The recognition of haemoglobin by antibodies raised for the immunoassay of β-amyloid

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Abstract Canine and porcine cerebrospinal fluid (CSF) were fractionated by size exclusion chromatography and analysed by a luminescence enzyme linked immunosorbent assay (ELISA) configured to detect β-amyloid. A peak of activity was observed in the CSF consistent with the molecular weight of β-amyloid. When CSF contaminated with blood was analysed an additional peak of immunoreactivity at a higher molecular weight was observed. The peak of activity was found to be derived from cross-reactivity of the immunoglobulins employed in the ELISA with haemoglobin. These findings are discussed with reference to primary and structural sequence homology between β-amyloid and haemoglobin from a number of species, the known properties of β-amyloid and recent clinical reports.

Key words: β-Amyloid; Alzheimer's disease; Hemoglobin; Cross-reactivity; Homology

1. Introduction

Alzheimers disease is the most common form of adult onset dementia. Approximately 5–10% of individuals in their 60s and more than 20% in their 80s show the symptoms of what is currently neither a curable or preventable disease [1]. The pathology of Alzheimer's disease is characterised by neuronal dysfunction and deposition of amyloid A4 protein (β-amyloid) as extracellular plaques and cerebrovascular amyloid [2].

β-Amyloid is a self-aggregating peptide which consists of 39–43 amino acids [3]. It is formed by cleavage of amyloid precursor protein (APP), a glycosylated receptor-like integral membrane protein, by two unidentified enzymes called tentatively β- and γ-secretase [4].

An ELISA has been configured in our laboratories comprising two monoclonal antibodies raised to different epitopes on β-amyloid. The two antibodies, named 2F12 and 1E8, recognise the epitope from amino acid residues 4–7, (FRHD) and 18–22, (VFFAE) respectively. β-amyloid-like immunoreactivity has been identified in the CSF of a number of species at $ng \cdot ml^{-1}$ concentrations [5]; however, little data exist to support the expectation that the ELISA specifically recognises β-amyloid protein.

2. Materials and methods

2.1. Fractionation of CSF and brain samples by low-pressure size-exclusion chromatography

CSF was fractionated by size exclusion using a calibrated Superdex 75 (Pharmacia, St. Albans, UK) column (16×750 mm) in 50 mM Tris-HCl (pH 8.0)+125 mM NaCl at 0.5 $ml \cdot min^{-1}$. A sample of synthetic β-amyloid 1–40 (Bachem, Saffron Waldon, UK) showed a peak with an apparent molecular weight of 5 kDa which is in good agreement with the expected value (4329 Da).

2.2. HPLC size-exclusion chromatography of synthetic amyloid

Synthetic β-amyloid 1–40 (Bachem, Saffron Waldon, UK) was chromatographed using a calibrated TSK3000GW (Anachem, Luton, UK) column (4.6×300 mm) in 50 mM Tris-HCl (pH 8.0) + 125 mM NaCl at 0.5 $ml \cdot min^{-1}$. Synthetic β-amyloid 1–40 ^{125}I-labelled at the lysine residue 16 (Penninsula Labs, St. Helens, UK) was added to the unlabelled synthetic β-amyloid 1–40 (Bachem, Saffron Waldon, UK) and mixed at a molar ratio of 1:1 with dog haemoglobin prior to incubation at ambient temperature for 24 h. Radioactivity was determined off-line using a gamma-counter (Wallac, Milton Keynes, UK).

