Amyloidogenic Potential of Plaque and Thrombus Proteomes and of Fold-Switching Metamorphic Proteins

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Article

Amyloidogenic Potential of Plaque and Thrombus Proteomes and of Fold-Switching Metamorphic Proteins

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Abstract

In previous work we have used the computer program AmyloGram to assess the amyloidogenic potential of proteins observed using mass spectrometry-based proteomics in thrombi extracted from individuals who had suffered an ischaemic stroke. As anticipated from our experimental observation of substantial amounts of amyloid in such clots, the AmyloGram scores were very high and entirely consistent with the amyloid nature of such thrombi. We here apply a similar strategy to assess the amyloidogenic nature of proteins in thrombi removed from venous thromboembolisms including pulmonary embolisms, similarly finding very high AmyloGram scores. The same is true for atherosclerotic plaques as determined from multiple studies in which the data were readily available. Amyloidogenesis is a specific activity or subset of a class of proteins known to adopt very different macrostates, in which amyloidogenesis to create insoluble fibrils is more or less irreversible. Another subset of multi-state proteins, whose conformational interchanges are much more reversible, involves what are referred to as 'fold-switching' or 'metamorphic' proteins'. We here use AmyloGram to analyse the amyloidogenic potential of these too, finding that while some are highly amyloidogenic their amyloidogenic potential is considerably more heterogeneous and little different from that of the overall proteome within Uniprot.

Keywords: amyloid; fibrinaloid; proteomics; inflammation; amyloidogenic sequences; embolism; atherosclerosis

Introduction

Following our discovery that blood can clot into an anomalous amyloid form [1, 2] to produce what we and others refer to as fibrinaloid microclots [3-17], we have more recently discovered experimentally that the macroclots retrieved from ischaemic stroke are also amyloid in nature [10, 18].

Among the conventional pathogenic amyloidoses [19-22], it is well known that amyloidogenic cross-seeding can be occurring [23-28]. This has led (i) to the recognition that this is also occurring in the microclots (as reflected in the complex proteomes so observed [15, 29-31]) and (ii) to predictions that this would also be true in macroclots [16].

A variety of computational programs exist for predicting the amyloidogenicity of a protein (summarised in [32] and in Table 1 of [15]), and we recently used one of these, AmyloGram [33, 34],

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to predict the amyloidogenicity of proteins observed in a variety of ischaemic stroke thrombi [35]. In that work [35], we 'calibrated' the system with proteins annotated by humans at Uniprot, where (with a generous margin) every one of these had an AmyloGram score exceeding 0.7 (as did 79% of all human polypeptides). Specifically, of the 83,567 proteins that were analysed, 66,190 (79.2%) had AmyloGram scores exceeding 0.7, while 45,169 (54.1%) exceeded 0.8 and 7409 (8.9%) were above 0.9. The implication was that any thrombus with a higher percentage than 0.7 (or a much higher median AmyloGram score) could or would effectively be enriched in amyloidogenic proteins, and this turned out very much to be the case [35], consistent with the experimental observations [10, 18].

As well as thrombi retrieved from ischaemic stroke, we also predicted that a variety of other thrombi, for which experimental proteomic data existed, including those from venous thromboembolisms including pulmonary embolism and deep vein thrombosis, and various cardiac issues, would also be amyloid in nature, though we did not test this with AmyloGram [16]. The purpose of the present work is, where the data are available in a suitable format, precisely to perform those analyses. We conclude that in all cases where proteomic data are available the thrombi are, like those from ischaemic stroke, expected to be amyloid in nature.

Materials and Methods

Just as with our previous endeavour [35] this work uses the online http://biongram.biotech.uni.wroc.pl/AmyloGram/ or R-based versions of AmyloGram (see https://github.com/michbur/AmyloGram or https://cran.r-project.org/web/packages/AmyloGram/index.html) to determine the AmyloGram scores; protein identification data are given in the original publications cited, or in Tables here.

