

Structural characterization of atrial natriuretic peptide amyloid fibrils from patients with atrial fibrillation

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Isolated atrial amyloidosis (IAA) is a localized cardiac disorder characterized by atrial natriuretic peptide (ANP) amyloids deposition in the atria, linked to aging and atrial fibrillation (AF). While monomeric ANP regulates blood pressure, its dimeric form is associated with cardiovascular conditions, including AF. The mechanistic link between ANP aggregation, IAA, and AF remains unclear. Here, we present the first high-resolution structural characterization of ANP fibrils extracted from AF patients, revealing two distinct fibril polymorphs. Both present covalent ANP dimers as building blocks but diverge in their structural architecture: one features antiparallel dimers stabilized by a single disulfide bond, while the other consists of parallel dimers bridged by two interchain disulfide bonds. These fibril morphologies were conserved across patients, suggesting a common aggregation mechanism in IAA. Overall, our findings ascribe to dimeric ANP a critical role in amyloid formation, offering promising directions for earlier detection and treatment of IAA.

Isolated atrial amyloidosis (IAA) is a localized form of amyloidosis characterized by the deposition of atrial natriuretic peptide amyloid fibrils (ANP) within the atria of the heart¹. Unlike systemic amyloidoses, which involve widespread amyloid deposition in multiple organs, IAA is confined to the atria and does not affect other tissues^{1,2}. IAA is primarily associated with aging and is frequently observed in the elderly^{3,4}. It is caused by the aggregation of ANP, a hormone produced by atrial cardiomyocytes⁵. ANP peptide (28 aa) can exist in three different forms: prepro-ANP, a 151 aa long protein; the pro-ANP, a 128 aa long polypeptide which is stored in the atrial granules; and ANP, the 28 aa long peptide which is the maturation

product of corin proteolytic cleavage of pro-ANP⁶. Mature ANP has a 17-amino acid ring linked by a disulfide bond between its two cysteine residues (Cys7 and Cys23), which is pivotal for the biological activity of the hormone⁶. ANP plays a crucial role in regulating blood pressure and fluid balance, by promoting renal excretion of sodium and water⁷. Additionally, in the event of heart failure or cardiovascular disorders, an antiparallel dimeric form of ANP (β -ANP) is found in circulation^{8,9}. Although the origin and mechanism of formation of β -ANP are still unknown, it has been reported that this form has a reduced physiological activity compared to monomeric ANP and may act as a trigger for aggregation *in vitro*⁸.

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Patients with IAA are known to have a higher susceptibility to atrial fibrillation (AF), an arrhythmia characterized by rapid and irregular beating of the atrial chambers of the heart^{3,10,11}. The potential role of IAA in the development of cardiac arrhythmias in the elderly is not yet fully understood, but a correlation has been observed between the incidence and severity of IAA in cardiac conditions and elevated ANP plasma levels, both monomeric and dimeric β -ANP forms^{12–14}. Indeed, deposition of ANP fibrils in the atria leads to structural remodeling of the atrial myocardium and, importantly, to the disruption of normal electrical conduction, which may be at the basis of the symptomatology observed in AF^{15,16}. Similarly, the constant stress placed on the atrial wall by persistent AF leads to overproduction and hypersecretion of ANP, whose local concentration increases dramatically promoting self-aggregation and eventually fibril deposition^{14–16}.

Although recent insights into the structural organization of amyloid deposits in cardiac tissues have advanced the understanding of fibrils' role in various cardiac amyloidoses^{17–23}, the mechanisms driving ANP aggregation and fibril formation in IAA remain unclear¹⁵. To try to shed light on ANP aggregation mechanisms and to pave the way for future studies aimed at correlating AF and IAA, we performed a structural characterization of ANP amyloid fibrils extracted from left atrial appendage of three AF patients. Our high-resolution analysis of amyloids extracted from one AF patient showed the presence of two distinct polymorphs of ANP fibrils, both characterized by the presence of covalent ANP dimers as amyloid building blocks. The two amyloid polymorphs differ both in the overall amyloid fold as well as in the building subunits, *i.e.* dimeric ANP, one polymorph is formed by anti-parallel dimers stabilized by a single disulfide bond (Cys7 – Cys23) while the other one is built by parallel dimers stabilized by two disulfide bonds (Cys7 – Cys7 and Cys23 – Cys23). We extended our analysis to two additional AF patients and observed a similar fold for both polymorphs, thus suggesting a common structural organization of ANP amyloids in IAA patients. In summary, we report here high-resolution reconstructions of the two preferred morphologies of ANP fibrils, revealing the crucial pathological role of dimeric ANP in promoting protein aggregation in atrial tissues.

Results and discussion

Isolation and characterization of ANP amyloid fibrils from patients

To gain insights into the structural organization of ANP amyloid fibrils *in vivo*, we characterized amyloid aggregates extracted from the left atrial appendage of three patients, hereafter named Pt1, Pt2, and Pt3, diagnosed with AF and undergoing open heart surgery requiring cardiopulmonary bypass (Supplementary Table 1). Histological analysis of atrial tissues showed positive staining for Congo red, consistent with extensive extracellular amyloid accumulation (Supplementary Figs. 1a, b, d, e, g, h). The presence of ANP within these deposits was confirmed through immunohistochemical analysis for all three patients (Supplementary Figs. 1c, f, i). Notably, ANP immunoreactivity was markedly elevated in regions containing Congo red-positive material, further supporting ANP's association with amyloid deposits (Supplementary Fig. 1).

Upon extraction from atrial tissues, amyloid fibrils were analyzed by liquid chromatography–tandem mass spectrometry (nLC–MS/MS). In all analyzed samples, human ANP (UniProt entry P01160) was among the top-scoring identifications. In Pt2, peptides mapped to residues 127–151 of the precursor protein, corresponding to residues 4–28 of the mature peptide (Supplementary Table 2 and Supplementary Fig. 2). Similarly, Pt1-derived fibrils contained peptides matching the mature ANP sequence; however, additional peptides corresponding to the pro-ANP region were also detected, suggesting the presence of some uncleaved precursor proANP within the amyloid deposits (Supplementary Fig. 2). Due to limited sample availability, nLC–MS/MS analysis could not be performed for Pt3.

Cryo-EM reconstruction of patient 2 amyloid fibrils

Amyloid fibrils were extracted from atrial appendage of Pt2 and cryo-EM data were collected at 300 kV Titan Krios (Supplementary Table 3). Visual inspection of the recorded images revealed two distinct fibril morphologies. The apparently dominant amyloid architecture, that corresponded to about 75% of fibrils did not display an evident helical organization likely the result of fibrillar coating (Fig. 1a). Conversely, the second morphology, instead, accounting for the remaining 25%, exhibited an evident helical periodicity, with measurable cross-over and width (Fig. 1d). Fibril manual picking and subsequent two-dimensional (2D) classification corroborated the existence of two distinct fibrillar populations (Supplementary Figs. 3, 4). For both polymorphs, the 2D class averages showed a clear 4.7 Å spacing (Supplementary Fig. 4c, f), typical of amyloid structures²⁴. Upon 2D classification, the two polymorphs were processed independently. After initial model generation from 2D class averages, 3D classification resulted in two predominant 3D classes showing two different fibril structures, termed hereafter PolA, the coated polymorph, and PolB, the naked polymorph (Supplementary Figs. 3, 6). The corresponding reconstructions were refined to spatial resolutions of 2.9 Å for PolA and 3.3 Å for PolB (Supplementary Table 4 and Supplementary Fig. 3), based on the 0.143 Fourier shell correlation (FSC) criterion (Supplementary Fig. 3)²⁵. Their local resolution varied in the fibril cross sections, with higher resolution occurring at the fibril center and lower resolution towards the edges (Supplementary Fig. 5). Both polymorphs were reconstructed with a pseudo-2₁ symmetry, with PolA presenting a twist value of 178.9° and a rise value of 2.35 Å, while PolB having a twist of 179.5° and a rise of 2.36 Å (Supplementary Fig. 3 and Supplementary Table 4).