2.3. βA4 ELISA

A Dynex Micolite II microtitre plate (Dynex Technologies, Billingshurst, UK), was coated overnight at 4°C with 1E8 monoclonal antibody (4 $\mu g \cdot ml^{-1}$) in PBS. The plate was blocked with 20% (w/v) fish gelatin (Amersham International, Little Chalfont, UK), 1.25 ml of goat serum (Sigma, Poole, UK) in 50 ml assay buffer (50 mM Tris-HCl (pH 7.4) + 0.0005% (v/v) Tween-20 + 2.18 M NaCl + 1.25 g bovine γ-globulins (Sigma, Poole, UK)) for 60 min at 37°C. βA4(1–40) (Bachem, Saffron Waldon, UK) standards were made up in assay buffer to give a final concentration range of 1.25–80 $ng \cdot ml^{-1}$. Fifty microlitres of CSF samples or standards were incubated with 50 μl of 4 $\mu g \cdot ml^{-1}$ of biotinylated 2F12 (Fab fragments) in assay buffer for 90 min at 37°C. After washing the samples were incubated with a 1:2000 dilution streptavidin/biotinylated horseradish peroxidase complex (Amersham International, Little Chalfont, UK), at 37°C for 60 min in assay buffer. Visualisation was by luminescence, generated by the addition of an immunoassay reagent kit containing luminol plus enhancer (Amersham International, Little Chalfont, UK), and quantified on a Dynatech luminometer (ML3000, Dynex Technologies, Billingshurst, UK). For the haemoglobin experiments, 50 μl of haemoglobin, final concentrations of 0–5 $mg \cdot ml^{-1}$ (Sigma, Poole, UK) from various species, was added concomitantly to the wells with 50 μl of 2F12 (4 $\mu g \cdot ml^{-1}$). Subsequent assay steps were as described above. All plate washings were with PBS + 0.05% (v/v) Tween-20 (Sigma, Poole, UK).

2.4. SDS-PAGE/blotting

10% (w/v) polyacrylamide gels were loaded and run with 20 μg protein per track and either visualised by silver stain or electroblotted. Gels were blotted onto PVDF membrane using a Tris/glycine transfer buffer (Kodak, Hemel Hempstead, UK) for 2 h at constant current 80 mA. For N-terminal sequencing, blots were stained with 0.2% (w/v) Amido Black and destained in water. For immunoblotting the blot was blocked for 1 h in Tris-buffered saline (TBS, pH 7.4) + 5% (w/v) bovine serum albumin (BSA) (Promega, Southampton, UK), washed in TBS + 0.5% (v/v) Tween-20 and incubated overnight with a polyclonal antibody to β-amyloid (B2) in TBS + 0.05% (v/v) Tween-20 + 2% (w/v) BSA. Following washing the blot was incubated for 1 h with anti-rabbit IgG (1:6000) in TBS + 2% (w/v) BSA. The blot was washed and incubated with rabbit peroxidase anti-peroxidase (PAP) solution (1:4000 in TBS + 0.05% (v/v) Tween-20 + 2% (w/v) BSA). The blot was washed and incubated with diaminobenzidine (Vector, Peterborough, UK) to visualise the bands.

For radioisotope studies ^{125}I-labelled synthetic β-amyloid (Peninsula Labs, St. Helens, UK) was introduced in the presence and absence

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of dog haemoglobin and run on 4–20% (w/v) acrylamide gels (R&D Systems, Abingdon, UK) under native or reducing SDS-PAGE conditions. Gels were stained with Coomassie Blue, dried under vacuum and analysed by both scanning laser densitometry and phosphoimagery (Molecular Dynamics, Chesham, UK).

2.5. Reverse-phase HPLC

Samples were analysed on a Hewlett Packard 1090L (Stockport, UK) by reverse-phase HPLC using 25 μl of injection onto an Aquapore RP-300, C_8, 7 μm (220×2.1 mm) column at 200 μl·min^{-1} (40°C). A gradient elution from 0.1% (v/v) TFA to 0.1% TFA + 70% (v/v) acetonitrile equivalent to a 1%·min^{-1} linear increase of acetonitrile was used. Detection was by UV at 214 nm.

2.6. N-terminal sequencing

Collected peptide peaks and blots were sequenced using an automated ABI 477A Pulsed Liquid Sequencer (Warrington, UK) with online 120A PTH-AA Analyser. Peptide solution (30 μl) was pipetted on to a cleaned glass-fibre disc, dried under nitrogen and sequenced using FAST-1 cycle conditions. Blots were sequenced using BLOTT-2 cycles. Derivatised amino acids were chromatographed using a PTH C_{18}, 5 μm (220×2.1 mm) column using a gradient elution from 0 to 90% acetonitrile in 3.5% (v/v) THF at 250 μl·min^{-1}.

3. Results and discussion

Pig CSF (5 ml) was separated by size-exclusion chromatography on a Superdex 75 column. Immunoreactivity profiles of fractions from the ELISA assay indicated two peaks of activity (Fig. 1). The later eluting of the two peaks represented activity at the expected molecular weight of β-amyloid (4 kDa). Fractions from the high molecular weight species recognised by the ELISA were separated in duplicate by SDS-PAGE and electroblotted on to PVDF membrane. These were either stained for total protein or probed with an antibody raised to β-amyloid. Bands from the total protein stain which corresponded to apparent β-amyloid immunoreactivity at 16 kDa in the Western blot were sequenced by automated Edman Chemistry. Two major sequences indicated the presence of pig haemoglobin α- and β-chains.

