The Utility of Mitochondrial Complex IV Analysis as a Biomarker of Carbon Monoxide Exposure*

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Summary

Carbon monoxide (CO) is a colourless, odourless gas that in high concentration can be fatal, and in low concentrations can lead to a variety of symptoms, including nausea, confusion, neurological problems and fatigue. One problem with human exposures to CO is that directly demonstrating CO toxicity is difficult as symptoms of CO poisoning are non-specific and CO can be rapidly eliminated from the body [1]. Once the level of suspicion in the medical practitioner is raised, confirmation of exposure in the patient needs to be made. Methods to confirm CO exposure require specialist equipment: blood gas or breath analysers, which tend to be utilised sometime after the patient has been removed from the source of exposure. This makes detection difficult and poisoning harder to confirm due to the half-life of CO in the body. It is considered likely that CO poisoning is under diagnosed, misdiagnosed and if diagnosed, CO poisoning is not necessarily detected or confirmed, making accurate morbidity and mortality statistics caused by exposure to CO, difficult to attain. There is a need therefore, to identify markers of exposure to CO that indicate both the presence and levels of toxicity for use by medical practitioners and Coroners, and for such markers to be robust and long lasting.

The terminal enzyme of the mitochondrial respiratory chain, cytochrome c oxidase (COX) is a major cellular target for CO leading to inhibition (a decrease) of cellular ATP production. Some studies indicate that CO may cause prolonged COX inhibition, long after exposure to CO has ceased. This indicates that it is likely that there are markers of exposure to CO that are still active at a cellular level when exposure to CO has ceased and possibly when symptoms of exposure have disappeared. We have therefore set out to determine if COX found in human tissue could be used initially in a forensic setting to provide evidence of CO exposure.

Using a cohort of 98 post mortem frontal cortex from normal donors, we have demonstrated that mitochondrial COX activity levels are stable post mortem under normal circumstances. COX activity expressed as a function of mitochondrial citrate synthase activity appears to be stable to post mortem interval up to about 100 hours, is unaffected by age at death of donor, relatively unaffected by tissue pH (used as a surrogate measure of pre-mortem agonal state), and independent of storage interval of tissue at -80^{o}C. Mitochondrial COX protein subunits COX1 and COX2 also appear unaffected by typical post mortem factors.

Using post mortem frontal cortex tissue from donors in whom it was confirmed, died from high level, acute CO exposure, we found that COX activity did not differ from normal control donor COX activity. Similarly, COX1 and COX2 protein levels were unchanged by rapid exposure to high levels CO. This would suggest that the tissue changes produced by high

level exposure to rapidly rising levels of CO are due to simple anoxia where CO displaces oxygen from haemoglobin preventing tissue oxygenation rather than prolonged inhibition of tissue COX activity. Brain COX activity would not therefore be a suitable marker of CO exposure in Coronial investigations of acute, high level CO poisoning.

Since CO also has physiological effects particularly in the regulation of blood pressure, and cellular effects as a gaseous signalling molecule, including interaction with a series of reactions linked to cyclic GMP, there may be indirect effects of toxic levels of CO on such systems. This was investigated. Levels of HIF1α and PGK1α were unchanged in brain tissue from carbon monoxide exposed cases, indicating that anoxia may not affect these systems acutely. sGCSβ is a protein which interacts with CO and is compatible with studies which show downregulation of sGCSβ in response to sustained CO exposure. On investigation, sGCSβ was found to be downregulated in brain tissue of CO exposed individuals. p38MAPK is another CO responsive protein, a signal enhancer which responds to a variety of cell stresses. Increased levels of p38MAPK may be a sign of neural stress that also appears to correlate with CO exposure. On investigation p38MAPK was found to be increased in the CO exposed individuals. The finding of changes in sGCSβ and p38MAPK may suggest a mechanism whereby CO exposure could be monitored using peripheral expression levels in for example lymphocytes from donors possibly exposed to both high and lower levels of CO.

Lay Summary

Carbon monoxide is a colourless and odourless gas produced because of burning fuel without an adequate supply of oxygen. At high levels, normally when there is poor ventilation, carbon monoxide can build up and cause a variety of symptoms including headache, tiredness, and nausea, and at very high levels can lead to coma and death. It is thought that around 30 people die every year due to carbon monoxide poisoning and over 4,000 people show milder symptoms of poisoning with carbon monoxide that require medical assistance, although this figure is widely considered an underestimate because carbon monoxide exposure is difficult to detect and if missed, will not be recorded in medical records. One problem is that by the time the suspicion of carbon monoxide poisoning has been raised, the carbon monoxide has been exhaled from the body and can no longer be detected. An additional problem is that symptoms often persist and it is thought that while carbon monoxide is removed from the blood, it still remains in tissues and organs at low levels and continues poisoning. Because of the problems of identifying carbon monoxide in the blood, it may be necessary to find a way of measuring carbon monoxide in tissues.

Carbon monoxide affects the body by preventing the uptake of oxygen by red blood cells and preventing the oxygen that is already being carried by the red blood cells from being released and reaching tissues and organs such as the heart and the brain. Carbon monoxide, in addition to affecting red blood cells also affects a key protein within human tissue called cytochrome oxidase. Cytochrome oxidase produces much of the cells' working energy. Therefore, by preventing cytochrome oxidase from working, carbon monoxide causes cells to stop working. In the case of the heart and the brain, a lack of energy causes heart cells to stop beating and the nerve cells in the brain that control breathing and heart rate to fail, leading to death. Potentially, if the effect of carbon monoxide on cytochrome oxidase could be measured, we may have a way of telling if someone has been exposed to carbon monoxide even if markers of exposure to carbon monoxide can no longer be measured in blood.

We measured cytochrome oxidase in human brain tissue from nearly 100 normal volunteer donors who had died without any known exposure to carbon monoxide and also in brain tissue from three donors who had died due to carbon monoxide poisoning. While we could measure cytochrome oxidase in brain tissue and it appears unaffected by different conditions associated with post-mortem preservation and experimental conditions, tissue from donors who had died from carbon monoxide poisoning did not show any differences in cytochrome oxidase from those who were not exposed. Therefore, contrary to what scientific understanding might lead

us to believe, measuring cytochrome oxidase does not appear to be useful in identifying someone poisoned by acute high level exposure to carbon monoxide.

Under normal circumstances, cells in the body produce tiny amounts of carbon monoxide, far below the levels that cause poisoning. This small amount of normally produced carbon monoxide is used to communicate signals between cells and it achieves this by interacting with several different proteins to have both short and long term effects on tissues. To see if some of these proteins are affected by inhaled carbon monoxide, we measured their relative levels in tissue from normal (unexposed) and carbon monoxide exposed individuals. Levels of HIF1α and PGK1α were unchanged in brain tissue from carbon monoxide exposed cases. The proteins sGCSβ, and p38MAPK were found to be decreased and increased respectively in brain tissue from carbon monoxide exposed individuals. sGCSβ is a protein which interacts with the small amounts of carbon monoxide produced by cells to increase the signalling molecule cGMP within cells. The reduced amounts of sGCSβ protein in carbon monoxide cases appears to be compatible with studies that show that in cells exposed to high levels of carbon monoxide there is a reduction of the levels of sGCSβ. This reduction of sGCSβ is thought to be an attempt by cells to reduce any prolonged damaging effects of carbon monoxide on the signalling systems. p38MAPK is a protein found in cells which reacts to and is involved in cell stress. The increase in the levels of p38MAPK in brain tissues could be a sign of stress but it appears to be specific to carbon monoxide exposure. The finding of changes in sGCSβ and p38MAPK might mean that we can detect if someone has been exposed to carbon monoxide, even after exposure has stopped and when conventional methods of detection are no longer of use, but this will require a different type of study to confirm this and to confirm whether such detection would be applicable in both exposures to high and low levels of CO.

1. Introduction

Carbon monoxide (CO) is a colourless, odourless and non-irritant gas which in high concentration can be fatal, and in low concentrations can lead to nausea, confusion, neurological problems and fatigue[2]. One problem with human exposures to CO is that demonstrating CO toxicity directly is often difficult as symptoms of CO poisoning are non-specific and CO can be rapidly eliminated from the body[1]. Once the level of suspicion in the medical practitioner is raised, methods to confirm CO exposure require specialist equipment. Blood gas or breath analysers tend to be utilised sometime after the patient has been removed from the source of exposure, thus making detection difficult and poisoning harder to confirm due to the half-life of CO in the body (75 ± 25 min)[3]. It is considered likely that CO poisoning is under diagnosed and if diagnosed, not necessarily detected or confirmed, making accurate morbidity and mortality statistics caused by exposure to CO, difficult to attain. There is a need therefore, to identify markers of exposure to CO that indicate both the presence and levels of toxicity for use by medical practitioners and Coroners, and for such markers to be robust and long lasting.

1.1 Carbon monoxide poisoning

Estimates suggest that up to 4,000 people each year attend hospital emergency departments presenting with a variety of symptoms due to accidental CO exposure, that require treatment, over 200 present requiring continued hospitalisation and there are around 30 potentially preventable deaths[4]. Malfunctioning gas appliances are a source of such CO exposures, although other fuel sources are also implicated. There is therefore a need to identify indoor sources of exposure to reduce ill health and potential fatalities[5]. For exposed individuals, there is additionally a need to identify suitable markers that indicate either current or past CO exposure. This is due to the fact that once individuals are removed from a source of CO, detectable levels of CO in the body fall rapidly in both blood and exhaled breath[6]. After inhalation, CO will rapidly diffuse across the alveolar capillary membrane and react with haemoglobin to produce carboxyhaemoglobin (HbCO) which is thought to underlie the toxicity of CO by causing tissue hypoxia by displacing oxygen from haemoglobin[7]. The affinity of CO for haemoglobin is 240 times greater than oxygen which can interfere with oxygen binding and its dissociation from haemoglobin when HbCO levels are high[8].

HbCO has been used extensively to monitor CO exposure. Over 4% of individuals presenting at accident and emergency departments in the UK with non-specific symptoms show HbCO

levels over 5% which may indicate CO exposure, although levels higher than this can be found in some normal non-presenting individuals who generally have HbCO levels of below 1%[9, 10]. Blood HbCO levels may however, be an unreliable indicator of exposure concentrations and HbCO levels of over 10% may only reliably indicate CO exposure[11]. This is further complicated by the high levels of CO and HbCO found in heavy smokers[10]. Based on several studies, toxic exposure to CO exposure has the potential to be considered by the clinician when HbCO levels are more than 20% which results in tissue and cellular hypoxia and clinically detectable symptoms[2]. Table 1.1 presents the effects of different concentrations of CO in humans and how this is reflected in HbCO levels as well as the respective clinical symptoms[12, 13].

Type of CO exposure	Concentration of CO (ppm)	HbCO levels (%)	Signs and symptoms
Low concentration, sub-toxic, sub-clinical	10 70-120 for 4 hours	2 10-20	Asymptomatic Relative asymptomatic, without signs of tissue hypoxia
Intermediate	200-800	30-50	Headache, dizziness, impaired judgement
High concentration,	>800	>60	Seizures, coma, death

Table 1.1 Effects of CO Exposure and Relation to Clinical Symptoms[12, 13].

This table should be considered as a guide only as effects will vary from subject to subject and with pattern of exposure. The levels of HbCO produced depends on the concentration of and the exposure time in the environment where CO exposure occurs. Sub-toxic concentration is referred to the threshold that is required to induce signs of hypoxia in tissues.

However, blood levels of HbCO can rapidly drop following removal from CO exposure and within a few hours, can return to normal. This makes clinical investigation difficult, particularly where patients are not suspected as being exposed. The reliability of methods for detecting CO poisoning are also unclear[14]. Whilst several studies have shown a correlation of HbCO levels with severity of injury in CO poisoning when measured acutely, one large major study failed to show any observable link between HbCO at first examination and the severity of impairment, and recommended abandoning the use of some existing scales using HbCO levels in suspected CO poisoning[15]. HbCO levels do not provide an indication of patient outcome following exposure. If HbCO cannot be used, a suitable marker of CO exposure is therefore required for

identifying exposed individuals in a number of scenarios: when they are no longer, or not presenting with symptoms; to confirm past exposure having not been re-exposed for some time after initial exposure. Furthermore, such a marker would be useful for monitoring the relationship between CO exposure and the severity of any clinical symptoms, and for highlighting the potential for the delayed appearance of neurological symptoms in particular.

1.2 Carbon monoxide poisoning and cytochrome *c* oxidase

High concentrations of CO can directly interfere with aerobic cellular respiration by binding to haem proteins within the cytosol and mitochondria[16]. One of the main routes of CO toxicity is thought to be via tissue hypoxia, where CO binding to the haem centres of haemoglobin prevents tissue oxygenation. CO needs to be activated by coordination with low-valence metals or iron to permit chemical reaction. In biological systems, Fe^{2+} of reduced haem proteins is the main target for CO binding, with a major protein being cytochrome *c* oxidase (COX) of the mitochondrial respiratory chain. Oxygen displacement from COX will result in a reduction in tissue mitochondrial activity and specifically of COX activity due to the reduced tissue oxygen availability. This causes a decrease in cellular energy levels and of adenosine triphosphate (ATP) production and ultimately cell death. This issue of reduced oxyhaemoglobin and tissue oxygen has however been challenged, since studies have shown that simply raising the levels of HbCO is not in itself lethal. It is known that 60-80% HbCO from intravenous or intraperitoneal administration of CO is not fatal, as dissolved blood CO is low and despite the reduced oxygen saturation, tissue oxygenation is sufficient[17-19].

COX is the terminal enzyme of the mitochondrial respiratory chain which utilises electrons donated from cytochrome *c*, the small molecular weight mobile haem component of Complex III of the respiratory chain. Under normal conditions for oxygen reduction, four electrons are transferred from reduced cytochrome *c* (Fe^{2+}-cytochrome *c*) to the Cu_A centre of the cytochrome *c* oxidase subunit 2 (COX2) and subsequently to the low spin haem *a* which is located in the COX1 subunit[20-23]. From haem *a*, electrons are transferred between molecules to the hemea_3-Cu_B centre where the bound molecular oxygen is located and which will be reduced to water. This transfer forms a proton gradient across the mitochondrial inner membrane, reducing the enzyme in the process (Equation 1.1). CO acts upon the reduced COX enzyme by directly competing with molecular oxygen (Ki~300nM) for the active site[24]. CO can form metal carbonyl complexes with many metals but in particular iron, forming a covalent bond as a comparatively stable metal carbonyl complex. The reaction of CO with the haema_3-Cu_B site at COX1 produces an intermediate carbonyl and, since the rate of reaction (Equation 1.2) is slow,

this complex is relatively stable[25, 26]. Under normal circumstances, the levels of molecular oxygen will compete against CO and allow for the oxidation of metal carbonyls, and metals can therefore remain active in an oxidised state (Equation 1.3).

$$4\ Fe^{2+}\text{-cytochrome } c + 8\ H^{+}_{in} + O_2 \rightarrow 4\ Fe^{3+}\text{-cytochrome } c + 2\ H_2O + 4\ H^{+}_{out} \quad \textbf{(1. 1)}$$

$$Cu^{2+}/Fe^{3+} + CO + H_2O \rightarrow Cu^{+}/Fe^{2+}\ CO_2 + 2H^{+} \quad \textbf{(1. 2)}$$

$$Cu^{+}/Fe^{2+} + \tfrac{1}{2}O_2 + 2H^{+} \rightarrow Cu^{2+}/Fe^{3+} + H_2O \quad \textbf{(1. 3)}$$

Since CO acts as a direct competitor of oxygen for COX, under normal tissue oxygen saturation (20-30 µM), there is no appreciable inhibition of cellular respiration at the tissue levels of CO typically seen. In the presence of CO intoxication however, the reduced oxygen in tissues due to the competition of CO for the haem sites of haemoglobin and thus, HbCO formation, leads to reduced oxygen saturation in the tissues. At levels of 1 µM CO, the Ki of CO (300 nM) is such that the effective concentration required for oxygen is moved to about 30 µM near the absolute tissue oxygen levels[24]. Under these conditions and in the presence of low levels of molecular oxygen within tissues, oxidation of the bimetallic centre cannot occur and the metal carbonyl complex remains with COX continuing to be inactive[24].

