states of the protein. The impact of a different membrane and/or extracellular matrix composition in different tissues on protein aggregation evokes the fascinating hypothesis that the combination of a particular conformational state (conformer) of the protein, as well as its interaction with a particular class of lipids, may be key elements to explain the tissue-specific deposition observed for ApoAI amyloidogenic isoforms.

In this scenario, the destabilization of protein hot spots, although a central element for the comprehension of ApoAI amyloidogenesis, would be a condition certainly necessary, but not *per se* sufficient to trigger amyloidosis, as the recognition of specific molecules (possibly lipids) on different cell types and in different cell contexts could be decisive to make particular cell types the natural target for the development of the pathology.

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Author contributions

All authors conceived and wrote the review.

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