Identification of Biomarkers in Peripheral Blood Lymphocytes Following Carbon Monoxide Exposure

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Synopsis

Making a diagnosis of CO poisoning is notoriously difficult, particularly if the healthcare professional does not have a raised suspicion that CO is the cause of their patient's symptoms. This is largely due to the symptoms of CO poisoning mimicking those of other, more common illnesses and because once exposure to CO ceases, the original symptoms of poisoning experienced by the patient diminish. The results of currently available tests for detecting CO following exposure then become increasingly ambiguous as time progresses following an exposure. Even when the results of such tests are useful, they are still limited to only confirming or refuting exposure to CO: information on levels of exposure, confirmation of effective treatment, the effects CO has had on the health of the patient and the long term health of the patient cannot be obtained.

This study forms part of an important and carefully considered portfolio of scientific research and events that have been funded by Gas Safety Trust (GST). It provides useful and significant results that will advance and inform several aspects of this portfolio of work.

This study to identify biomarkers in blood lymphocytes, took forward the findings from a previous GST funded study that identified the effects of CO on the normal functioning of cellular signalling systems in post mortem brain tissue. Using the findings from the earlier

study, this study has successfully developed the work to establish a test for CO that is less time critical and can identify exposures to lower levels of CO. Such exposure would be of the type experienced by someone who is being chronically exposed and whose symptoms are vague or perhaps have ceased by the time they are attended to by a healthcare professional. The identification of 22 potential biomarkers is important: it shows that in blood lymphocytes there is specificity associated with changes due to CO poisoning, and that finding the best one or combination of markers to not only confirm exposure, but also to provide insight into what effects on the body the exposure is likely to have caused, is important. These findings also suggest the possibility that the protein changes observed can be translated into a clinical test that is superior to those that are currently reliant on recent or higher levels of exposure.

This study and the potential future work suggested, therefore touches many aspects of the portfolio of work funded by GST: it enhances the collection of work that is investigating new biomarkers of exposure for improved detection of CO in patients, and it develops the work taking place in parallel to progress an understanding how CO effects health and the mechanisms that cause ill health both in the short and long term. The improvement in this understanding is important if healthcare professionals are to be advised on how best to treat their patients. The results of this study therefore progresses the work being undertaken to improve the evidence base required by NHS England, Department of Health, the Alliance of Royal Collages, National Poisons Information Service and others on the identification of and treatment strategies for CO exposure to protect public health.

However, it is clear that further work is required. The important next steps to develop a clinical test given the evidence base presented, should be considered.

Lay Summary

Carbon monoxide is a colourless, odourless, tasteless gas that at high levels can be deadly and at low levels can make people feel as though they have the flu, feel sick, tired, and confused. These symptoms are also often found in people suffering from other, more common illnesses, making it less likely that carbon monoxide is considered as being responsible for these symptoms [1] . There are ways to show that carbon monoxide is the cause of these symptoms, but the special equipment that is needed is often used by health practitioners long after the patient has left the place where carbon monoxide was present. At this point, there is little chance of identifying carbon monoxide poisoning because carbon monoxide quickly leaves the body when fresh air, not containing carbon monoxide is breathed in. It is very likely that carbon monoxide poisoning is under diagnosed and even when it is suspected as a cause of illness, carbon monoxide poisoning is not always confirmed. This makes it difficult to find out how many people suffer ill health or are poisoned by carbon monoxide. Knowing that people have been poisoned by carbon monoxide is important: being poisoned by carbon monoxide can harm many parts of the body and is known to damage the brain. This sort of damage can cause psychological problems in those who are poisoned and issues that can affect the patient's wider family for a number of years after poisoning has stopped. Without understanding that carbon monoxide was the cause of a problem, it is harder for the patient to receive the correct medical help [COMED REPORT 2017][1]. What is needed is a method which lets hospital and medical staff find out if someone has been poisoned by carbon monoxide, even if carbon monoxide is no longer present in the body. This method will

[1] COMED (2017) Carbon monoxide poisoning: saving lives, advancing treatment – a call for action across the healthcare sector. Policy Connect www.policyconnect.org.uk

preferably show the amount of carbon monoxide that was breathed in and also if carbon monoxide has had any toxic effects on the body.

Although carbon monoxide is poisonous at high levels, carbon monoxide is produced deliberately by cells in the body at very low levels where carbon monoxide passes signals from cell to cell. This normal carbon monoxide system allows the cells to communicate with each other and leads to both short and long term changes to cells. However, during carbon monoxide poisoning, this normal carbon monoxide signalling system is likely to be altered. Our previous work supported by the Gas Safety Trust, showed that carbon monoxide might be responsible for changes in the enzymes sGCSβ and p38MAPK found in brain tissue from individuals who had died from carbon monoxide poisoning. The changes in sGCSβ and p38MAPK might be because high levels of carbon monoxide alter the normal carbon monoxide cell communication system. It is this change to the communication system that might provide a way of showing carbon monoxide poisoning had occurred even if no carbon monoxide was found in the patient's body. The purpose of the current study was therefore to:

- See what changes occur in normal white blood cells when they are exposed to a poisonous level of carbon monoxide.
- Identify a set of changes (a carbon monoxide calling card or "signature") that indicates carbon monoxide poisoning.

This would allow us to identify a pattern of changes characteristic of carbon monoxide poisoning from a normal, venous blood sample taken from a patient who had been exposed to carbon monoxide. This pattern of changes might be of use when poisoning by carbon monoxide is suspected, but when there is no current evidence of exposure. This might allow

a doctor to use the carbon monoxide signature to find out if their patient has been exposed to carbon monoxide if they attend an Accident and Emergency department or a GP surgery because they feel unwell.

