B-AMYLOID PRECURSOR PROTEIN STAINING OF THE BRAIN IN SUDDEN INFANT AND EARLY CHILDHOOD DEATH

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ABSTRACT

Aims

To develop and validate a scoring method for assessing β-amyloid precursor protein (APP) staining in cerebral white matter and to investigate the occurrence, amount and deposition pattern based on the cause of death in infants and young children.

Methods

Archival cerebral tissue was examined from a total of 176 cases (0 to 2 years of age). Each of the APP-stained sections was graded according to a simple scoring system based on the number and type of changes in eight anatomical regions.

Results

Examination of the sections revealed some degree of APP staining in 95% of the cases. The highest mean APP scores were found in cases of head trauma, and the lowest scores were found in the cases of drowning. APP staining, although sometimes minimal, was found in all 48 cases of and sudden infant death syndrome (SIDS). Patterns of APP staining (the amount and distribution) were different in cases of head trauma, infection and SIDS but were similar in the SIDS and asphyxia groups.

Conclusion

This study demonstrates the use of an integrated scoring system that was developed to assess APP staining in the brain. APP staining was seen in a high proportion of cases, including relatively sudden deaths. The amount of APP was significantly higher in cases of trauma than in non-traumatic deaths. However, APP was detected within all groups. The pattern of APP staining was similar in infants who had died of SIDS and from mechanical asphyxia.

Keywords: β-APP, APP, axonal injury, SIDS, immunohistochemistry, sudden infant death, head trauma, asphyxia

List of abbreviations

APP	β-Amyloid precursor protein
CNS	Central nervous system
H&E	Haematoxylin and eosin
IC	Internal capsule
LOA	Limits of agreements
PBS	Phosphate buffered saline
SD	Standard deviation
SIDS	Sudden infant death syndrome
WM	White matter

INTRODUCTION

β-amyloid precursor protein (APP) is a widely expressed membrane glycoprotein found in most mammalian tissues, but primarily within the brain [1,2]. In normal neurons, APP is present at low, undetectable levels and performs functions relating to neuronal stem cell proliferation, synaptogenesis and neuroprotection [3-6]. However, when neurons are damaged, APP production is rapidly up-regulated, resulting in its accumulation within axons due to fast axoplasmic transport inhibition [7-12]. APP-positive changes have been recorded as early as 35 minutes after injury in humans and the increase in APP remains the most effective marker of axonal injury in formalin-fixed, paraffin-embedded material [13-17]. In addition to survival time, the extent of brain damage influences the amount of APP present, while the age of the decedent has been shown not to affect the amount of APP staining [14,18]. Though primarily used as an indicator of traumatic injury, several studies have demonstrated that accumulation of APP can also be caused by hypoxic-ischaemic injury and various other causes, in the absence of head injury, such as sudden infant death syndrome (SIDS) [14,16,19-29]. In fact, the traditionally believed higher amount of APP in cases of head injury has not been possible to demonstrate statistically [29].

The assessment of APP in white matter has been addressed in different ways, mostly by using semi-quantitative methods in which immunopositivity is graded from mild to moderate or severe (Table 1) [16,19,20,30]. Not all studies have, however, specified the methods used for grading the amount of APP [22,31,32]. Most studies of APP expression have concentrated on axonal injury due to traumatic causes [14,16,27-29]. In contrast, some authors have found APP changes to be

more discrete or non-existent in SIDS or other non-traumatic causes of death [17,26]. The previously used methods of assessing APP staining do not report reproducibility and are not suitable for statistical analysis because categorized variables are used instead of continuous variables. Other methods such as the Axonal Injury Sector Scoring method and variations thereof are accurate and objective but require detailed and uniform sampling of tissue and are therefore only suitable when whole brains are available [14,28].

As APP accumulation is an energy-dependent process, the amount of APP staining is related to the length of patient survival after the traumatic event [14,18,29]. When a neuron is injured, the cytoskeleton breaks down, possibly due to calcium influx [33,34]. Because the cytoskeleton plays an important role in axonal transport, this is consequently impaired [11,13,20]. Shortly after injury, APP begins to accumulate as small pinpoint areas of staining or granules. The size of these small punctate areas increases with time, forming axonal swellings [13,35] which have the potential to progress into retraction bulbs when the distal part of the axons is disconnected [13]. The following report describes a uniform, reproducible and sensitive technique to assess APP changes in white matter that can be performed on archival tissue and which is suitable for statistical analysis. Furthermore, we demonstrate the application of this method on archival tissues of infants and young children dying of traumatic and non-traumatic causes.

MATERIALS AND METHODS

A total of 182 children from 0 to 2 years of age were autopsied at Forensic Science South Australia in Adelaide, South Australia over a 7.5 year period from July 1999 to December 2006. All cases had undergone full police and coronial investigations with full autopsies and a varying range of ancillary testing. Information concerning cause of death, post mortem interval, and circumstances of death such as resuscitation attempts and survival time from the incident to death were reviewed and entered into a database using EpiData version 3.1 software. Estimates of survival time from the life-threatening incident or finding of the decedent to death were based on police reports, hospital records and autopsy reports.

The cases were grouped into different categories as follows. The San Diego criteria were used to classify cases of SIDS [36,37]. Cases described as "undetermined consistent with asphyxia" and not meeting the SIDS criteria were categorised as "undetermined". Other categories included head trauma, mechanical asphyxia, infection, drowning, dehydration, perinatal death, congenital heart disease, pre-existing brain pathology, failure to thrive, poisoning, burns/incineration, and other/combination.

Brain and spinal cord tissue were taken from 176 (97 %) of the 182 cases of sudden infant death included in the study. After evaluation of the method, twelve cases with only one or two sites were excluded. Of the remaining 164 cases, a formal examination had been performed by a neuropathologist in 132 cases (80 %). The brain tissue had been investigated by forensic pathologists in the remaining 32 cases (20 %). Of the 164 cases, 121 subjects (74 %) were less than one year of age, 25 (15 %) were between 1 and 2 years of age, and 18 (11 %) were between 2 and 3

years old. A total of 100 (61 %) of the cases were male and 64 (39 %) were female. Prematurity (gestational age less than 37 completed weeks) was noted in 28 (30 %) of the cases, but information concerning gestational age was not available in 72 (44 %) of the cases. The number of sites sampled from the brain varied considerably and ranged from 3-64 (mean 23) per case. The highest mean number of sites was sampled from cases of SIDS and dehydration (mean 27) and the lowest from the two cases of poisoning (carbon monoxide and morphine), with a mean of five sites. A total of 3834 sites were examined.