Results

Venous Thromboembolism

In the case of thrombi removed from individuals following a venous thromboembolism, we identified the 18 proteins given in the data from Stachowicz and colleagues (their Table 1, that included concentrations) [36], and ran the list on the AmyloGram server, with the results shown in Figure 1. With one exception, each scores above 0.7, with a median score exceeding 0.86, strongly implying that these clots are amyloid in nature.

Protein IDs	Protein names	Gene names	AmyloGram score	Average concn (nmol/g)
P08697	Alpha-2-antiplasmin	SERPINF2	0.8627	39.2436
P01023	Alpha-2-macroglobulin	A2M	0.8508	16.1977
P01008	Antithrombin-III	SERPINC1	0.8661	1.6678
P02749	Beta-2-glycoprotein 1	APOH	0.7307	0.1782
P00740	Coagulation factor IX	F9	0.9176	0.004
P12259	Coagulation factor V	F5	0.888	0.0125
P00451	Coagulation factor VIII	F8	0.8902	0.0005
P00748	Coagulation factor XII	F12 0.0135	0.8451	0.0135
P00488	Coagulation factor XIII A chain	F13A1	0.8898	13.554
P05160	Coagulation factor XIII B chain	F13B	0.8811	0.0347
P02671	Fibrinogen alpha chain	FGA	0.8276	303.171
P02675	Fibrinogen beta chain	FGB	0.8627	377.274
P02679	Fibrinogen gamma chain	FGG	0.9222	324.323
P02751	Fibronectin	FN1	0.9176	62.3088
P00747	Plasminogen	PLG	0.8755	2.8753
P00734	Prothrombin	F2	0.7237	2.348

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P04004	Vitronectin	VTN	0.8475	1.3471
P04275	von Willebrand factor	VWF	0.9176	0.2985

Figure 1. AmyloGram scores of 18 proteins given in the data from Stachowicz and colleagues [36] (their Table 1) that included concentrations in the thrombi. The colour scale reflects the AmyloGram score.

Pulmonary Embolism

The data we used for these studies came from a study of Bryk and colleagues [37], whose Table (their Supplementary Information Table S1) contained 198 polypeptides that were both in thrombi retrieved following a pulmonary embolism and were also differentially expressed relative to controls (normal clots more-or-less reflect the standard plasma proteome [15]). Six proteins (SERPINA1, IGKC;IGKV1-8, IGHM, IGLL5, IGHG3, SRRM1) would not run with the R code for some reason and were added via the Web server at http://biongram.biotech.uni.wroc.pl/AmyloGram/. The data are illustrated in Figure 2, where it may be observed that 190/198 have AmyloGram scores exceeding 0.7, 148/198 exceeding 0.8, 108 exceeding 0.85, and 22 (labelled in Fig 2) exceeding 0.9. The highest AmyloGram score is held by the human telomerase reverse transcriptase TERT (Uniprot O14746) with a value of 0.956. Again it is very clear that the amyloidogenicity of proteins enriched in the clots taken following a pulmonary embolism are very much greater than the average, a finding consistent with recent studies from other thrombi [10, 15, 16, 18, 35] and – since insoluble amyloids are well known to be much more refractory to proteolysis than are soluble proteins – one that plausibly underpins their resistance to fibrinolysis.