Following CO exposure, it is possible that tissue COX activity remains low, despite the declining levels of CO and HbCO even in the presence of oxygen. Experimental studies have suggested that CO exposure causes reduced COX activity even in the presence of declining HbCO levels[16, 27]. The extended inhibition of COX after CO exposure may underlie some of the persistence of clinical symptoms following exposure including persistent headache, nausea, abdominal pain, fatigue, confusion and cognitive impairment. Similar findings have been suggested for cyanide intoxication which also inactivates COX by a similar mechanism to CO, where COX activity in the brain was found to be significantly inhibited even in the presence of low tissue cyanide levels, and remained inhibited for at least two days[28]. However, some studies suggest that CO improves COX activity by stimulating the expression of haem oxygenase-1 (HO-1) or hypoxia inducing factor-1α (HIF-1α) which results in improvement of mitochondrial function and COX activity[29, 30]. Other studies have shown that the effects of CO on COX activity depend on the concentration and on the period of CO exposure. Mice that have been exposed to 1000 ppm CO for 3 hours showing elevated HbCO (~60%), which is considered as being a toxic dose, show decreased myocardial COX activity[31]. However, a two-step response was observed in liver mitochondria and brain astrocytes where initially there was a decrease in COX activity, which was then followed by an increase in COX activity[32, 33].

Currently, it is not clear if COX activity is reduced in tissues following CO exposure as some studies suggest no change during acute phases although others show reductions in the oxidative state of COX[16]. As such, it would suggest that COX activity might be a useful indicator of CO exposure, particularly when CO exposure has ceased or when direct CO determination cannot take place[34]. Indications are that COX activity is stable within tissues post mortem and could therefore be used as a measure of CO exposure during forensic investigations[34]. Lymphocyte COX activity appears to be altered in a similar way to brain COX activity, showing near normal levels during the acute phase of exposure, but then demonstrating persistent inhibition long after CO levels return to normal. This suggests blood lymphocyte COX activity may also be a suitable marker for CO toxicity[35]. We will therefore investigate the potential utility of COX activity determination as a marker of CO exposure in human tissues when there is acute exposure.

1.3 Cell signaling pathways of carbon monoxide

The exogenous uptake of high levels of CO might be considered toxic however CO is normally produced as an elimination product of cellular metabolism in cells and tissues. CO is produced in cells as byproduct of the activity of haem oxygenase enzymes (HO) which catabolizes haem molecules into biliverdin, ferrous ion and CO[36]. Recent studies have shown that the CO produced at physiological levels has profound effects on intracellular pathways, which culminate in anti-inflammatory, anti-proliferative and anti-apoptotic effects[37]. The most studied physiological effects of CO are on cellular signaling pathways including the modulation of soluble guanylate cyclase β (sGCSβ, *GUCY1B3*) activity and consequent stimulation of cyclic guanosine monophosphate (cGMP) production[38]. CO binds directly to the haem iron in sGCSβ, activating the enzyme which results in several fold increased production of cGMP[39]. The increased production of cGMP also induces the inactivation of cGMP-dependent protein kinase (PKG1α, *PRKG2*) which is also involved in various intracellular signaling pathways[40]. Interaction of CO with this cGMP dependent pathway may cause some of the effects of CO seen clinically. Increased production of cGMP causes vasodilation both directly and indirectly. Other mechanisms include the modulation of various mitogen-activated protein kinase (MAPK) pathways channels[37]. The MAPK pathways is not known to contain any haem containing proteins which are the normal targets of CO, with the effects of CO on their pathways most likely representing a downstream event, perhaps due to cGMP production, rather than a primary target[41, 42]. CO can however, directly affect the stimulation of haem containing large conductance Ca^{2+}-dependent K^{+} channels (*KCNMA1*) to cause acute vasodilation, which may again relate to some of the clinical symptoms observed with short term CO exposure. It has

been suggested that this route via Ca^{2+}-dependent K^+ channels can lead to cerebral vessel vasodilation and may relate to the headache observed with CO exposure[43, 44]. CO may therefore have effects other than on COX or Hb.

1.4 Aims and plan of investigation

Our main aim is guided by the central hypothesis that 'COX activity inversely reflects CO exposure in key areas of the brain'. This specifically relates to the loss of COX activity observed due to specific binding of CO to haem a/a_3 redox centres of COX located at COX1 and that this process is only slowly reversible. A secondary aim is to identify the effects of CO in intracellular proteins that are potentially involved in CO regulated signalling pathways and to determine if increased levels of CO can effect expression.

The specific aims arising from the central hypotheses are:

1. To identify if COX is stable in post mortem human frontal cortex brain tissue.
2. To assess the effects of known CO exposure on human frontal cortex COX.
3. To measure protein levels related to COX protein subunits and CO signalling pathways in the human frontal cortex of normal and CO exposed brain tissue.

The key specific outcomes relating to the specific aims are:

1. To assess changes in COX activity and specific proteins due to post mortem factors.
2. To assess if CO exposure reduces COX activity or the expression levels of specific proteins in brain tissue.

In order to accomplish the set goals, a plan of investigation was formed which included investigating the use of COX activity in post mortem human brain tissue as a marker for CO exposure. Post mortem frontal cortex brain tissue and measure COX activity using a validated assay was utilised. Protein levels are determined semi-quantitatively by following a standard Western blot approach. Frontal cortex tissue was used since it is relatively simple to identify at post mortem compared to globus pallidus, and also has higher levels of COX activity[45]. Furthermore, the protein levels of the core catalytic subunits of COX, COX1 and COX2, as well as proteins that are implicated in cell signalling pathways (sGCSβ1, p38MAPK, PKG1α and HIF1α) involving of CO were determined.

The majority of the tissue was obtained from the Medical Research Council funded Newcastle Brain Tissue Resource (NBTR) at Newcastle University which is a UK Human Tissue Authority licensed human tissue bank working under the Human Tissue Act (2006). One CO

exposed case was obtained from the Addenbrookes Cambridge Brain Bank. Cases with CO exposure were also identified from the MRC Brain Banks Network database however, on further investigation, most cases were unsuitable due to complicated pathology (1 case at Oxford), associated infection (1 case at Edinburgh), and lack of suitable tissue (2 cases at Kings College London). NBTR holds human brain tissue obtained from over 1,500 donors, predominantly those with different neuropsychiatric disorders, but also with donor tissue from over 250 normal individuals. Tissue is obtained at post mortem following full assent of the next of kin and in line with donor consent and wishes according to National Research Ethics Service approvals. Tissue is stored as snap frozen (at -120^{o}C) tissue samples. Age at death, sex, and time interval from death to tissue storage is known for all donors along with other demographic data. Given normal perfusion levels, it is unlikely that endogenous CO[46] or lower level exogenous CO will interfere with COX activity (see above).

2. Material and methods

2.1 Clinical material

To investigate the possible role of CO toxicity in the brain a study involving post-mortem human brain tissue was undertaken. Brain tissues from frontal cortex was obtained from NBTR, Newcastle University and the Addenbrookes Cambridge Brain Bank. Three different groups were included in the present study: healthy control cases which showed an absence of any clinically significant neurological or psychiatric history and who showed age related neuropathology according to standard accepted criteria. Approximately 0.5 g of snap frozen grey matter tissue was dissected from the lateral frontal cortex (Brodmann Area 9). Tissue was rapidly thawed, the overlying meninges removed, and the majority of the white matter dissected off and the remaining grey matter was homogenised for enzyme/protein analysis (see section 2.2). Demographic information on the groups studied is shown in Table 2.1.

2.2 Sample preparation

The preparation of the brain homogenate was based on protocol by Spinazzi *et al.* with slight modifications in order to achieve maximum COX activity[47]. Initially, the thawed tissue was used to measure pH by using pH Flat Surface Electrode (Hanna). The meter was calibrated before use on a daily basis. Afterwards, the thawed brain tissue was dissected with a scalpel

blade, weighed on a three place balance and 10 volumes of ice-cold CCA buffer[1] (20mM Tris-HCl, 250mM sucrose, 40mM KCl, 2mM EGTA, pH 7.4, containing protease inhibitor tablets (Complete ethylenediaminetetraacetic acid (EDTA) free; Roche) was added. Brain tissue was homogenized in Teflon-plunger motor-driven 10 ml borosilicate glass tissue grinder, with 16 strokes and the rotational speed placed at the lowest setting. A small amount of the homogenate was used for the measurement of the COX and CS activity immediately and the remaining tissue was aliquoted and stored at -80^{o} C. Before assay, brain samples were further sonicated for 10 seconds with a sonic probe and then for 40 minutes in a sonic bath on ice in order to achieve maximum release of the membrane bound mitochondrial complex.

[1] Buffer was stored at -20^{o}C until use.

Case	Diagnosis	Age at death	Post mortem delay (hours)	Gender
1	Control	65	14	M
2	Control	94	20	F
3	Control	47	15	M
4	Control	63	24	F
5	Control	99	19	F
6	Control	87	14	F
7	Control	87	21	M
8	Control	53	19	M
9	Control	71	32	M
10	Control	75	27	F
11	Control	105	52	F
12	Control	87	8	M
13	Control	64	64	M
14	Control	69	16	F
15	Control	68	54	M
16	Control	72	17	M
17	Control	103	21	F
18	Control	58	39	F
19	Control	78	23	F
20	Control	89	24	F
21	Control	72	27	F
22	Control	74	45	F
23	Control	59	19	F
24	Control	99	111	M
25	Control	83	19	M
26	Control	55	41	M
27	Control	96	95	F
28	Control	77	83	M
29	Control	86	75	F
30	Control	74	53	F
31	Control	94	15	F
32	Control	88	22	F
33	Control	70	72	M
34	Control	67	22	M
35	Control	98	59	F
36	Control	89	34	F
37	Control	78	34	F
38	Control	95	66	F
39	Control	81	43	M

Case	Diagnosis	Age of death	Post mortem delay (hours)	Gender
40	Control	73	25	M
41	Control	74	49	F
42	Control	86	45	F
43	Control	89	98	F
44	Control	87	77	F
45	Control	96	29	F
46	Control	86	50	M
47	Control	97	21	F
48	Control	65	47	F
49	Control	99	5	F
50	Control	80	16	M
51	Control	85	57	M
52	Control	88	26	M
53	Control	96	16	M
54	Control	64	93	M
55	Control	88	28	M
56	Control	80	31	F
57	Control	85	45	M
58	Control	80	25	F
59	Control	52	102	M
60	Control	81	40	F
61	Control	90	63	F
62	Control	101	104	F
63	Control	91	72	M
64	Control	71	25	M
65	Control	93	12	F
66	Control	94	25	M
67	Control	81	75	F
68	Control	91	24	M
69	Control	84	45	M
70	Control	21	30	M
71	Control	70	24	F
72	Control	50	7	M
73	Control	94	74	F
74	Control	75	11	M
75	Control	78	42	M
76	Control	75	27	M
77	Control	57	21	M
78	Control	96	30	F

Case	Diagnosis	Age of death	Post mortem delay (hours)	Gender
79	Control	46	24	M
80	Control	77	38	F
81	Control	78	43	M
82	Control	73	24	M
83	Control	51	32	M
84	Control	50	82	F
85	Control	75	31	F
86	Control	88	65	M
87	Control	72	32	F
88	Control	95	50	M
89	Control	82	53	F
90	Control	76	24	F
91	Control	72	30	F
92	Control	94	50	F
93	Control	100	40	F
94	Control	101	19	F
95	Control	82	26	F
96	Control	56	11	M
97	Control	75	64	M
98	Control	94	50	F
99	CO	60	48	M
100	CO	44	12	M
101	CO	32	76	M

Table 2.1 Demographic Information on the Control and CO exposed cases.

Age, post mortem delay and gender are reported and where F: female and M: male.

2.3 Activity assays

2.3.1 Mitochondrial activity assays

Samples were prepared and assayed on the same day. Cytochrome *c* oxidase (COX) and Citrate synthase (CS) activity assays were performed based on previously described protocols which are in use within the National Health Service Mitochondrial Disorders Diagnostic Laboratories[48]. The assays were performed on a Cary WinUV 300 spectrophotometer with a circulator set at 30^{o} C.

2.3.1.1 Cytochrome *c* oxidase (COX) activity assay

The specific activity of mitochondrial COX was measured by following the oxidation of reduced cytochrome *c* (Fe^{2+}-cytochrome *c*) at 550 nm (extinction coefficient for the reduced Fe^{2+}-cytochrome *c* at 550 nm is E_{550} = 29.0 mM^{-1} cm^{-1}). As the apparent affinity constant K_m for the substrate (reduced Fe^{2+}-cytochrome *c*) and the dissociation constant K_i for the product (oxidised Fe^{3+}-cytochrome *c*) are similar, the reaction rate decays pseudoexponentially during the assay as the substrate is oxidized, so the activity is expressed as an apparent first-order rate constant (K/sec) rather than an initial rate.

Initially, the Fe^{2+}-cytochrome *c* was prepared by reducing the oxidised Fe^{3+}-cytochrome *c* with ascorbic acid, whereby 50 milligrams of Fe^{3+}-cytochrome *c* from equine heart (Sigma Aldrich) was dissolved in 500 µl 20 mM potassium phosphate (KH_2PO_4; Sigma Aldrich) pH 7.4 (assay buffer). To the Fe^{3+}-cytochrome *c* solution, a few grains of solid L-ascorbic acid (Sigma Aldrich) was added to reduce the Fe^{3+}-cytochrome *c* to Fe^{2+}-cytochrome *c*. The reaction was considered complete when the colour of cytochrome *c* was turned to a bright red colour. L-ascorbic acid was removed from the mixture by desalting the cytochrome *c* solution on a PD10 column (GE healthcare). The column was equilibrated with the assay buffer before loading Fe^{2+}-cytochrome *c*. The bright red fraction of the eluted Fe^{2+}-cytochrome *c* was collected and stored at -30^{o} C and was used within two weeks. The concentration of Fe^{2+}-cytochrome *c* was estimated by measuring the absorbance at 550 nm and the quality of Fe^{2+}-cytochrome *c* from the ratio of the absorbance at 550 nm and at 565 nm. In order to determine the Fe^{2+}-cytochrome *c* concentration the absorbance was set to zero by using the assay buffer and then a baseline measurement was recorded from 600 nm to 500 nm. The reduced Fe^{2+}-cytochrome *c* was diluted 200-fold with assay buffer and then the absorbance was recorded. In order to determine the quality of the reduced Fe^{2+}-cytochrome *c*, the ratio of Delta A_{550}/ Delta A_{565} (Delta A=Abs_{peak} - $Abs_{baseline}$) was calculated. If the ratio was <6 the Fe^{2+}-cytochrome *c* was not used due to oxidation. The above procedure was repeated in triplicate and the average absorbance was used to determine the concentration of the reduced Fe^{2+}-cytochrome *c* which was calculated based on equation 2.1:

$$Fe^{2+}\text{-cytochrome } c \text{ concentration (mM)} = \frac{\text{Delta Abs}_{550}}{29} * \frac{1000}{\text{sample volume}} \quad \textbf{(2. 1)}$$

where Delta Abs_{550} is the difference in absorbance between the peak and baseline of the trace at 550 nm, 29 is the extinction coefficient for reduced Fe^{2+}-cytochrome *c* (mM^{-1} cm^{-1}) at 550 nm and sample volume is in µl.