PolA is built by an anti-parallel dimeric ANP with a single disulfide bond

PolA is formed by an anti-parallel dimer of ANP stabilized by a single disulfide bond. Mass spectrometry analysis (Supplementary Fig. 2) confirmed the ANP sequence, allowing unambiguous modeling of the entire PolA structure. The model exhibits strong map-model agreement, supported by 2D class averages and map projections (Fig. 1, Supplementary Fig. 5A–F, and Supplementary Table 4), validating its physical consistency within the reconstructed map. The fibrils consist of two twisted pair of protofilaments, each formed by stacks of covalent ANP dimers, and adopting the cross- β architecture characteristic of amyloid fibrils (Fig. 1a–c)²⁴. The two pair of protofilaments are related by pseudo-2₁ symmetry, resulting in identical cross-sectional organization. Within each pair of protofilament, the two chains are identical and related by a local 2-fold rotational symmetry. Residues 5–28 of ANP could be modeled in the density, indicating that only the first four residues of mature ANP lie outside the rigid amyloid core (Figs. 1c, d, 2a). In the dimeric arrangement, the two monomers adopt an anti-parallel conformation, with the C-terminal region of one chain facing the N-terminal region of the other (Fig. 2b). Each monomer contains two β -strands: the N-terminal β 1 extends between residues Ser6–Gly9, and β 2 is spanning residues Ile15 to Gly20 (Fig. 2a). A steric zipper motif stabilizes the dimer interface, mediated by the two anti-parallel β -strands β 2 (Figs. 2b, 3a, b). This motif is stabilized by hydrophobic (Ile15, Ala17, Ser19, Leu21) and hydrophilic (Ile15, Gly16, Ala17, Ser19) interactions, forming a compact fibrillar core (Fig. 3a, b). A single disulfide bond links Cys7 of one monomer to Cys23 of the other (Figs. 1d, 2b). Notably, the two remaining Cys residues do not form a disulfide bond, as evidenced by discontinuous density in the map (Fig. 1d). Similar to the polypeptide forming other *ex vivo* amyloid structures^{18,22,23}, the ANP dimer is not planar along the fibril axis (z-axis): while the *termini* close to the disulfide bond lie on the same plane, the distal *termini* are distanced 14.5 Å along the z-axis (Fig. 2c). This arrangement positions the Cys7 of the dimer on layer (i) facing Cys23 from a neighboring dimer on layer (i-1); thus, the presence of the

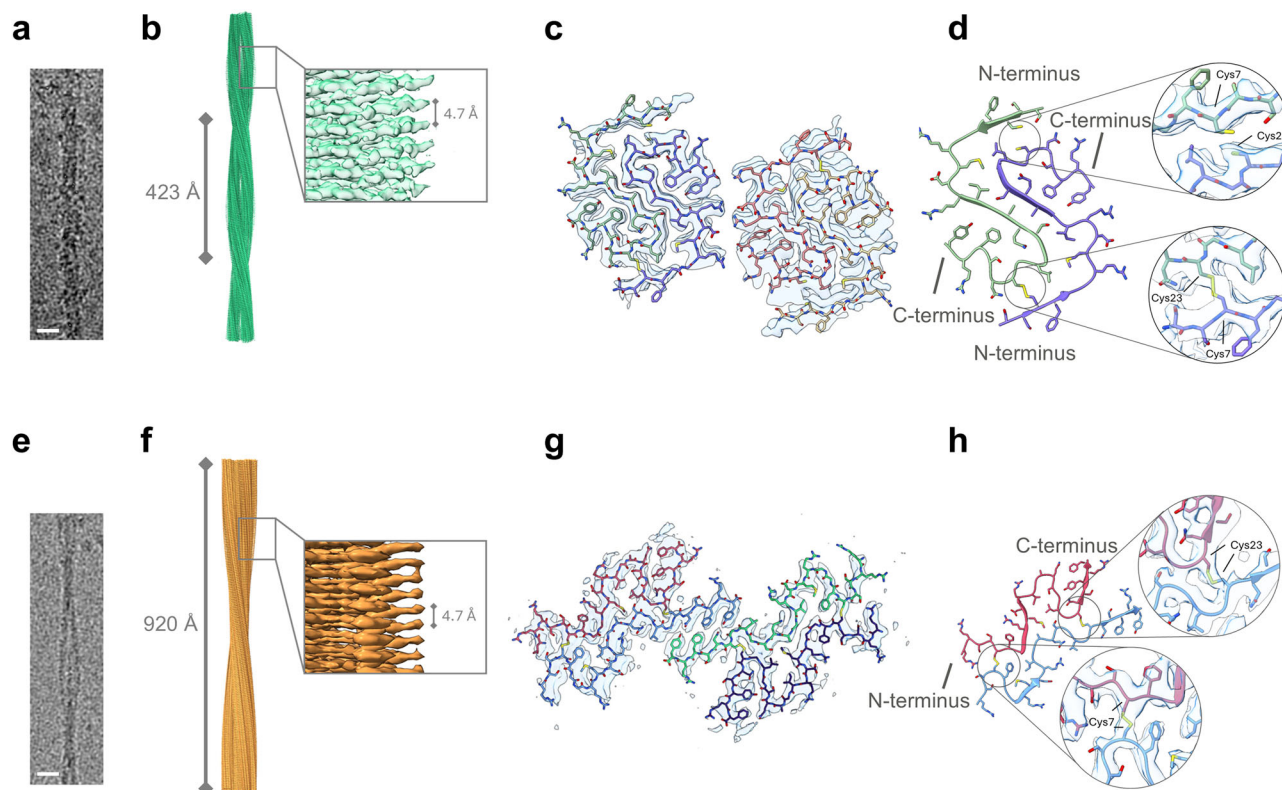


Fig. 1 | Cryo-EM structures of ex vivo ANP fibrils. **a** Representative coated fibril image (Scale bar 10 nm). **b** Side-view of the reconstructed PolA 3D map, with a zoom-in highlighting the 4.7 Å distance between layers. **c** Cross-section of the PolA 3D map (cyan) overlaid with the atomic model. **d** Structure of dimeric ANP in PolA with zoomed insets showing Cys residues forming or non-forming disulfide bond, together with a continuous or non-continuous potential map. **e** Representative

naked fibril image (Scale bar 10 nm). **f** Side-view of the reconstructed PolB 3D map, with a zoom-in highlighting the 4.7 Å distance between layers. **g** Cross-section of the PolB 3D map (cyan) overlaid with the atomic model. **h** Dimeric ANP in PolB with zoomed insets showing Cys residues forming disulfide bonds, as confirmed by the continuity of the potential map.

second disulfide bond would covalently link ANP dimers making each pair of protofilament a covalent polymer (Fig. 2C). In PolA, the interactions between the two pair of protofilaments are mediated primarily by an ionic interaction between Arg14 and Asp13, resulting in binding interface of $\sim 40 \text{ Å}^2$ (Fig. 3c).