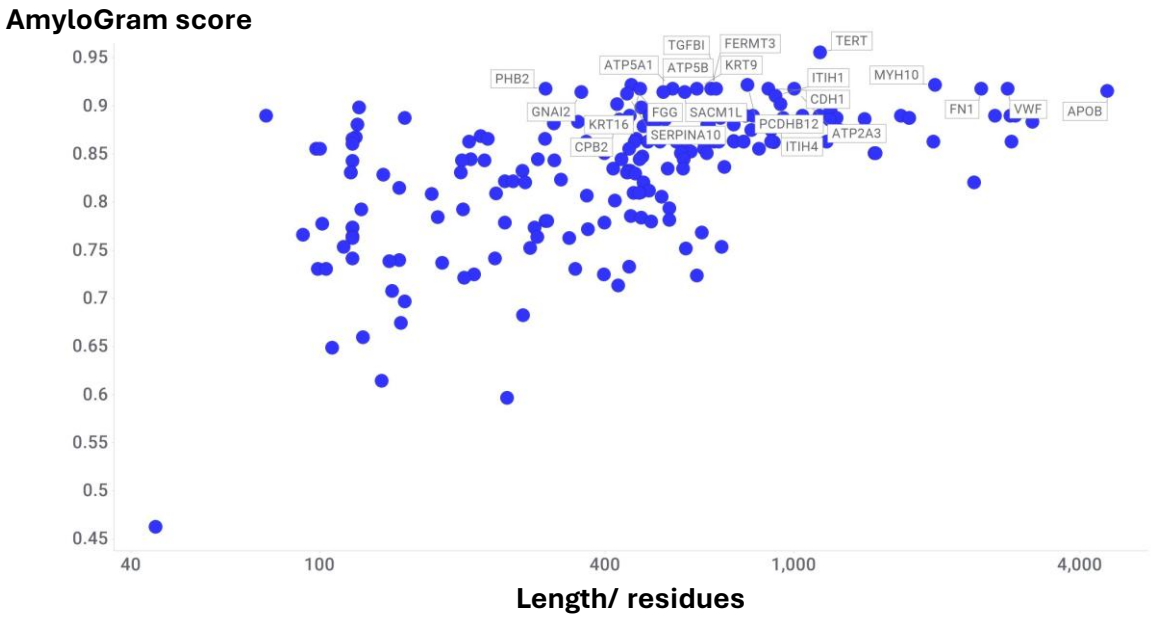

Figure 2. AmyloGram scores for 198 polypeptides that were both found in thrombi retrieved following a pulmonary embolism and were also differentially expressed relative to those of control clots. Those 22 with an AmyloGram score exceeding 0.9 are labelled.

Atherosclerosis and Acute Myocardial Infarction

Lipoprotein proteomes have been reviewed by [38]. However, our interest here is in atherosclerotic plaques, whose proteomes have been studied by several groups [38-51], with some papers providing more easily accessible/analysable data than others.

Langley and colleagues [43] sought biomarkers of high-risk atherosclerotic plaques, and identified a 4-biomarker signature (matrix metalloproteinase 9, S100A8/S100A9 (calprotectin), cathepsin D, and galectin-3-binding protein). Their AmyloGram scores are respectively 0.9148, 0.7768

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(for S100A8), 0.7941, and 0.8661. Clearly, each is highly amyloidogenic, and we previously highlighted galectin-3-binding protein (LG3BP, Uniprot Q08380) [16] as being a major player in essentially every kind of persistent thrombus.

Rocchiccioli and colleagues [52] found 31 proteins that were differentially secreted from atherosclerotic plaques. ELISA assays of plasma samples confirmed a significantly higher concentration of thrombospondin-1 and vitamin D binding protein in atherosclerotic subjects; as with LG3BP above, we had previously highlighted thrombospondin-1 as being a major player in essentially every kind of persistent thrombus [16], not least as it has long been known [53-55] that it is actually incorporated into fibrin during thrombus formation.

Theofilatos and colleagues [46] also determined a 4-protein atherosclerotic plaque signature for high-risk cardiovascular mortality, consisting of calponin-1 (Uniprot P51911), Vitamin K-dependent protein C (Uniprot P04070), serpin H1 (Uniprot P50454), and versican (Uniprot P13611). Their AmyloGram scores are, respectively, 0.6934, 0.9148, 0.8789, and 0.8627.

Alonso-Orgaz and colleagues [42] assessed the proteome of the human coronary thrombus in patients with ST-segment elevation acute myocardial infarction, using means of three different approaches involving a separation step followed by mass spectrometry. 46 proteins could be identified using all three methods [42], and these are illustrated in Figure 3. All 46 have an AmyloGram score exceeding 0.7, 35 have scores that exceed 0.8, while 29 (labelled in the Figure) exceed more than 0.85, and three exceed 0.9. The median value (between proteins scoring 0.856 and 0.863) is 0.86. Interestingly, talin-1, with a score of 0.851 is among them and is a known amyloidogen [56]. Obviously these are again very high scores, serving to underline the fact that such thrombi are likely to be amyloid in nature, and this recognition adds a major means of explaining why they are so resistant to proteolysis. Amyloid deposition in the thrombus associated with cardiac amyloidosis is of course known [57], and (although seemingly not widely recognised) amyloid deposition is in fact an established feature of atherosclerotic plaques [58-63], so in one sense the high AmyloGram scores here are unsurprising.