When dog CSF was fractionated on the Superdex column, a peak of immunoreactivity was observed at the expected molecular weight for β-amyloid. A second higher molecular weight peak of immunoreactivity analogous with the pig CSF was observed. The lower molecular weight peak was collected and further analysed by reverse-phase HPLC. A peak of immunoreactivity of similar retention time to synthetic β-amyloid standard was observed; however, the levels were below the level of detection of automated N-terminal sequencing even when an equivalent to 30 ml of CSF was processed. When a sample of dog CSF was specifically collected avoiding blood-derived contamination only one peak of immunoreactivity was identified with an apparent molecular weight of approximately 4 kDa. Possible explanations for the immunoreactivity of the higher molecular weight peak included β-amyloid aggregates, β-amyloid in association with another protein, a larger fragment of APP and/or antibody cross-reactivity with other proteins.

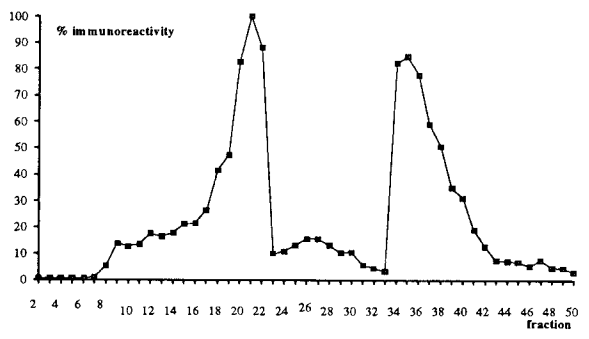
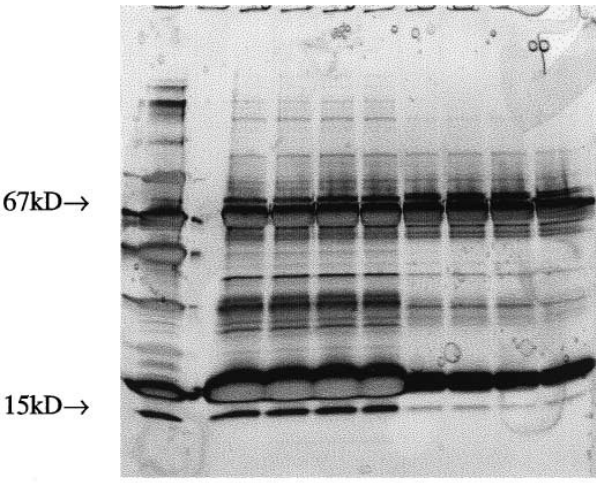


Fig. 1. ELISA analysis of pig CSF gel filtration samples.



Markers ---Contaminated CSF---- -----Clean CSF------

Fig. 2. SDS-PAGE of clean and contaminated dog CSF.

Samples of clean and blood-contaminated dog CSF were analysed by SDS-PAGE under reducing conditions and silver staining (Fig. 2). Two major bands were observed with apparent molecular weights of 67 and 15 kDa. In the case of the contaminated sample an increase in the 15 kDa band was noted. In the uncontaminated canine CSF the major sequence at 15 kDa (VPAGTDGPN) showed 33% sequence identity with the N-terminal sequence of human transthyretin. It is noteworthy that the sequence for canine transthyretin is currently unavailable in the public sequence databases. In the contaminated dog CSF two additional sequences were identified as the α- and β-chains of dog haemoglobin which have a subunit molecular weight of 15217 Da (α-chain) and 15996 Da (β-chain).

