Autoantibody Markers of Neural Degeneration are Associated with Post-Mortem Histopathological Alterations of Neurologically-Injured Pilot

Mohamed B Abou-Donia [a] *FRW van de Goot* [b] *MFA Mulder* [c]

Abstract

Introduction: *There are numerous concerns regarding the neurotoxicity of contaminated air inside pressurised aircraft. Neurological symptoms have been seen in many aircrew personnel who have reportedly been exposed to the potentially toxic breathable air in airliners. Symptoms, allegedly contracted by aircrew and passengers, are thought to be caused by a single large exposure or repetitive cumulative low-level exposures to toxic chemicals in the airliner internal air. Genetic variation plays a role.*

Case Presentation: *We report the case of a 43-year-old airline pilot who presented with neurological deficits and other symptoms. The pilot died without regaining good health. Ante-mortem in vivo blood had been collected before death. Analysis of the serum confirmed grossly elevated levels of serum autoantibody biomarkers for neuronal cell degeneration compared with a control group. At autopsy, various tissues underwent histopathological assessment. Brain and spinal tissues exhibited axonal degeneration and demyelination. Peripheral nerves showed T-lymphocyte infiltration and demyelination. T-lymphocytes had infiltrated the heart muscle tissue.*

Conclusions: *The postmortem tests and pathological examination of the nervous system, confirm autoantibodies biomarkers results. Differential diagnosis showed that work environment, clinical condition, histopathological examination and the serum biomarkers for nervous system injury of the subject's nervous system is consistent with organophosphate-induced neurotoxicity. The results also showed that exposure to organophosphates rendered the nervous system and heart tissue sensitive and predisposed to further injury.*

INTRODUCTION

This report presents the case of a 43-year old man in the United Kingdom, a non-smoker and non-drinker, who complained of chronic ill health, and died without regaining good health. The subject, just before death, attributed his symptoms to repeated exposure to engine oil fumes during the course of his employment as a commercial airline pilot. We present the results of routine medical evaluations, specialized tests, autopsy results, and the level of serum biomarkers for brain injury. The results of these tests were correlated with his *ante-mortem* clinical condition, and those of *post mortem* examination of brain tissues. As far as the authors are aware, this is the first case study of a pilot with chronic ill health following exposure to contaminated air, which reports findings from autopsy, including histopathological examination of brain tissues.

a Duke University Medical School, USA b Forensic Pathologist, Netherlands c Medical Consultant, Netherlands

BACKGROUND

The internal breathing air of all airliners (with the exception of the relatively new Boeing 787) is drawn in from outside by the aircraft's engines or auxiliary power unit (APU), using the compressor sections of these gas turbine engines. This "bleed-air" is used to heat the cabin air and pressurise the cabin altitude. It is also used to pressurise the potable water tank, as well as the hydraulic system. It is alleged that the engine seals leak in daily use, or fail, allowing heated oil mist to escape into the bleed air [1-3, 29, 49, 51-55, 65]. The only air that enters such aircraft during operation is this "bleed air." Inadequate or improper maintenance practices, including an overfilled engine-oil reservoir, a worn or defective oil seal, a defective APU, or a failed bearing can each individually, or in combination, result in air emissions (gaseous, vapor, and particulate constituents of pyrolyzed engine oil and hydraulic fluid) [2]. These emissions contaminate the air-conditioning ducting [1] and are supplied to the cabin and flight deck [3,4]. The engine lubricating oil contains tri-cresyl-phosphate (TCP) (2–6% by weight), of which the tri-*ortho*-cresyl phosphate (TOCP) content is less than 0.1%[1] of the total TCPs. The oil also contains *n*-phenyl-1-naphthylamine, alkylated diphenyl amines and phenol dimethyl-phosphate [5]. Hydraulic fluid contains tributyl phosphate (TBP), dibutyl phenyl phosphate (DPP), or butyl diphenyl phosphate (BDP) or a mixture of all [2-4, 6, 65].

It has been more than a decade since some pilots and flight attendants started to complain of nervous system-related symptoms following alleged exposure to air emissions inside aircraft [8]. The symptoms were hypothesized to have resulted from exposure to the organophosphates present in engine oil and hydraulic fluid [8]. There are three neurotoxic actions caused by organophosphates: First, cholinergic neurotoxicity that is caused by inhibition of acetylcholinesterase, followed by over-stimulation of muscarinic and nicotinic acetylcholine receptors, with subsequent development of cholinergic toxicity [9]. Second, organophosphorus ester-induced delayed neurotoxicity (OPIDN) which is a central-peripheral axonopathy, characterized by primary Wallerian-type axonal degeneration of the central (CNS) and peripheral (PNS) nervous systems, followed by secondary demyelination [10-13]. The clinical picture for

1 *A published paper has recently called this proportion into question due to the much higher proportion of the more neurotoxic ortho isomers which has been detected in reported studies* [7]

OPIDN is manifested initially by mild sensory disturbances, ataxia, weakness, and muscle fatigue and twitching, which may progress to paralysis. Third, organophosphorus ester-induced chronic neurotoxicity (OPICN) is characterized by long-term neurological and neurobehavioral deficits accompanied by brain neuronal cell death [9].

MATERIALS AND METHODS

Materials

For tests performed in USA, NFP (bovine spinal cord), tau protein (human), MAP-2 (bovine serum), tubulin (bovine brain), and MBP (human brain), from Sigma-Aldrich (Saint Louis, Missouri); GFAP (human) from Biotrend Chemikalien GmbH (Cologne, Germany); and S100B (human brain) from American Qualex International Inc. (San Clemente, California); Horseradish peroxidase-conjugated goat anti-human IgG, and enhanced chemiluminescence reagent were obtained from Amersham Pharmacia Biotech (Piscataway, New Jersey); SDS gels, 2-20% gradient (8x8), and tris-glycine 15 mM were obtained from Invitrogen (Carlsbad, California). All other materials were purchased from Amersham.

Methods

Some peripheral nerves and parts of the central nervous system (CNS) were removed. Histopathological investigation for peripheral nerves was carried out locally in the Netherlands.

A few months before the subject's death, blood was drawn, serum prepared and stored at minus 70°C. It was tested, after death, for circulating autoantibodies specific to seven proteins associated with the nervous system. Under a protocol approved by the Institutional Review Board at Duke University Medical Center, the results were compared with those of controls who were age-matched males, who had no connections with aviation, and did not report air emission exposures or any neurological symptoms.

Using Western Blot Assay, all proteins were loaded as 10 ng/lane except for albumin, which was loaded as 100 ng/lane, using one gel for each serum sample, in triplicate [14]. Proteins were denatured and electrophoresed in SDS-PAGE (4% to 20% gradient). The proteins were transferred

into polyvinylidene fluoride (PVDF) membranes (Amersham). Nonspecific binding sites were blocked with Tris-buffered Saline-Tween (TBST) (40 mM Tris [pH 7.6], 300 mM NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk for 1 h at 22°C. Membranes were incubated with serum samples at 1:100 dilutions in TBST with 3% non-fat dry milk overnight at 4°C. After five washes in TBST, the membranes were incubated in a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-human IgG. The membranes were developed by enhanced chemiluminescence using the manufacturer's protocol and a Typhoon 8600 variable mode imager. The signal intensity was quantified using Bio-Rad image analysis software (Hercules, California). All tests were performed with the investigators masked to diagnosis.

Levels of Autoantibodies against neural proteins were obtained by dividing the optical density arbitrary units for subjects and controls and by serum albumin density concentration; this value for each subject was normalized to healthy controls and expressed as fold change from the controls [14].

Grouped data are reported as mean ± SE. The values from subjects were compared to the control group using a paired t-test. Mean values from the subjects' group were compared within groups using two-way ANOVA (SigmaStat; Systat Software). A P value < 0.05 was accepted as statistically significant [14].

THE SUBJECT

An outline of the subject's relevant life-events during his career is presented in Table 1. He began his flying career as an airline pilot in 1996. His career lasted for 15 years during which he flew a total of about 8,000 hours. Although he never reported a documented air emission, he said that he noticed that the engines, on start-up, would create puffs of smoke (BAe ATP) inside the aircraft accompanied by an oily smell. After three years of flying, in 1999, he started feeling that his brain was slow and he had some signs of confusion. In the year 2000, he changed over to the Embraer 145 Jet. In 2006, while driving home after a flight, he had scintillating vision in his eye, moving from center to side of the eye, and thereafter experienced three days of mental confusion. In 2007, the company he worked for was taken over by a major UK airline and he carried on flying for them, only now as an Airbus 319/320/321 line pilot. On 17 August 2011, while driving his car, he

suddenly stopped near a junction, without a reason, and his car was run into by a following car, but at slow speed.