Slides from each sample of formalin-fixed, paraffin-embedded brain tissue had been stained with haematoxylin and eosin (H&E) to evaluate non-APP changes such as necrosis or apoptosis.

Immunohistochemistry

All paraffin-embedded brain and spinal tissue blocks were cut at 5 µm and incubated with a monoclonal antibody (clone 22C11, Chemicon (Millipore), Billerica, MA, USA) to APP according to standard protocols [38]. Archival slides had been manually stained including overnight incubation of the sections with the APP antibody at a dilution of 1/2000 while slides from 2006 were stained using the Dako auto-stainer with Envision+ Dual Link (Dako, Denmark) including incubation with APP antibody at a dilution of 1:150 for 30 minutes. Comparison of auto-stained and manually stained slides from the same tissue block in 43 blocks showed no significant differences (group level, p=0.8556, Kruskal-Wallis equality-of-populations rank test; paired slide-to-slide comparison: p=0.3029, Wilcoxon signed-ranks test).

Grading of the amount of APP staining

The amount and pattern of APP staining was recorded for each slide, and the slide was scored

according to a grading system that was developed specifically for this study (Table 2). The entire slide was examined. Small granules were defined as APP positive axonal changes of ≤ 5 µm, equivalent to one small unit on a 10 mm/100 unit micrometer at 200 times magnification (Fig. 1A). The granules were often disposed in a linear arrangement in the line of an axon, and in those cases the granules were only scored as one change (Fig. 1B). A maximum of 1 to 5 granules per field of view (view field diameter 1,100 µm at 200 times magnification) was assigned a score of one. Six to 20 granular changes/field of view were assigned a score of 2, and more than 20 received a score of 3. APP positive axonal changes larger than 5 µm with no definite sign of transection were defined as axonal swellings (Fig. 1A) and were scored in the same way as the granular changes.

Axonal swellings with definite signs of transection, i.e., a well-defined bulb with a loss in continuity followed by a continuation of the axon, was defined as a retraction bulb. The presence of axonal bulbs (independent of the amount of APP) were assigned a score of 1 because a preliminary examination of slides from approximately 40 cases showed that there were usually less than five retraction bulbs present per field (1C). Brainstem and spinal cord sections were not scored for retraction bulbs, as many brainstem and spinal cord axons travel in the longitudinal plane, and transverse sections make it difficult to assess retraction bulbs. Finally, sections were scored for broad bands of axonal lengths under low power magnification (Fig. 1D). A score of 1 was assigned if the lengths were without granular changes, and a score of 2 was assigned if the bands had multiple granular changes. Increased background staining without a clear band-like pattern was not scored to avoid false positives. Finally, the sections were divided into eight

anatomical regions: 1. corpus callosum, 2. internal capsule, 3. hemispheric white matter (internal capsule excluded), 4. cerebellum, 5. midbrain, 6. pons, 7. medulla oblongata and 8. spinal cord. The highest scores in each category (granular changes, axonal swellings, retraction bulbs and bands) and from each region were then added, and the results in each category were divided by the maximum obtainable score for each region to give a percentage. There was a maximum score of nine (3 (granular changes) +3 (swellings) +1 (retractions bulbs) +2 (bands)) for the corpus callosum, internal capsule, hemispheric white matter (internal capsule excluded), cerebellum, and a score of 8 for the brainstem and spinal cord as retraction bulb scoring was not included (Tables 2 and 3). If sections were sampled from all regions, the maximum score would be 68 and less if sections were missing from a region. The result of this grading system is an APP staining score as a percentage of the maximum possible for each anatomical region and for the regions combined (see Table 3).

The grading of APP staining was performed by LLJ, blind to the case diagnosis and clinical history.

Twenty cases were randomly selected to evaluate the consistency of scoring by the same observer (LLJ) and across different observers. The time interval between the two assessments for intraobserver variability was approximately three years. This interval was chosen to ensure that the observer would not remember the cases. For the interobserver assessment, a senior neuropathologist (BPU), who was not otherwise connected with the development of the method, was chosen to evaluate the same 20 cases. The grading of APP staining was done blind to the diagnosis, previous review of APP and the clinical history. A total of 348 sites were reviewed for the intra- and inter-variability analysis.

Stata software version 11 was used for statistical analysis.

The study was approved by The University of Adelaide Human Ethics Committee (#H-008-2007)

RESULTS

With a three-site minimum, the maximum inter- and intraobserver difference in APP scoring was 17%, and in the majority of the cases it was less than 10%. Reducing the maximum inter- and intraobserver difference to less than 13 % would have necessitated excluding any cases with fewer than 25 sites. Thus, we chose the three-site minimum because a higher minimum would have required the exclusion of an unacceptable number of cases. To meet the three-site criteria, five cases with one slide and seven cases with two slides were excluded. However, the amount of APP did not increase or decrease with increasing number of sites sampled (Fig. 2). Evaluation of repeatability showed that the intraobserver variability was within the limits of agreement in all but one case when excluding cases with only one or two sites sampled. The differences between the two intraobserver measurements were not significant (t-test of the mean, *p=0.5098*). Evaluation of the interobserver variability showed no significant differences in the total APP scores between the two observers (t-test of the mean, *p=0.7438*), and all but one difference between the paired observations were within the limits of agreement. The mean of the differences (bias) was found to be 1.28 and 0.66 in the intraobserver and interobserver assessments respectively. This means that the cases on average received an APP score that was approximately one score higher the second time/by the second observer compared to the first time/first observer.