One millilitre plastic cuvettes were filled with an appropriate volume of assay buffer which had been placed in a 30^{o} C water bath to equilibrate. The following reaction reagents, 0.345 mM DDM (Sigma Aldrich), 15 µM Fe^{2+}-cytochrome *c* (kept on ice), were added, mixed gently and placed in the spectrophotometer. The absorbance was set to zero and baseline rate (non-enzymatic blank) was recorded for 30 to 50 seconds, then 20 µl of a brain homogenate was added (the brain homogenate was diluted 3-fold for the assay with CCA buffer), gently mixed and the decrease in absorbance recorded for 90 seconds in order to allow the measurement of the enzyme-catalyzed oxidation of Fe^{2+}-cytochrome *c*. At the end of the reaction a few grains of potassium ferricyanide (Fluka) were added to oxidize the remaining Fe^{2+}-cytochrome *c*, the sample was gently mixed and the absorbance change was recorded until it became a flat line which was used as an endpoint reading for the final activity calculation. Before analysing the brain samples a validation of the assay was performed daily by assaying mitochondria from pig heart muscle and only if the measured activity was in the determined range (73.22±12.36 K/sec) the analysis of the brain samples was performed. The final COX activity of each sample was taken as the average of three independent measurements.

In order to calculate the COX activity, 6 time points were chosen together with their respective absorbance values ($AbsT_0$-$AbsT_5$) while the curve was in the exponential phase along with the end point absorbance value (Figure 2.1A). The final COX activity was calculated based on equation 2.2:

$$\text{COX activity } (10^{-3}\ \text{K/sec}) = \left[\frac{\left(\log\frac{A}{X_5} - \log\frac{A}{X_1}\right)}{\text{time period}}\right] * 2.303 * \frac{1000}{\text{sample volume}} * \text{dilution factor} * 1000 \qquad \textbf{(2. 2)}$$

where A (absorbance change) = $AbsT_{end}$ -$AbsT_0$, X_1=$AbsT_{end}$ –$AbsT_1$, X_5=$AbsT_{end}$ –$AbsT_5$, time period (sec) is the time difference between $AbsT_1$ and $AbsT_5$, 2.303 is the conversion factor for log10 to natural log, sample volume is in µl and K is the first-order rate constant.

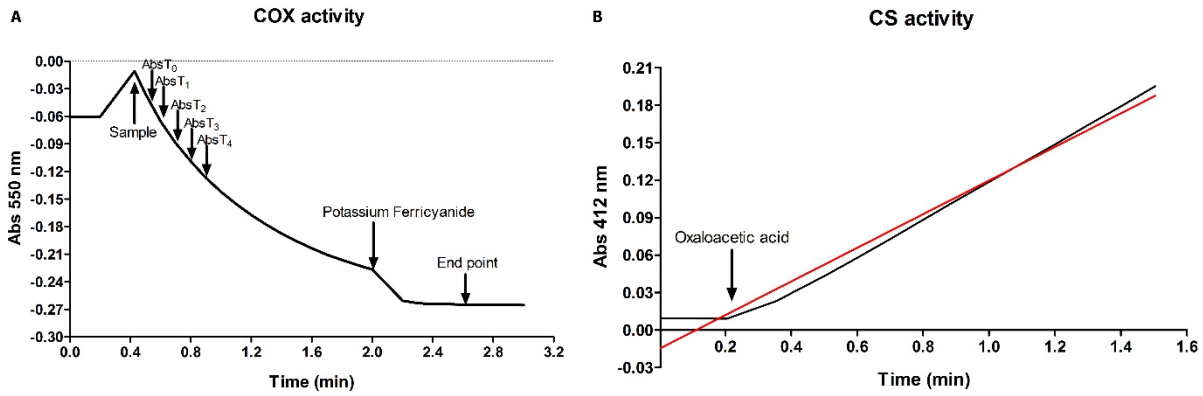


Figure 2.1 Representative graphs of COX and CS Activity.

(A) Graphical representation of COX activity which indicated the time points that can be used for the calculation of the activity. **(B)** Representative graph of CS activity where the slope has been fitted in order to determine the activity.

2.3.2 Citrate synthase (CS) activity assay

CS is the most commonly assayed mitochondrial matrix marker enzyme to assess mitochondrial content. The activity of CS is routinely used as a reference when expressing activities of respiratory chain complex enzymes, and the ratio of COX/CS is the preferred way of expressing COX activity data. The specific activity of mitochondrial CS is measured by following the CoASH released from acetyl-CoA during the enzymatic synthesis of citrate, which reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to yield the 5-thio-2-nitrobenzoate ion at 412 nm (extinction coefficient at 412 nm is E_{412}=13.6 mM^{-1} cm^{-1}).

The CS activity was determined by following the absorbance change at 412 nm. In 1 ml cuvettes 0.1 M TrisHCl pH 8 (assay buffer), 100 µM DTNB (10 mM DTNB stock solution was diluted in assay buffer; Sigma Aldrich), 1% Triton (Sigma Aldrich), 100 µM Acetyl-CoA (Sigma Aldrich) and 20 µl of brain homogenate were added (the brain homogenate was diluted 3-fold for the assay with CCA buffer). The contents of the cuvette were mixed gently and placed in the spectrophotometer. The absorbance was set to zero and a baseline rate was recorded for 10 to 50 seconds after which 250 µM of oxaloacetic acid (Sigma Aldrich; 1 M oxaloacetic acid was freshly diluted with 2 M $KHCO_3$ (Sigma Aldrich) and then a working solution of 50 mM oxaloacetate was prepared by diluting it with assay buffer) was added into the cuvette and gently mixed to start the reaction and record the slope (Abs/min) of the increase in absorbance over 1.5 min (Figure 2.1B). Similar to the COX assay a validation of the assay was performed daily before analysing the brain samples and only if the measured activity of the pig heart muscle was in the determined range (49.52±10.94 mmoles/min) were assays

conducted with the brain samples. CS activity was calculated (equation 2.3) as the slope (Abs/min) of the enzyme-catalysed formation of 5-thio-2-nitrobenzoate ion activity and expressed as nmols 5-thio-2-nitrobenzoate ion/min as follows:

$$\text{CS activity (nmols/min)} = \frac{\text{slope (Abs/min)}}{13.6} * \frac{1000}{\text{sample volume}} * \text{dilution factor} * 1000 \quad \textbf{(2. 3)}$$

where slope (Abs/min) = Abs_{T2} - Abs_{T1}/ $T_2 - T_1$, 13.6 is the is the extinction coefficient for DTNB in Tris buffer ($mM^{-1}\,cm^{-1}$) at 412 nm and sample volume is in µl.

2.4 Protein concentration determination with Bradford assay

Protein concentration within homogenized samples was determined by using Bradford's method[49] and a bovine serum albumin (BSA) standard curve. Standards of BSA (New England Biolabs) were prepared with concentrations of 1, 0.8, 0.6, 0.5, 0.4, 0.2, 0.1, and 0 mg/ml. Brain homogenates were diluted 1:10 v/v in water. Five microliters of BSA standards and 5µl of sample were loaded into a 96-well plate in triplicate. In each well, 250 µl of Bradford reagent (Sigma Aldrich) was added and incubated for 20 min at room temperature. The absorbance was measured at 595 nm on a plate reader (Synergy HT Reader). The sample concentration was determined based on the BSA standard curve.

2.5 Western blot analysis

Western blot analysis was performed by using one-dimensional SDS gel electrophoresis of proteins with NuPAGE Novex (Invitrogen, Thermo Scientific) pre-cast gels. Ten micrograms protein extract from brain homogenates were prepared containing 1×NuPAGE LDS sample buffer (Invitrogen) and 1×NuPAGE sample reducing agent (Invitrogen). Samples were vortexed and heated for 10 min at 70^{o} C, and then loaded into NovexNuPAGE 4-12% precast gels (Invitrogen) along with SeeBlue pre-stained standard (Invitrogen) molecular weight marker. The gels were electrophoresed in 1×NuPAGE MOPS running buffer. To the inner chamber of the electrophoresis tank 0.25% antioxidant (Invitrogen) was added and the samples electrophoresed for 20 min at 120 V and for 60 min at 160 V. Samples were transferred to nitrocellulose membranes with iBlot transfer stacks (Invitrogen) at 20 V for 8 min. Membranes were stained with Ponceau S (Sigma Aldrich) for 1 min and rinsed with MilliQ water. Membranes were cut in strips based on molecular weight of the protein of interest (Table 2.2) and washed twice for 10 min with 1×Tris buffered saline (TBS: 50 mM TrisHCl, pH 7.4 and 150 mM NaCl; Santa Cruz Biotechnology) containing 0.2% Tween 100 (TBS-T; Sigma

Aldrich). Membranes were blocked for 1 h in 5% non-fat dry milk in TBS-T at room temperature and then incubated for 1 h at room temperature (RT) or overnight (O/N) with the primary antibody. Primary antibodies were diluted (Table 2.2) in 5% non-fat dry milk in TBS-T for the incubations. Blots were given two 10 min washes followed with TBS-T and then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.3) for 30 min at RT.

Protein of interest	***Molecular Weight***	***Dilution***	***Incubation***	***Supplier***	***Secondary Antibody***
VDAC1	30/35 kDa	1:8,000	1 h RT	Abcam	Mouse IgG-HRP
COX1	35 kDa	1:12,000	1 h RT	Abcam	Mouse IgG-HRP
COX2	25 kDa	1:10,000	1 h RT	Abcam	Mouse IgG-HRP
sGCSβ1	65 kDa	1:5,000	1 h RT	Santa Cruz Biotechnology	Rabbit IgG-HRP
PKG1α	78 kDa	1:1,000	1 h RT	Cell Signalling	Rabbit IgG-HRP
p38 MAPK	40 kDa	1:2,000	1 h RT	Cell Signalling	Rabbit IgG-HRP
HIF1α[2]	92 kDa	1:500	O/N	Novus	Mouse IgG-HRP
GAPDH-HRP	36 kDa	1:1,000	1 h RT	Santa Cruz Biotechnology	

Table 2.2 Molecular Weight of the Analysed Proteins and Dilutions of the Primary Antibodies.

Secondary Antibodies	***Dilution***	***Supplier***
Horseradish peroxidase (HRP)–conjugated anti-	1:4,000	Abcam
Horseradish peroxidase (HRP)–conjugated anti-	1:4,000	Dako

Table 2.3 Dilutions of Secondary Antibodies.

Membranes were then washed with TBS-T for approximately 1 h and developed by using the enhanced chemiluminescence system Pierce ECL plus substrate (Thermo Scientific) according to the manufacturer's instructions. Substrate A and Substrate B were mixed in a 40:1 ratio and incubated with the membranes for 1 min and then the membranes were transferred to cassettes. Membranes were exposed to autoradiography film (Santa Cruz Biotechnology) to detect the chemiluminescent signal. For proteins with a molecular weight close to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which was used as a loading control, membranes were

[2] Samples used for HIF1a probing used 40 μg protein loading and gels were transferred for 10 min.

stripped of the initial antibodies by incubating them with 60 mM TrisHCl (Sigma Aldrich) pH 6.8, 2% SDS (Santa Cruz Biotechnology) and 0.6% mercaptoethanol (Sigma Aldrich) for 1 h at 50^{o} C. Membranes were then washed with TBS-T followed by blocking in 5% non-fat dry milk and incubation with the new antibody as described previously. The X-ray films of the western blots were scanned using a flatbed scanner and band intensity was analysed using ImageJ software[50]. The GAPDH band was used to normalise the results and the ratio for the protein of interest/GAPDH ratio was calculated for all samples

2.6 Statistical analysis

Statistical analysis was performed in GraphPad Prism 5, all values are presented as mean ± SD. The data were initially analysed with a D'Agostino and Pearson omnibus normality test and F-test for variances which showed that certain data sets were not normally distributed. Spearman's rank test was utilized to define correlations between the examined variables such as age at death, post mortem delay and activity/protein levels. A non-parametric t-test (Mann-Whitney U test) was used to analyse between two group variables (controls versus CO exposed cases) and one way ANOVA followed by Dunnett or Tukey post-test for more than two groups. In all cases p values less than 0.05 were considered as being statistically significant.

3. Results

Initially, 98 brain samples from normally frontal cortex and three CO cases were assayed to determine the COX and CS activity. Both assays were conducted on the same day, by using the same amount of protein to ensure comparability. In order to identify if COX or CS activities were affected by variables such as age at death, post mortem delay, pH or years of tissue storage, Spearman's rank correlation was used as the data failed to pass the D'Agostino & Pearson omnibus normality test. All the determined variables and the measured activities of COX and CS for the control cases are presented at Table 3.1 and for the CO cases in Table 3.2 where the results are arranged with increasing age at death of the case.

3.1 Effects of age, pH, post mortem delay, storage and gender in COX activity

Analysis revealed that the COX/CS activity ratio in the healthy brain did not present any significant correlation with the age at death (r_s=-0.136, p=0.180, n=98), post mortem delay (r_s=-0.018, p=0.861, n=98), pH of the tissue (r_s=0.120, p=0.240, n=98) or the years that the tissue was stored (r_s=-0.112, p=0.274, n=98; Figure 3.1 and Table 3.3). The results were also analysed relative to gender where no significant difference was identified for the male group although in the female group a small but significant negative correlation was shown between COX/CS and time that the brain was in storage (r_s=-0.322, p=0.019, n=98; Table 3.4-5).

Since the final COX/CS activity was not affected by post mortem delay, age at death or storage time, we investigated the enzyme activities independently when normalized to the amount of protein used for the assay. COX/µg protein was not significantly affected by post mortem delay (r_s=0.127, p=0.214, n=98; Figure 3.2). Age at death also did not affect COX/µg activity (r_s=0.033, p=0.747, n=98; Figure 3.2). A significant effect of time in storage was observed with increased storage time leading to decreased COX/µg activity (r_s=-0.271, p=0.007, n=98; Figure 3.2). The CS/µg protein activity showed a positive correlation with post mortem delay (r_s=0.182, p=0.074, n=98; Figure 3.2) and a significant increase in the CS/µg activity was seen with age at death (r_s=0.237, p=0.018, n=98; Figure 3.2). Storage time of the brain tissue also significantly decreased CS/µg activity over time (r_s=-0.273, p=0.007, n=98; Figure 3.2).