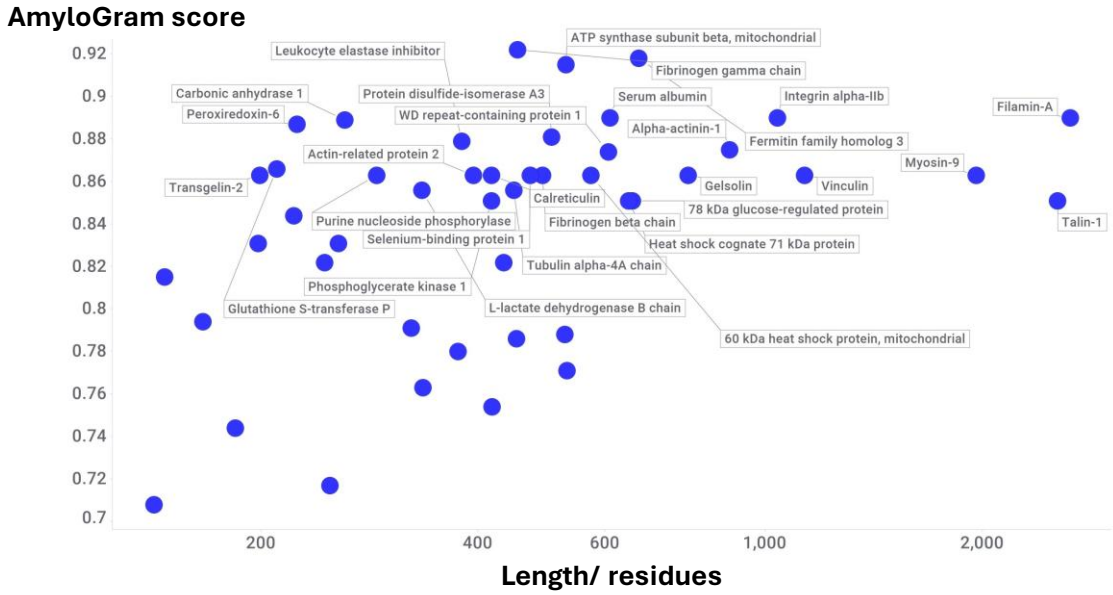

Figure 3. AmyloGram scores of proteins in the proteome of the human coronary thrombus in patients with ST-segment elevation acute myocardial infarction. 46 proteins were found present in each case when assessed with three different separation/mass spectrometric methods. Data taken from the Supplementary information provided with reference [42].

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Wang and colleagues [51] identified 11 proteins associated with coronary atherosclerosis (Their Table 1), and their AmyloGram scores are given in Figure 4. Every single entry has an AmyloGram score exceeding 0.75.

Protein	UniProt	Length/ residues	AmyloGram Score
PCSK9	Q8NBP7	692	0.7906
CELSR2	Q9HCU4	2923	0.8898
APOE	P02649	317	0.8349
LPA	P08519	2040	0.874
IL6R	P08887	468	0.8776
FN1	P02751	2477	0.9176
APOA5	Q6Q788	366	0.7995
AGER	Q15109	404	0.9322
CD4	P01730	458	0.9113
TGFB1	P01137	390	0.8021
SPARCL1	Q14515	664	0.7515

Figure 4. 11 proteins identified by Wang and colleagues (Table 1 of [51]) as being associated with coronary atherosclerosis. The colouring reflects the AmyloGram score.

Hansmeier *et al*. [64] identified 20 proteins that were raised in atherosclerotic plaques (their Supplementary Table S3), and these are tabulated, along with their AmyloGram scores and lengths, in Figure 5. All proteins again have AmyloGram scores exceeding 0.7, many being considerably greater.