The three most common categories of death were SIDS (48 cases, 29%), head trauma (20 cases, 12%), and mechanical asphyxia (16 cases, 10%) (Table 4). "Undetermined" causes of death comprised 27 cases (16%). The head injury cases resulted from 9 (45%) motor vehicle accidents, 8

cases (40%) of non-accidental injury (NAI), 2 cases (9%) with other accidental causes of head injury, and 1 case (6%) with an undetermined cause. The small group with previous brain pathology included a case with a history of epilepsy, a case of pseudo-TORCH syndrome, and a case with leukomalacia resulting in seizures and developmental delay. In all of these cases, subjects were found dead in bed with no other apparent causes of death. The five cases included in the 'perinatal' group were stillbirths, or subjects had become unresponsive during labour due to perinatal events such as cord prolapse or antepartum haemorrhage due to vasa previa. Three cases were preterm with gestational ages of 24, 28 and 30 weeks. All cases of head trauma had evidence of meningeal and/or intra-parenchymal macroscopic haemorrhage. In the cases of dehydration, two subjects died from lack of water due to the death of their caretaker, four had gastroenteritis and one had a fever and feeding difficulties.

Only eight (5%) of the cases were completely APP negative, and 25 (15%) had very low APP scores ranging from 1 to 9. Fifty per cent of the cases had total APP scores between 12 and 45. The mean APP score was 30.9 (SD 24, range 0 to 98). The causes of death in the APP negative cases were mechanical asphyxia (2), drowning (2), undetermined (1), poisoning (1), and infection (1). A total of 117 cases had no reported verifiable survival time, although some survival time cannot be completely excluded.

In 37 cases, the reported survival time was between 25 minutes and 19 days. A figure showing the relationship between the amount of APP and survival time in the group with the highest amount of APP, head trauma, is shown in Fig. 4, demonstrating and an increase in APP with increasing survival time. In another 10 cases, hereof 7 with non-accidental injury, survival was highly

probable based on the history and the findings, but the verifiable information was missing.

The sampling of six the eight regions varied only marginally between most of the diagnostic groups. Hemispheric white matter was sampled in all but one case, and internal capsule, cerebellum, pons, midbrain and medulla oblongata were all sampled in more than 85% of the cases. Corpus callosum was sampled in 74 % of the included cases, with a range from 60 % to 94 % in the major diagnostic groups (ten cases or more). A total of 47 % of all of the included cases had sections from spinal cord. In the major diagnostic groups the range was from 18% (infection) to 70% (drowning), but SIDS, undetermined, head trauma and mechanical asphyxia had sections from the spinal cord included in 44-50% of the cases.

The group with head trauma had the highest mean APP score (59), and the APP scores in this category were significantly higher than in the cases of SIDS, mechanical asphyxia, undetermined cause of death, infection, and dehydration, again in order of decreasing significance (Kruskal-Wallis, *p=0.0004-0.0494*) (Fig. 3). The groups with the lowest mean scores were those where death was attributed to drowning (mean APP score 10, SD 20) or poisoning (mean APP score 13, SD 18) (Table 4). The APP scores in the drowning cases were significantly lower than in the cases of head trauma, SIDS, dehydration, undetermined causes of death, infection, other/combined causes of death, perinatal causes, and mechanical asphyxia, in order of decreasing significance (Kruskal-Wallis, *p=0.0001-0.0324*). If the two regions with the most variable sampling frequency, the corpus callosum and the spinal cord, were excluded from the computations in the major diagnostic groups, head trauma remained the diagnostic group with the highest mean APP level compared to the above-listed groups and the order of decreasing significance did not change

(Kruskal-Wallis: (*p=0.0002-0.0098*)). Equally importantly, drowning remained the diagnostic group with the lowest mean APP score and neither the order of decreasing significance did not change here (Kruskal-Wallis: head trauma (*p=0.0002-0.0345)*). Indeed, the differences in sampling had no significant impact on the final results and only very little impact on the p-values in the groups with ten cases or more.

The SIDS group showed a mean APP score of 28 (SD 17) (Table 4), and none of the SIDS infants were APP negative.

The amount of APP was unevenly distributed in the eight regions. The mean APP score was highest in the cerebral sections, with a mean APP score of 43 in the internal capsule and 42 in the corpus callosum and hemispheric white matter (internal capsule excluded). The lowest mean scores were 20 in the medulla oblongata and 21 in the cerebellum (Fig. 5 and Table 5). In all eight anatomical regions, the range of the APP score was 0-100.

A significant feature emerged when the APP staining in each region was plotted for the four major diagnostic groups that included 10 or more cases (SIDS, head trauma, mechanical asphyxia and infection). While the patterns of APP staining (i.e., the amount and distribution) were different in the cases of head trauma, infection and SIDS, there was striking similarity between the regional pattern in the SIDS and mechanical asphyxia groups (Fig. 6).

DISCUSSION

This study presents a new and sensitive grading method for assessing the expression of APP in central nervous system tissue and demonstrates its use in the analysis of infant and early childhood deaths from a range of causes. The highest APP scores were found in cases of head trauma, demonstrating increasing staining with prolonged survival. The lowest were found in cases of drowning. In addition, a similarity was found between the regional patterns in SIDS and mechanical asphyxia.

APP positive axons have been recorded as early as 30 minutes after injury in animal studies and 35 minutes in human adults using normal light microscopy, but most human studies record minimum lengths of survival from 90 minutes to 3 hours [11,13,15,27,28,30]. In this study, head injury cases with verifiable survival times had increasing amounts of APP with increasing survival time. This is in agreement with Wilkinson's (1999) and Gorries (2002) studies on the relationship between survival time, the size of axonal swellings and the amount of APP [14,18]. In our study, only eight cases had APP scores of zero, and none had reported survival times or reported head injuries. Although some survival time cannot be completely excluded, this emphasises the relationship between APP deposition and duration of the lethal episode.

In addition, we were able to demonstrate a significantly higher amount of APP in the group with head injury compared to several of the non-head injury groups. While some studies claim to have found no APP in cases without head injury, other studies with more non-head injury cases have verified that APP is indeed common in cases such as SIDS, although not as pronounced as in cases with cerebral trauma [15,17,20,26,29,39]. While this difference in staining was expected we are, to our knowledge, the first to document it to be statistically significant. Oehmichen's (2000) study,

which included 252 cases, concluded that no statistically significant difference was found in the amount of APP between traumatically and non-traumatically induced axonal injuries [29]. One possible explanation for this discrepancy in findings, is the fact that Oehmichen used a three-tiered scoring method, which requires much larger number of cases to reach statistical significance compared with a model with a continuous variable as in the present study [40]. Despite this significant difference, we also report an overlap in the amount of APP found in cases with and without head injury, demonstrating that APP alone cannot be used to differentiate between cases with injury from those without. Consequently, even though significant differences in the amount of APP have been demonstrated between for instance the group of SIDS and the group of dead trauma, it is not possible to determine the cause of death based on the amount of APP. It has to be emphasized that the strength and the purpose of the scoring system is to enable statistical comparisons between diagnostic groups and comparisons between the amount of APP and certain risk factors. However, it is evident that APP is a helpful adjunct, and that the diagnosis of non-traumatic injury should be made with caution especially in cases where the amount of APP exceeds 70 (Fig. 3).