Age at death	*Post mortem delay (hrs)*	*pH units*	*Storage time (yrs)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3} K/sec/ µg)*	*CS/ µg protein (nmoles/min*	*COX/CS (10^{-3} $K.sec^{-1}$/*
21	30	6.66	34	2078	1458	59.48	41.73	1.42
46	24	6.75	23	2672	1692	67.00	42.43	1.57
47	15	6.54	21	1863	1544	61.46	50.92	1.20
50	7	6.93	33	1364	943	41.509	28.72	1.44
50	82	6.46	23	2576	1692	71.43	46.93	1.52
51	32	7.00	23	1962	1632	58.815	48.93	1.20
52	102	6.23	2	2677	1619	162.33	98.22	1.65
53	19	7.16	19	2933	1855	85.93	54.36	1.58
55	41	5.72	7	2034	1884	54.88	50.82	1.07
56	11	6.73	17	3120	1891	79.76	48.36	1.64
57	21	6.55	23	2916	2068	84.51	59.94	1.40
58	39	5.65	10	1391	1334	56.54	54.24	1.04
59	19	6.01	8	2201	1703	71.86	55.61	1.29
63	24	6.54	21	1695	1877	49.87	55.21	0.90
64	93	7.05	2	2436	1861	77.54	59.25	1.30
64	64	6.43	13	3170	1930	90.35	55.00	1.64
65	47	6.19	3	2585	1772	78.73	53.98	1.45
65	14	6.98	23	2495	1865	61.96	46.32	1.33
67	22	7.05	6	2454	1772	67.96	49.07	1.38

Age at death (years)	*Post mortem delay (hrs)*	*pH units*	*Storage time (yrs)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3} K/sec/ µg)*	*CS/ µg protein (nmoles/min*	*COX/CS (10^{-3} $K.sec^{-1}$/*
68	54	5.98	11	1810	1444	77.44	61.79	1.25
69	16	6.73	11	2882	1999	84.07	58.32	1.44
70	72	6.55	6	2624	1835	77.13	53.95	1.42
70	24	5.46	34	2706	2024	63.07	47.18	1.33
71	32	6.30	19	2593	2249	50.17	43.51	1.15
71	25	6.00	1	2529	1830	67.16	48.61	1.38
72	27	7.31	9	2557	1809	71.16	50.35	1.41
72	17	6.53	11	1910	1288	72.18	48.68	1.48
72	32	7.13	22	1899	1787	59.51	56.01	1.06
72	30	6.30	21	1570	2035	44.58	57.79	0.77
73	24	6.19	23	2639	1456	71.59	39.50	1.81
73	25	6.77	5	2932	2080	96.92	68.78	1.40
74	49	5.82	5	2604	1833	74.34	52.35	1.42
74	45	6.73	9	2816	1722	85.26	52.16	1.63
74	53	6.89	6	2989	1744	90.51	52.82	1.71
75	11	6.53	29	1991	1534	56.61	43.64	1.29
75	31	6.59	23	1968	1751	82.30	73.22	1.12
75	27	6.36	18	2432	1586	82.20	53.61	1.53
75	64	6.23	12	2254	1827	91.64	74.29	1.23
75	27	7.22	27	2938	1896	93.15	60.13	1.54

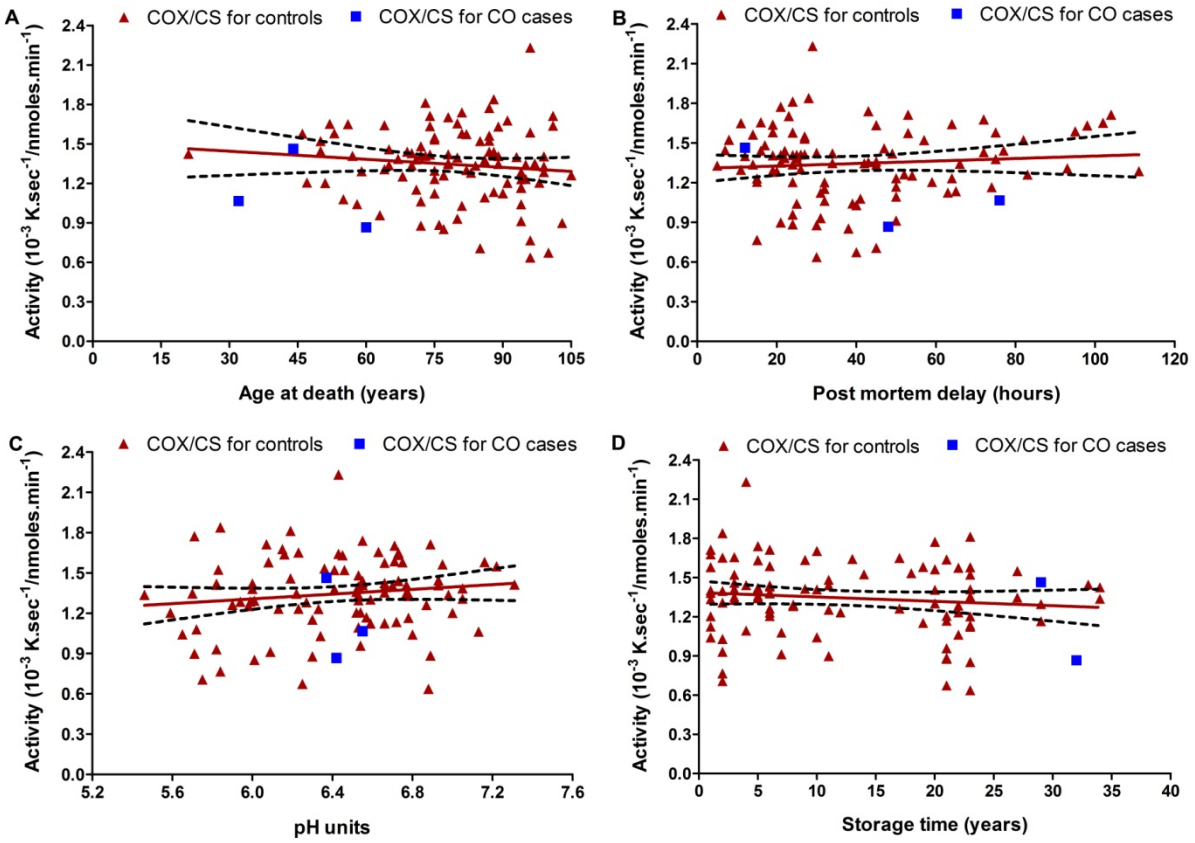
Age at death (years)	*Post mortem delay (hrs)*	*pH units*	*Storage time (yrs)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3} K/sec/ µg)*	*CS/ µg protein (nmoles/min*	*COX/CS (10^{-3} $K.sec^{-1}$/*
76	24	6.89	21	1819	2055	50.42	56.98	0.88
77	83	6.87	6	1529	1212	64.84	51.42	1.26
77	38	6.01	23	1637	1920	49.26	57.78	0.8
78	42	6.22	27	2280	1693	67.05	49.79	1.34
78	23	6.71	10	3319	1948	92.62	54.37	1.70
78	34	6.00	5	2873	2022	90.69	63.83	1.42
78	43	6.66	23	2699	1992	81.60	60.22	1.35
80	31	5.82	2	1658	1780	49.88	53.56	0.93
80	25	6.66	2	2141	1533	73.99	52.99	1.39
80	16	6.63	3	3338	2015	110.28	66.60	1.65
81	43	6.55	5	3373	1938	87.77	50.42	1.74
81	40	6.34	2	1865	1812	45.13	43.83	1.02
81	75	6.08	1	2597	1644	90.11	57.04	1.57
82	26	6.59	20	2381	1828	66.52	51.09	1.30
82	53	6.66	22	2891	1835	74.79	47.48	1.57
83	19	6.27	8	2030	1584	60.39	47.12	1.28
84	45	6.53	1	2187	1638	78.59	58.86	1.33
85	57	6.41	3	2270	1492	77.72	51.09	1.52
85	45	5.75	2	1407	1905	45.68	61.83	0.73
86	45	6.52	5	2615	1923	79.87	58.74	1.35

Age at death (years)	*Post mortem delay (hrs)*	*pH units*	*Storage time (yrs)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3} K/sec/ µg)*	*CS/ µg protein (nmoles/min*	*COX/CS (10^{-3} $K.sec^{-1}$/*
86	75	6.41	6	2194	1592	64.49	46.80	1.37
86	50	6.53	4	2003	2047	55.03	56.24	0.97
87	21	5.71	20	2802	1581.25	78.20	44.12	1.77
87	8	5.83	14	2928	1920	86.97	57.06	1.52
87	14	6.95	20	3257	2082	93.91	60.05	1.56
87	77	6.55	4	2237	1552	68.91	47.80	1.44
88	22	6.73	6	2693	1870	135.65	94.22	1.43
88	65	6.72	23	2060	1814	73.16	64.44	1.13
88	28	5.84	2	3490	1898	99.25	53.98	1.83
88	26	5.82	3	2496	1768	69.66	49.35	1.41
89	98	6.45	5	2645	1620	99.55	61.00	1.63
89	24	6.93	10	3014	2139	87.01	61.76	1.40
89	34	5.7	5	2619	1944	79.21	58.81	1.34
90	63	6.66	1	1843	1639	65.99	58.68	1.12
91	72	6.15	1	2584	1540	103.12	61.44	1.67
91	24	5.59	1	2106	1749	70.73	58.74	1.20
93	12	6.53	1	2803	2000	85.46	60.99	1.40
94	25	6.8	1	1371	1317	52.91	50.82	1.04
94	74	6.78	29	2093	1796	54.99	47.20	1.16
94	50	6.09	7	1329	1455	46.08	50.48	0.91

Age at death (years)	*Post mortem delay (hrs)*	*pH units*	*Storage time (yrs)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3} K/sec/ µg)*	*CS/ µg protein (nmoles/min*	*COX/CS (10^{-3} $K.sec^{-1}$/*
94	20	5.94	22	2330	1813	61.67	47.99	1.28
94	15	6.33	6	2250	1827	69.52	56.46	1.23
94	50	6.57	21	2176	1861	62.07	53.07	1.16
95	50	6.13	22	1968	1595	57.70	46.78	1.23
95	66	6.78	5	2622	1954	86.83	64.70	1.34
96	29	6.43	4	3186	1427	86.10	38.57	2.23
96	30	6.88	23	1282	2013	40.74	63.95	0.63
96	95	6.71	6	2413	1519	85.51	53.85	1.58
96	16	5.84	2	1255	1884	41.00	61.55	0.66
97	21	6.77	3	2604	1927	71.26	52.76	1.35
98	59	6.52	6	2366	1963	62.19	51.59	1.20
99	5	6.78	3	2247	1691	70.47	53.04	1.32
99	19	6.7	20	2758	1962	78.29	55.70	1.40
99	111	5.99	8	1893	1471	82.15	63.84	1.28
100	40	6.25	21	1254	1852	41.59	61.43	0.67
101	19	6.16	21	2678	1634	72.36	44.17	1.63
101	104	6.07	1	2568	1498	163.27	95.28	1.71
103	21	5.71	11	1641	1825	57.06	63.47	0.89
105	52	5.90	17	2224	1762	76.63	60.71	1.26

Age at death (years)	***Post mortem delay (hrs)***	***pH units***	***Storage time (yrs)***	***COX activity (10^{-3} K/sec)***	***CS activity (nmoles/min)***	***COX/µg protein (10^{-3} K/sec/ µg)***	***CS/ µg protein (nmoles/min µg)***	***COX/CS (10^{-3} K.sec^{-1}/ nmoles.min^{-1})***
32	76	6.55	NA	1844	1730	48.77	45.75	1.06
44	12	6.37	29	3301	2254	96.43	65.85	1.46
60	48	6.42	32	1899	2189	58.28	67.20	0.86

Table 3.1 COX and CS activity in the Frontal Cortex of CO exposed cases.



By examining changes between the two genders we identified that neither post mortem delay nor age at death caused any significant effect on the COX/µg protein in both genders, whereas storage time of female brain samples presented a statistical significant negative correlation (r_s=-0.379, p=0.005, n=98; Table 3.4-5). CS/µg protein did not show any change in the female brain, whereas in the male brain a significant CS/µg protein increase with increasing post mortem delay (r_s=0.453, p=0.002, n=98; Table 3.4-5) and age at death (r_s=0.297, p=0.047, n=98; Table 3.4-5) was identified. Time in storage for the male group showed a correlation with decreasing CS/µg activity with increasing time (r_s=-0.436, p=0.003, n=98; Table 3.4-5).

Figure 3.1 Effects of Age, Post Mortem delay, pH and Storage Time in the COX/CS Activity in the Healthy and CO exposed Frontal cortex.

The measures COX/CS activity were examined with Spearman rank test in order to identify correlations with **(A)** age at death (r_s=-0.136, p=0.180, n=98), **(B)** post mortem delay(r_s=-0.018, p=0.861, n=98), **(C)** pH units (r_s=0.120, p=0.240, n=98) and **(D)** storage time (r_s=-0.112, p=0.274, n=98). The presence of CO exposed cases (blue squares) in the range of the controls is also indicated. Spearman r_s correlation for n=98 samples is reported and p values less than 0.05 were considered statistically significant. For each studied correlation the 95% confidence interval of the linear regression is also presented.

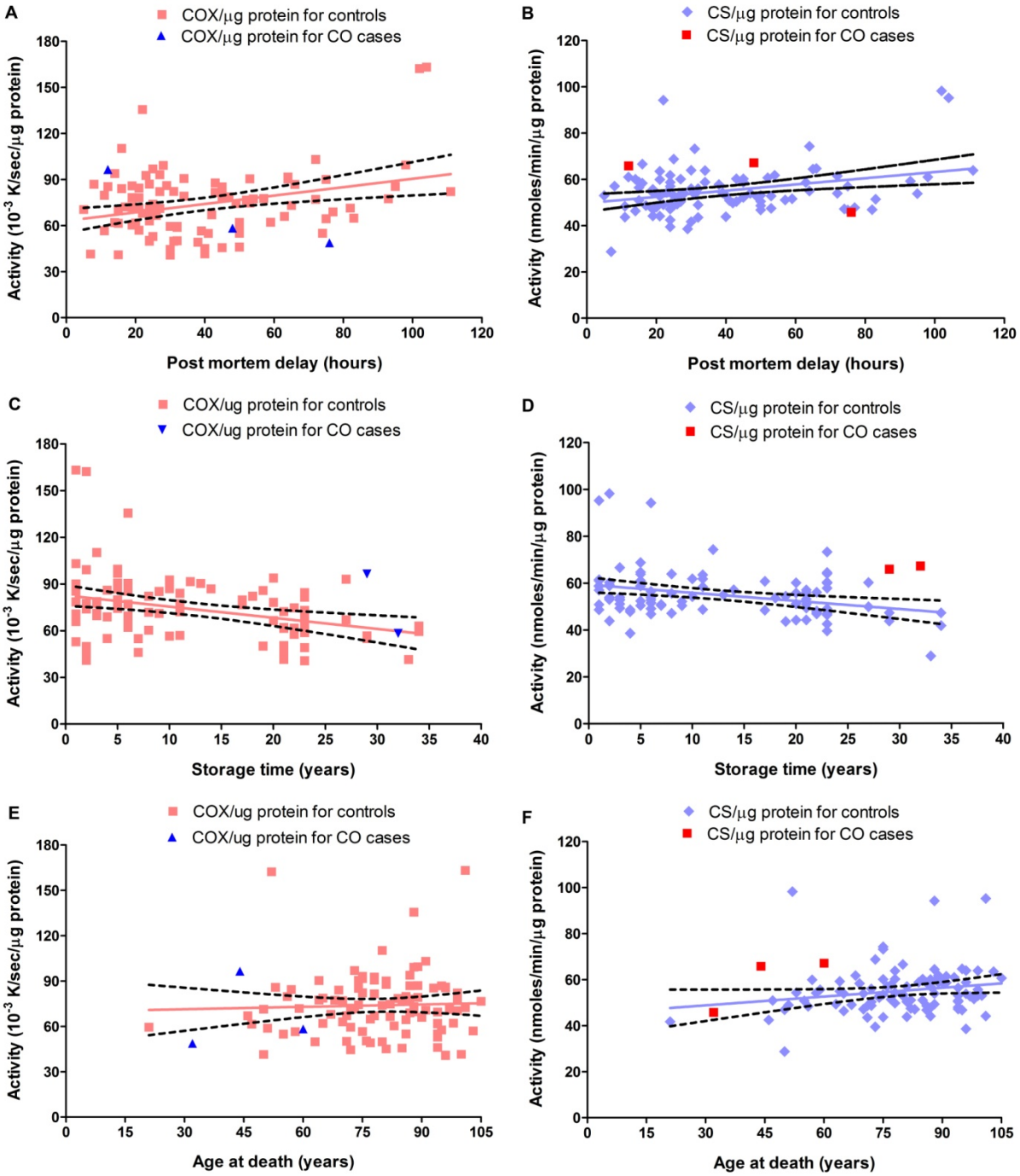


Figure 3.2 Effects of Different Variables in COX/µg and CS/µg activity.