His last flight was on September 2, 2011. On 17 September 2011 he went to the Accident & Emergency Department (A&E) (ER), as he felt severe pain and tightness in his chest. Standard A&E cardio tests did not reveal the cause; he was kept in overnight and discharged the next morning with no diagnosis. A week later he went back to A&E as he "felt uncoordinated" and he said all his symptoms had become worse. A&E discharged him with no diagnosis and scheduled (non urgent) scans. At examination on 22 September 2011, his family doctor noted *"Numbness or pain all limbs. Headaches occipital area. Finds walking and co-ordination difficult and veers to the right. Brain doesn't know what legs are doing."* An appointment was made for him to see a neurosurgeon in a week. He was prescribed Zopiclone 7.5mg, one at bedtime. The next day he was prescribed Amitriptylene 10 mg 1 or 2 at night. This was later stepped up to 3 to 4 at night as he couldn't get to sleep because of pains. The medical notes record instances of sleep-apnea, and he was referred to a sleep clinic. He was of athletic build (185cms/70 kg) and bore no excess fat. He was therefore not a typical sleep-apnea patient. The neurosurgeon later reported that no surgical intervention was appropriate.

On 17 November 2011, he was referred for evaluation to a consultant psychiatrist, whom he saw several times and, in January 2012, he was admitted to a psychiatric hospital. He remained an inpatient there for four weeks and then he discharged himself. There are no recorded psychiatric symptoms in his medical notes. The subject felt that because "his doctors couldn't ascribe a diagnosis at all, they may as well evaluate his mental condition". He was prescribed antidepressants and benzodiazepines. He had undergone many tests in the UK, but all results were negative and did not offer any diagnosis. These tests included blood, with no remarkable result.

On February 23, 2012, which is just after he discharged himself from the psychiatric hospital, a test (for which he self-referred) showed low levels of intra-cellular Adenosine Triphosphate (ATP) in addition to a subcutaneous fat biopsy which found organophosphate metabolites; this is approximately six months after his last flight.

All in all, he was examined by no less than 15 specialists, including neurologists while still resident in the UK. In spite of this there was no diagnosis.

On April 5, 2012, he showed up, unannounced, in an Amsterdam clinic still having insecure, staggering and heavy gate, walking difficulty and neck stiffness. He remained in the Netherlands until he died some 9 months later. He also experienced severe fatigue with constant pain and was desperate; he suffered severe headaches, slow mental process, sharp decline in memory, and pain when moving his eyes. He continued to have difficulty walking. He had ataxia. He would fall off his bicycle for no reason.

MRI scans, on July 23, 2012, and on December 6, 2012 showed no structural defects to explain the loss of functions in the patient. He also had an fMRI scan, but no abnormalities were found to explain his symptoms. On September 27, 2012 he was still suffering from serious neurological complaints. On that date his neurologist expressed serious doubts that he would ever be in a better shape to fly in the foreseeable future. On October 19, 2012 his clinical psychologist gave him extensive neuro-psychological tests and concluded that he had sub-standard performance. At the Center for Neurotherapy (NCH Hilversum) from May 5 to December 11, 2012, at his own request, he had regular Quantitative Electro-Encephalograph (QEEG) daily neuro-therapy which provided substantial relief of the complaints in his head.

On December 12, 2012 the subject was found dead in his hotel room. When he died, he was a 43 year old airline pilot on sick leave and was still on full pay. His condition prior to his sudden death was lucid, puzzled, and inquisitive. He had lost weight. On occasions he was in extreme pain, mainly in the head. He was waiting for an appointment at the pain clinic at Amsterdam University Hospital. He had acquired a "sleep-angel" (an electronic device which sounds an alarm when breathing is not detected for a determinate period). This had been "armed" by him before he went to bed. When hotel staff entered his room, the alarm was still sounding. In bed, he wore a mouth-guard, the effect of which was to keep the lower jaw proud of the upper jaw during sleep. When he was found dead in his bed he was still wearing it. These two devices are often recommended for persons suffering from sleep-apnea.

The subject had been athletic, and had been a champion at paragliding, which he loved doing.

DIAGNOSIS

While in the U.K., the subject was never diagnosed despite his chronic illness, constant pain and a long list of complaints from 1999 to 2012, including constant severe headaches, occasional severe

chest pain, short-term memory loss, confused mental process, cognitive dysfunction, apnea, numbness that he described as "pins and needles", clumsiness, tendency to fall easily, and fatigue. He was seen by many specialists, and underwent several tests and was admitted more than once to hospital but was never diagnosed as suffering from any disease, while in the UK. He was only diagnosed, shortly before he died in the Netherlands, of having symptoms related to exposure to organophosphates. The primary basis of diagnosis was because his symptoms were consistent with those caused by exposure to the organophosphates present in jet engine oil and hydraulic fluid. The first sign of neurological deficits that were consistent with OPIDN had been in 2008 when he experienced a slow onset of numbness in hands and feet, creeping up as far as the elbows and knees respectively. It is unknown whether he went to a doctor at this time with these complaints. These symptoms are typical as the earliest manifestations of OPIDN. In August 2011 he had paresthesia in both legs and both arms; that is a hallmark of OPIDN. The application, in the Netherlands, of differential diagnosis techniques, together with the extensive consultations and tests already carried out there and in the UK, offered no alternative diagnosis.

POST MORTEM REPORTS

There were two autopsies carried out, one for the police and another for the family.

Brain Weight

An autopsy report indicated that there were signs of fluid accumulation in the brain that resulted in his brain weighing 22% more than an average healthy adult brain [15].

Toxicology Report - Pentobarbital levels

Toxicology tests on the blood revealed the presence of pentobarbital (also known as pentobarbitone) at a potentially lethal level. The Netherlands Forensic Institute (NFI), where the subject's first autopsy took place, reported that the level of pentobarbital in the femoral blood was 27 mg/L, and its concentration in heart blood was 32 mg/L. Pentobarbital concentration in eye fluid was 14 mg/L. The second, family-instructed, autopsy reported the femoral blood level at 22.3 mg/L (average for femoral blood: 24.65 mg/L) and in the hair pentobarbital was detected at 3.84 ng/μg. Hair testing demonstrated that he had taken this medication before at therapeutic doses. The brain was not tested for pentobarbital.

TABLE 1 Timeline of Events Related to the Subject's Career

N°	Date	Events	Remarks
1	1996	Starting Flying Career: BAe ATP turbo prop.	He was healthy and fit to fly
2	1996-1999	No documented fume event; when starting engines, they would create puffs of smoke inside the aircraft (BAe ATP); Experienced an oily smell.	No Symptoms
3	1999	First started feeling that his brain was slower than normal, and some early signs of confusion soon began (After 3 years of flying).	Cholinergic
4	2000	Changed over to Embraer 145 Jet.	
5	2006	While driving home after a flight, had scintillating vision in eye moving from center to side of the eye; 3 days of mental confusion.	Cholinergic
6	2007	Went on to fly Airbus 319/320/321 as a line pilot for major UK airline.	
7	2008	Slow symmetric onset of numbness in hands and feet, creeping up as far as elbows and knees.	OPIDN
8	August 22, 2011	He had been rear ended from behind by a car. He braked suddenly at a T-junction for no apparent reason. Whiplash was suspected. Prescribed Naproxen 500.	
9	August 22, 2011	Continued to fly for his airline.	OPIDN
10	September 2, 2011	Last Flight	
11	September 8, 2011	No improvement in his condition and not sleeping at night; Zopiclone 7.5mg before bedtime. Blood was taken with no remarkable results. An MRI was prescribed. Paresthesia in both legs and both hands	Cholinergic/OPIDN
12	September 17, 2011	Symptoms became worse. He arranged his own CT and MRI scans; Went to see an osteopath without referral. Went to the ER (A&E) because of severe tightness and pain in his chest. Continued to have difficulty walking; had ataxia.	OPIDN
13	September 25, 2011	Attended again at the ER as he felt "uncoordinated".	OPIDN/OPICN
14	January 23, 2012	A psychiatrist referred him to a psychiatric hospital. Inpatient for a month.	
15	February 23, 2012	Discharged himself from the psychiatric hospital. Thereafter he self-referred and obtained a subcutaneous fat biopsy: OP metabolites (6 months after his last flight). Test: low level of ATP.	
16	April 5, 2012	Consultation in the Netherlands: Insecure staggering and heavy gait, walking difficulty, signs of being in severe and constant pain and desperate, pain in moving eyes, headache, tremors, some neck stiffness, slow mental process, sharp decline in memory. Accepted in an outpatient clinic.	OPIDN/OPICN
17	July 23, 2012	Amsterdam: MRI. No structural deficits were found to explain his loss of functions.	
18	September 27, 2012	Amsterdam: Neurologist; Still suffering from serious neurological complaints. Serious doubt that he will ever fly in the foreseeable future.	
19	October 19, 2012	Amsterdam. Extensive neuro-psychological tests; sub-standard performance. Memory was too poor and seemed to always try to be masking effort to hide his deficits.	OPICN
20	December 6, 2012	Amsterdam, fMRI; No diagnosed abnormalities.	OPICN
21	May 5– December 11, 2012	Amsterdam. Regular QEEG daily neuro-therapy provided substantial relief of complaints in his head. He was lucid, very puzzled, and inquisitive. He had been losing weight. No one was aware that he was taking pentobarbital or for how long. He was waiting for an appointment at a pain clinic at Amsterdam University Hospital.	OPICN
22	December 12, 2012	Found dead in hotel room. He was a 43-year old airline pilot on sick leave and was still on full pay.	