A total of 156 (95%) of the 164 were APP positive including all 48 SIDS cases. This proportion of APP positive cases is relatively high compared to other studies in the literature, especially in light of the high number of cases (117) with no recorded survival time [20,26,29,30,32]. Possible explanations for this variability in APP amount could be differences in the study inclusion criteria and to some extent, varying definitions of what constitutes APP staining between and among investigators and research institutions, as some investigators do not record smaller and less frequent lesions, or they do not regard mild focal APP staining as significant [26,30,41]. In

contrast, we included both small and focal APP positive axons. Furthermore, the traditional focus on traumatic head injury may also lead to an underestimation of the amount of APP present in non-head injury cases, where the investigators may be less likely to note low and/or focal levels of staining [30]. As to the cases with no reported survival time, the lack of evidence of survival does not preclude survival for a shorter or longer period of time. In addition, the time of death is difficult to define as death may be a lengthy process where cells are known to die at different rates rather than a single event [42]. As APP has been found to persist for up to 99 days after head injury, it is also possible that at least some of the APP staining may have originated from previous episodes of non-lethal trauma or hypoxia [25]. Certainly, in one case, the finding of multifocal areas of APP staining in an infant who died of SIDS enabled the identification of significant apnoea in his subsequent sibling, which raised the possibility of a familial central apnoea syndrome [38].

Concerning the anatomical distribution of the APP changes, our study showed that the amount of APP was highest in the corpus callosum, internal capsule and hemispheric white matter (internal capsule excluded), with lesser amounts in the brainstem, cerebellum and spinal cord. The regional differences found in this study are in accordance with cases following head injury that used scoring methods where the brains were meticulously and uniformly sampled and the amount of APP mapped [28,41].

Other researchers have differentiated patterns due to hypoxia-ischaemia from those due to traumatic brain injury [20,30]. However, hypoxia-ischaemia is the probable end-stage of a number of conditions including traumatic brain injury; thus, a significant overlap of the APP patterns is not unexpected, and a diagnosis based only on APP staining fails in almost two thirds

of cases [43]. An interesting observation concerned the amount and distribution of APP staining in cases grouped under different causes of death. A striking similarity between the SIDS group and the group with mechanical asphyxia was demonstrated in contrast to that of infection and head injury. Whether this similarity results from a preceding long period of terminal asphyxia in SIDS cases, as suggested by others requires further evaluation [44-46]. Perhaps the pattern that was identified by the current method of APP staining scoring in SIDS cases sheds some light on the lethal mechanism?

In conclusion, this study has demonstrated a new scoring method for assessing APP deposition in archival central nervous system tissue. The data confirm that APP staining is found in a high proportion of paediatric cases, even when death appears to have been relatively sudden. The scoring system identified the highest amount of APP staining in head trauma cases, with an increase in the amount of staining with prolonged survival time (although this was not an absolute finding). While the regional APP pattern was quite different in cases of head trauma, infection and SIDS, there was notable similarity between the SIDS and mechanical asphyxia groups. Whether this finding supports an asphyxia-based mechanism of death in SIDS cases requires further investigation.

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BP Ulhøi participated in validation of the method and helped edit the manuscript;

J Banner participated in the design of the study, the validation of the method, and helped edit the manuscript;

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The Authors declare that there is no conflict of interest.

Figures and Tables

Table 1 Different methods of assessing APP immunopositivity in cerebral white matter

Author	Scoring system
Otsuka 1991[11]	(-) negative (+) weak (++) moderate (+++) strong
Sherriff 1994[19]	(-) Absolute count of APP-positive axons in a section with 0-5 axons (+) Absolute count of APP-positive axons in a section with 6-150 axons (++) Absolute count of APP-positive axons in a section with 150-300 axons (+++) Absolute count of APP-positive axons in a section with over 300 axons
Gentleman 1995[16]	1: Any staining of the axons, however slight 2: Scattered patches of axonal damage 3: Extensive axonal damage
Blumbergs 1995[28]	Axonal injury sector scoring (AISS) method APP-positive axonal swellings recorded on a series of line diagrams of standard brain sections divided into 116 sectors and an AISS score ranging from 0-116 Requires an accurate sampling of the cerebral hemispheres at 11 coronal levels separated by 10-mm intervals, 6 cross-sections of brainstem and one section from each cerebellar hemisphere
Oehmichen 1998[29]	(0) No recognisable expression (+) Isolated or disseminated APP-positive axons and/or fragments or bulbs (++) APP-positive axons, fragments or bulbs occurring in groups, sometimes in foci and sometimes diffusely
Kaur 1999[20]	Mild: Occasional focal axonal varicosities Moderate: Not described Severe: Diffuse, well-formed axonal staining with abundant bulbs
Gorrie 1999[41]	Each field (0.05 cm2) with at least three positive axons was scored as positive and marked. The marked sections were then scanned into a computer and, using image analysis software, overlaid with a sector grid. The proportion of injury in each sector was determined. This method requires highly accurate sampling of the brain.
Reichard 2003[30]	(-) No staining (+) Mild staining: pinpoint, typically requiring a high-power objective (40x) (++) Moderate staining: axonal swellings/bulbs, often in "patches" (+++) Severe staining: extensive axonal staining, readily apparent with a low-power objective (2x) In addition, a clinicopathological diagnosis is based on a combination of the clinical history and pattern of APP immunopositivity: mTAI (multifocal traumatic axonal injury), dTAI (multifocal traumatic axonal injury), MAI (metabolic axonal injury), VAI (vascular axonal injury) or PAI (penumbral axonal injury).
Thornton 2006[47]	(0) no APP-immunoreactive axons (+) 1-25 APP-immunoreactive, damaged axons (++) 26-50 APP-immunoreactive, damaged axons (+++) 51-75 APP-immunoreactive, damaged axons (++++) 76-100 APP-immunoreactive, damaged axons (+++++) over 100 APP-immunoreactive, damaged axons
Johnson 2011[26]	Negative