It was considered feasible that interactions between amyloid and haemoglobin in vivo may occur. To investigate this β-amyloid was spiked with ^{125}I-labelled β-amyloid and the retention of radioactivity in the presence of dog haemoglobin monitored using size exclusion HPLC (not shown). Analysis of fractions indicated that a small amount of radioactivity (< 10%) was associated with the haemoglobin. However, this binding was non-saturable and could not be competed out by unlabelled β-amyloid in a 1000 molar excess suggesting non-specific interaction. Binding with similar characteristics was observed with bovine serum albumin. Analysis by native PAGE and SDS-PAGE followed by phosphorimagery indicated that in the presence of haemoglobin an additional minor band of radioactivity in native format was observed, but was not stable to SDS (Fig. 3). This was in contrast to the ap-

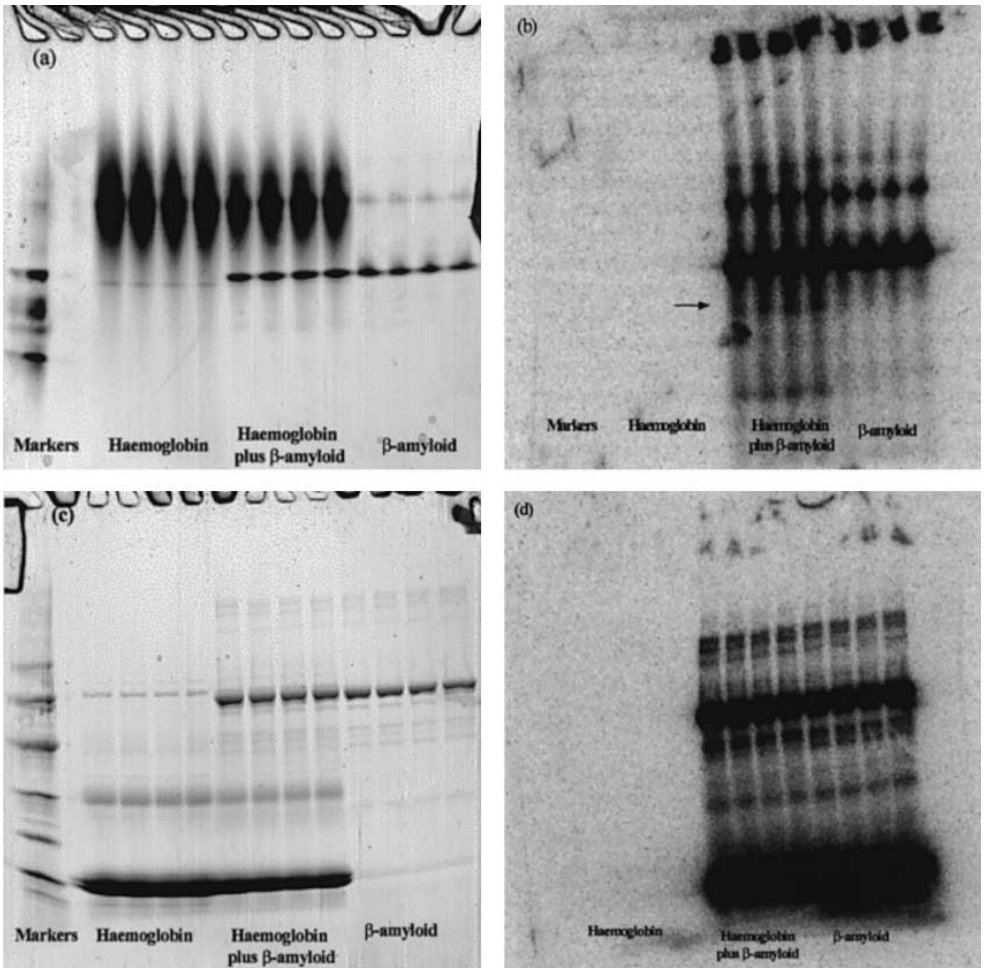


Fig. 3. Native and SDS-PAGE analysis of canine haemoglobin in the presence and absence of ^{125}I-labelled β-amyloid 1–40. Native PAGE gels are shown in Coomassie stain (a) and phosphoimage (b). The observed extra band in the haemaglobin plus β-amyloid band is denoted by the arrow (→). Reducing SDS-PAGE gels are shown in Coomassie stain (c) and phosphoimage (d).

parent immunoreactivity which as described earlier was stable to reducing SDS-PAGE as visualised by Western blotting.