Pentobarbital is generally a prescription-only medicine. It is a short-acting (half-life is 21-42 hours) sedative-hypnotic barbiturate. At therapeutic doses, barbiturates reversibly depress activity of all excitable tissues, with the central nervous system (CNS) being the most sensitive, and very little effect on skeletal cardiac or smooth muscle occurs. Only in acute intoxication, depression is extended and serious deficits in cardiovascular and other peripheral functions occur. Oxygen consumption in various tissues, and respiration in the mitochondria, can be depressed by barbiturates in high concentration [16].

There was no evidence that the subject had been prescribed this medication and those treating him were unaware that he was apparently taking it. The police did not find any medicine container for this drug in his possessions. This raises two reasonable presumptions: (i) there was nothing to indicate to him a safe dose, and (ii) he may not even have been aware that this substance was pentobarbital. The drug can be purchased on the black market or via the internet. It is thought, however, that he was taking it because he had difficulty going to sleep and, or, to relieve excessive pain.

Summary of Autopsy Findings

Summarised, the two autopsy reports indicated the following findings (there were no material discrepancies between the two):

1. The subject's death "can well be explained by functional disorders of the brain and the heart, on the basis of tissue damage in both these organs."
2. "The pentobarbital found in the blood of the subject at the levels observed contributed to death by its depressant effects on the central nervous system. On the basis of the levels observed in the toxicological examination performed, the death of the subject can be attributed to pentobarbital."
3. The autopsy yielded indications of a recent oxygen deficiency in the heart and brain which caused damage to the cardiac muscle with signs of herniation of the brain. "As such, this person's death can well be explained by functional disorders of the heart and brain."
4. The autopsy cardio-pathological examination revealed pathological abnormalities in the heart, i.e. inflammation of the cardiac muscle and narrowing of the coronary arteries. These abnormalities may have resulted in cardiac dysfunction leading to damage to the cardiac

muscle. The damage to the cardiac muscle and dysfunctions may subsequently have led to an oxygen deficit in the brain, resulting in brain herniation and death.

5. Two causes can be identified which, either independently or in combination, may have led to the oxygen deficiency resulting in the subjects' death. These two causes are the following:
   a. In the toxicological examination, pentobarbital was found in the body. The toxicologists concluded that the measured concentrations of pentobarbital can explain the subject's death. Due to its effects as a central nervous system depressant in the concentrations established, pentobarbital may have caused an imbalance between oxygen supply and removal to the brain and the heart, resulting in a lack of oxygen, tissue damage and damage to the cardiac muscle, herniation of the brain, organ dysfunctions and death. The findings of fluid in the lungs are unspecific but may still be consistent with toxicological influences as referred to above, as a result of pentobarbital.
   b. The autopsies and supplementary cardiopathological examinations revealed pathological abnormalities in the heart. i.e., inflammation of the cardiac muscle and narrowing of the coronary arteries, which may have resulted, separately or in combination, in cardiac dysfunction leading to damage to the cardiac muscle.

HISTOPATHOLOGICAL ASSESSMENTS

Heart Tissues

Examination of the heart tissues revealed myocardial autolysis and an excess of lymphocytes in many places in the myocardium, mainly loose between muscle fibres, but also grouped and present particularly in the epicardium. Myocytolysis was noted. Striking was the presence of a relatively high amount of lymphocytes in the myocardium. There was thickening of the arterial walls with lymphocytic infiltrate. Both pathologists therefore reported lymphocytic myocarditis. Photo 1 demonstrates lymphocytic infiltration in the heart muscle.

The mediastinum including the thorax and the associated fatty tissues demonstrated an excessive number of lymphocytes. The diaphragm appeared normal, but in the adjacent connective tissue, blood vessels and nerve branches there was extensive lymphocytic activity, with a large number of T-lymphocytes and a reduced number of B-cells.

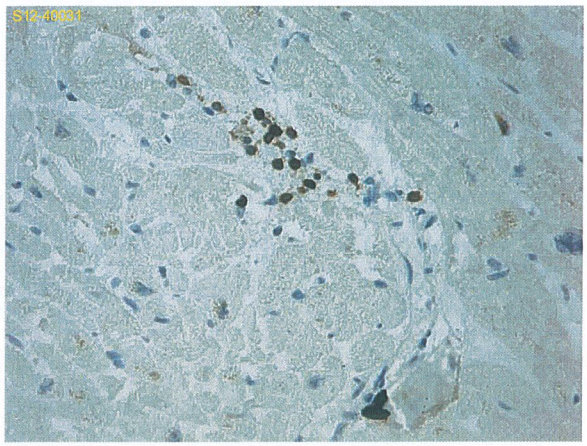

Photo 1
Heart Muscle Infiltrate of T-Lymphocytes

Sciatic and Femoral Nerves

The sciatic and femoral nerves showed an endoneural T-lymphocyte invasion. A pathological determination of peripheral endoneuritis was made. In addition the peripheral nerves showed gross axonal demyelination. This is demonstrated in photos 2 and 3.

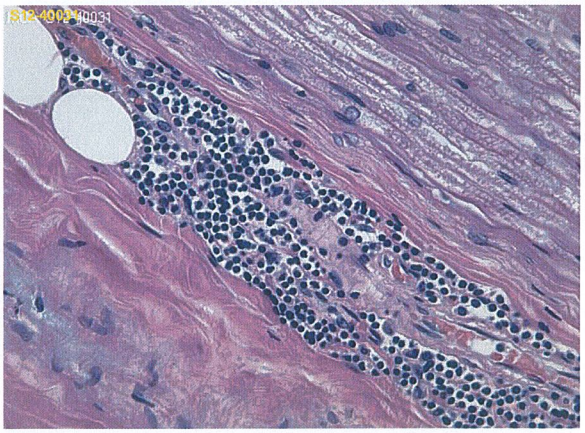

Photo 2
Demyelination (absence of more white material) and lymphocytic invasion (black dots) of peripheral nerve.

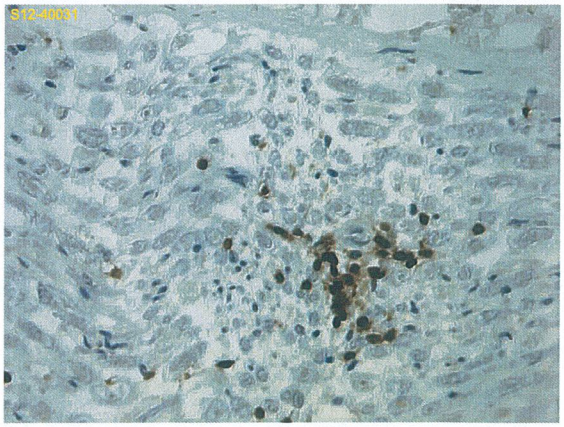

Photo 3
T-Lymphocyte infiltration of peripheral nerve

Absence of axonal degeneration in peripheral nerves is consistent with results of OPIDN in laboratory animals. Experimental studies of OPIDN have shown absence of peripheral nerve pathological changes in animals long after developing OPIDN. This is consistent with the regenerative capacity of the peripheral nervous system. Previous studies have indicated that

although damage to both the CNS and PNS may contribute to neurological dysfunction in OPIDN, CNS lesions are more significant because they are irreversible, whereas PNS can regenerate. This conclusion is consistent with the spasticity seen in human patients exposed to TOCP that suggests the presence of injury in the central nervous system [17]. In experimental studies, ataxia correlates with consistent early occurrence of spino-cerebellar tract degeneration in the posterior and lateral columns of affected cats [18].

There was therefore a confirmation of the *pre mortem* diagnosis of some neuronal deficits.

Histology Examination of the Central Nervous System (CNS)

Various sections of the central nervous system (brain, brain stem and spinal cord) were subjected to histological examination. Samples were drawn from frontal cortex, hippocampus, cerebellum and spinal cord. The tissues had been fixed in formalin. They were dehydrated and embedded in paraffin wax. Sections were stained with haematoxylin-eosin (H&E) alone or in combination with luxol fast blue (LFB). The test looked for neuronal cell death and demyelination. The H&E stains the tissues pink and dead cells are remarkable as dark-stained matter. After staining the material with H&E, the LFB is used to stain the myelin blue. Where demyelination had occurred, areas of myelin which should then have been blue are seen as areas of pink.

Cortex

The frontal cortex exhibited clear neuronal cell loss and prominent spongiosis. The prefrontal cortex showed increased glia cells and macrophages. Spongiosis was also present here as well as

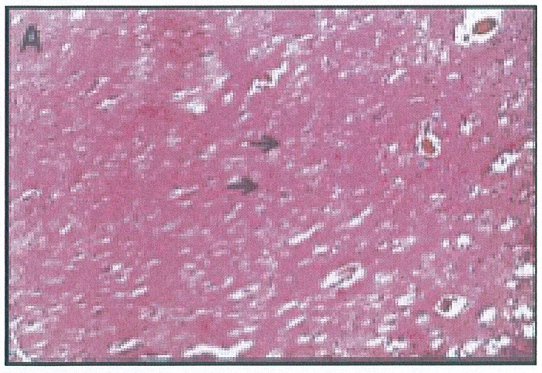

Photo 4
Section of frontal cortex at X4 showing neuronal cell loss and prominent spongiosis (arrows)

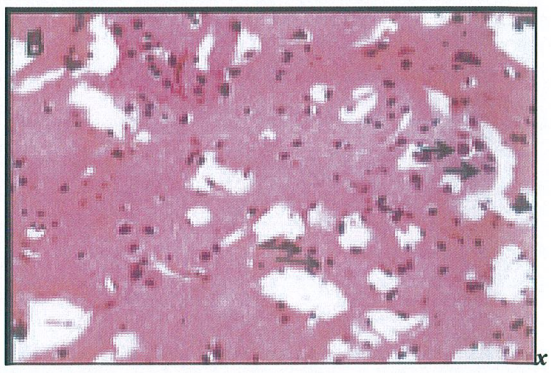

Photo 5
Sections through pre-frontal cortex at X20 noting increased glial cells (long arrows) and macrophages (short arrows)

shrunk and dying neuronal cells *(Photos 4 to 7)*. This material also demonstrated demyelination under low and high magnification.