Name	Gene	Uniprot	Length/ Residues	AmyloGram Score
Complement C1q subcomponent subunit B	C1QB	A0A0A0MSV6	228	0.7546
Asporin	ASPN	Q9BXN1	380	0.7915
Vitronectin	VTN	P04004	478	0.8475
Insulin-like growth factor-binding protein 7	IGFBP7	Q16270	282	0.7643
Serum amyloid P-component	APCS	P02743	223	0.9148
Alpha-1-antichymotrypsin	SERPINA3	P01011	423	0.8627
Pigment epithelium-derived factor	SERPINF1	P36955	418	0.8627
Tetranectin	CLEC3B	P05452	202	0.7225
Apolipoprotein A-IV	APOA4	P06727	396	0.7249
Complement C1q subcomponent subunit C	C1QC	P02747	245	0.7792
Plasminogen	PLG	P00747	810	0.8755
Cysteine and glycine-rich protein 2	CSRP2	Q16527	193	0.8074
Alpha-1-antitrypsin	SERPINA1	P01009	418	0.802
Vitamin D-binding protein	GC	P02774	474	0.8988
Biglycan	BGN	P21810	368	0.8452

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Antithrombin-III	SERPINC 1	P01008	464	0.8661
Glutathione S-transferase omega-1	GSTO1	P78417	241	0.9132
Lumican	LUM	P51884	338	0.8096
Kininogen-1	KNG1	P01042	644	0.8559
Beta-2-glycoprotein 1	APOH	P02749	345	0.7307

Figure 5. 20 proteins that were raised in atherosclerotic plaques (Supplementary Table S3 of [64]) and their AmyloGram scores.

The data from the 2025 study of Lorentzen and colleagues [49] (their Supplementary Information Table S2, provided there as an Excel sheet, with 128 peptides and 83 unique proteins) analysed proteins in atherosclerotic plaque from the perspective of their ease of degradation/ plaque stability or otherwise, though our interest here is simply which proteins were present and their AmyloGram scores. The data are given in Figure 6. All but 5 proteins (some represented by multiple peptides) have AmyloGram scores exceeding 0.7.

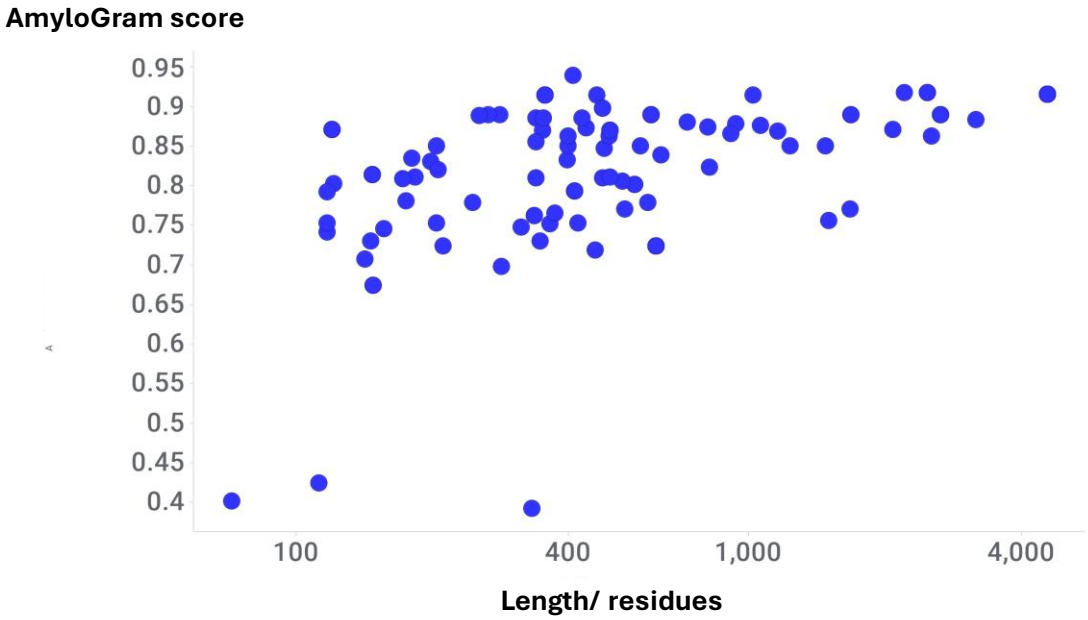

Figure 6. 83 proteins enriched differentially in atherosclerotic plaque from the perspective of their ease of degradation/ plaque stability. Protein identification data are obtained from Supplementary Table S2 of a study of Lorentzen and colleagues [49]. Again almost all have an AmyloGram score in excess of 0.7.