The measured activities of COX and CS normalised with amount of protein used for the assay and correlated with **(A, B)** post mortem delay (COX/µg: r_s=0.127, p=0.214, n=98; CS/µg: r_s=0.182, p=0.074, n=98), **(C, D)** storage time (COX/µg: r_s=-0.271, p=0.007, n=98; CS/µg: r_s=-0.273, p=0.007, n=98) and **(E, F)** age at death (COX/µg: r_s=0.033, p=0.747, n=98; CS/µg: r_s=0.237, p=0.018, n=98) in order to identify significant effects. The presence of CO exposed cases (blue squares) in the range of the controls is also indicated. Spearman r_s correlation for n=98 samples is reported and p values less than 0.05 were considered statistically significant. For each studied correlation the 95% confidence interval of the linear regression is presented.

	pH units	*Storage time (years)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3}*	*CS/ µg protein (nmoles/min/µg)*	*COX/CS (10^{-3} K.sec^{-1}/ nmoles.min^{-1})*
Age at death (years)	r_s=-0.189, p=0.063, n=98, NS		r_s=-0.083, p=0.417, n=98,NS	r_s=0.010, p=0.922, n=98, NS	r_s=0.033, p=0.747, n=98, NS	r_s=-0.233, p=0.018, n=98, significant negative	r_s=-0.136, p=0.180, n=98, NS
Post mortem delay (hours)	r_s=-0.118, p=0.247, n=98, NS		r_s=-0.121, p=0.236, n=98, NS	r_s=-0.216, p=0.033, n=98 significant negative	r_s=0.127, p=0.214, n=98, NS	r_s=0.182, p=0.074, n=98, NS	r_s=-0.018, p=0.861, n=98, NS
pH units		r_s=0.221, p=0.029, n=98, significant positive	r_s=0.158, p=0.120, n=98, NS	r_s=0.128, p=0.208, n=98, NS	r_s=0.102, p=0.316, n=98, NS	r_s=-0.038, p=0.712, n=98, NS	r_s=0.120, p=0.240, n=98, NS
Storage time (years)			r_s=-0.069, p=0.500, n=98, NS	r_s=0.072, p=0.480, n=98, NS	r_s=-0.271, p=0.007, n=98, significant negative	r_s=-0.273, p=0.007, n=98, significant negative	r_s=-0.112, p=0.274, n=98, NS

Table 3.2 Effects of Ageing, Post Mortem delay, pH units and Storage Time in the Mitochondrial COX and CS activities in control brain.

In order to identify the effects of the different variables in the COX, CS and COX/CS activity, Spearman rank test was used for the 98 control cases and p values less than 0.05 are considered statistically significant. NS: non-significant

Female group	*pH units*	*Storage time (years)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3} K/sec/ µg)*	*CS/ µg protein (nmoles/min/µg)*	*COX/CS (10^{-3} K.sec^{-1}/ nmoles.min^{-1})*
Age at death (years)	r_s=0.016, p=0.911, n=53, NS		r_s=-0.016, p=0.908, n=53, NS	r_s=-0.034, p=0.808, n=53, NS	r_s=0.032, p=0.821, n=53, NS	r_s=0.158, p=0.257, n=53, significant negative	r_s=-0.029, p=0.837, n=53, NS
Post mortem delay (hours)	r_s=-0.116, p=0.410, n=53, NS		r_s=-0.141, p=0.314, n=53, NS	r_s=-0.417, p=0.002, n=53, significant negative	r_s=0.039, p=0.784, n=53, NS	r_s=-0.099, p=0.480, n=53, NS	r_s=0.136, p=0.330, n=53, NS
pH units		r_s=0.105, p=0.455, n=53, NS	r_s=0.231, p=0.096, n=53, NS	r_s=0.218, p=0.116, n=53, NS	r_s=0.193, p=0.167, n=53, NS	r_s=0.043, p=0.759, n=53, NS	r_s=0.157, p=0.261, n=53, NS
Storage time (years)			r_s=-0.189, p=0.174, n=53, NS	r_s=0.301, p=0.028, n=53 significant positive	r_s=-0.376, p=0.005, n=53 significant negative	r_s=-0.077, p=0.584, n=53, NS	r_s=-0.322, p=0.028, n=53 significant negative

Table 3.3 Effects of Ageing, Post Mortem delay, pH units and Storage Time on Mitochondrial COX and CS Activities in control female brain.

In order to identify the effects of the different variables in the COX, CS and COX/CS activity, Spearman rank test was used for the 53 healthy female cases and p values less than 0.05 are considered statistically significant. NS: non-significant

Male group	*pH units*	*Storage time (years)*	*COX activity (10^{-3} K/sec)*	*CS activity (nmoles/min)*	*COX/µg protein (10^{-3} K/sec/ µg)*	*CS/ µg protein (nmoles/min µg)*	*COX/CS (10^{-3} K.sec^{-1}/ nmoles.min^{-1})*
Age at death (years)	r_s=-0.495, p=0.001, n=45, significant negative		r_s=-0.138, p=0.367, n=45, NS	r_s=0.007, p=0.965, n=45, NS	r_s=0.056, p=0.715, n=45, NS	r_s=0.297, p=0.047, n=45, significant positive	r_s=-0.178, p=0.239, n=45, NS
Post mortem delay (hours)	r_s=-0.110, p=0.472, n=45, NS		r_s=-0.080, p=0.600, n=45, NS	r_s=-0.045, p=0.767, n=45, NS	r_s=0.223, p=0.141, n=45, NS	r_s=0.453, p=0.002, n=45, significant positive	r_s=-0.156, p=0.307, n=45, NS
pH units		r_s=0.334, p=0.025, n=45, significant positive	r_s=0.109, p=0.475, n=45, NS	r_s=0.037, p=0.811, n=45,NS	r_s=0.033, p=0.830, n=45, NS	r_s=-0.107, p=0.485, n=45,NS	r_s=0.088, p=0.566, n=45, NS
Storage time			r_s=0.028, p=0.853, n=45, NS	r_s=-0.106, p=0.489, n=45, NS	r_s=-0.174, p=0.253, n=45. NS	r_s=-0.436, p=0.003, n=45, significant negative	r_s=0.086, p=0.575, n=45, NS

Table 3.4 Effects of Ageing, Post Mortem delay, pH units and Storage Time in the Mitochondrial COX and CS Activities in the control male brain.

In order to identify the effects of the different variables in the COX, CS and COX/CS activity. Spearman rank test was used for the 45 healthy male cases and p values less than 0.05 are considered statistically significant. NS: non-significant.

The activity of the three CO exposed cases was determined and compared with the healthy controls (Figure 3.3). No statistically significant difference was observed in the COX/CS activity (non-parametric t-test, p=0.25), or in the independent COX/µg (non-parametric t-test, p=0.582) and CS/µg (non-parametric t-test, p=0.362: Figure 3.3) activities. To determine how the activity of the CO cases was distributed when compared with variables such as age at death, post mortem delay, pH or time of storage, the measured activities where plotted together with the controls (Figure 3.1-2). Post mortem delay, storage time and pH of the CO cases did not seem to have any impact in the activity as measured activity is in the same range as control cases with the same characteristics. However, when looking at the effect of the age at death, we can see that potentially the COX/CS activity of the CO cases is slightly lower compared to aged matched controls, but not significantly (Figure 3.1A).

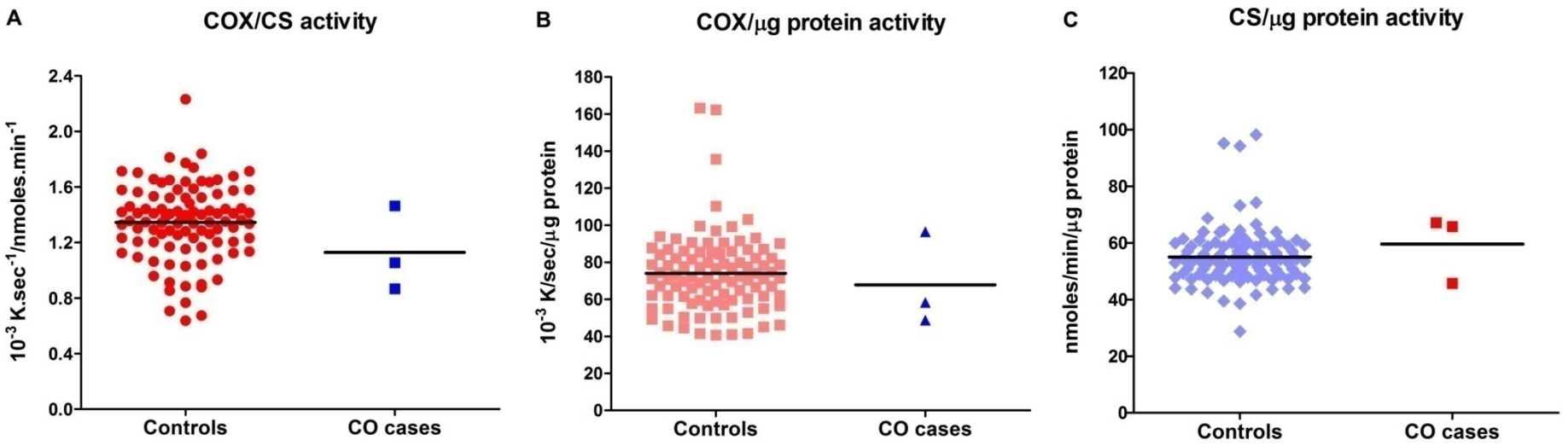


Figure 3.3 Comparison of the Mitochondrial Enzymes Activities in the Healthy and CO Exposed Frontal cortex.

The measured **(A)** COX/µg protein and **(B)** CS/µg protein activity as well as the **(C)** COX/CS activity in the brain of the healthy controls compared to CO poisoned brain. Non-parametric t-test was used and no statistical significant change was observed.

3.2 Stability of COX in brain tissue.

To ensure the measured activity in the CO exposed cases was stable and repeatable due to the low number of cases, the samples were assayed twice at different time points. By examining the standard operating protocol we identified that the only potential difference was the time between homogenization and assay. In order to further examine if the activity of the CO and control cases was stable over a specific time period we measured COX activity at 1, 2, 3 and 4 hours after the samples had been thawed with storage on ice. The analysis showed that, over a period of 4 hours, COX activity was relatively stable with a non-specific pattern of increase/decrease over that time period (Figure 3.4).

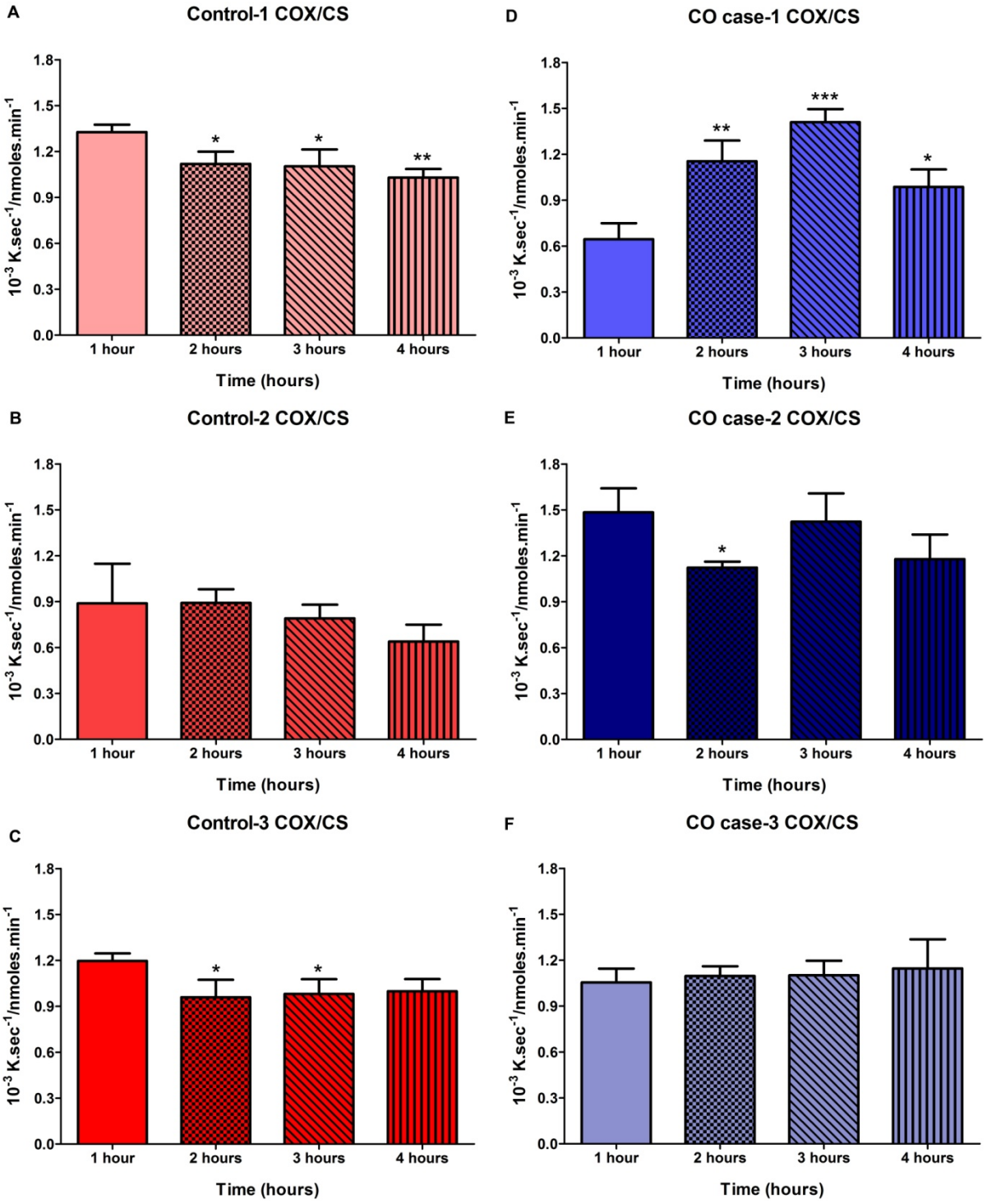


Figure 3.4 Comparison of COX/CS activity in Controls and CO Cases over a Period of 4 hours.

The COX/CS activity as determined over a period of 4 hours in **(A)** Control-1, **(B)** Control-2, **(C)** Control-3, **(D)** CO case-1, **(E)** CO case-2 and **(F)** CO case-3. Data were analysed with one way ANOVA followed by Dunnett post-test and p values less than 0.05 are considered statistically significant. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$.

3.3 Changes in protein levels in the Control and CO exposed brain

In order to determine if the protein levels of the two core subunits of COX, COX1 and COX2, mitochondria levels via VDAC1 as well as proteins that are implicated in intracellular CO signalling pathways such as sGCSβ1, p38MAPK, PKG1α and HIF1α, were altered by CO exposure, we conducted Western blot analysis. Due to the limitations of sample loading on protein gels and since no direct comparison can be performed amongst different gels, we selected 22 control cases which were representative of the entire age range (46-105 years), pH, post mortem delay, storage time and the measured COX/CS activity. Levels of the protein of interest were measured in the CO exposed samples and compared with the healthy controls. Levels of sGCSβ1 did not change following CO exposure compared to the full age range controls (non-parametric t-test, p=0.900; Figure 3.5B). Levels of PKG1α, a downstream target of sGCSβ1, were decreased slightly but not significantly by 18% in the CO exposed samples (non-parametric t-test, p=0.194; Figure 3.6B). The protein levels of COX1, COX2 and VDAC1 were not significantly altered by CO exposure compared to the full range controls (non-parametric t-test, p>0.05; Figure 3.6C-E). A small decrease (11%) in the protein levels of p38 (another target of the CO signalling pathway) were observed in the frontal cortex, compared to healthy full range controls, although this was not significant (non-parametric t-test, p=0.645; Figure 3.6C). The protein levels of HIF1α were also measured and showed a non-significant 10% decrease (non-parametric t-test, p=0.769; Figure 3.7B).

Consequently, the measured protein levels of the full range healthy controls and CO cases proteins were subjected to further analysis in order to identify significant correlation between their levels and other factors such as age at death, pH or post mortem delay. No significant correlation was observed between age at death of the full range control cases and the protein levels of COX1 (r_s=-0.348, p=0.112, n=22), COX2 (r_s=-0.207, p=0.354, n=22) and VDAC1 (r_s=-0.128, p=0.569, n=23; Figure 3.8 and Table 3.6). However, with age at death, sGCSβ1 (r_s=-0.496, p=0.018, n=22; Figure 3.9A) and HIF1α (r_s=-0.646 p=0.0011, n=22; Figure 3.9D), decreased with age at death, whereas the protein levels of p38 increased with age at death (r_s=0.862, p<0.0001, n=22; Figure 3.9C). The levels of PKG1α showed no significant change with age at death (r_s=0.182, p=0.416, n=22; Figure 3.9B and Table 3.6).