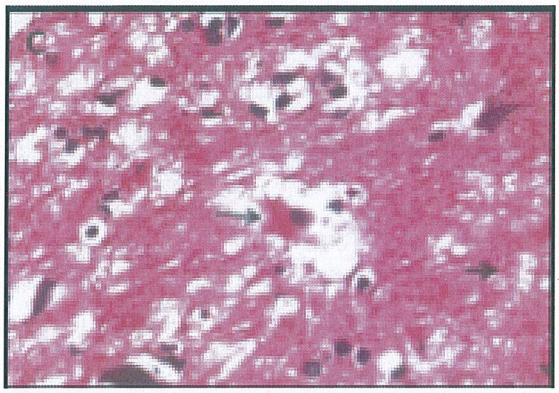

Photo 6
X 40 Sections through pre-frontal cortex showing dying neuronal cell indicated by long arrow. Spongiosis shown by the short arrow

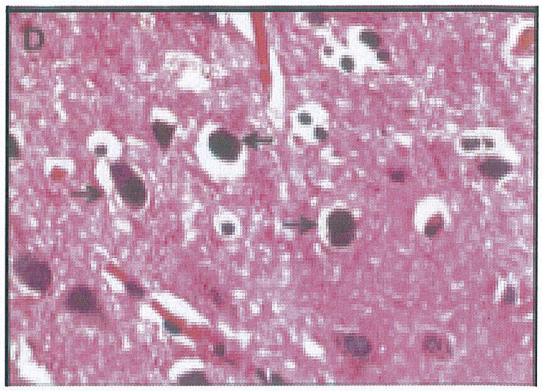

Photo 7
X40 section through pre-frontal cortex showing shrunk And dying neuronal cell indicated by arrows. Note the dense chromatin in the dying cells

Demyelination was noteworthy in the cortex sections. This is demonstrated in Photos 8 & 9.

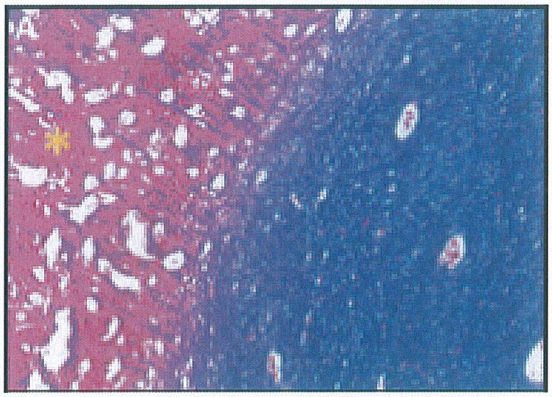

Photo 8
X 4 Section of Cortex stained with H&E and LFB with yellow asterisk demonstrating area of demyelination

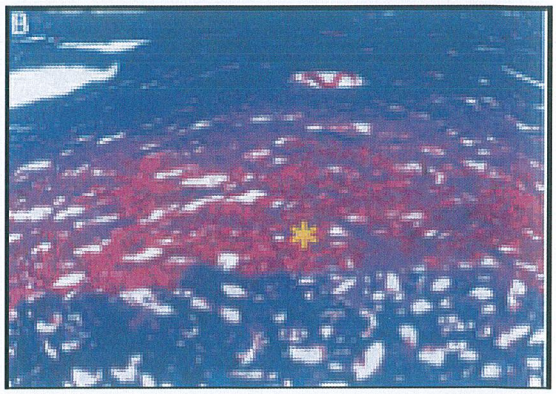

Photo 9
X20 section of cortex stained with H&E and LFB with Yellow asterix demonstrating area of demyelination

Hippocampus

The hippocampus tissue was also stained using the same staining and the same technique.

Dentate gyrus of hippocampus tissue showed slight spongiosis in the outer zone and in the molecular layer. With higher magnification there was clear evidence of apoptotic cells and astrocytes. These are demonstrated at Photos 10 & 11.

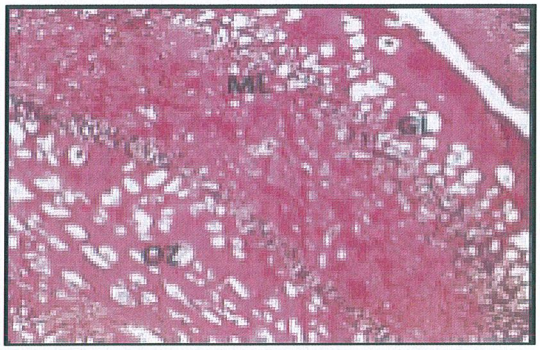

Photo 10
Section of dentate gyrus (X10) showing spongiosis in the outer zone (OZ) and in the molecular (ML) and granular (GL) layers

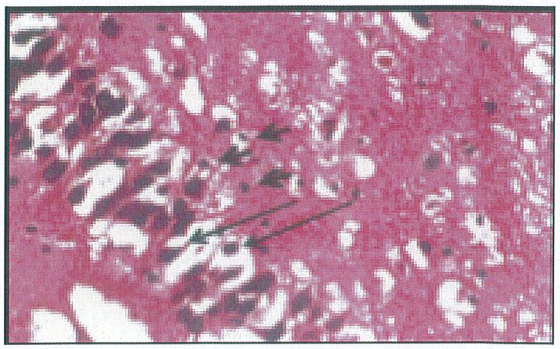

Photo 11
This is a photomicrograph of a section of dentate gyrus at X40 showing apoptotic cells with chromatin dense nuclei (long arrows) and astrocytes (short arrows)

The hippocampus also had demyelinated cells. This is demonstrated in Photos 12 & 13.

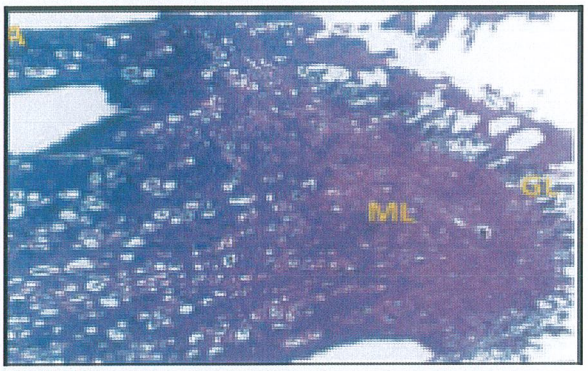

Photo 12
Section of hippocampus outer layer and molecular layer (ML) at X4 showing demyelination where the blue staining is missing and allows the pink staining to remain.

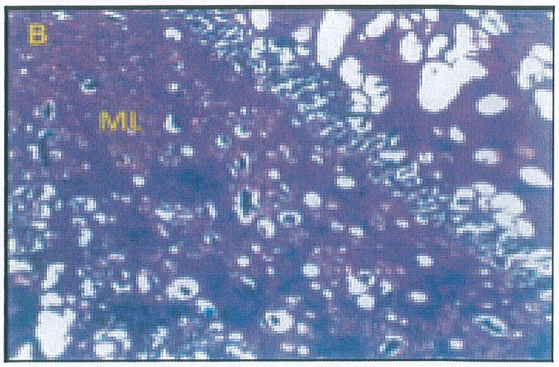

Photo 13
Section of hippocampus dentate gyrus showing areas of demyelination. The blue staining gives way to pink, where the myelin is missing

Cerebellum

Stained and examined in the same way, the cerebellum showed substantial cell loss in the Purkinje cell layer, the molecular layer and the granular layer. Clear evidence was observed of degenerated, damaged and shrunken Purkinje cell layer. The damaged Purkinje cells were hyperchromatic. The damage to the Purkinje layer is depicted in Photos 14 to 16.

Staining the cerebellum with H&E demonstrated Purkinje cells that are damaged, shrunken and hyperchromatic (darkly stained) basophilic perikaryon and are indicated in the affected cells by the arrows in Photo 16.