Focally (staining within a single area of the slide) Mild: 1-3 axonal profiles per high-power field (hpf, 40x) Moderate: 3-10 profiles per hpf Severe: more than 10 profiles per hpf Multi-focally (multiple areas of the same microscopic slide separated by a power field greater than 10x) Mild: 1-3 axonal profiles per hpf Moderate: 3-10 profiles per hpf Severe: more than 10 profiles per hpf

Table 2 Scoring chart based on type and amount of APP

Amount per field at 200x magnification	Score
Granular changes	
1-5	1
6-20	2
> 20	3
Swellings	
1-5	1
6-20	2
> 20	3
Retraction bulbs	
Present	1
Bands	
Nongranular	1
Granular	2

Table 3 An example case scoring APP in various anatomical regions and in total

Region	Score	Possible Maximum	%
Corpus callosum	8	9	89
Internal capsule	6	9	67
Hemispheric white matter (excluding the internal capsule)	7	9	78
Cerebellum	2	9	22
Midbrain	4	8	50
Pons	5	8	63
Medulla	2	8	25
Spinal cord	3	8	38
TOTAL	37	68	54

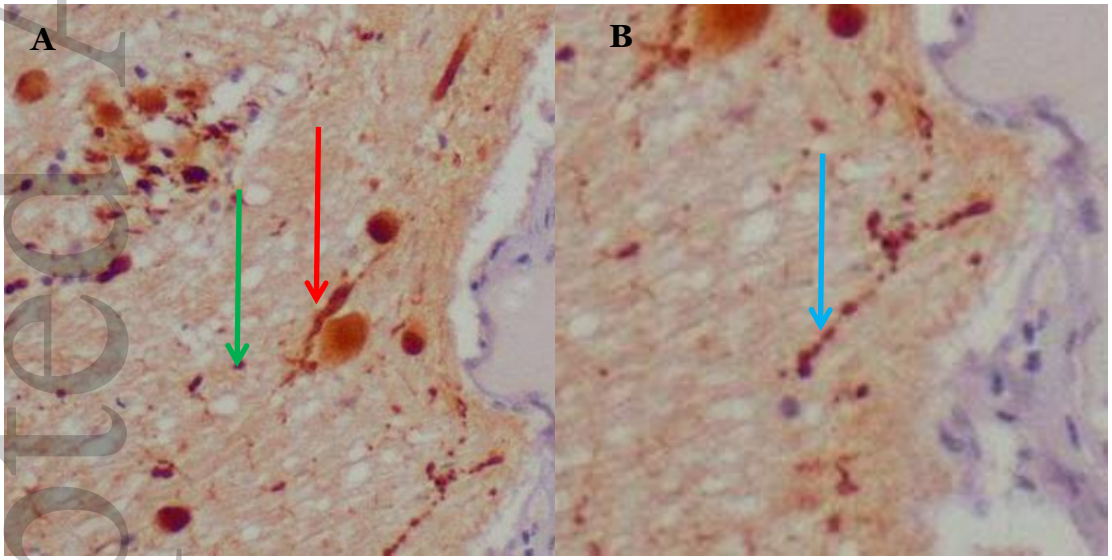
Table 4 Distributions of cause of death and number of tissue sections in relation to mean APP score in 164 cases of sudden infant and early childhood death. The highest mean APP was found in the head trauma group and the lowest mean APP was found in the group with drowning as cause of death.

Cause of death	# of cases (%)	Sex, m/f (%)	Mean age (weeks)	Mean APP (SD)	Mean # of sections (range)
SIDS	48 (29)	32/16 (67/33)	38	28 (17)	27 (3-45)
Undetermined	27 (16)	14/13 (52/48)	32	28 (20)	25 (5-61)
Head trauma	20 (12)	12/8 (60/40)	60	59 (31)	23 (3-46)
Mechanical asphyxia	16 (10)	10/6 (63/38)	31	23 (20)	22 (3-42)
Infection	11 (7)	6/5 (55/45)	30	30 (25)	20 (3-28)
Drowning	10 (6)	6/4 (60/40)	75	10 (20)	16 (9-26)
Other/combination	8 (5)	4/4 (50/50)	46	32 (23)	26 (12-41)
Dehydration	7 (4)	5/2 (71/28)	85	32 (20)	27 (7-59)
Perinatal death	5 (3)	4/1 (75/25)	0.5	52 (41)	9 (3-16)
Congenital heart disease	3 (2)	1/2 (33/67)	46	18 (18)	20 (10-27)
Pre-existing brain pathol	3 (2)	1/2 (33/67)	108	33 (26)	22 (20-24)
Failure to thrive	3 (2)	2/1 (67/33)	8	16 (10)	21 (15-24)
Poisoning	2 (1)	2/0 (100/0)	124	13 (18)	5 (4-5)
Burns/incineration	1 (1)	1/0 (100/0)	77	30 (-)	6 -
Total (%)	164 (100)	100/64 (61/39)	38	30 (25)	23 (3-61)

Table 5 APP scores in the eight anatomical regions and sampling of the eight regions. Hemispheric white matter was sampled in all but one case, and internal capsule, cerebellum, pons, midbrain and medulla oblongata were sampled in 85%-99% of the cases.

LOCATION	# of cases w sections (% of total number of cases)	Mean number of sections	Mean APP score (SD)
Corpus callosum	121 (74)	2.8	42 (29)
Internal capsule	158 (96)	2.5	43 (30)
Hemispheric wm (IC excl)	163 (99)	7.8	42 (31)
Cerebellum	152 (93)	2.0	21 (28)
Midbrain	139 (85)	1.8	24 (29)
Pons	149 (91)	2.3	28 (31)
Medulla	149 (91)	1.9	20 (27)
Spinal cord	77 (47)	8.3	24 (25)

Fig. 1 Examples of types of axonal APP changes. **A**: The red arrow shows an axonal swelling and the green arrow a granular change (200x magnification). Granular changes in the absence of larger swellings were still considered positive. **B:** Close-up of a section of Fig. 1A showing granules in a linear arrangement along an axon. **C:** Axonal swellings with definite signs of transection, i.e., a well-defined bulb with a loss in continuity, followed by a continuation of the axon. These were defined as retraction bulbs (400x magnification). **D**: The black arrow shows what the authors have termed a band (40x magnification).