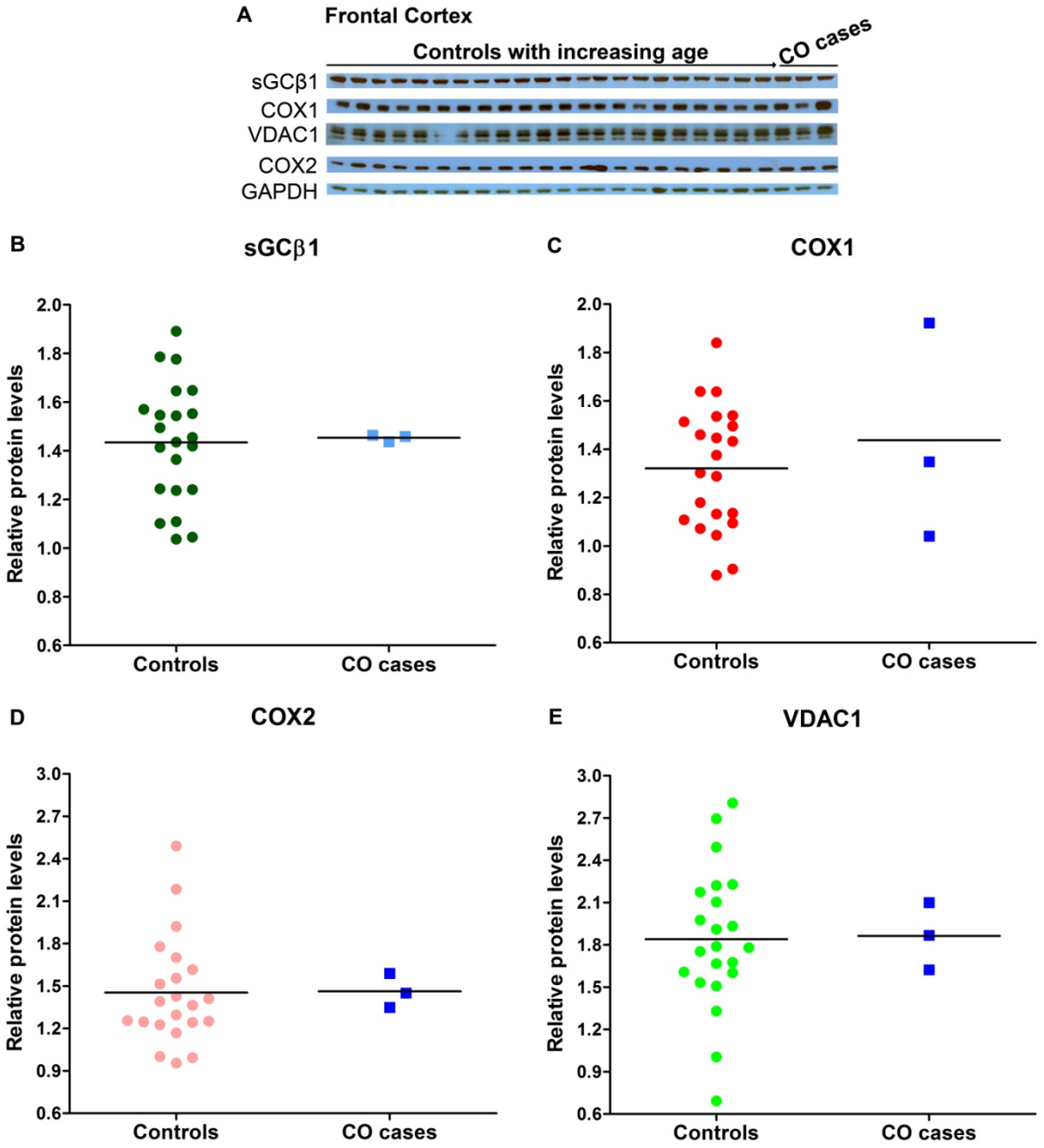


Figure 3.5 Protein levels of sGCβ1, COX subunits and VDAC (Mitochondria) levels in the Frontal cortex of Control and CO Exposed Cases.

The protein levels of sGCSβ1 (sGCβ1), COX1, VDAC1 and COX2 were measured by **(A)** Western blot analysis which was followed by densitometric analysis of **(B)** sGCSβ1, **(C)** COX1, **(D)** COX2 and VDAC1 normalised with GAPDH.

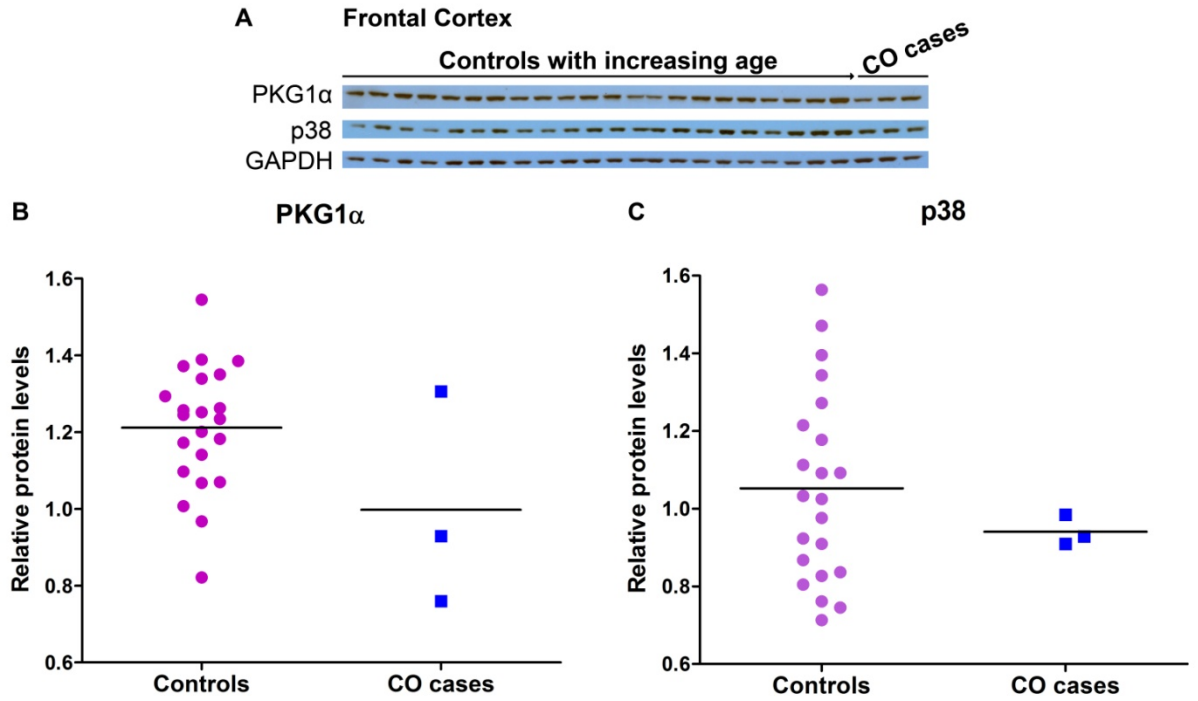


Figure 3.6 Protein Levels of PKG1α and p38 in the Frontal cortex of Control and CO Exposed Cases.

The protein levels of PKG1α and p38 were measured by **(A)** Western blot analysis which was followed by densitometric analysis of **(B)** PKG1α and **(C)** p38 normalised with GAPDH.

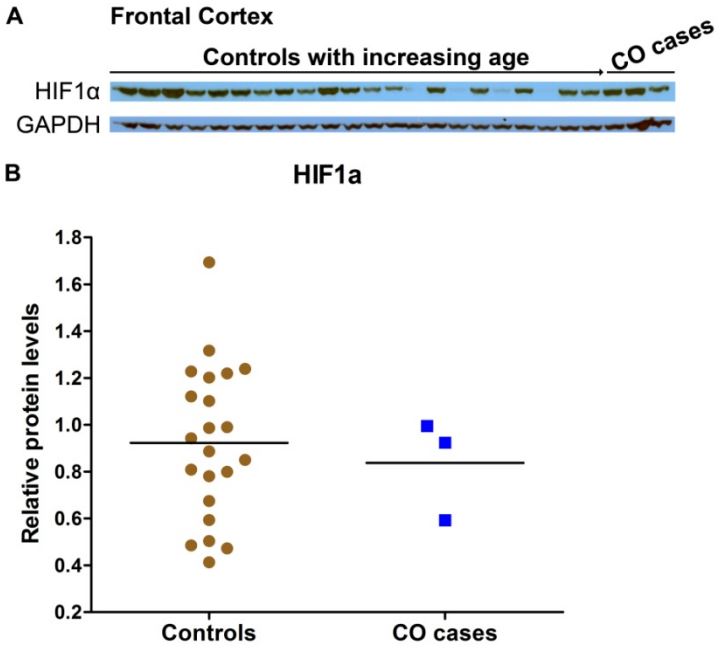


Figure 3.7 Protein Levels of HIF1α in the Frontal cortex of Healthy Controls and CO Cases.

The protein levels of HIF1α were measured by **(A)** Western blot analysis which was followed by densitometric analysis of **(B)** HIF1α normalised with GAPDH. A non-parametric t-test was used to identify changes where no statistical significant change was observed.

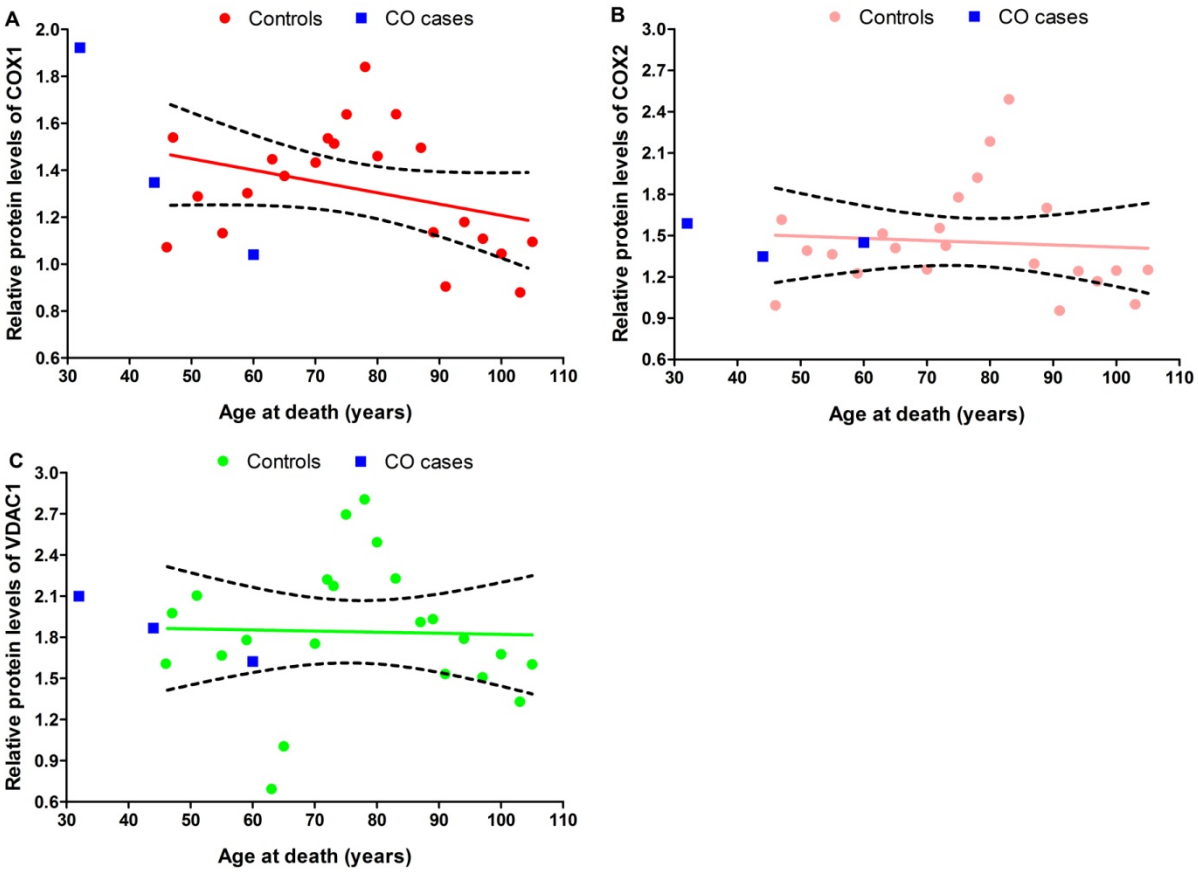


Figure 3.8 Graphical representation of Correlations between COX subunits, VDAC1 and Age at death in the Control Frontal Cortex.

Age at death of the control cases was correlated with the protein levels of **(A)** COX1 (r_s=-0.348, p=0.112, n=22), **(B)** COX2 (r_s=-0.207, p=0.354, n=22), **(C)** VDAC1 (r_s=-0.128, p=0.569, n=2). The presence of CO exposed cases (blue squares) is also indicated. Spearman r_s correlation for n=22 samples is reported and p values less than 0.05 were considered as statistically significant. For each correlation the 95% confidence interval of the linear regression is presented.

Protein levels of COX1, COX2 and VDAC1 of the CO exposed cases were within in the same range as their full range controls (Figure 3.8A-C). Protein levels of sGCSβ1, PKG1α and HIF1α of the CO cases were in the lower range of full range control cases (Figure 3.9A, B and D). The protein levels of p38 in the CO exposed brain appear to be slightly higher compared to full range controls (Figure 3.9C). No significant correlation was observed between post mortem delay or pH and the levels of the studied proteins (Table 3.6).

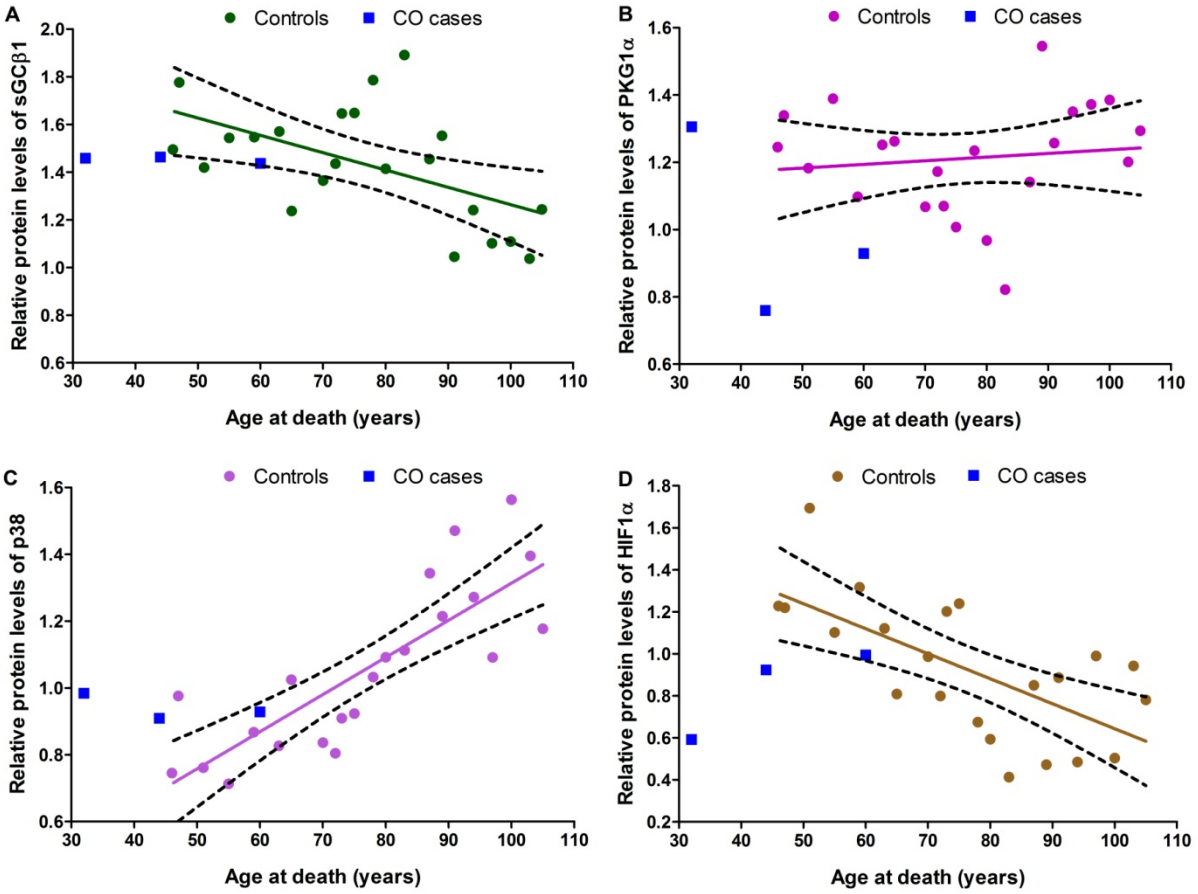


Figure 3.9 Graphical representation of Correlations between the Protein Levels of sGCβ1, PKG1α, p38 and HIF1α with the Age at Death in the Control Frontal cortex.