PolB is built by a parallel dimeric ANP with two disulfide bonds

Like PolA, PolB consists of two twisted pair of protofilaments connected by pseudo- 2_1 symmetry. However, each pair of protofilament contains two structurally distinct chains forming a parallel asymmetric ANP dimer, contrasting with the symmetric anti-parallel ANP dimers observed in PolA (Fig. 2a–c). The density allowed the modeling of residues 3–28, nearly covering the entire mature ANP sequence (Fig. 2a). Within each monomer, two distinct β -strand are present, one spanning from residue Gly10 to Arg14, while the second covering the last 5 residues (from Asn24 to Tyr28) (Fig. 2a). However, unlike in PolA, in PolB ANP dimers lack an internal steric zipper motif, resulting in a less compact fibrillar core. Intra-dimer interactions are predominantly non-polar, involving Phe8, Ile15, Ala17, and Gln18 from one monomer and Gly9 and Met12 from the other (Fig. 3d, e). Nevertheless, the fibril fold is further stabilized by polar backbone contacts, including Gly9–Gly16 and Gly22–Ser19, and a hydrogen bond between side chains of Arg3 and Ser6 (Fig. 3d, e). Dimer stability is also secured by two disulfide bonds (Cys7–Cys7 and Cys23–Cys23) between the ANP monomers, which are supported by continuity in the potential map (Fig. 1h). The presence of two disulfide bonds per ANP dimer results in a flat arrangement of the dimer along the z-axis, placing all residues belonging to the dimer on the same fibrillar layer (Fig. 2e). PolB displays a more extended contact surface between the two pairs of

protofilament compared to PolA: a steric zipper motif stabilizes the interface, where the C-terminal β -strands $\beta 2$ create a tightly packed set of interactions via both polar and non-polar contacts through residues Asn24, Phe26, and Tyr28 (Fig. 3f).

PolA and PolB: ANP dimer organization defines different amyloid structures

Both ANP fibrillar assemblies here described present two pair of protofilaments whose building block is dimeric ANP (Fig. 2). In both polymorphs, the two pair of protofilaments are structurally identical and related by pseudo- 2_1 symmetry (Fig. 1c, g and Supplementary Fig. 3). However, the different chemical nature of the covalent ANP dimers results in two fully different amyloid structures (Fig. 2). At the secondary structure level, this is reflected in significant differences in the length and position of β -strands between the two polymorphs (Fig. 2a). While in PolA a steric zipper motif by the two facing strands $\beta 2$ contributes to the formation of a compact dimer, PolB lacks such intra-dimer motif and the interactions between the two monomers within the covalent ANP dimers are rather limited to the two disulfide bonds. While the antiparallel ANP dimers forming PolA consist of two structurally identical protofilaments, PolB lacks this symmetry, resulting in two non-equivalent ANP monomers within the dimer (Fig. 3). Despite these differences, both polymorphs rely on similar hydrophobic residues to stabilize the dimeric interface. In PolA, interactions - both polar and non-polar - are primarily mediated by residues belonging to strand $\beta 2$, notably Met12, Ile15, Ala17, Ser19, and Leu21, resulting in an extensive interface (Fig. 3). In PolB, a comparable core is involved, but with a lower overall compactness and number of contacts (Fig. 3d, e). Indeed, the dimeric interface in PolA is more

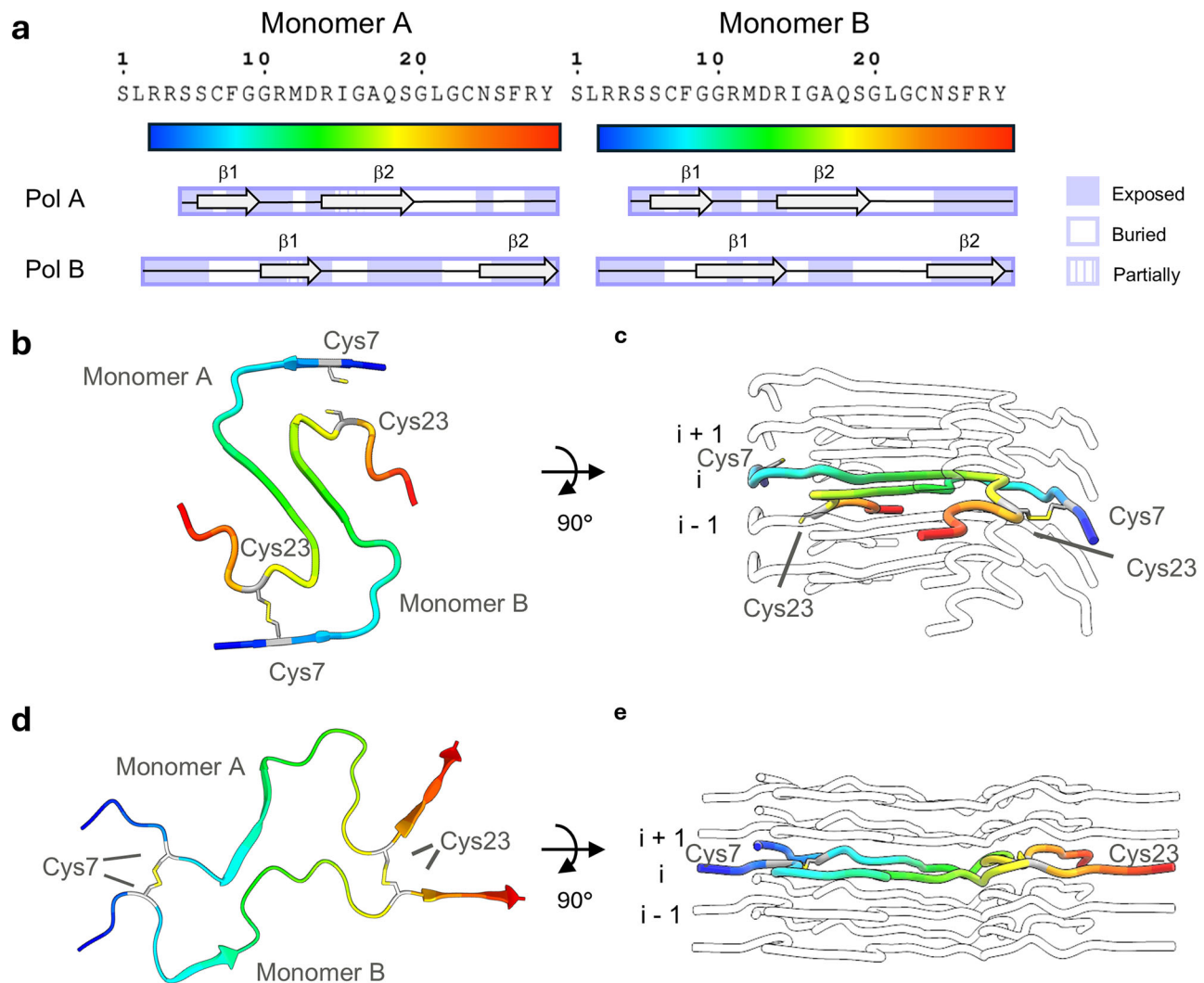


Fig. 2 | Secondary structure and disulfide bond in ANP PolA and PolB fibrils. **a** Sequence of two ANP chains (Monomer A and B) as found in each layer of the pair of protofilaments in both PolA and PolB. Below the sequence a schematic representation of the secondary structural elements color-coded according to residue exposure to solvent. **b** Ribbon diagram of ANP dimer as folded in PolA fibrils. Each monomer is rainbow colored from N- to C- terminal regions, Cys residues are shown in gray. **c** Side-view of the one protofilament model comprising five dimeric