Aragonès and colleagues [40] compared the proteome of carotid atherosclerotic plaque and non-diseased mammary artery; 25 proteins showed statistically significant differences. Their median AmyloGram score is 0.845, and they are listed, along with their AmyloGram scores, in Figure 7.

Uniprot	Protein	Gene	Length/ residues	AmyloGram Score
P51911	Calponin-1	CNN1	297	0.6943
P09493	Tropomyosin α-1 chain	TPM1	284	0.699

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P00325	All-trans-retinol dehydrogenase (NAD(+)) ADH1B	ADH1B	375	0.7872
Q01995	Transgelin	TAGLN	201	0.8836
P07951	Tropomyosin beta chain	TPM2	284	0.5363
P21333	Filamin-A	FLNA	2647	0.8898
P30086	Phosphatidylethanolamine-binding protein 1 (RKIP)	PEBP1	187	0.9176
P63267	Actin, gamma-enteric smooth muscle	ACTG2	376	0.7797
P18206	Vinculin	VCL	1,134	0.8627
P08670	Vimentin	VIM	466	0.7466
O43707	Alpha-actinin-4	ACTN4	911	0.8195
P12814	Alpha-actinin-1	ACTN1	892*	0.8755
P08107	Heat shock 70 kDa protein 1A/1B	HSPA1A / HSPA1B	641	0.8508
P04075	Fructose-bisphosphate aldolase A	ALDOA	364	0.7532
P00915	Carbonic anhydrase 1	CA1	259	0.8887
P32119	Peroxiredoxin-2	PRDX2	198	0.8313
P40925	Malate dehydrogenase, cytoplasmic	MDH1	334	0.9113
P04264	Keratin, type II cytoskeletal 1 (epidermal)	KRT1	644	0.8627
P08294	Extracellular superoxide dismutase (Cu-Zn)	SOD3	240	0.8146
Q9Y490	Talin-1	TLN1	2,541	0.8508
P59665	Neutrophil defensin 1 (Defensin alpha-1)	DEFA1 / DEFA1B	94	0.8451
P01766	Immunoglobulin heavy variable 3-13	IGHV3-13	116	0.7645
P02649	Apolipoprotein E	APOE	317	0.8349
P10909	Clusterin (Clusterin precursor)	CLU	449	0.872
P25311	Zinc-alpha-2-glycoprotein (precursor)	AZGP1	298	0.8661

Figure 7. 25 proteins that were raised in the proteome of carotid atherosclerotic plaque relative to non-diseased mammary artery [40], along with their Amyogram scores (that are also encoded in colour).

Overall, there is a high enrichment of amyloidogenic proteins in each of the eight studies of atheromatous plaques as reviewed here.

Fold-Switching Proteins

Amyloidogenic proteins, including prions, clearly represent a set of proteins that can exist in multiple stable macrostates under a given set of conditions. The usual form or conformational ensemble, as synthesised at the ribosome, commonly has abundant α-helices whereas the amyloid forms are much richer in β-sheets, specifically the crossed-β structure [65-70], that is the characteristic of amyloid and the one to which fluorogenic stains such as thioflavin T bind [71-74]. While the amyloid form is significantly more stable thermodynamically (amyloidogenesis, involving accretion

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of multiple molecules leading to insoluble fibril formation, is essentially irreversible), the two forms are separated via a substantial energy barrier of some 36 kJ.mol^{-1} [75-80].