The age at death of control brain tissue was correlated with the protein levels of **(A)** sGCβ1 (r_s=-0.496, p=0.018, n=22), **(B)** PKG1α (r_s=0.182, p=0.416, n=22), **(C)** p38 (r_s=0.862, p<0.0001, n=22) and **(D)** HIF1α (r_s=-0.646, p=0.0011, n=22). The presence of CO exposed cases (blue squares) is also indicated. Spearman r_s correlation for n=22 samples is reported and p values less than 0.05 were considered statistically significant. For each correlation the 95% confidence interval of the linear regression is presented.

The protein levels of the COX subunits and mitochondrial VDAC levels were also correlated with the measured COX activity. COX1 protein levels only showed a non-significant positive trend with COX/CS activity (r_s=0.200, p=0.371, n=22; Figure 3.10A) with no significant correlation for COX2 (r_s=-0.003, p=0.986, n=22; Figure 3.10B). A significant positive correlation was identified between the protein levels of VDAC1/mitochondria with both COX1 (r_s=0.730, p=0.0001, n=22; Figure 3.10C) and COX2 (r_s=0.704, p=0.0003, n=22; Figure 3.10D). The protein levels of the two COX subunits also showed a positive correlation (r_s=0.821, p<0.0001, n=22; Figure 3.10E) possibly indicating the assembly process of the complex. The VDAC1 levels showed a non-significant positive trend with COX/CS activity (r_s=0.172, p=0.443, n=22) which implies that increased levels of mitochondria will result in

higher COX/CS activity (Table 3.6). Table 3.6 presents a detailed list of the correlations which were studied for proteins, activity and other variables such as post mortem delay or pH.

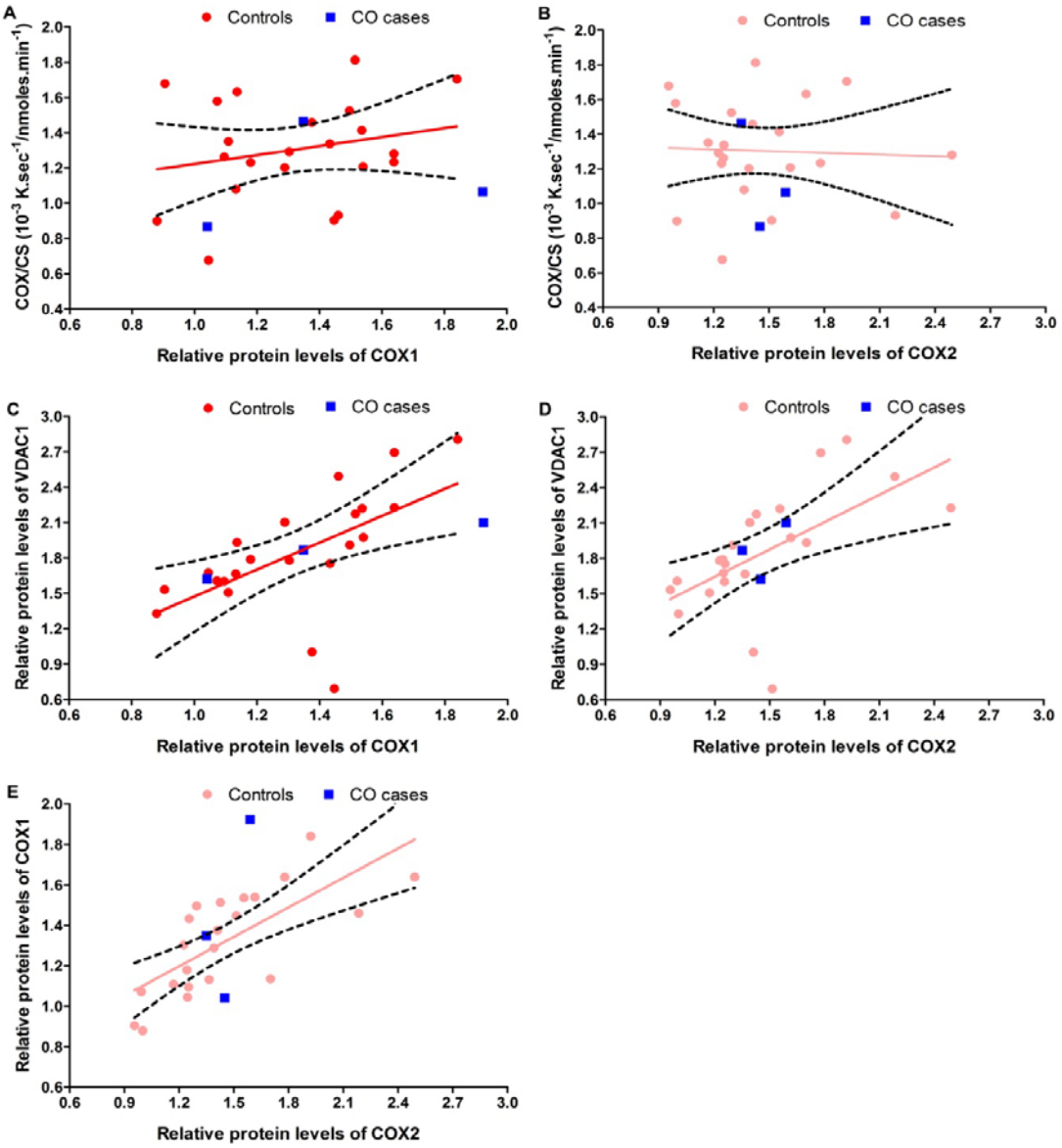


Figure 3.10 Graphical representation of Correlations between COX or Mitochondrial proteins and Activity in Control Frontal Cortex.

The measured COX/CS activity was correlated with **(A)** COX1 (r_s=0.200, p=0.371, n=22) and **(B)** COX2 (r_s=-0.003, p=0.986, n=22) protein levels. The mitochondria levels as indicated by VDAC1 were positively correlated with **(C)** COX1 levels (r_s=0.730, p=0.0002, n=22) and **(D)** COX2 (r_s=0.704, p=0.0003, n=22) protein levels. (E) COX1 and COX2 protein levels also presented a significant positive correlation (r_s=0.821, p<0.0001, n=22). The presence of CO exposed cases (blue squares) is also indicated. Spearman r_s correlation for n=22 samples is reported and p values less than 0.05 were considered statistically significant. For each correlation the 95% confidence interval of the linear regression is presented.

	sGCSβ1 protein levels	*COX1 protein levels*	*COX2 protein levels*	*VDAC1 protein levels*	*p38 protein levels*	*PKG1α protein levels*	*HIF1α protein levels*
Age at death (years)	r_s=-0.494, p=0.018, n=22, significant negative	r_s=-0.384, p=0.121, n=22, NS	r_s=-0.207, p=0.354, n=22, NS	r_s=-0.128, p=0.569, n=22, NS	r_s=0.862 p<0.0001, n=22, significant positive	r_s=0.182, p=0.416, n=22, NS	r_s=-0.646, p=0.001, n=22, NS
Post mortem delay (hours)	r_s=-0.188, p=0.401, n=22, NS	r_s=-0.299, p=0.176, n=22, NS	r_s=0.078, p=0.729, n=22, NS	r_s=-0.099, p=0.658, n=22, NS	r_s=0.035, p=0.874, n=22, NS	r_s=0.233, p=0.295, n=22, NS	r_s=-0.062, p=0.78, n=22, NS
pH units	r_s=0.265, p=0.233, n=22, NS	r_s=0.206, p=0.357, n=22, NS	r_s=0.184, p=0.411, n=22, NS	r_s=0.201, p=0.369, n=22, NS	r_s=-0.269, p=0.225, n=22, NS	r_s=0.2198, p=0.325, n=22, NS	r_s=0.076, p=0.735, n=22, NS
COX/μg protein		r_s=0.130, p=0.562, n=22, NS	r_s=-0.018, p=0.934, n=22, NS	r_s=0.144, p=0.522, n=23, NS			
COX/CS		r_s=0.200, p=0.371, n=22, NS	r_s=-0.003, p=0.986, n=22, NS	r_s=0.172, p=0.443, n=22, NS			
COX1 protein levels			r_s=0.821, p<0.0001, n=22, significant positive	r_s=0.770, p=0.0001, n=22, significant positive			
COX2 protein levels				r_s=0.704, p=0.0003, n=22, significant			

Table 3.5 Correlations between Protein, Activity, Age at Death, Post Mortem delay and pH in Control Frontal Cortex.

In order to identify the effects of the different variables in the protein levels of sGCSβ1, COX1, COX2, VDAC1, PKG1α, p38 and HIF1α as well as with the measured COX/CS activity, Spearman rank test was used for the 22 control cases and p values less than 0.05 were considered statistically significant. NS: non-significant.

3.4 Determination of protein changes in CO Exposed Cases and Age Matched Normal Cases

Changes in protein levels were apparent with age at death of the individual in normal brain with an increase observed of p38MAPK, a slight increase in PGKα and a decrease in sGCSβ and HIF1α seen. It was therefore necessary to analyse the CO exposed cases with a more restricted age at death range of normal controls to determine if CO exposure led to protein changes which may have been masked by the effects of age at death. We therefore used a group of 8 normal brain tissue samples and compared these with CO exposed cases. Determination of COX activity in CO exposed cases and comparison to the restricted age at death matched group showed no significant changes, with a slight reduction in COX/CS activity in CO exposed cases and no major change in COX/ug protein activity or CS/ug protein activity (Fig 3.11)

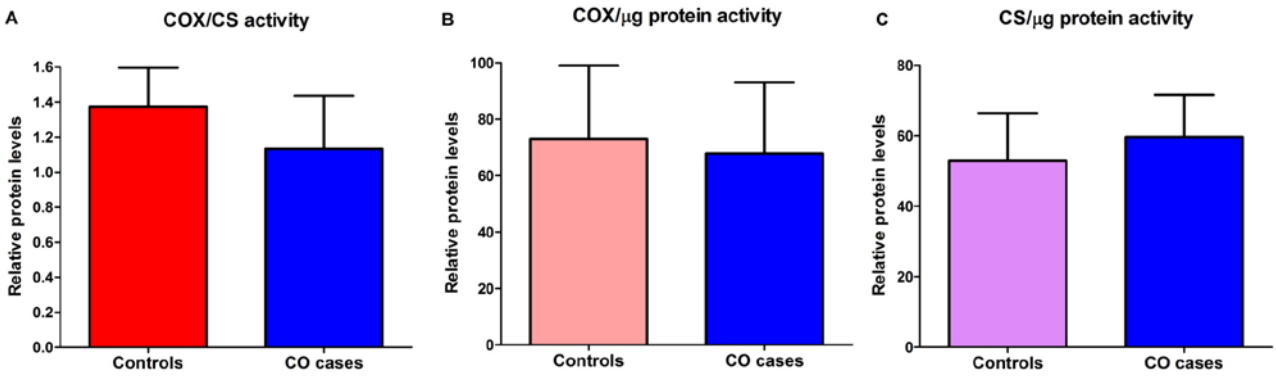


Figure 3.11 Comparison of the Mitochondrial Enzymes Activities in Healthy Aged Matched Controls and CO Exposed Cases.

The measured **(A)** COX/CS activity as well as the independent **(B)** COX/µg protein activity and the **(C)** CS/µg protein activity in the brain of the aged matched healthy controls (n=8) compared to CO exposed brain (n=3). Non-parametric t-test was used and no statistical significant change was observed.

Using western blotting, determination of the levels of COX protein subunits (Figure 3.12) showed no significant change in the levels of COX1 or COX2 subunit when compared to age at death matched normal controls and similarly no change in mitochondrial protein mass assessed by VDAC1 protein levels. Levels of sGCSβ1 in CO exposed frontal cortex were reduced compared to age at death matched controls ($P<0.05$).

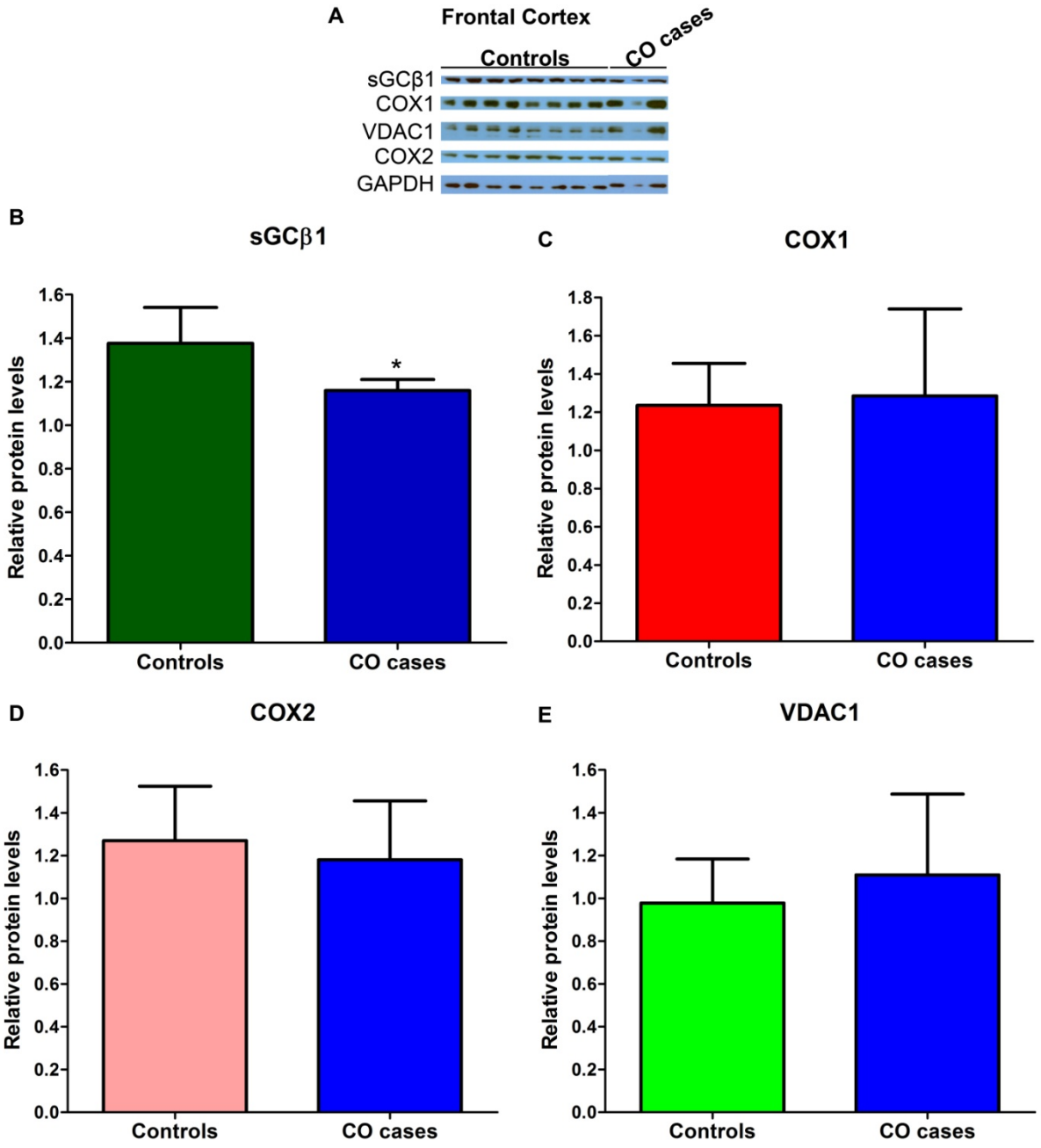


Figure 3.12 Protein levels of sGCSβ1, COX subunits and Mitochondria levels in the Frontal cortex of Aged Matched Healthy Controls and CO Exposed cases.