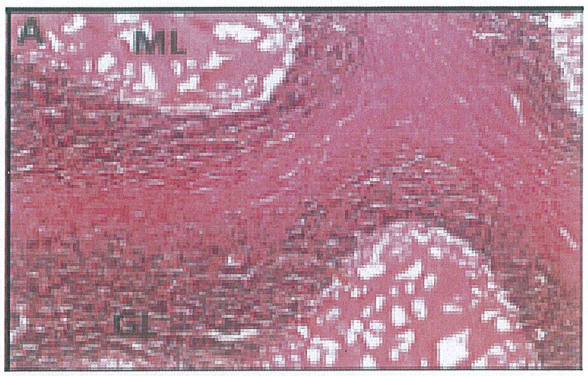

Photo 14
X4 Section of cerebellum showing substantial cell loss in the Purkinje, molecular and granular layers

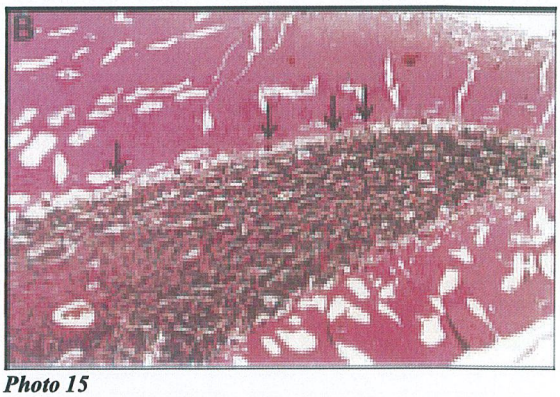

Photo 15
X10 section of cerebellum showing substantial loss of Purkinje cells

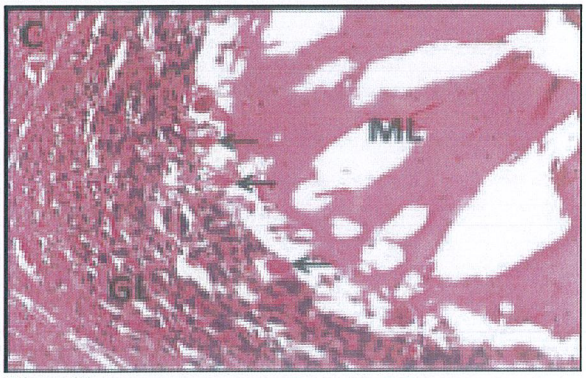

Photo 16
X20 Degenerated Purkinje neurons in a pinkish colour are denoted by the arrows.

The cerebellum was also demyelinated when stained with LFB. See Photos 17 & 18.

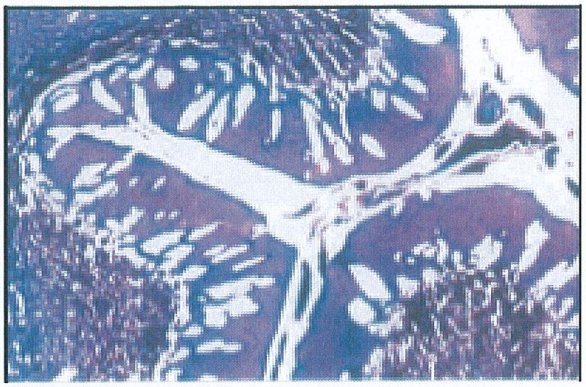

Photo 17
A X4 magnification showing demyelinated cells where blue gives way to pink

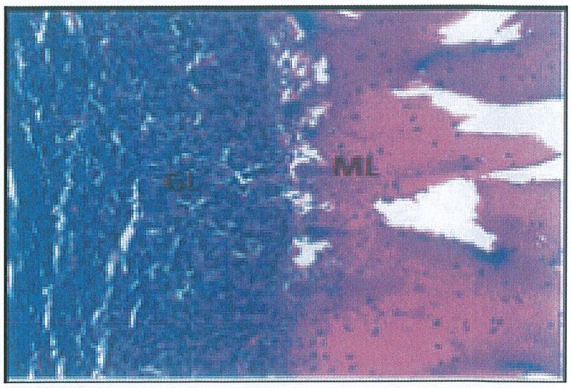

Photo 18
A X20 section demonstrating slight demyelination of the granular layer (GL) and pronounced demyelination of the molecular layer (ML)

Spinal Cord

Areas of spinal cord examined were grey matter, white matter and central canal. Significant findings included areas of grey matter that contained motor neurons with normal Nissl substance. This grey matter, however, contained macrophages and lymphocytes, and shrunken and dying hyperchromatic cells.

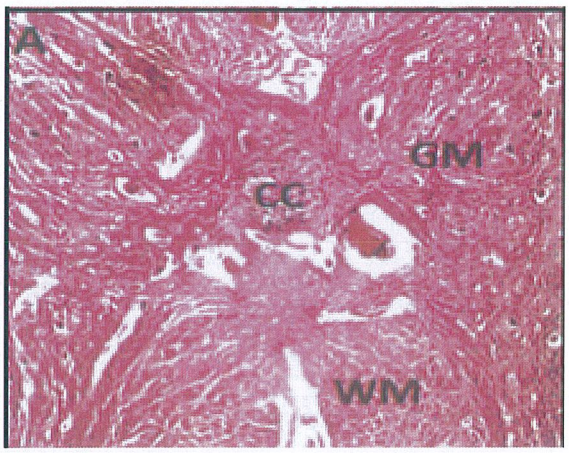

Photo 19
At X4 showing areas of white matter (WM) grey matter (GM) and central canal (CC).

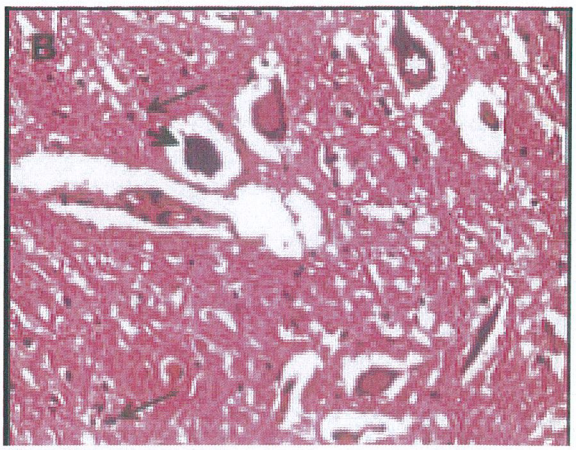

Photo 20
X40 grey matter contains motor neurons (see asterisk), macrophages (long arrow)with presence of lymphocytes. Example of shrunken and dying hyperchromatic cell is shown by the short arrow

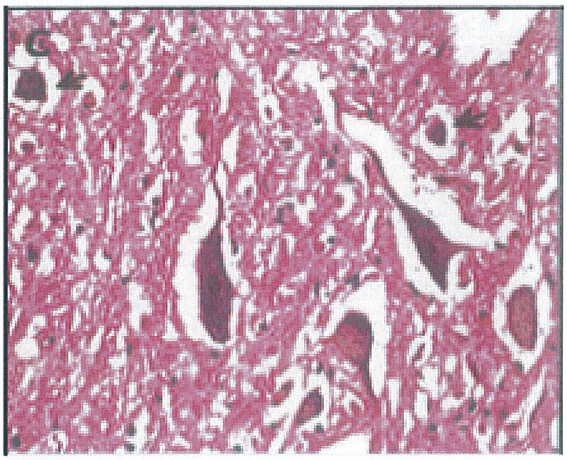

Photo 21
X40 slide of grey matter showing shrunken and hyper-chromatic dying cells (arrows)

Areas of spinal cord, when stained also with LFB showed clear evidence of demyelination. This is demonstrated at Photos 22 & 23.

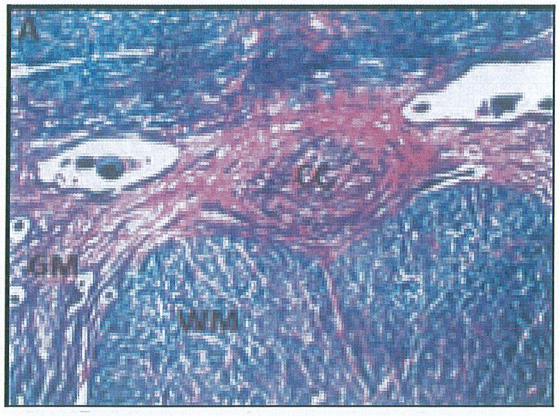

Photo 22
X4 section of spinal cord shows demyelination of white matter (WM), grey matter (GM) and central canal (CC)

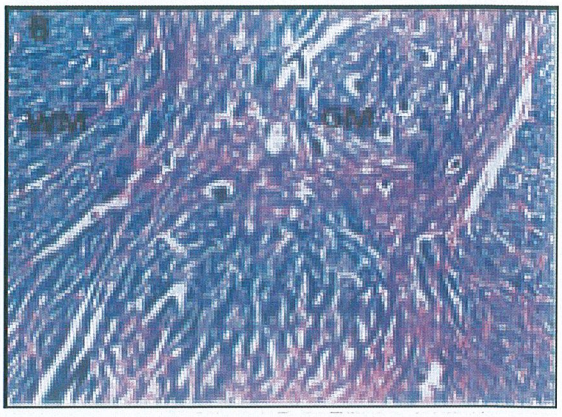

Photo 23
This slide at X20 shows demyelintion more pronounced in the grey matter (GM) than the white matter (WM)

The above histology results are also confirmatory of the *ante mortem* diagnosis of CNS deficits.