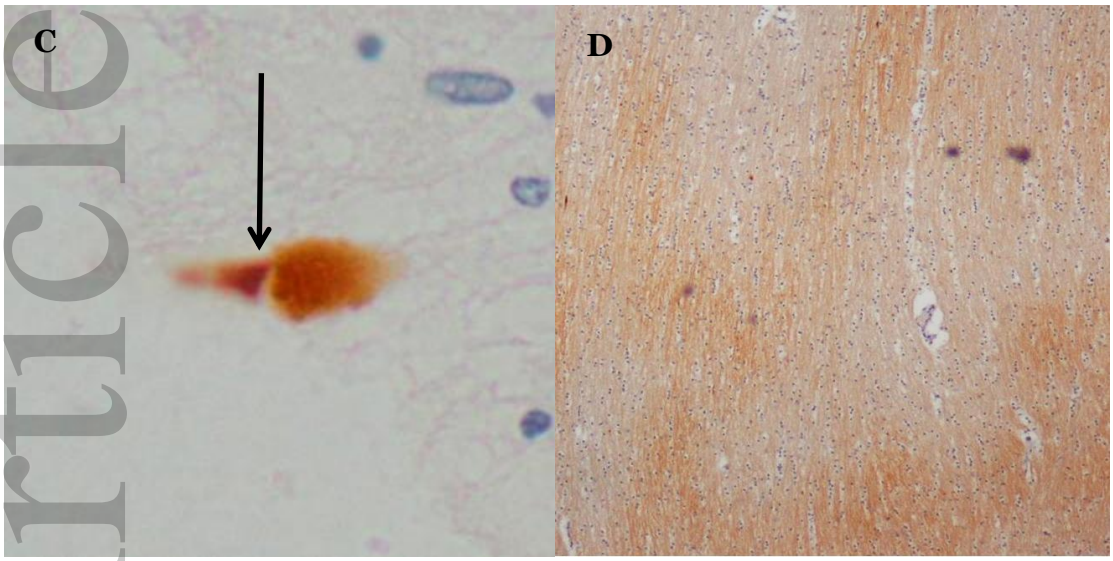


Fig. 2 The relationship between APP score and the number of sites sampled, showing no relation between the two. The horizontal line represents the mean APP score (30.9). Cases with one or two section have been excluded.

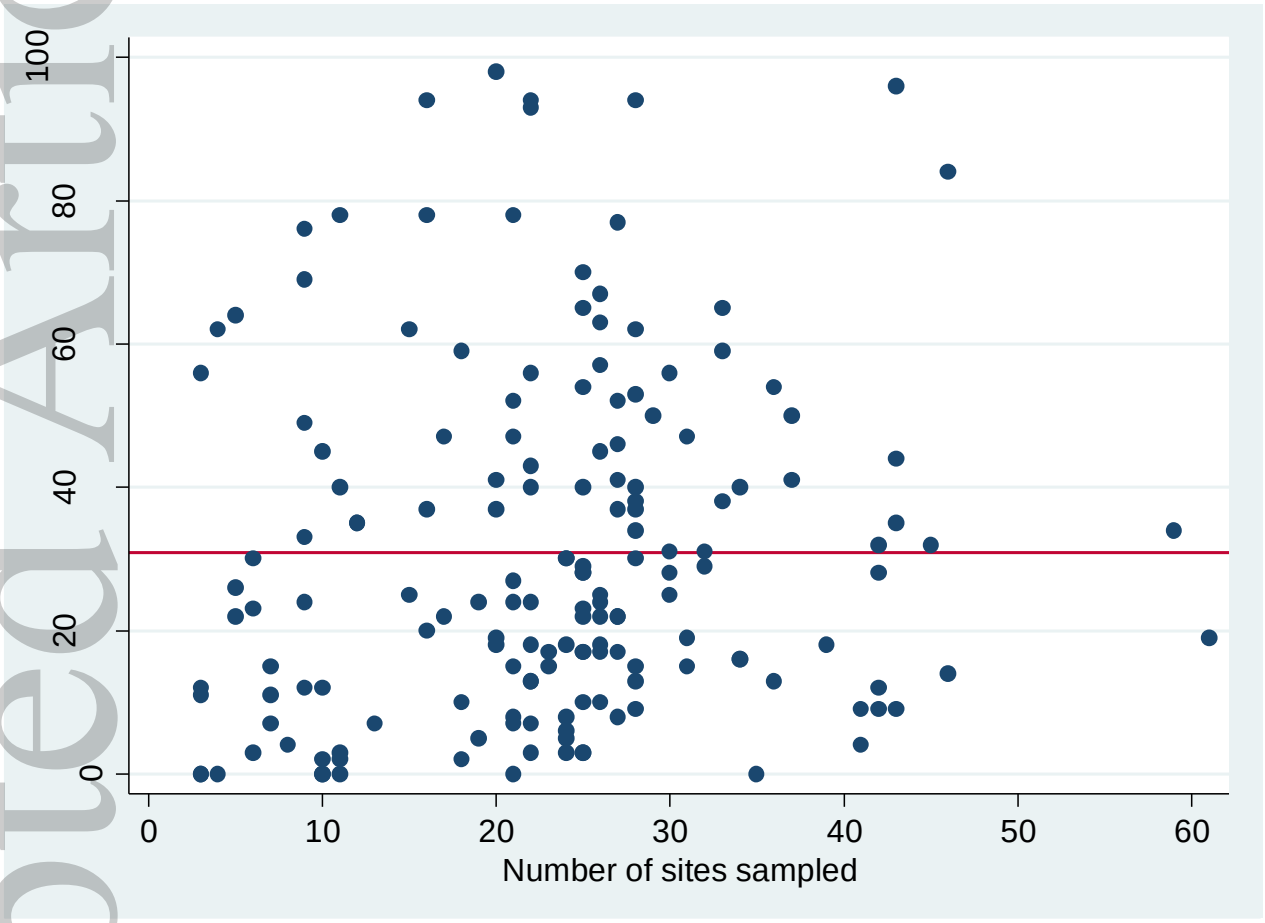


Fig. 3 Fig. 3 APP scores in relation to cause of death. The groups are in descending order of mean total APP score and the vertical lines of the boxes are medians. The red vertical line represents the mean overall APP score of 30.9. The dots represent outliers.