Reports exist that transthyretin (pre-albumin) found in the CSF plays a role in amyloidogenesis with a constantly growing list of single point mutations of transthyretin resulting in familial amyloidosis [6–8]. Data described earlier indicated the presence of transthyretin in both clean and contaminated dog CSF (Fig. 2). On separation of both clean and contaminated canine CSF by RP-HPLC, peaks were noted in the contaminated CSF with sequences showing 60% and 64% identity with human transthyretin. Although transthyretin has been identified in CSF samples, the lack of β-amyloid immunoreactivity in the uncontaminated sample (containing transthyretin) suggests that transthyretin is not responsible for the observed reactivity. A possible explanation for failure to determine any complexation of amyloid–transthyretin may be that amyloid associated with transthyretin is sterically hindered from being detected in the immunoassay.

Table 1
Sequence homology between β-amyloid epitopes and haemoglobin (Hb) from human, bovine and dog. Identity and similarity are defined in the text

Epitope	Species	Chain	Sequence	% Identity	% Similarity
	Human Hb	α	^{47}D–^{60}K	28.6	57.1
2F12		β	^{76}A–^{95}K	33.3	60.0
(1–16)	Dog Hb	α	^{47}D–^{60}K	21.4	50.0
		β	^{112}C–^{125}Q	28.6	35.7
	Bovine Hb	α	^{77}G–^{92}R	12.5	37.5
		β	^{79}D–^{94}K	25.0	37.5
	Human Hb	α	^{103}H–^{116}E	28.5	50.0
1E8		β	^{99}D–^{112}C	0	57.1
(13–27)	Dog Hb	α	^{15}D–^{27}E	30.8	69.2
		β	^{99}D–^{112}C	0	57.1
	Bovine Hb	α	^{103}H–^{116}D	35.7	57.1
		β	^{110}V–^{120}E	27.3	63.6

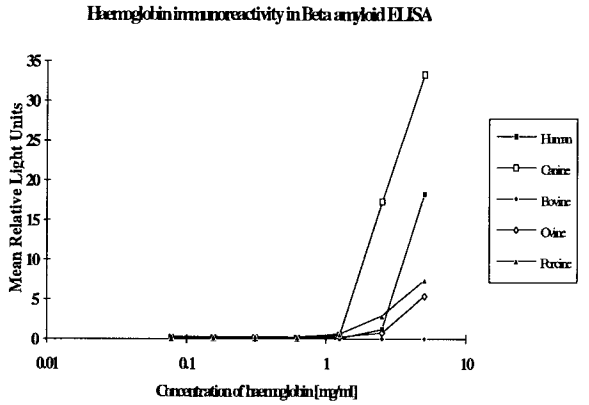


Fig. 4. Haemoglobin immunoreactivity in β-amyloid ELISA.

Apolipoprotein E (ApoE) may be involved in amyloid transport [9]. ApoE has been identified in association with amyloid in plaque formations and there is evidence of the epsilon 4 allele of ApoE as a risk factor for familial Alzheimer's disease [10–12]. No evidence was found to support the association between endogenous ApoE and amyloid in this study. The bands of immunoreactivity observed, when probing the immunoblot with antibodies raised against β-amyloid, corresponded with both the presence of haemoglobin ($\approx$15 kDa) as determined by sequencing and a lower band at the expected molecular weight of amyloid ($\approx$4 kDa). No band immunoreactive with anti-β-amyloid corresponded with bands highlighted when probing with antibodies raised against ApoE. The lack of detectable ApoE–amyloid complex is not unexpected as the published levels of complex are low even in the presence of excess β-amyloid [11].

To test the hypothesis that the antibodies 1E8 and 2F12 recognise haemoglobin, samples of haemoglobin (Sigma, Poole, UK) from various species was assayed by ELISA (Fig. 4). There was an apparent cross-reactivity of the antibodies used in the amyloid ELISA with haemoglobin at levels >1 mg·ml^{-1}; however, the cross-reactivity is species dependent with the order of reactivity canine > human > porcine > ovine > bovine.

The antibodies used in the assay, 1E8 and 2F12, were raised against the β-amyloid derived peptides ^{13}H–^{27}N and ^{1}D–^{16}K, respectively. The epitopes were later mapped to ^{18}V–^{22}E (1E8) and ^{4}F–^{7}D (2F12). Primary sequence homology was compared between the peptide immunogen sequences and haemoglobin α- and β-chains from various species. Using BestFit comparison on the GCG protein sequence analysis software (Genetic Computer Group, Madison, USA), the identity varied widely from 0% to 35.7% depending on the haemoglobin species and subunit, whereas the similarity as defined by the Smith Waterman algorithm ranged from 35.7% to 69.2% (Table 1). These data therefore demonstrate a relatively low primary sequence homology between β-amyloid and mammalian haemoglobin. The epitope sequences recognised by the antibodies were not present in the primary sequence of α- or β-haemoglobin chain of the three species. Consideration of the homology of the primary sequence of β-amyloid and haemoglobin is therefore not satisfactory to explain the apparent cross-reactivity observed. In addition primary sequence homology does not correlate well with the observed species differences in cross-reactivity.