It has long been known that the same peptide sequences can adopt quite different conformations in different proteins [81-84](and, for that matter, that similar conformations can have very different dynamics [85, 86]). In particular, this ability to adopt multiple stable conformations or macrostates, usually referred to as polymorphs [4, 78, 87-113], is very much true of amyloids. Equally, there is a more general class of proteins that can adopt multiple macrostates, in this case often often – but not always – reversibly. These macrostates or conformations probably have different evolutionarily selected functions, and proteins exhibiting this are known as fold-switching [114-128] or metamorphic [117, 129-141] proteins. Some 6% are known to be fibril-forming [120], and the question thus arose as to whether these might also have a tendency to be more amyloidogenic.

A list of fold-switching proteins (see [122]) was kindly provided by Dr Lauren Porter. It is based on PDB references, some of which are different 3D structures of the same protein, and we have cut this down to provide one representative of each as per Table S1 of the Supplementary Information. This leaves 121 examples, and their AmyloGram scores and lengths are plotted in Figure 8.

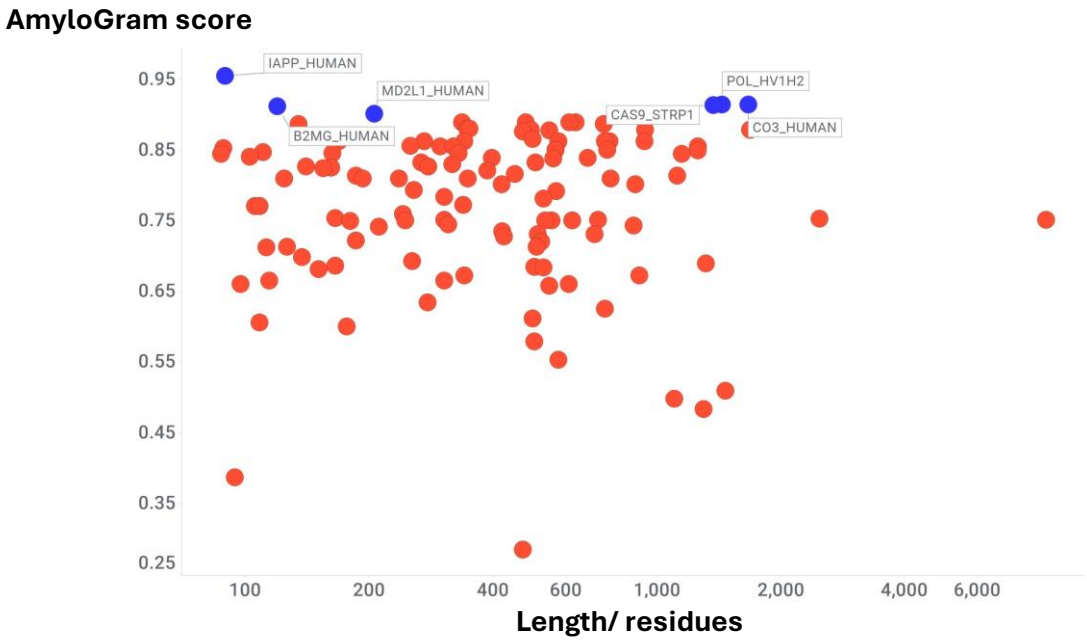

Figure 8. AmyloGram scores of 121 fold-switching proteins. Those six with an AmyloGram score exceeding 0.9 are marked in blue. 95 of the 121 have an AmyloGram score exceeding 0.7.

For context, the median AmyloGram score of the 204 proteins labelled at Uniprot as amyloid (following human analysis) was 0.88, while the median score for all human proteins was 0.81 [35]. The median score for fold-switching proteins was 0.81, meaning that in general they did not tend overall to be unusually amyloidogenic or otherwise. This is reasonable, as fold-switching is based on a relatively short subsequence ('fold-switching regions') of the protein of interest [118, 142]. This said, the distribution of overall AmyloGram scores is significantly heterogeneous, since some of them, with AmyloGram scores in excess of 0.9, such as the well-known amyloidogenic proteins amylin (islet amyloid polypeptide) [143-145] and $\beta_2$microglobulin [146-148], certainly are amyloid in nature, while complement C3 seems to be involved in cross-seeding and thus guilty by association [149-151]. HIV reverse transcriptase is also of interest, as it has been implicated in amyloidogenesis as part of Alzheimer's dementia [152]. The numbers (out of 121) of polypeptides with AmyloGram scores exceeding 0.7, 0.75, 0.8, 0.85 and 0.9 are, respectively, 95, 83, 64, 35 and 6.