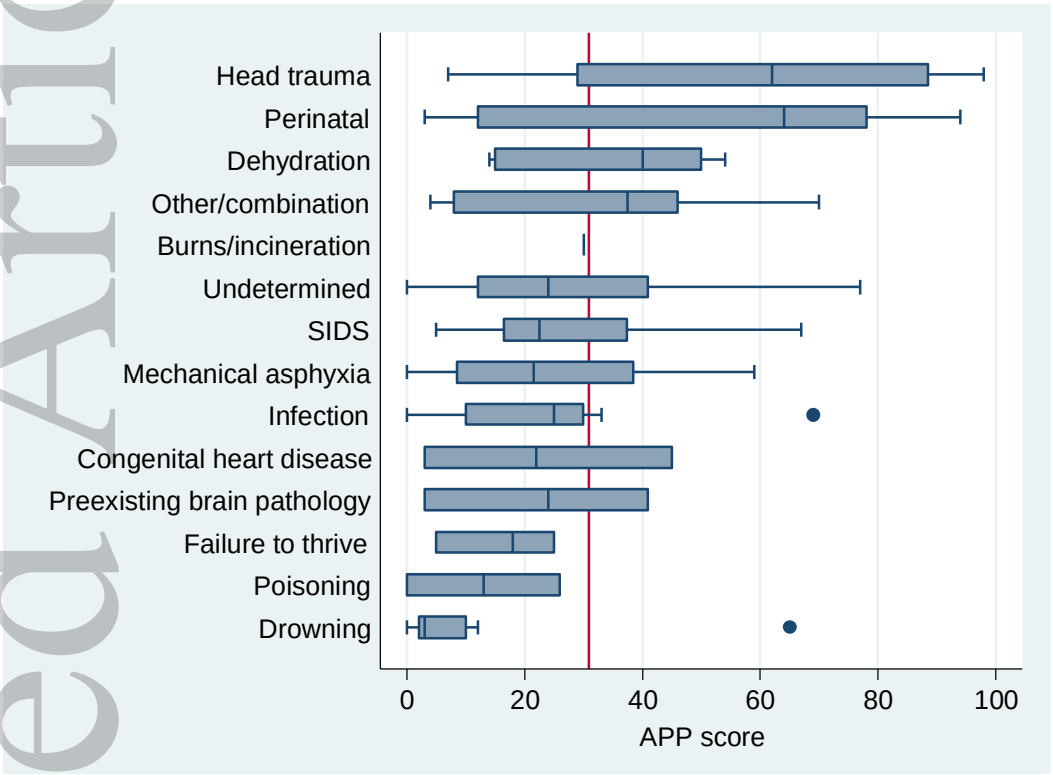


Fig. 4 The relationship between APP score and the survival time in cases of traumatic brain injury, showing an increase in the amount of APP with increasing survival time

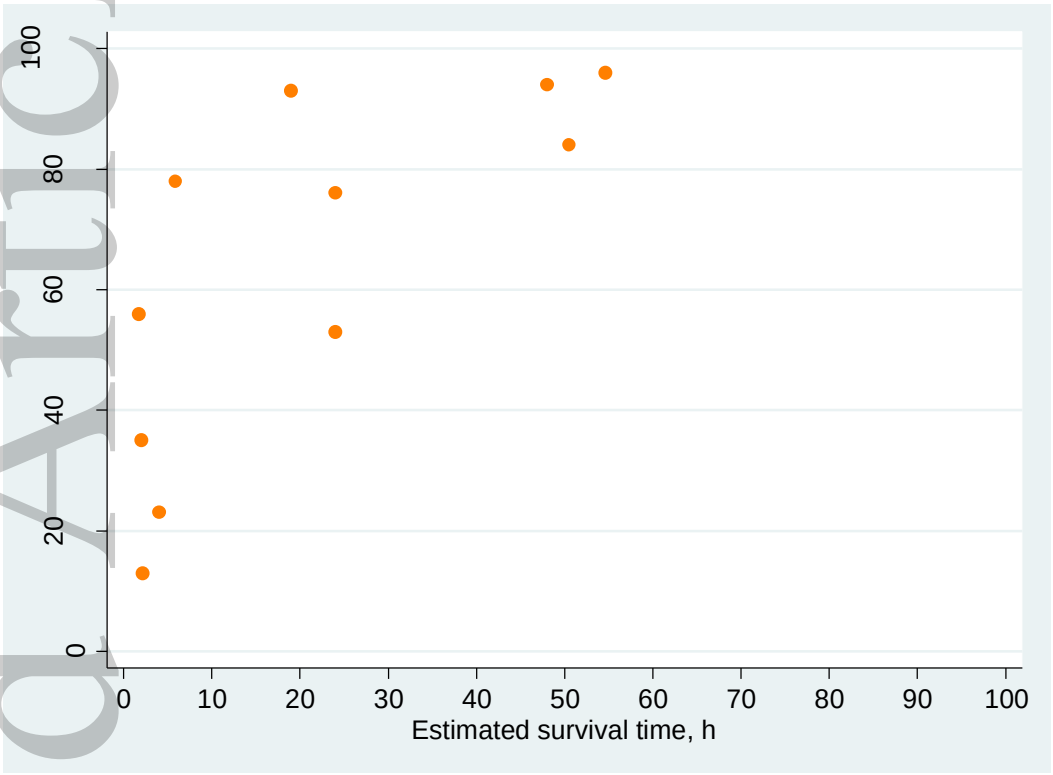


Fig. 5 APP score distributions in the eight anatomical regions, showing the highest amounts APP to be located in the cerebrum and the lower amounts to be located in the cerebellum, brainstem and spinal cord. The red horizontal line represents the mean APP score (30.9), and the horizontal lines of the boxes are medians. The dots are outliers.