subunits of PolA. Layers are indicated by i , $i + 1$ and $i - 1$. Each ANP dimer spans two layers. Cys residues are labeled and colored in gray. **d** Ribbon diagram of a single cross-section of PolB fibril with Cys residues labeled and colored in gray. Each monomer is rainbow colored from N- to C- terminal regions. **e** Side-view of the one protomer model comprising five subunits of PolB. Layers are indicated by i , $i + 1$ and $i - 1$. All residues of one ANP dimers belong to one fibrillar layer. Cys residues are labeled and colored in gray.

extensive and tightly packed, measuring $\sim 870 \text{ \AA}^2$ compared to $\sim 634 \text{ \AA}^2$ in PolB (Fig. 3a, b, d, e).

In both polymorphs the interactions between the pairs of protofilaments are rather limited; however, their number and nature greatly differ in the two cases (Fig. 3c, f). PolA exhibits minor interprotofilament contacts, restricted to a salt bridge between Arg14 and Asp13 residues (interdimer interface of $\sim 40 \text{ \AA}^2$) (Fig. 3c). In PolB the two facing C-terminal $\beta 2$ strands form a steric zipper comprising Asn24, Phe26, and Tyr28, forming a hydrophobic and polar cluster (interdimer interface of $\sim 181 \text{ \AA}^2$) (Fig. 3f); in PolA such C-terminal regions interact with adjacent ANP dimers.

All these structural differences lead to differential residue exposure to solvent (Fig. 2a): in PolA, residues 4–15 are buried within the inter-dimer interface, while the segments comprising the two reduced Cys residues remain solvent-accessible (Fig. 2a). Conversely, PolB exhibits reduced solvent accessibility at both N- and C- terminal regions, with increased exposure of the extended central section (Fig. 2a).

Conserved polymorphism in different patients

To understand whether the degree of polymorphism observed in Pt2 was also present in the two other patients, Cryo-EM data were also collected on fibrils extracted from Pt1 and Pt3 using a 200 kV Talos Artica (Fig. 4a, b and Supplementary Fig. 6). In both patients, similarly to what observed for Pt2, two distinct fibril morphologies—i.e. coated and naked fibrils—were observed at raw micrograph level (Fig. 4a, b). Particularly, Pt3 presented a dominant population of coated fibrils (around 75%) similar to what observed in Pt2, while the fraction of coated fibrils increased up to 87% in Pt1 (Fig. 4c).

The 2D classification of manually picked coated fibrils in Pt1 revealed shared structural features with Pt2 PolA fibrils (Fig. 4d–f). Pt1 PolA was reconstructed at $3.7 \text{ \AA}$ resolution, yielding a map with overall poorer quality than the one reported for Pt2 (Fig. 4d–f). However, despite lacking some side chain features, the potential map closely resembled the Pt2 one (Fig. 4f). Indeed, superimposition of Pt1 and Pt2 maps highlights the

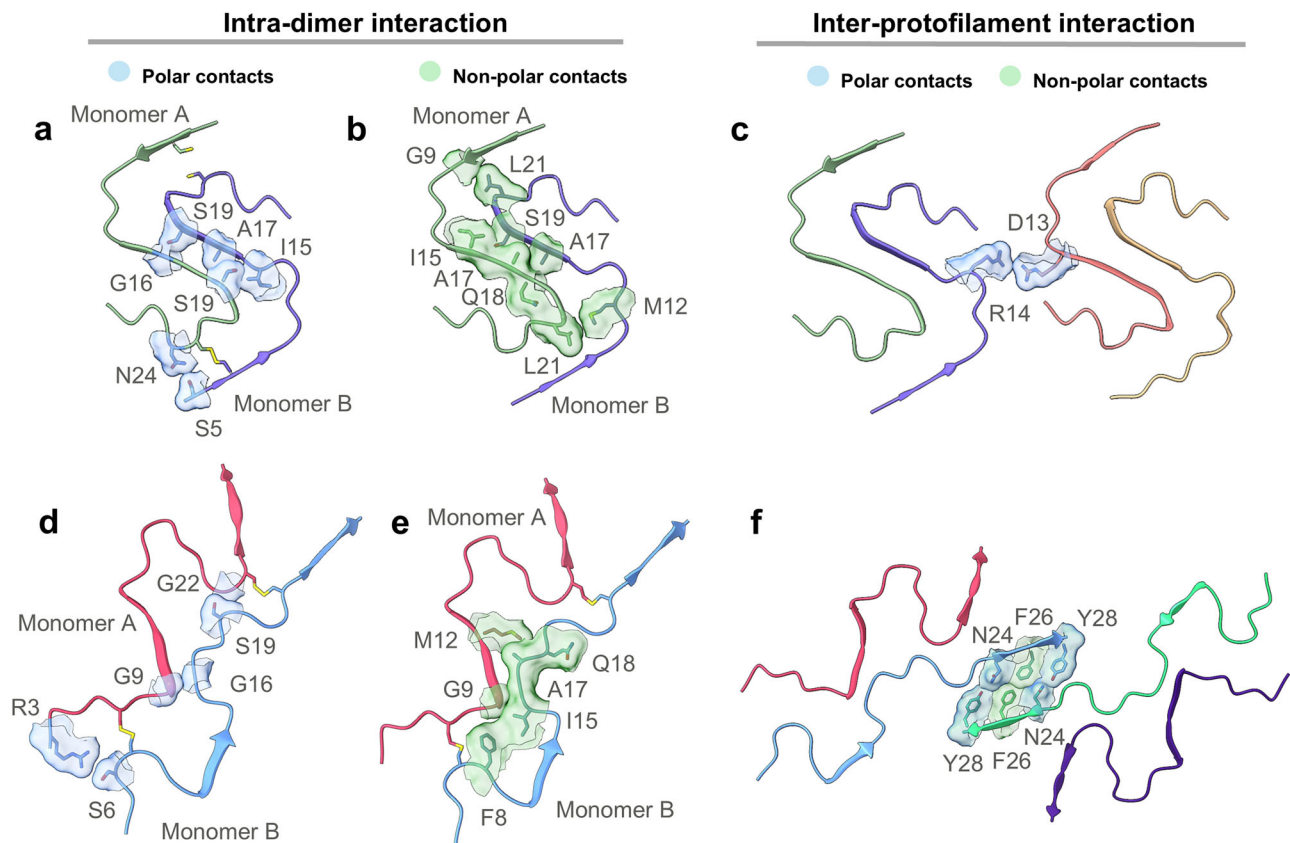


Fig. 3 | PolA and PolB intra and inter-fibrillar stabilizing interactions. a, b PolAr (a) and non-PolAr (b) interactions stabilizing PolA fibril fold at dimer level. **c** PolAr contacts (blue) mediate the interaction between PolA protofilaments. **d, e** PolAr (d)

and non-PolAr (e) interactions stabilizing PolB fibril fold at intra-dimer level. **f** PolAr (blue) and non-PolAr (green) contacts mediate the interaction between PolB protofilaments.

similarity between the two patients' fibril organizations (Fig. 4f). This, combined with evidence from 2D and 3D comparisons, strongly suggests an identical fold for PolA in Pt1 and Pt2 (Fig. 4d). In Pt3, instead, despite at micrograph level similar-looking coated fibrils being present, the low number of fibrils, thus of particles, did not allow any high-resolution information.