The consideration and comparison of the primary sequence homology does not reflect the 3-dimensional spatial orientation of the amino acid residues in the haemoglobin tertiary structure. Experimental crystal structures do exist for both the bovine (1hda) and human (1hga) haemoglobin entities and the resolution of these structures is 2.2 Å and 2.1 Å, respectively. At present, there is no experimental structure for canine haemoglobin; however, the homology of this sequence with the bovine/human sequences is good and as such, the canine structure is predicted to have the same fold motifs as the other family members. The structures were overlaid (RMS 0.57 Å, based on superimposition of the backbone atoms) and all the interatomic distances between the surface residues calculated. These distances were used to highlight several β-amyloid antibody epitope regions that are close in 3-dimensional space and that also occur on the surface of the haemoglobin structure. For example, the 2F12 epitope, FRHD, has an analogue in the haemoglobin structure, namely, ^{41}F–^{40}R–^{89}H–^{85}D, these residues are both surface accessible and occur in close proximity to each other. Clearly these relationships are only apparent upon consideration of the actual 3-dimensional structure and cannot be derived solely from the primary sequence information. Similar binding regions exist for the 1E8 antibody epitope, ^{98}V–^{42}F–^{45}F–^{62}A–^{21}E. Indeed, basing the analysis solely on the binding regions, the β-amyloid antibodies have molecular recognition sites on the haemoglobin structure that are close in space; however, no inter-species differences are predicted. But assuming that the canine sequence has the same overall fold as the other haemoglobins, then ^{82}A, which exists in both canine and human, is changed to ^{82}E in bovine haemoglobin. This changed site is close in space to the 2F12 binding site reported earlier and it is not unreasonable that such a significant surface amino acid point mutation could have a major effect on the preferential binding of the antibody.

It should also be noted that the limit of detection for β-amyloid (ng·ml^{-1}) compared to that for haemoglobin (mg·ml^{-1}) indicates a 10^6 fold selectivity for β-amyloid. It is apparent that the relatively high concentration of haemoglobin in the circulatory system (14 g·dl^{-1} in human blood) ensures that even low levels of contamination in the CSF due to traumatic tap or cerebral bleeding can have a significant impact on the bioassay of β-amyloid.

These data support the view that the immunoreactivity is the result of genuine cross-reactivity between β-amyloid and haemoglobin and has direct relevance for ex vivo measurement of β-amyloid in the presence of relatively low levels of haemoglobin. Such cross-reactivities are not without precedent. Human β-amyloid peptide has been shown to display cross-reactivity with human glyceraldehyde-3-phosphate dehydrogenase [13] and rat amyloid with rat calcitonin gene-related peptide [14], despite displaying only 19% and 17% primary sequence identity, respectively.

The apparent cross-reactivity between β-amyloid and haemoglobin has clear implications for the detection and quantitation of β-amyloid in biological tissues and fluids. Although the potential clinical relevance of such observations is less clear some reports have postulated a link between haemoglobin and Alzheimer's disease.

Increased levels of haemoglobin-derived peptides have been reported in the brain tissue of Alzhemier patients [15]. Of several hundred peaks analysed by RP-HPLC, 15 peaks were elevated in Alzheimer's patients. Of these, 9 peaks

were found to be fragments of haemoglobin. Entry of haemoglobin-derived peptides into the CNS may be a manifestation of a loss of blood–brain barrier integrity [16,17]. It appears that oxidative stress may have a role to play in the pathology of Alzheimer's disease. An elevated activity of the inducible form of haem-oxygenase-1, catalysing the conversion of the pro-oxidant haem to biliverdin which is ultimately converted to the anti-oxidant bilirubin has been observed in Alzheimer's disease [18,19]. Further work will be needed to fully elucidate the role of haemoglobin in Alzheimer pathology.

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