2 NEURO-SPECIFIC AUTOANTIBODIES

We identified and quantified IgG-class autoantibodies in the serum of the subject compared to controls against cytoskeletal proteins associated with neuronal and glial degeneration (Table 2). These were: (1) neurogenesis, i.e., neurofilament triplet proteins (NFP), tubulin, microtubule associated protein-tau (tau protein), and microtubule associated protein-2 (MAP-2); (2) myelinogenesis, i.e., myelin basic protein (MBP); and (3) astrogliogenesis, i.e., glial fibrillary acidic protein (GFAP) and S100B. Both GFAP and S100B are secreted by the astrocytes.

Table 2 lists the levels of autoantibodies against neural proteins for control and the subjects and the folds increase of the subject's autoantibodies relative to healthy control. The test shows that the pilot's autoantibodies were highly significantly elevated against nervous system-specific proteins than those of the controls. This finding is consistent with neuronal damage.

Levels of Autoantibodies

The levels of autoantibodies of the subject exhibited highly significant increases over that of controls as shown in Table 1. Folds increase in autoantibodies compared to healthy controls is in descending order: MBP, 18.54 > MAP-2, 14.31 > NFP, 12.21 > GFAP, 10.24 >; Tubulin, 6.13 > Tau, 4.62 > S100, 6.56. Therefore this test too was confirmatory of the *ante mortem* diagnosis of neural damage.

TABLE 2 **Levels [a] of Serum Autoantibodies (AA) in Controls and the Subject and Subject's folds increase relative to Healthy Controls**

Brain Specific Protein	Neurological Function	AA level Control Mean ±SE	AA level Subject Mean ±SE	Subject (Fold of Healthy Control)	Location of Tissue Injury	Associated Neurological Deficits
Neurofilament Protein (NFP)	Neurogenesis Axonal Development and Axonal Transport	0.59 ± 0.17	7.20± 0.54	12.21	Axonal Degeneration	**1. Cerebral Cortex** Weakness, Deficits in Posture, Locomotion; deficits in movement of fingers, speech and facial expression. **2. Limbic System** Learning, Memory Deficits
TAU Proteins (TAU)		0.86 ± 0.25	3.97 ± 0.38	4.62		
Tubulin	Axonal Transport Present in Other Tissues	1,54 ± 0.23	9.44 ± 0.86	6.13	Axonal Degeneration and Damage to Other Tissues	
Myelin Basic Protein (MBP)	Myelino-genesis Myelin Development	0.75 ± 0.13	13.91 ± 1.10	18.54	Demyelination	
Microtubule Associated Proteins-2 (MAP-2)	Neurogenesis Dendrite Development of Nerve Cell	1.51 ± 0.13	21.61 ± 1.23	14.31	Dendrite Degeneration	**Purkinje Cells (Cerebellum)** Inco-ordination, Staggering Ataxia
Glial Fibrillary Acidic Protein (GFAP)	Gliogenesis Forms Scar in Injured Axons	0.84 ± 0.25	8,58 ± 1.34	10.24	Axonal Injury	Chronic Axonal Injury, Blockage
S-100B Protein	From Astrocytes in Acute Injury	0.25 ± 0.06	1.64 ± 0.12	6.56	Acute, Traumatic Brain Injury	Acute Axonal Injury

(Footnotes to Table 2)

[a] ***The results are expressed as mean values of triplicate assays of optical density arbitrary units normalized to albumin optical density as fold of healthy controls.***

Notes: ***The values from subjects were compared to the control group using paired t-test and were all Highly Significant $p < 0.001$***

DISCUSSION

This report presents the results of tests performed on a pilot of commercial aircraft who flew for 15 years. Three years after he started flying, he began complaining of chronic ill health that he attributed to breathing toxic substances in the flightdeck air. He gave a sample of his blood to evaluate autoantibodies against specific proteins that are biomarkers for brain injury, a few months before his sudden death at age of 43. During his 12-years of chronic illness, he was examined by several physicians, admitted to hospital several times, and underwent many tests; but he was never diagnosed as suffering from any disease. Shortly before his death he went to the Netherlands where he was diagnosed as having organophosphate-induced neurotoxicity. We deal later with the matter of differential diagnosis. Here it is worth mentioning a few medical pointers, in discussing the case. The subject's symptoms are consistent with the cholinergic effects of organophosphates, particularly the relatively early reported episode of scintillating vision. The first sign of neurological deficits that were consistent with OPIDN was in 2008 when he experienced a slow onset of numbness in hands and feet, creeping up as far as the elbows and knees respectively. These symptoms are typical as the earliest manifestations of OPIDN [11]. In August 2011 he had paresthesia in both legs and both arms; that is a hallmark of OPIDN [11]. The symptoms reported to the doctors in the Netherlands are consistent with those of OPICN [9]. In Table 1, showing the summary of life-events, we have added remarks to elucidate such pointers.

Autoantibodies against nervous system specific proteins as biomarkers for brain injury

Nervous system injury results in neuronal degeneration, demyelination, and glial damage. Subsequently, nervous system-specific proteins are released into circulation. These proteins are short-lived because they ultimately reach the liver where they are degraded [14]. Nervous system-derived proteins act as antigens and react with plasma cells (derived from B lymphocytes) to form autoantibodies. Initially, after a time-lag of approximately four days, plasma cells produce small amounts of the short-lived IgM type that accounts for approximately 10% of immunoglobulin.

Exposure of the memory plasma cells to the same antigen at a later time, results in a secondary immune response, and it rapidly switches to produce greater quantities of IgG, IgA, or IgE. IgG is

the major circulating antibody accounting for approximately 70% of immunoglobin. The early appearance and long survival of autoantibodies to these proteins permit practical surveillance of exposure and toxicity. Therefore neurologic symptoms, along with IgG, IgM, and/or IgA autoantibodies against neurotypic and glyotypic specific proteins, are important in the pathogenesis and diagnosis of nervous system injury.

The autoantibody results show significantly increased autoantibodies against brain-specific cytoskeletal proteins, consistent with neuronal and glial degeneration. The levels of autoantibodies against nervous system-specific proteins were much higher, except for tau and tubulin than the mean levels of the 34 cases previously reported [14]. This case showed the following folds change over the ones reported in published paper 14: NFP: 4.48; Tau: 0.74; Tubulin: 1.08; MB: 4.44; MAP-2: 4.20; GFAP: 3.30 and S100B:14.13. Cytoskeletal proteins are major targets of chemical-induced injury of the brain. Neuro-filaments (NF) are major constituents of the axon, accounting for 80% of its protein [18-20]. NFs provide the rigidity and support through assembly of the three subunits in a sub-stoichiometric ratio. Microtubule associated protein, Tau an axon-specific cytoskeletal protein that binds to and stabilizes microtubules [21]. Besides maintaining neuronal architecture, tau plays a pivotal role in brain development and synaptic plasticity [22]. Loss of tau results in neuro-degeneration and cognitive deficits [23]. MAP-2, the most abundant protein in the brain is located in neuronal cell bodies and dendrites. MAP-2 helps stabilize microtubules and mediate interactions with other organelles with microtubules [22]. Increased serum autoantibodies against MAP-2 are consistent with injury of neurons belonging to the cerebral cortex and CA1 subfield of the hippocampus induced by organophosphates [24]. Previous reports showed that degradation of MAP-2 following exposure to neurotoxic chemicals in the cerebral cortex and hippocampus is the result of global ischemia [25]. Abnormal phosphorylation of MAP-2 by organophosphate-induced activation of Calcium Calmodulin kinase II (CaMKII) may impair their normal structure and function of neurons [26]. Myelin is produced by oligodendrocytes, supporting cells located in the central nervous system (CNS). The loss or damage of myelin is associated with demyelinating signaling and nervous system diseases such as multiple sclerosis (MS) and the release of myelin basic protein (MBP). GFAP plays an important role in the long-term maintenance of brain cytoarchitecture, proper functioning of the blood brain barrier, and modulation of neuronal function [27]. The finding of highly significant increased autoantibodies against GFAP is consistent with axonal degeneration and previous reports that individuals with

neuropsychiatric disorders have elevated levels of GFAP [27]. S-100B, a small calcium-binding protein is produced mostly by astroglial cells of the central nervous system, exerts both detrimental and neurotrophic effects, depending on its concentration in brain tissues. Traumatic acute injury results in great destruction of astrocytes leading to massive release of S100B protein (up to 50 folds) into plasma, whereas its levels in psychiatric disorders were higher in patients compared to controls, correlating well with its neuro-protective action [28].

It is realized that the test detects only damage to the nervous system. It is also accepted that this test is unable to confirm whether the cause of the neural damage was neurotoxic contamination of the aircraft cabin air or not. Nevertheless it is possible to form a view as to whether the extracted results are consistent with damage observed time and time again in aircrew following known and documented exposure to organophosphorus compounds. It is also possible to correlate the results to the neurological deficits and symptoms reported by a surfeit of aircrew that are affected and grounded [14, 29, 30, 65]. Aircrew are said to be at risk because they happen to fly frequently, not because they manipulate the controls or apparatus of an airliner. So passengers too may be at risk if they fly frequently.