Interestingly, both Pt1 and Pt3 fibrils present a minor population of fibrils with evident helical assembly as observed in Pt2. Manual measurement of fibrillar features (crossover, maximum width, and minimum width) confirmed the structural similarities in Pt1 and Pt2 (Fig. 4h): similar cross-over and width parameters were observed, strongly suggesting a common structural organization of PolB in Pt1 and Pt2 (Fig. 4h). Moreover, the high-resolution reference-free 2D classes of PolB from Pt1 showed clear similarities with PolB projections from Pt2, further corroborating the hypothesis of a common PolB structure in the two patients (Fig. 4g). The limited number of naked fibrils found in the dataset from Pt3 did not lead to statistically meaningful measurements of helical properties. Overall, these data suggest that the co-existence of PolA and PolB may be a constant feature of ANP amyloid deposits.

Unfortunately, neither mass spectrometry nor structural data could suggest the nature of PolA coating, whose nature remains unknown. It could be speculated that the coating consists of one or more components of the extracellular matrix interacting and maybe stabilizing the fibrils, as recently observed for other cardiac amyloids¹⁹. However, further studies are needed to elucidate the identity and the functional implications of such coating. Even though helical architecture of PolB fibrils was evident from raw images, it cannot be ruled out that also PolB fibrils are also coated, though more sparsely than the ones of PolA.

Monomeric ANP aggregates in vitro under non-reducing but not under reducing conditions

Lastly, to assess the intrinsic amyloidogenic potential of the ANP sequence, in vitro aggregation experiments of native ANP monomers—i.e. presenting an intramolecular SS bond between Cys7 and Cys23—were performed (Supplementary Fig. 7a). Under physiologically relevant conditions, monomeric ANP exhibited substantial aggregation propensity, as demonstrated by Thioflavin T (ThT) fluorescence assays, which produced characteristic sigmoidal aggregation kinetics (Supplementary Fig. 7a)²⁶. Both tested concentrations (200 μ M and 300 μ M) displayed a lag phase of ~24 h, consistent with data previously reported⁸. Transmission electron microscopy (TEM) of the 300 μ M aggregated samples further confirmed the formation of fibrillar structures, supporting the ability of monomeric ANP to form amyloid fibrils (Supplementary Fig. 7b). To investigate the role of the disulfide bond in modulating ANP aggregation, parallel aggregation experiments were conducted under reducing conditions in presence of dithiothreitol (DTT). Remarkably, the presence of DTT completely inhibited amyloid formation, as evidenced by the absence of ThT fluorescence even after 60 h (Supplementary Fig. 7a). These findings not only reinforce the inherent aggregation propensity of the ANP peptide but also underscore the critical role of the disulfide bond in driving amyloid formation. Thus, these data indicate that ANP is intrinsically prone to aggregate into cross- β structures and the presence of disulfide bond(s) is essential for fibril formation, both in vitro and in vivo.

In summary, this study presents high-resolution structural data on ANP amyloid fibrils found in atrial tissue of AF patients, revealing critical insights into their formation. In vivo, ANP amyloid deposits consist exclusively of covalent dimers, which can