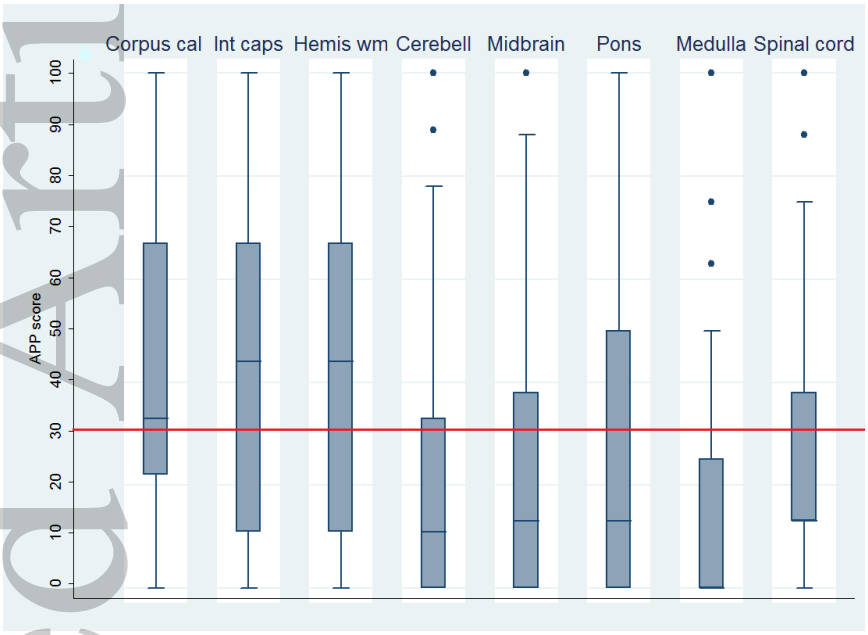
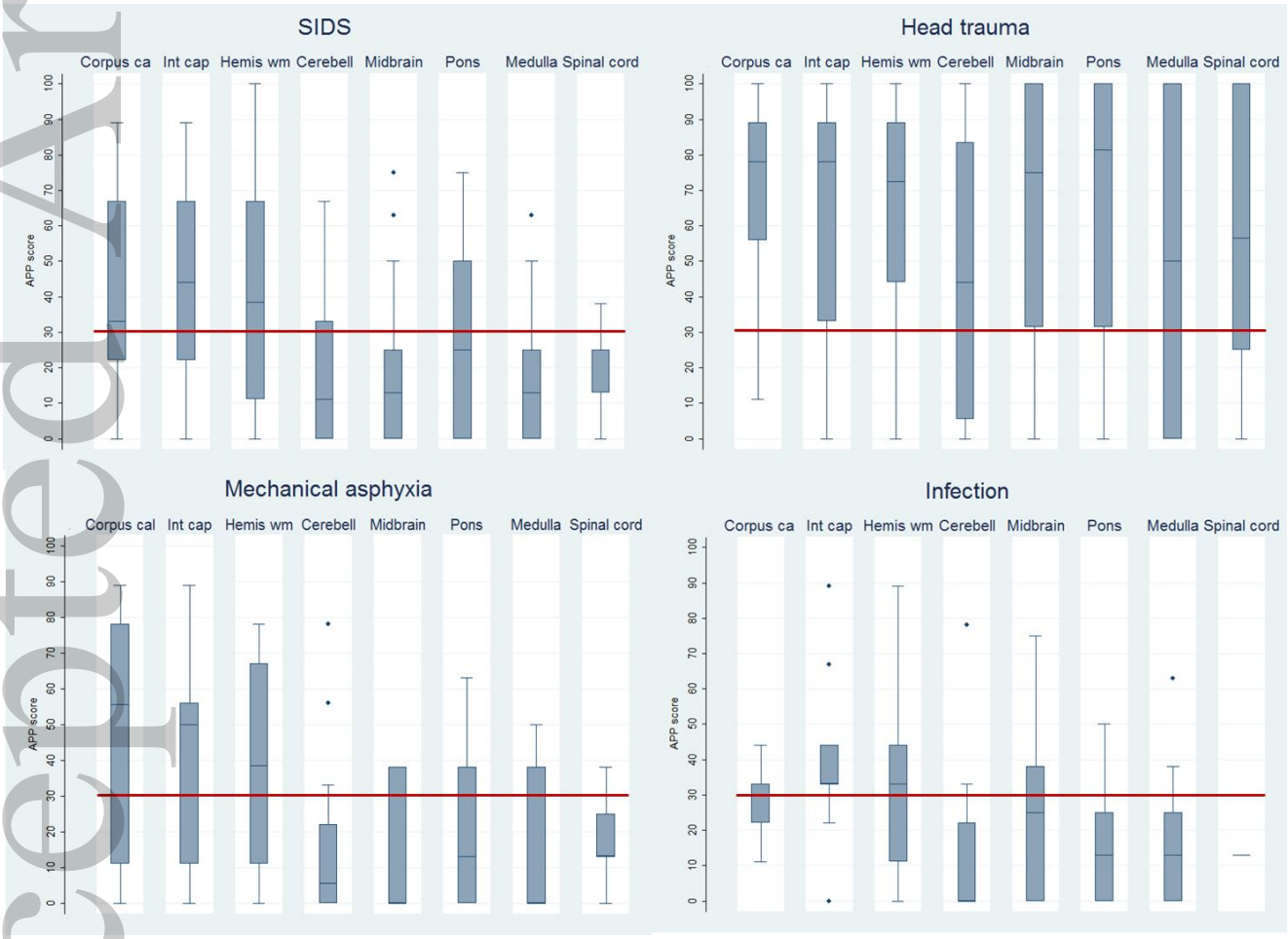


Fig. 6 APP scores in the eight anatomical regions for the four major diagnostic groups (SIDS, head trauma, mechanical asphyxia and infection). While the patterns of APP staining (i.e., the amount and distribution) were different in the cases of head trauma, infection and SIDS, there was striking similarity between the regional pattern in the SIDS and mechanical asphyxia groups. The red horizontal line represents the mean APP score (30.9), and the horizontal lines of the boxes are medians. The dots are outliers.



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