The protein levels of sGCSβ1 (sGCβ1), COX1, VDAC1 and COX2 were measured by **(A)** Western blot analysis which was followed by densitometric analysis of **(B)** sGCSβ1, **(C)** COX1, **(D)** COX2 and VDAC1 normalised with GAPDH. A non-parametric t-test was used to identify changes where p values less than 0.05 are considered as significant. *, $p<0.05$.

Levels of PKG1α in frontal cortex from CO exposed cases showed no significant changes when compared to age at death matched control cases (Figure 3.13). Using western blotting, levels of p38MAPK were however elevated in frontal cortex brain tissue from CO exposed cases compared to controls (P<0.05; Figure 3.13). Levels of HIF1α were unchanged in CO exposed cases (Figure 3.14).

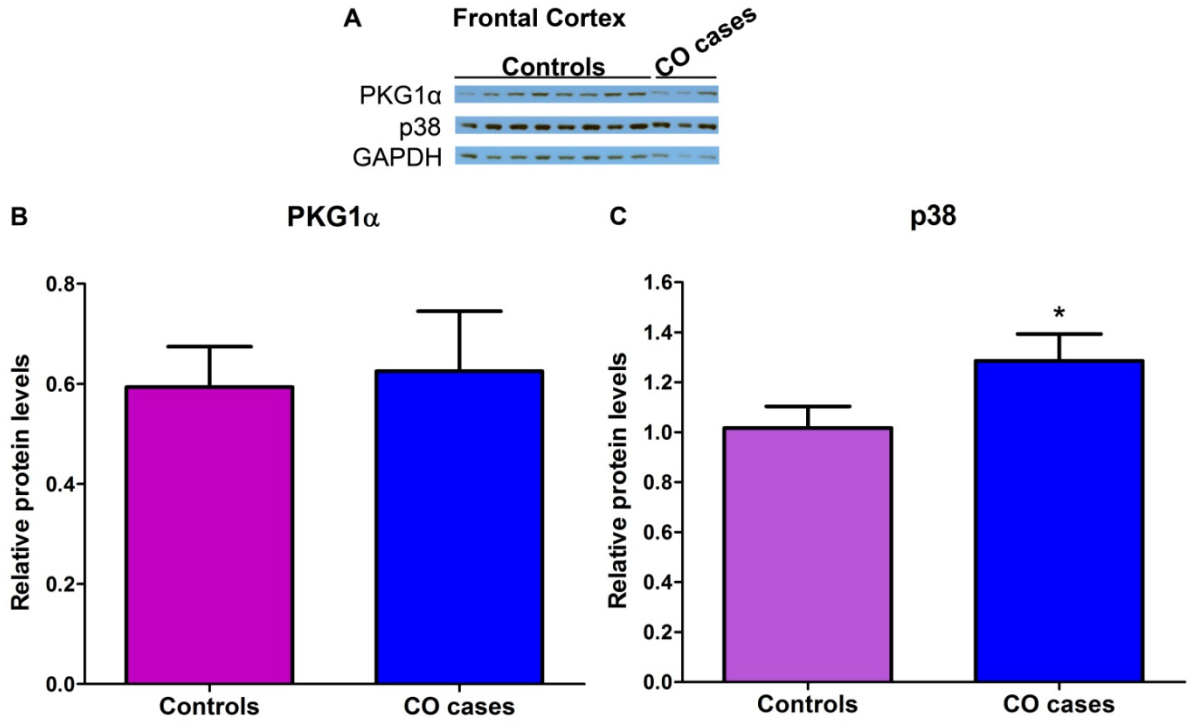


Figure 5.13 Protein Levels of PKG1α and p38MAPK in the Frontal cortex of Age Matched Healthy Controls and CO Exposed Cases.

The protein levels of PKG1α and p38MAPK (p38) were measured by **(A)** Western blot analysis which was followed by densitometric analysis of **(B)** PKG1α and **(C)** p38MAPK normalised with GAPDH. A non-parametric t-test was used to identify changes where p values less than 0.05 were considered as significant. *, p<0.05.

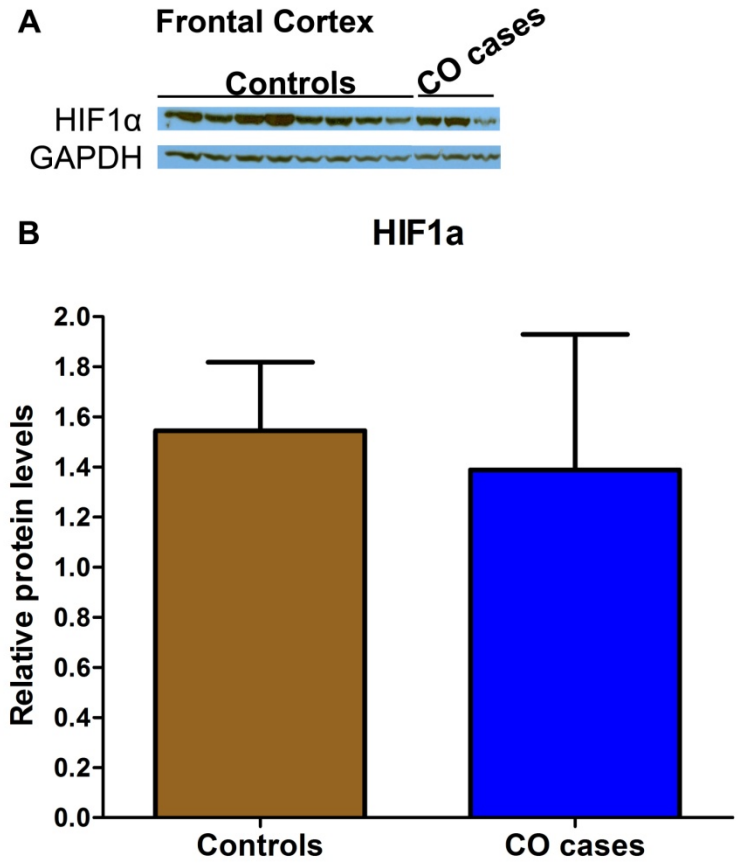


Figure 3.14 Protein Levels of HIF1α in the Frontal cortex of Age Matched Healthy Controls and CO Exposed Cases.

The protein levels of HIF1α were measured by **(A)** Western blot analysis which was followed by densitometric analysis of **(B)** HIF1α normalised with GAPDH. A non-parametric t-test was used to identify changes where no statistical significant change was observed.

Summary data for the determination of enzyme activity and protein levels in CO exposed cases compared to age at death matched controls is presented in Table 3.7.

Variable	**P (non-parametric t-test)**	**Change**	**Sample Number**
COX2 protein levels	0.9212	7% decrease	8 controls/3 CO
COX1 protein levels	0.6303	4% increase	8 controls/3 CO
sGCβ1 protein levels	**0.0242**	16% decrease	8 controls/3 CO
VDAC2 protein levels	0.7758	14% increase	8 controls/3 CO
p38 protein levels	**0.0121**	27% increase	8 controls/3 CO
PKG1α protein levels	0.6303	5% increase	8 controls/3 CO
HIF1α protein levels	0.9212	10% decrease	8 controls/3 CO
COX/CS activity	0.2090	16% decrease	18 controls/3 CO
COX/μg protein activity	0.6511	7% decrease	18 controls/3 CO
CS/μg protein activity	0.3397	13% increase	18 controls/3 CO

Table 3.7 Summary Data for Comparison of Protein and COX Activity Determination and Protein Determination in CO Exposed Cases and Age Matched Controls.

4. Discussion

Demonstrating CO exposure and any associated toxicity is difficult as CO can be rapidly eliminated from the body making its detection difficult and symptoms of CO poisoning are non-specific and therefore difficult to directly attribute to CO[1]. CO exposure is widely regarded as being under diagnosed and even when exposure is suspected, it is often not confirmed. Having a marker which would confirm CO exposure, particularly when CO is not significantly elevated as determined when using either the standard biomarker carboxyhaemoglobin (HbCO) or CO itself, would be a significant step forward for improving both diagnosis and public health monitoring. Since CO can interact with reduced haem proteins containing Fe^{2+}, haem proteins could be used as indicators of CO exposure. As mitochondrial cytochrome *c* oxidase (COX) is a major CO target in tissues, we sought to determine the activity and levels of CO in exposed tissues, with a particular view to using COX in Coronial investigations where CO might be suspected as contributing to the cause of death.

Our investigation utilised a large sample of frontal cortex tissue from normal brain tissue donations as a suitable source of post mortem tissue that could be used in suspected CO exposure. These normal samples were compared with tissue samples from individuals with a known acute CO exposure which was given as the cause of death. COX activity was determined using a standard clinical assay for the determination of mitochondrial function, and this activity normalised to the activity of citrate synthase (CS), a mitochondrial mass marker. Mitochondrial COX activity appeared to be stable over a range of post mortem intervals from 5 to 111 hrs after death, and unaffected by tissue pH between 5.46 and 7.31 (see Figure 3.1). Freezer time also showed no effect on measured COX/CS activity in normal control samples indicating that COX/CS activity is stable across a range of different measures and could be used as a metabolic marker in post mortem investigation. Analysis of samples from cases with known CO exposure resulting in death within a relatively acute time frame shown no major changes compared to the control group using any of the measures (see Figure 3.1). This would indicate that COX activity, whilst stable using typical post mortem material, is relatively unaffected by levels of CO exposure that cause death. Acute exposure to high levels of CO therefore appears to cause death by anoxia and mitochondrial respiratory chain arrest rather than direct chemical inhibition of COX by CO. This further confirms that at such levels CO would appear to displace oxygen from haemoglobin to produce HbCO causing cerebral and peripheral tissue hypoxia[7,8].

Whilst tissue hypoxia is likely to be the cause of death in CO exposure, there are additional symptoms experienced by people exposed to CO at non-lethal levels. Such cases of poisoning can be long lasting with persistent symptoms during exposure times. These include headache, nausea, fatigue, and confusion. These symptoms may be due to the effects of CO as a signaling molecule[37], where acute production of CO causes cerebral vasodilation and abnormal firing patterns of both peripheral and central nervous system neurones. Haem containing large conductance Ca^{2+}-dependent K^+ channels (*KCNMA1*) on cerebral arterioles, are a target of CO and are activated acutely in response to CO to cause vasodilation, although longer term exposure to elevated CO is known to cause vasoconstriction by reducing nitric oxide production through nitric oxide synthase inhibition leading to vasoconstriction and headache observed with CO exposure[43, 44, 51]. There are likely to be other cellular effects of CO since soluble guanylate cyclase β (sGCSβ) which is activated by CO, leads to increased production of cGMP with consequent downstream signaling effects[38,39] on several cellular systems including p38MAPK, HIF1α, and PI3K-Akt pathways [52].

We therefore investigated the effects of CO exposure on several proteins which show interaction with CO using a western blotting approach on available tissue samples. The results of protein determination in CO exposed tissues is consistent with exposure to CO as acting via sGCSβ and MAPK signaling cascades. Allowing for the effects of age at death which appears to affect protein expression, CO exposure was associated with decreased protein expression of sGCSβ (see Fig 3.12). Nitric oxide which activates sGCSβ can, following prolonged exposure, lead to downregulation of certain forms of sGCS[53, 54] and it is possible that high level CO exposure can achieve similar downregulation of sGCSβ. This effect is likely to be a normal physiological response to CO exposure whereby the intracellular signaling effects of increased cGMP can be limited by downregulation of the major biosynthetic enzyme. Whether this occurs at a posttranslational level by increased degradation of sGCSβ or by decreased transcription/translation is unclear, although studies with nitric oxide on the related sGCSα suggest transcription and translation may be the route[54].

Elevated p38MAPK protein levels were observed in brain tissue from CO exposed individuals (see Figure 3.13). Applying CO using a CO releasing drug, leads to upregulation of p38MAPK in the eye in response to injury[55] and also in cerebral vessel derived cells[56], ostensibly through increasing p38MAPK phosphorylation but also by increasing protein levels. Activation and upregulation of p38MAPK appears to be a protective mechanism to prevent cellular stress in response to cell damage or in response to supra-physiological levels of CO and therefore the

increased levels of p38MAPK observed in the current investigation may, as with downregulation of sGCSβ, be a mechanism whereby cells respond to reduce the effects of cell damage. Whereas CO directly interacts with sGCSβ via the haem group, for p38MAPK, CO interaction is likely to be indirect due to the absence of a haem group within the protein and evidence suggests that it is increased cGMP that leads to the upregulation of p38MAPK, as with nitric oxide[57, 58]. In this case, p38MAPK upregulation may be a downstream event caused by CO exposure. It is possible that other downstream mediators of physiological CO effects may be altered by CO exposure, and whilst HIF1α and PGK1α do not show alterations in CO exposed individuals, other mediators such as Akt or caveolin-1 may be altered and could be targets for future investigation[58, 59].

5. Conclusion

Our investigations have shown that COX activity is relatively stable to a wide range of agonal and post mortem factors which may impact Coronial investigations and therefore COX could be used as a marker if a need can be identified. Using the unique access to tissues from individuals acutely exposed to high levels of CO, we have demonstrated that COX activity is unaffected by CO exposure, indicating that tissue hypoxia is the likely cause of death under these circumstances. Extending these investigations has shown that the major protein subunits of COX, COX1 and COX2, are also unaffected by high level acute CO exposure. Whilst these studies indicate that acutely CO mediates its effect through hypoxia, chronic lower level exposures which are known to cause neurological and cognitive effects[60, 61] may act via different mechanisms over and above those of hypoxia. Although investigation of such low level chronic exposures should be a priority, it is unlikely that novel biochemical investigations such as those undertaken as part of this study could be repeated due to the absence of available tissue.

Extending the work using the available CO exposed tissues to investigate the physiological CO signalling system we have shown that acute high level CO exposure causes a reduction in the CO target enzyme soluble guanylate cyclase (sGCSβ) and an increase of the downstream effector p38 mitogen activated protein kinase (p38MAPK). These novel findings suggest that the system which normally responds to low levels of endogenously produced CO, may at high exogenous CO levels be altered in an attempt to reduce the toxic effects of CO. These findings indicate that by investigating changes in the physiological CO system, it may be possible to monitor CO exposure. Understanding the chronic effects of CO exposure would be of use since

many symptoms of CO exposure such as fatigue, headache, loss of concentration may stem from prolonged CO exposures and from longer term changes to cells and tissues.

The finding of an altered physiological CO response system lends itself to further investigations into the effects of CO. Using *in vitro* approaches with human cells (peripheral blood cells, lymphocyte cell lines), it should be possible to determine the acute and subacute effects of CO exposure by profiling gene expression to identify the relevant proteins and systems which are altered in response to CO exposure. These could then be carried forward to *in vivo* animal studies where confirmation of changes could be obtained in a controlled environment. Such markers of CO exposure would enable clinical studies to be put in place to determine the expression of these proteins in blood to demonstrate CO exposure in the absence of raised carboxyhaemoglobin, particularly where the non-specific symptoms of exposure are present (e.g. headache, nausea, dizziness fatigue), and where there is a suspicion of CO exposure. Such a test may also be useful as an adjunct for monitoring individuals who are known to have been exposed to CO to determine how rapidly tissues return to normal following removal from exposure, thus also providing some insight into why delayed effects from CO exposure are experienced by patients after apparent recovery.

6. References

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