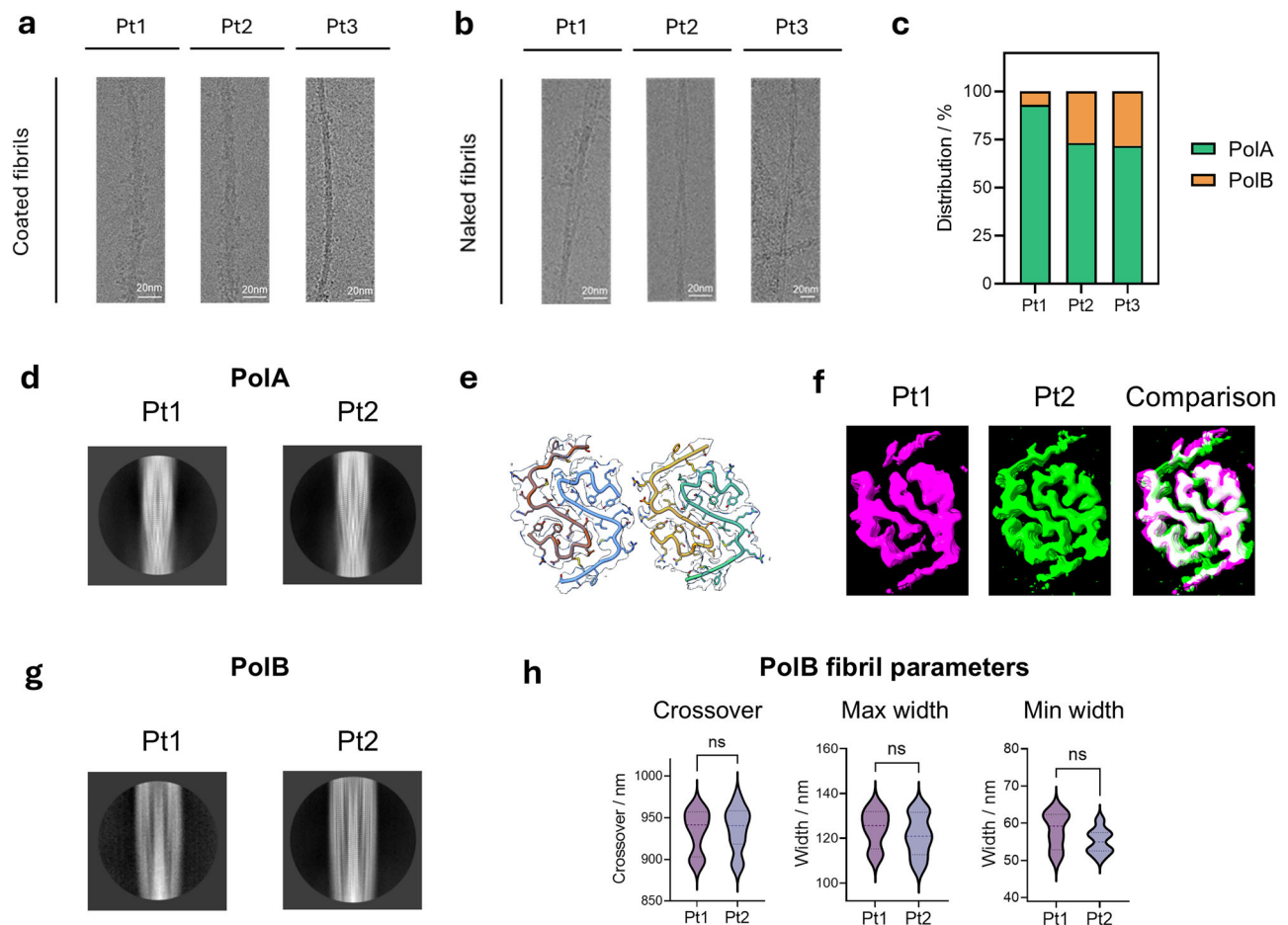


Fig. 4 | PolA and PolB folds are conserved in Pt1, Pt2, and Pt3. **a, b** Representative images of Pt1, Pt2, and Pt3 coated (**a**) and naked (**b**) ANP fibrils. **c** Histograms reporting distribution of PolA and PolB in the three analyzed patients. **d** Reference-free 2D class averages of PolA fibrils in Pt1 and Pt2. **e** Pt2 model fits into 3.7 Å resolution map of PolA from Pt1. **f** Cross-sectional maps of Pt1 (magenta) and Pt2 (green) and their superimposition. **g** Reference-free 2D class averages of Pt1 and Pt2 PolB fibrils. **h** Cross-over, maximum width and minimum width measurements of PolB from Pt1 and Pt2. Crossover is defined as the distance in Å between two

consecutive turnovers and it corresponds to half of the fibril pitch; maximum width is the longest distance between the edges of the fibril; minimum width is defined as the intersection between the two protofilaments. Statistical analysis was performed using a two-sided unpaired t-test, with significance set at $p < 0.05$. The results were as follows: crossover ($p = 0.876$; $n_{Pt1} = 11$ vs $n_{Pt2} = 11$), maximum width ($p = 0.607$; $n_{Pt1} = 4$ vs $n_{Pt2} = 7$), and minimum width ($p = 0.248$; $n_{Pt1} = 4$ vs $n_{Pt2} = 8$). None of the comparisons reached statistical significance.

adopt either parallel or antiparallel configurations, leading to two distinct fibril types. Notably, while monomeric ANP can form amyloid fibrils in vitro, it is absent in in vivo atrial amyloid deposits, suggesting that dimerization is essential for the formation of pathologic amyloid plaques in vivo. Although this study does not clarify how these dimers form via two-disulfide-bonded (parallel) and one-disulfide-bonded (antiparallel) dimers in vivo, it strongly supports the idea that dimeric ANP drives IAA development. A plausible explanation is that chronic ANP overexpression - common in heart failure and cardiovascular stress⁹ - leads to abnormal dimer formation in the endoplasmic reticulum of atrial cells. Under these conditions, elevated dimeric ANP levels may promote protein crowding and aggregation, facilitating amyloid deposition.

Thus, the study suggests that blood level of dimeric ANP - rather than of total ANP - should be carefully monitored as a potential biomarker for amyloid deposition risk in atrial tissue. This could have significant diagnostic and prognostic value, particularly in patients with heart failure, AF, or other cardiovascular disorders predisposing them to IAA. Overall, this work not only advances our molecular understanding of IAA pathogenesis but also opens new avenues for its early detection and intervention.

Methods

Patients' samples

Pt1, Pt2, and Pt3 were diagnosed with persistent atrial fibrillation (AF) and were scheduled for corrective or palliative cardiac surgery requiring extracorporeal circulation at the Division of Cardiac Surgery, Policlinico San Donato. As part of the surgical procedure, a small fragment of the left atrial appendage was excised. This tissue, which would otherwise be discarded, was collected for histological and structural analysis. Patients' clinical characteristics are reported in Supplementary Table 1. The three tissue samples were entirely used for the described experiments.

Histological analysis

The samples of the left atrial appendage were fixed in 10% neutral buffered formalin and prepared for paraffin embedding. Tissues were dehydrated through a standard graded ethanol series (70%, 80%, 95% and 100%) and then cleared in xylene and embedded in paraffin blocks. The paraffin-embedded tissues were cut into 3–6 μm thick sections using a precision rotary microtome. The sections were mounted on positively charged glass slides to improve tissue adhesion and prepared for subsequent histological and immunohistochemical analyses.

To assess the presence of amyloid-like deposits, the tissue sections were deparaffinized, rehydrated and stained with Congo red alkaline solution (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's instructions. Haematoxylin counterstaining was performed to visualize the cell nuclei. Amyloid deposits were identified using both standard brightfield and fluorescence light microscopy.

Immunohistochemistry for ANP was performed using the HRP/DAB Detection IHC Kit (Abcam) according to the manufacturer's protocol. Sections were deparaffinized in xylene, rehydrated through a descending ethanol gradient and subjected to heat-induced antigen retrieval in 10 mM citrate buffer (pH 6.0) at 98 °C for 20 min. To block non-specific binding, the sections were incubated with the blocking solution provided. Slides were then incubated for 1 h at room temperature with the primary mouse anti-ANP antibody (BioRad, 0200–0648, 1:200 dilution in blocking solution), followed by a 10-min incubation with the biotinylated secondary antibody as recommended in the kit. Signal detection was performed using the avidin–biotin–peroxidase complex and 3,3'-diaminobenzidine (DAB) as chromogenic substrate. Nuclei were counterstained with Mayer's haematoxylin (Bio-Optica) according to standard procedures.

Images were acquired using a DM6 M upright fluorescence microscope (Leica) equipped with LAS X Navigator software. Full slides scans were performed at 20x magnification.

Fibril extraction from left atrial appendages

After excision, non-fixed left atrial appendages were flash frozen in liquid nitrogen and stored at –80 °C until amyloids were extracted as described previously²⁷. Briefly, 100 mg of tissue were minced with a scalpel and washed in Tris calcium buffer (20 mM Tris, 140 mM NaCl, 2 mM CaCl₂, pH 8.0). The tissue was digested with 5 mg *Clostridium histolyticum* collagenase (Sigma Aldrich) dissolved in 1 ml of Tris calcium buffer, for 16 h at 37 °C at 750 rpm. Then, the homogenized tissue was washed through 10 washing cycles in Tris EDTA buffer (1 ml, 20 mM Tris, 140 mM NaCl, 10 mM EDTA, pH 8.0). Supernatants were discarded after each wash step. The remaining pellet was then repeatedly homogenized in 50 µL ice-cold water, storing supernatants.

For all three patients, water extract fraction number 3 was selected for cryo-EM analysis. For Pt1 and Pt2 fraction 2 was used for mass-spectrometry analysis.