Increased autoantibodies against neural proteins in the present study are in agreement with previous reports in humans and experimental animals. Autoantibodies against neurofilament proteins were detected in the serum of some individuals who were exposed to arsenic and developed neurologic deficits [31] and in a child who became quadriplegic after exposure to the TCP [32]. Autoantibodies against NFP, GFAP, and MBP were detected in the serum of a teenager who was exposed to the organophosphorus insecticide methamidophos [33]. An experimental study showed that autoantibodies to NFP, MBP, and GFAP were elevated in hens exposed to the active metabolite of TOCP, i.e., phenyl saligenin phosphate and they developed OPIDN [32].

Nervous System Inflammation

In the central nervous system only very few T-lymphocytes are found under healthy conditions. Accumulation of infiltrating T-cells can occur under inflammatory conditions characterized by increased activation and proliferation of microglia cells. The T-cell infiltration occurs initially synchronous to the induced neuro-degeneration and contains more $CD4^+$ than $CD8^+$ T-cells, suggesting complementary roles in the disease [35]. Dying cells and/or the accumulation of debris

or aggregated proteins can occur in the CNS, e.g. as a result of exposure to neurotoxic compounds. Resting microglia cells become activated, become profound antigen-presenting cells and start to secrete pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) and mediators (ROS-Reactive Oxygen Species), and resulting in the recruitment of T-lymphocytes. Activated T cells are capable of extravasating into the CNS where they perform immune surveillance. Chronic elevation of myeloid suppressor cells (MSC), while not the primary cause of the disease, might contribute to the lack of recovery and to further exacerbation of the disease conditions [36]. Cytokines, like TGF-ß1, is a major regulator of the immune response by exerting both anti-inflammatory and pro-inflammatory effects in a context-dependent manner. In neurological diseases Treg-derived systemic TGF-ß1 inhibits T-cell mediated disease, whereas locally increased TGF-ß1 at the site of antigen presentation exacerbates disease. This is consistent with the chemotactic effects of TGF-ß1 on T-lymphocytes and also its pro-inflammatory Th1 polarizing effects. In addition, TGF-ß1 induces tissue repair and recruits microglia to the site of damage [37]. The combination of chronic and persistent neurological damage, increased antigen presentation by microglia cells, and a suppressed immune regulation by increased numbers of Treg and myeloid suppressor cells, will result in activation and recruitment of pro-inflammatory Th1 cells and Th2 cells promoting the development of autoantibodies directed against neural tissue. These factors strongly contribute to the development and aggravation of neurological disease. In future histological analysis of such patients, T-cell subsets and cytokines, and B-cells and autoantibodies should be detected and quantified.

Involvement of Ca^{2+}/calmodulin-dependent Kinase II (CaMKII)

The NFI autopsy reported that "*pentobarbital caused imbalance between oxygen supply and removal to the brain and the heart, resulting in a lack of oxygen, tissue damage and damage to the cardiac muscle, herniation of the brain organ dysfunctions and death*". The results of this single case-study do not contradict the conclusion of the autopsy reports that death resulted from damage to the heart and the brain. In fact a plausible explanation can be offered as to how the heart and brain were injured that agrees with the conclusions of the report. It is postulated that exposure to organophosphates in the aircraft caused activation of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) that resulted in heart damage and contributed to the subject's death. CaMKII, is a multifunctional heteromeric serine/threonine protein kinase [38]. An early event in organo-

phosphate-induced neurotoxicity is increased Ca^{2+} concentration in neuronal mitochondria [39], followed by enhanced autophosphorylation [40, 41], activity [29, 30], and increased mRNA expression [43,44] of CaMKII. Recent studies have shown that CaMKII (more specifically CaMKIIβ) is a regulator of oligodendrocytes myelination and maturation. Overexpression of CaMKIIβ demonstrated a decrease in the process network of oligodendrocytes. Thus organophosphate-induced expression and activity of CaMKII results in release of myelin basic protein. CaMKII is also involved in the apoptotic death in early stages of cardiac [45] and nervous system diseases [11,12]. This enhancement is also accompanied by increase in apoptosis (Bax/Bcl-2 ratio and TUNEL positive cells) associated with an enhancement of CaMKII activity. CaMKII is a pre-apoptotic protein [46]. Activated CaMKII promotes heart failure by mediating pathological efforts of ischemia reperfusion (IR) through induction of both apoptosis and necrosis [47]. Activated CaMKII-induced cell death involves mitochondrial pro-death pathways [47]. This explanation is supported by the finding that inhibition of CaMKII attenuates cell death in the heart that results from catecholamines, myocardial infarction or IR. Mitochondrial-triggered cell death results from activated CaMKII by Ca^{2+} overload or excess reactive oxygen species (ROS) production in the mitochondria [48]. This explanation is consistent with the autopsy report implicating imbalance of oxygen supply in causing damage to the heart and the brain.

Differential Diagnosis

A debate has been ongoing, for several years, regarding the cause of symptoms such as the subject's health complaints and whether they are due to exposure to engine oil fumes or other factors [30]. Establishing a causal link with exposure is not easy; the main reason is there was (and still is) no on-board monitoring of aircraft cabin-air contamination. This is in spite of the deep concern that has been expressed over decades [1-4, 8, 9, 49, 50, 65], and also in spite of the fact that many *ad hoc* detection tests, and well-resourced studies, have reported contamination [51-55]. Clinicians have to rely on the patients' history to determine whether their symptoms relate to exposure. On the other hand, processes such as recall bias and attribution error can make patient testimony unreliable. To complicate matters further, patients usually see physicians long after exposure has ceased when toxic substances may have been eliminated from the body and results from routine medical investigation often fail to identify any abnormalities. Generally, clinicians are unaware of the potential toxic air contamination within aircraft. In order to find out the cause

of the subject's chronic ill health and his eventual death, we carried out differential diagnosis or "detective toxicology" by considering his use of pentobarbital, alleged exposures to chemicals, the results of symptoms and complaints of the patient, routine medical evaluations, specialized tests, autopsy results and autoantibodies results and other possible nervous system diseases.

Involvement of pentobarbital in the subject's toxicity, integrity of the Blood-Brain Barrier (BBB) and neuronal cell death

Pentobarbital-induced toxicity

Pentobarbital has been implicated in the poisoning and sudden death of the subject because it was found in his body during autopsy. Pentobarbital can induce death when used in high doses, i.e. 10g. Death occurs in 0.5 to 12% of cases. Most of the cases are the result of deliberate attempts at suicide. It is believed that poisoning often results from "drug automatism". This refers to the situation when a patient who could not go to sleep after the first or second dose, becomes confused, and without being aware, ingests an overdose; if recovered, there is no memory of having taken an additional dose. A study of 488 cases of intoxication, classified approximately one fourth of these cases to be automatism [57]. The automatism cases showed higher proportion of cerebral lesions than did the patients with suicidal intent, and were thus probably more disposed to confessional state during mild intoxication. In the current case, histopathological assessment of the brain indicated the presence of cerebral lesions that are consistent with automatism and suggest that his death might have been an overdose instead of suicide; this is in agreement with the absence of a suicide note [56]. Even if the subject consumed an overdose of pentobarbital that contributed to his death, it might be suggested that this was because of exposure to organophosphates that caused severe cortical damage leading to drug automatism.

Effect of pentobarbital on the integrity of the Blood Brain Barrier

In the present investigation it is hypothesized that exposure to neurotoxic chemicals in the aircraft caused a breakdown of the Blood Brain Barrier (BBB) and neuronal death in the brain and spinal cord. A question has arisen, whether these effects on the BBB and neuronal cells are caused by pentobarbital. The postmortem report indicated that the subject had used pentobarbital as a sedative; however, it is not known for how long or how much. The autopsy report revealed the

presence of pentobarbital in the femoral blood and in the hair. Pentobarbital was found in the lower part of a 2 cm hair, suggesting that he did not use it over a long time. It was also present at a high enough concentration in the blood for the post mortem report to conclude that it contributed to his death. A computer literature search failed to find publications on the effect of long-term low-level, or acute large dose of pentobarbital on neuronal cells or the integrity of the BBB. In contrast, a sedative dose of pentobarbital was found to protect both the BBB as well as neuronal cells from death. These studies suggested that when the BBB is disrupted, pentobarbital may be effective in protecting the BBB. An infusion of 20 or 50 mg/kg pentobarbital attenuated the degree of leakage of the BBB [58, 59]. Thus, when the BBB is disrupted, pentobarbital plays a significant role in decreasing the leakage.

Effect of pentobarbital on neuronal cell death.

Barbiturates are known to prevent post ischemic cell death in selected vulnerable regions in the brain including CA1 pyramidal cells in experimental animals [58]. Moreover, inducing coma treatment with barbiturates has been an effective therapeutic method for cerebral ischemia [59]. Pentobarbital resulted in complete protection against CA1 cell death in the hippocampus CA1 subfield on Day 14 in accordance with previous reports [60]. Pentobarbital (50 mg/kg, IP) protected CA1 pyramidal cells from death. The neuroprotective mechanism of pentobarbital is generally considered through the CNS depression, or through enhancement of the receptor binding or through $GABA_A$ receptor binding [61]. It is concluded that neither the integrity of the BBB or neuronal cell death had been affected or caused by pentobarbital.