Discussion

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The amyloidogenic clotting of blood to make fibrinaloid microclots (commonly with an equivalent diameter of 2-200 μm) is now well established, and has been described in dozens of papers from multiple laboratories (e.g. [1-17, 31, 153-156]). More recently, it was established that the macroclots (of over 1 mm diameter) that can be thrombectomised following an ischaemic stroke are also amyloid in character [10, 18], and there is also evidence that amyloid is a feature of atherosclerotic plaques [60-63]. Cross seeding, in which an amyloid protein induces amyloid formation in other amyloidogenic proteins that can then become part of the same fibril, is also commonplace [15, 23-25, 27, 28, 109, 157-182], as are amyloid-nucleic acid interactions [183-188].

Consequently, one can predict (correctly) that insoluble amyloid structures will tend to accumulate preferentially those proteins that are themselves more amyloidogenic than normal [15, 16]. In a recent study [35] we used the amyloid prediction program AmyloGram [33, 34] to assess this for the proteome of macroclots extracted following an ischaemic stroke, finding that the AmyloGram scores for the proteins in the stroke thrombus proteome (as measured by a number of groups) were indeed noticeably greater than the average for proteins [35].

The purpose of the present study was to assess this kind of phenomenon for macroclots taken from other diseases, such as venous thromboembolism and pulmonary embolism, and also for the many examples in which the proteome of insoluble atherosclerotic plaques had been analysed. The conclusion from the analyses above was again that in all cases the proteomes displayed a very strong tendency towards amyloidogenicity, consistent with self-seeding and providing a ready explanation both for why they are insoluble and – since amyloids are notoriously resistant to proteolysis (e.g. [70, 189-193]) – for why the thrombi are rather resistant to the normal mechanisms of fibrinolysis.

There are no necessary changes in the primary sequence of proteins following their amyloid formation; notwithstanding, amyloids can form multiple, stable variants known as polymorphs, and the insolubilisation of amyloids when they form fibrils is thermodynamically more-or-less irreversible. However, another class of proteins that can switch conformation dramatically but reversibly, including from α-helices to β-sheets, are referred to as fold-switching or metamorphic proteins. It was thus of interest to assess whether or not these too tended to be unusually amyloidogenic. The answer is that while some examples such as amylin and $\beta_2$microglobulin are indeed highly amyloidogenic, the median amyloidogenicity as reflected in their overall AmyloGram score was more or less identical to that of the proteins in Uniprot. Since it is recognised that relatively short subsequences of amino acids are actually responsible for the fold switching, this is possibly not surprising, but it was worth assessing.

Conclusion

Having established the fact that the AmyloGram scores of proteins embedded in the thrombi extracted following an ischaemic stroke are sufficient to predict that they are highly amyloidogenic [35], as they are experimentally [10, 18], it was of interest to assess the amyloidogenic potential in thrombi from other thrombi such as those involving venous thromboembolisms [16]. In every case it was found that the proteomes of these thrombi involved highly amyloidogenic proteins. The same was true in a series of studies of atherosclerotic plaques. Given that the simple presence of these plaques and thrombi indicates clearly (by definition) that they are resistant to the normal methods of fibrinolysis, it is clear that novel means will be required to effect their removal. This provides exciting opportunities.

Author contributions: All authors contributed to the conceptualization of the study, data analysis, manuscript drafting, and final editing.

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presented here are the sole responsibility of the authors and do not necessarily reflect the views of the funding agencies.

Data availability: All data supporting the findings of this study are available within the article. Stroke thrombus proteomic datasets were obtained from previously published studies and are cited accordingly.

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Conflicts of Interest: E.P. is a named inventor on a patent application related to the use of fluorescence-based methods for microclot detection in Long COVID. The funding bodies had no involvement in the design of the study, data collection or analysis, manuscript preparation, or the decision to submit for publication. The other authors have no disclosures.

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