Mass-spectrometry analysis

Proteomic analysis was performed using nano-liquid chromatography electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS) in a shotgun, label-free approach on water extracts obtained from Pt1 (*n* = 1) and Pt2 (*n* = 1). Briefly, fibrillar proteins were solubilized in 8 M urea for 1 h at room temperature and protein concentration was determined using Bradford assay. Protein samples were reduced with 13 mM dithioerythritol (DTE) for 30 min at 50 °C and alkylated with 26 mM iodoacetamide (IAA) for 1 h at RT, in the dark. Alkylation was quenched with 1 mM aqueous methylamine. Samples were subsequently diluted with 20 mM ammonium bicarbonate (pH 8) and digested overnight at 37 °C using sequencing-grade trypsin (Sigma Aldrich, Saint Louis, MO, USA) at a 20:1 protein-to-enzyme ratio. The digestion was blocked by acidification to inactivate the protease. Prior to nLC-MS/MS analysis, peptides were desalted and purified using Pierce C18 Tips (Thermo Fisher Scientific). Peptides were then analyzed using a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA, USA) coupled to an Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Scientific, Bremen, Germany) equipped with nano electrospray ion source. The peptide mixture was loaded onto an Acclaim PepMap 100 0.3 × 5 mm C18 (Thermo Scientific) and separated on an EASY-Spray ES900 analytical column (15 cm × 75 µm ID) packed with 3 µm, 100 Å Acclaim PepMap RSLC C18 resin (Thermo Scientific). Chromatographic separation was achieved using a binary solvent system consisting of mobile phase A (0.1% formic acid in water) and

mobile phase B (0.1% formic acid in 80% acetonitrile), at a flow rate of 300 nL/min and a column temperature of 35 °C. Each sample was analysed in a single injection. The mass spectrometer was operated in positive ion mode with data-dependent acquisition (DDA). Full MS scans were acquired in the Orbitrap over the *m/z* range 375–1500 at a resolution of 120,000 (at *m/z* 200), followed by higher-energy collisional dissociation (HCD) MS/MS of the most intense precursor ions, with a collision energy set at 35 eV and a cycle time of 3 s between master scans.

Acquired raw files were processed using Proteome Discoverer software (version 2.5). Database searching was performed against the *Homo sapiens* reference proteome from UniProt (45,451 target sequences, downloaded on 18th June 2025). Search parameters included semi-trypsin cleavage specificity, with carbamidomethylation of Cys as a fixed modification, and methionine oxidation and N-terminal carbamylation as a variable modifications. Precursor and fragment mass tolerances were set to 10 ppm and 0.02 Da respectively, and up to two missed cleavages were allowed. Percolator node was used to filter peptide spectral matches (PSMs) and peptides to a false discovery rate (FDR) of <1%. Peptide identifications were further filtered to include only those with XCorr ≥ 1.5²⁸.

Raw MS data have been deposited in the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner depository with the dataset identifier PXD066795.

Single-particle cryo-EM sample preparation and data collection

Samples were mixed by vortexing for 10 s at room temperature. Droplets of 3 µl were incubated for 30 s at 30 mA using a GloQube system (Quorum Technologies) on freshly glow-discharged holey thick carbon grids (C-flat 1.2/1.3 C, Protochips), and plunge-frozen in liquid ethane using a Vitrobot Mk IV (Thermo Fisher Scientific), operated at 4 °C and 100% humidity. Dataset for Pt1 and Pt3 were collected automatically on a Talos Artica 200 kV (Thermo Fisher Scientific), equipped with a Falcon 3 direct electron detector operated in electron counting mode (Supplementary Table 2). For Pt1, a total of 1818 movies were recorded at a nominal magnification of 120000, corresponding to a pixel size of 0.889 Å/pixel and a total dose of 40 e[−]/Å², equally distributed over 40 fractions. For Pt3, instead, a total of 191 movies were recorded at a nominal magnification of 76000, corresponding to a pixel size of 1.43 Å/pixel.

Pt2 dataset was collected at ESRF Grenoble at Beamline CM01 on a Titan Krios (Thermo Fisher Scientific), equipped with a Gatan K3 direct electron detector on a Gatan Bioquantum LS/967 energy filter and a FEI Volta phase plate. A total of 16251 movies were recorded at a magnification of 105000, corresponding to a pixel size of 0.839 Å/pixel and a total dose of 41 e[−]/Å², equally distributed over 40 fractions.

Helical reconstruction

Pt1. Fibrils were picked manually from dose-weighted, motion- and CTF-corrected image micrographs in RELION 3.1^{29–32}. After manual picking, a first set of 53,415 segments was extracted in 800-pixel boxes with 3.6 Å/pix. The tube diameter, rise and number of asymmetrical units were set to 150 Å, 5 Å and 15, respectively. Reference-free 2D classification was performed to select a single large class average belonging either to PolA or PolB for initial model generation.

Pt1–PolA. Initial model generation was performed using Relion 3.1 software *relion_helix_inimodel2D* from a single large class average (box size 800 pixel and pix size 3.6 Å/pix) with an estimated cross-over distance of 420 Å. A second extraction of the same particles yielded 303,729 smaller segments applying a box size of 400 pixel and pix size of 0.889 Å/pix with tube diameter, rise and asymmetrical unit values of 150 Å, 4.75 Å and 3, respectively. The initial model was re-scaled and re-windowed to match the un-binned particles and low-pass-filtered to 10 Å. Consecutive rounds of 3D autorefinement

applying C1 symmetry, angular sampling, helical twist and rise values of 3.7° , -2.1° and $4.9 \text{ \AA}$, respectively, eventually lead to a $5 \text{ \AA}$ resolution map. Following 3D classifications, a total of 50,018 segments with similar cross-section densities were selected. After additional steps comprising Bayesian polishing, CTF refinement and masked 3D refine, segments were aligned by the final 3D autorefinement with solvent-flattened FSC for map reconstruction. The final map was reconstructed with helical twist and rise values of -2.1° and $4.87 \text{ \AA}$ to an estimated resolution of $3.7 \text{ \AA}$.

Pt1 – PolB. Initial model generation was performed from a single large class average with an estimated crossover distance of $920 \text{ \AA}$ using *relion_helix_inimodel2D* software implemented in *Relion* 3.1. 21,245 smaller segments were extracted for the refinement by applying a box size of 400 pixel and pix size of $0.889 \text{ \AA/pix}$ with tube diameter, rise and asymmetrical unit values of $150 \text{ \AA}$, $5 \text{ \AA}$ and 3, respectively. The initial model was re-scaled and re-windowed to match the un-binned particles and low-pass-filtered to $10 \text{ \AA}$. After 3D classification, a total of 9498 segments with similar cross-section densities were selected. A 3D autorefinement was then performed applying C1 symmetry, angular sampling, helical twist and rise values of 3.7° , -0.96° and $4.9 \text{ \AA}$, respectively, eventually lead to a $9 \text{ \AA}$ resolution map.

Pt2. Fibrils were manually picked from dose weighted, motion- and CTF-corrected image micrographs in *RELION* 3.1. After manual picking, a first set of 499,324 segments were extracted in 800 pixel boxes and pix size of $3.36 \text{ \AA/pix}$. The tube diameter, rise and number of asymmetrical units were set to $200 \text{ \AA}$, $4.75 \text{ \AA}$ and 3, respectively. Reference-free 2D classification was performed to separate PolA (total of 285254 segments) and PolB (total of 88401 segments).

Pt2–PolA. The homogeneous set of PolA segments was extracted for the refinement applying a box size of 400 pixel and pix size of $0.839 \text{ \AA/pix}$ with tube diameter, rise and asymmetrical unit values of $150 \text{ \AA}$, $4.75 \text{ \AA}$ and 3, respectively, yielding a total of 285,254 segments. Pt1–PolA map was used as reference map in two consecutive 3D classification jobs to increase segments homogeneity. A total of 67547 segments with similar cross-section densities were selected. A first 3D autorefinement applying C1 symmetry, angular sampling, helical twist and rise values of 3.7° , -2.1° and $4.9 \text{ \AA}$, respectively, yielding a $3.9 \text{ \AA}$ map. After additional steps comprising Bayesian polishing, CTF refinement, and masked 3D refinement segments were aligned by the final 3D autorefinement imposing a pseudo- 2_1 symmetry with solvent-flattened FSC for map reconstruction. The final map was reconstructed with helical twist and rise values of 178.9° and $2.35 \text{ \AA}$ to an estimated resolution of $2.9 \text{ \AA}$.