Other Disease

After the autopsy was done, the subject's pathology results was briefly considered as "reminiscent" of Guillain–Barré Disease but was discounted. Guillain–Barré disease is an acute polyneuropathy, and a disorder affecting the peripheral nervous system [62, 63]. Ascending paralysis, weakness beginning in the feet and hands and migrating towards the trunk, is the most typical symptom. The disease is usually triggered by an infection. With prompt treatment by intravenous immunoglobulins or plasmapheresis, together with supportive care, the majority will recover completely. Guillain–Barré syndrome is rare, at one to two cases per 100,000 people annually. Guillain–Barré, unlike disorders such as multiple sclerosis (MS) and Lou Gehrig's

disease (ALS), is a purely peripheral nerve disorder and does not in general cause nerve damage to the brain or spinal cord.

Organophosphate-Induced peripheral and central nervous system injury

Nervous system damage may result from acute traumatic injury following a single large chemical exposure that causes neurological deficits. It can also result from repeated, continuous low-level, chronic chemical exposure, causing small neural increments of injuries that accumulate and result in neurological deficits. Another single low-level exposure that may not result in the development of symptoms, but which occurs on the top of the pre-existing chronic exposure, may push neural injury to the level above the threshold level, leading to the development of symptoms of neurological deficits. Although neurological deficits may result from a single chemical exposure, combined chemical exposure is more effective in causing nervous system injury. Also, such injury may result from sequential exposure to chemicals such as pesticides. Multiple chemicals compete with each other for the body's defensive mechanism, with subsequent increased delivery of each chemical to the neurotoxicity target. Stress has been shown to enhance chemical-induced nervous system damage. Chronic or subchronic exposures to small doses of organophosphorus compounds are more neurotoxic and more efficient in producing OPIDN than large single doses [64]. Whereas the threshold dose for a single oral dose of TOCP that produces OPIDN in hens was determined to be 250 mg/kg, 36 daily 0.5 mg/kg doses, totaling 18 mg/kg induced OPIDN; in other words, the single dose that caused OPIDN was 14 times greater than that of divided doses for the same effect. Daily small doses were seven times more effective then a single large dose in producing OPIDN.

The subject had been working as a pilot for 15 years, during which he was reputedly exposed to organophosphates that led to his illness. Dermal exposure to organophosphates is more effective than oral administration in producing OPIDN. An example is the finding that it took 64 daily oral doses of 1.0 mg/kg (total dose is 64 mg/kg) of the organophosphorus insecticide, leoptophos to cause OPIDN, compared to 25 dermal doses of 0.5 mg/kg (total dose is 12.25 mg/kg leptophos to achieve the same effect [64]. In other words, the total oral dose that caused OPIDN was 5 times that of a dermal dose. Although there is no data regarding inhalation exposure, this route is the most efficient route of delivery of Organophosphates to the nervous system. Also, the minimum daily dermal dose was 10 times as effective as the daily oral dose in producing OPIDN.

Although the precise identity or quantity of chemicals that the subject was exposed to is not known (due to the absence of monitoring), their presence aboard planes has been reported. TCP, TOCP and TBP were detected in cabin air on commercial and military aircraft [1,3,51-53, 55, 65]. A study on behalf of the UK Government found levels of TCP in cabin air which were alleged to be within safe limits [53]. This study, however, was undertaken during routine flight and not in a fume event.

Aircrew and some passengers have been reporting ill health following air emissions for many years, the immediate effects being eye, ear, nose and throat irritation, respiratory problems, headaches, nausea and cognitive impairment, which usually recede on cessation of exposure; but a number of individuals report persistent chronic ill health including chronic fatigue, cognitive impairment, headaches, and muscle weakness [8, 14, 30, 65].

Genetic Variation

It has been established that individual sensitivity to neurotoxicity induced by chemicals including organophosphates is genetically and environmentally controlled [66, 67]. Furthermore, a certain segment of the population is either effectively tolerant to exposure, while others will have a reduced, or non-existent, tolerance. This observation is related to the individual's genetic makeup resulting in having individual differences for sensitivity to chemical induced injury. Following entry into the human body, an organophosphate undergoes metabolic processes including absorption, binding, distribution, storage, metabolic biotransformation and excretion [66]. Detoxification of organophosphate (OP) esters is carried out by specific enzymes mostly present in the liver and blood. In the liver the major enzymes that metabolize organophosphates into less toxic metabolites are cytochrome P450 isozymes, via dearylation of aromatic OP's such as TCP and TBP, that are present in engine oil and hydraulic fluid, respectively [66, 67]. In blood, the first line of defence against organophosphate-induced toxicity are the enzymes paraoxonase1 (PON1) and plasma butyrylcholinesterase (BChE). PON1, a plasma enzyme tightly associated with high-density lipoprotein particles and also found in liver [67]. PON1 is polymorphically distributed in human populations with an amino acid substitution glutamine/arginine (Gln/Arg) at position 192 of this 354-amino acid protein. In addition, catalytic efficiency is determine by the position 192 amino acid. Protein levels of PON1 vary by as much as 15-fold among individuals with the same PON1(192) genotype. Both the plasma PON1 level and the position 192 amino acid are important.

On the other hand, BChE protects exposed individuals by acting as a scavenger by binding to organophosphates, and by subsequent hydrolysis that result in their removal from circulation and leading to less of the OP's reaching neurotoxicity targets such as the brain [67]. There are genetic variants of this enzyme known as "atypical" BChE that have less ability to hydrolyze organophosphates [68, 69]. The atypical enzyme in which an aspartate at position 70 is substituted by glycine [70], is incapable of hydrolyzing organophosphates [71]. The homozygous usual enzyme (E^U, E^U) is 80% inhibited by dibucaine and is present in most of the Caucasian population [70]. The heterozygous atypical enzyme (E^U, E^a) with 60% inhibition by dibucaine is present in about 4% of the population. The homozygous atypical enzyme (E^a, E^a) that is 20% inhibited by dibucaine occurs with an incidence under 0.04% - 0.6%. Individuals with such a variant, are more sensitive to organophosphate–induced neurotoxicity than individuals with the normal variant and are considered predisposed and at high risk to organophosphate-induced neurotoxicity. In the present case, no attempt was made to find out if the subject had any genetic BChE variants that contributed to his organophosphate-induced neurotoxicity.

Brain Weight

The autopsy report indicated that there were signs of fluid accumulation in the brain (his brain weighed 22% more than an average healthy adult brain). Increased water in the brain was a hallmark of brains, autopsied in the 1930s, of victims of TOCP poisoning known as "Ginger Jake". Old autopsy reports contained phrases like "brain described as water-logged" [72] and "there was considerable oedema of the cortex and the meninges appeared thickened" [73].

Symptoms and Complaints of the Subject

The symptoms and complaints of the subject in this case represent very strong evidence for the source of illness. Although these symptoms may seem "non-specific" to a layman, or even a physician who is not familiar with organophosphate-induced neurotoxicity, their temporal occurrence and action are highly specific for neurotoxicity induced by organophosphates. Considering the fact that the subject was not a neurotoxicologist, it is safe to assume that he was not familiar with organophosphate induced neurotoxicity, its literature, or even to his exposure to it, since he did not report any air emissions, until questioned. Nevertheless, his symptoms were not

only consistent with, but also identical to, known and documented effects of organophosphates poisoning [9]. His low-level chronic exposure to organophosphates that initially caused un-noticed small increments of nervous system injury eventually reached above the threshold level for symptoms to become evident. This is consistent with the subject not complaining initially, then as time went by, he suffered symptoms of cholinergic neurotoxicity, followed by symptoms of OPIDN and finally OPICN. The time course and complaints were consistent with organophosphates poisoning. The subject's description of his symptoms which relate to OPIDN, such as paresthesia and ataxia [9-13], are very specific and so technical, that he could not have invented them.

Autoantibodies Against Nervous system specific Proteins

Increased autoantibodies against nervous system-specific proteins are very strong evidence for nervous system injury. They indicate neuronal and glial degeneration consistent with OPIDN and OPICN. They also explain the specific region that is injured, such as the spinal cord and cerebellum that are injured in OPIDN. Also, injury and degeneration of the cortex, hippocampus and cerebellum usually accompany OPICN.

CONCLUSION

The results of the present case-study show that histopathological alterations in the brain confirm and validate the results of increased serum autoantibodies against brain-specific proteins. Also, both of these tests are consistent with organophosphate-induced neurotoxicity. Our results offer an explanation for the autopsy report that death resulted from damage to the heart and brain via organophosphate-induced activated CaMKII. This study demonstrates that exposure to the aircraft environment that allegedly contained organophosphates, rendered the subject susceptible and predisposed to injury by pentobarbital. Organophosphates have been shown to cause overexpression, increased phosphorylation, and increased activity of CaMKII, a pre-apoptotic protein that causes apoptotic cell death to both the brain and the heart.

In the absence of any competing diagnosis, the negative results of all other tests and examinations, and in the light of the discovery of very strong autoantibody markers for brain damage that is

confirmed by histopathological examination, one is drawn to the conclusion that the most likely cause of the subject's illness is organophosphate induced neurotoxicity.

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