Pt2–PolB. The homogeneous set of PolB segments was extracted for the refinement applying a box size of 400 pixel and pix size of $0.839 \text{ \AA/pix}$ with tube diameter, rise and asymmetrical unit values of $150 \text{ \AA}$, $4.75 \text{ \AA}$ and 3, respectively, yielding a total of 93,973 segments. Low resolution patient 1 – PolB map was used as reference map in a 3D classification to increase segments homogeneity. A total of 31454 segments with similar cross-section densities were selected. A first 3D autorefinement applying C1 symmetry, angular sampling, helical twist and rise values of 3.7° , -2.1° and $4.9 \text{ \AA}$, respectively, yielding a $4.4 \text{ \AA}$ map. After additional steps comprising Bayesian polishing, CTF refinement, masked 3D refinement segments were aligned by the final 3D autorefinement imposing a pseudo- 2_1 symmetry with solvent-flattened FSC for map reconstruction. The final map was reconstructed with helical twist and rise values of 179.5° and $2.36 \text{ \AA}$ to an estimated resolution of $3.35 \text{ \AA}$.

Pt3. Fibrils were picked manually from dose weighted, motion- and CTF-corrected image micrographs in *RELION* 3.1. After manual picking,

a first set of 19,590 segments were extracted in 800-pixel boxes and pix size of $3.6 \text{ \AA/pix}$. The tube diameter, rise and number of asymmetrical units were set to $220 \text{ \AA}$, $4.75 \text{ \AA}$ and 3, respectively. Reference-free 2D classification was performed to identify PolA (total of 14,065 segments) and PolB (total of 5,525 segments). The same set of 19,590 segments were also extracted in 400-pixel boxes and pix size of $0.889 \text{ \AA/pix}$. Reference-free 2D classification was performed to identify PolA and PolB.

Cross-over and width determination

Raw images for Pt1 and Pt2 data collection were analysed in *ImageJ* software for cross-over and width determination. Cross-over was defined as the distance in $\text{\AA}$ between two consecutive turnovers of the helical fibril and corresponds to half of the fibril pitch. Instead, width was measured either as the maximum width, i.e. the longest distance between the edges of the fibril, and the minimum width, corresponding to the intersection between the two pairs of protofilaments. Statistical analysis was performed using the unpaired T-test in *GraphPad prism* software.

Model building

PolA. The model was built de novo starting from a map region featuring two close in space residues with an associated bulky side-chain volume, identified as Phe26 and Tyr28. The model was built and refined in *Coot* and *Phenix* real-space refinement. The final model comprises five mature ANP dimeric molecules in each pair of protofilament, obtained by refinement with additional non-crystallographic (NCS) restraints. For each monomeric ANP, 24 residues were model, resulting in a 48 amino-acid dimer stabilized by a single disulfide bond. *Phenix* and *Molprobit* validation revealed map-model cross-correlation (CC_{mask}) and *Molprobability*-score values of 0.79 and 1.74, respectively (Supplementary Table 4), indicative of a physically valid model with definite map support.

PolB. The model was built de novo starting from a map region featuring two close in space residues with an associated bulky side-chain volume, identified as Phe26 and Tyr28. The model was built and refined in *Coot* and *Phenix* real-space refinement. The final model comprises five mature ANP dimeric molecules in each pair of protofilament, obtained by refinement with additional non-crystallographic (NCS) restraints. For each monomeric ANP, 26 residues were modeled, resulting in a 52 amino-acid dimer stabilized by two disulfide bonds. *Phenix* and *Molprobit* validation revealed map-model cross-correlation (CC_{mask}) and *Molprobability*-score values of 0.69 and 2.78, respectively (Supplementary Table 4).

Aggregation assay

The mature synthetic ANP peptide with a disulfide bond between Cys7 and Cys23 was purchased from Genscript. Lyophilized ANP peptide was dissolved in 20 mM Hepes pH 7.4, 200 mM NaCl, at 2 mM with or without 10 mM DTT. The solution was centrifuged at 4°C for 10 min at $20800 \times g$. The stock solution was eventually diluted to the final concentrations for the experiments (200 μM and 300 μM) in 20 mM Hepes pH 7.4, 200 mM NaCl, with or without 10 mM DTT. Freshly prepared thioflavin T (ThT) was added to a final concentration of 20 μM . 75 μL of each condition were then pipetted into black polystyrene 96-well half-area plates with clear bottoms and polyethylene glycol coating (Corning). Each condition was performed in triplicate in each experiment. Plates were sealed to prevent evaporation and incubated at 37°C under quiescent conditions in a Varioskan Lux plate reader (Thermo Fisher Scientific). Upon excitation at 450 nm, fluorescence at 480 nm was recorded through the bottom of the plate every 8 min. All the experiments were performed in triplicates. The mean ThT fluorescence values from the independent experiments were plotted against time.

TEM analysis

Freshly prepared ANP fibrils were analyzed by TEM. For the analysis, the ending point of the aggregation assay at 300 μ M in 20 mM Hepes pH 7.4, 200 mM NaCl was used. 4- μ l droplet of sample was applied onto a 400-mesh copper carbon-coated grids (Agar Scientific). After 1-min incubation, excess of sample was removed and the grid was stained with 2% (w/v) uranyl acetate solution, blotted dry, and imaged on a Talos L120C transmission electron microscope (Thermo Fisher Scientific) operating at 120 kV. Morphological characterization of the fibrils was performed using the software ImageJ.

Ethical statement

This study was approved by the Ethical Committee of IRCCS Policlinico San Donato (CE: CET 224–2024) and was performed in accordance with the Declaration of Helsinki. We have written informed consent of the patient to publish clinical information potentially identifying the individual.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The Cryo-EM data for polA generated in this study have been deposited in the EMPIAR, EMDDB and RCSB databases under accession code [EMPIAR-12800](#), [EMD-53891](#) and [9RBD](#). The Cryo-EM data for polB generated in this study have been deposited in the EMPIAR, EMDDB and RCSB databases under accession code [EMPIAR-12800](#), [EMDB-53910](#) and [9RBW](#). Additionally, cryo-EM polA and polB potential maps and structural models have been uploaded in figshare (<https://doi.org/10.6084/m9.figshare.29234984>). The mass spectrometry proteomic data have been deposited in the PRIDE partner depository for the ProteomeXchange Consortium with dataset identifier: [PXD066795](#). Source Data are provided with this paper and contains raw data for graphs reported in main manuscript and supplementary file. Source data are provided with this paper.

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Author contributions

Conceptualization and Supervision by L.A. and S.R. Investigation and Analysis by L.B., M.P., A.C.S., D.M.B., F.C., C.V., F.S., P.S., I.V., M.M., and S.N. Funding acquisition and Resources by L.M., G.C., C.P., L.A., and S.R. Original draft by L.B., A.C.S., and S.R. Review and editing by all the authors. Data visualization by L.B., M.P., and S.N. Edited and reviewed by L.B., S.N., and S.R. Contribution and approval of the submitted version by all authors.

Competing interests

The authors declare no competing interests.

Additional information

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