Following ethical approval, we approached healthy volunteers and obtained blood samples after their agreement. The white blood cells were separated from the red blood cells and the white cells placed in a sterile solution. The white blood cells were then placed in a specially designed chamber so that they could be exposed to either a normal gas (air) mixture or a controlled mixture of air and carbon monoxide. A level of 200 parts per million (200ppm) carbon monoxide was chosen since this level of carbon monoxide causes severe symptoms in people, but won't cause death of the white cells. Following the exposure of cells to either carbon monoxide or normal air for 6 hours, all of the chemical messages found in the cell contained in the RNA, were extracted. Individual RNA messages were then determined and the number of messages (typically 20,000 in a cell) were counted. The total number of messages found between the white cells exposed to carbon monoxide and those given normal air were compared. Using specialised computer programs several changes in the cells given carbon monoxide were identified:

1) A decline in the white blood cell system that would normally help white blood cells stick to blood vessels. This may be, because the enzyme NOX, which allows cells to signal to each other, is directly prevented from doing so by carbon monoxide.
2) A specific response given by the cell that showed it was lacking energy. Carbon monoxide may prevent the cells from producing energy because it binds to the cell's energy factory (the mitochondria).

3) A reduction in the ability of the white blood cells to respond to inflammation. This is the system that allows the white cells to fight something harmful such as bacteria. This signal may be observed for two reasons:
    a. because carbon monoxide binds to a protein that causes blood-clotting (NOS3);
    b. because carbon monoxide binds to proteins called cytochrome P450s. Some cytochrome P450s are involved in responding to inflammation, particularly in blood vessels.

    By binding to NOS3 and some cytochrome P450s, carbon monoxide reduces the availability of these proteins to take part in the processes that contribute to a response to inflammation.
4) An increase of the system that allows cells to change their shape (differentiate), meaning that they could do this more easily and frequently. When a cell changes shape, its normal function also changes. How exactly carbon monoxide could do this is not entirely clear. However, some scientists have found similar effects when they exposed cells to special carbon monoxide releasing molecules (CORMs).

These changes in the white blood cells are likely to provide pieces of evidence that collectively provide the basis for the carbon monoxide signature that will allow us to determine if someone has been poisoned by carbon monoxide.

We will, as part of future experiments, see if we can reproduce these specific findings in white blood cells. Moreover, we will see how long these specific changes lasts once the carbon monoxide is no longer present. This will allow us to see how reliable the test for a new blood carbon monoxide signature could be. If this carbon monoxide signature works, we will then go on to find out if it can detect carbon monoxide poisoning in blood from people who are

known to have been poisoned by carbon monoxide (a positive test), and those whom we know have not been poisoned (negative test). This will provide us with the information needed to establish if the carbon monoxide signature might work in practice and provide information to assist with not just diagnosis of poisoning, but also the effect of treatment and longer term health of people exposed to CO.

Technical Summary

Carbon monoxide (CO) is a colourless, odourless, tasteless gas which at high concentrations can be fatal, and at low concentrations can lead to 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]. CO exposure is usually confirmed using either measurements of expired CO in breath or percentage concentrations of carboxyhaemoglobin, a specific biomarker of CO exposure. Methods to confirm CO exposure require specialist equipment and these approaches are frequently used long after the patient has left the source of CO, when the likelihood of detecting CO is low due to the short half-life of CO in the body. It is considered likely that CO poisoning is under diagnosed and if diagnosed, not necessarily confirmed through detection, making accurate morbidity and mortality statistics caused by exposure to CO, difficult to attain. There is a need therefore, to identify markers which demonstrate the specific effects of CO on cells and tissues. These markers will be required to show both the level of CO exposure and the toxic effects of CO, whilst also being robust and long lasting for practical use.

CO, whilst toxic at high levels, also acts as a physiological gaseous cellular messenger at very low levels, causing a variety of changes to cells and tissues. These normal effects of CO cause elevations of the cell signalling molecule cGMP in cells and cause both acute changes to cells, but also long term changes to cell physiology. The Gas Safety Trust has supported previous investigations of CO exposure that have shown reductions in the CO target enzyme sGCSβ and concomitant elevations of the enzyme p38MAPK in post mortem brain tissue from individuals exposed acutely to CO. These changes may be as a direct consequence of the CO acting

through the normal cellular signalling systems utilised by CO, but could additionally provide a signature of the effects of CO. Being able to define this CO signature could provide a mechanism by which the effects of CO could be detected even in the absence of CO itself (i.e. long after exposure has ceased and carboxyhaemoglobin levels have returned to normal). The purpose of this study will therefore be to:

1) Determine what changes in gene expression (a CO signature) occur in peripheral blood lymphocytes *in vitro* following high level (200ppm) CO exposure.

These studies have the potential to allow us to identify new biomarkers of CO exposure that will be of use when there is suspicion of CO exposure at fatal and non-fatal levels. A particular focus within this study will be the identification of new biomarkers associated with non-fatal levels of carbon monoxide exposure. It is envisaged that these biomarkers will present themselves in patients whose immediate symptoms of poisoning have ceased, but who are either suffering from delayed neurological sequelae or other post poisoning non-specific symptoms. Such a test would provide a suitable measure of CO exposure for use in accident and emergency departments and in blood taken at GP surgeries.

Following ethical approval we obtained healthy donor peripheral blood mononuclear cells (PBMC) from 6 volunteers. PBMC were placed in pre-warmed gas exposure chambers with circulating air movement and exposed to 200ppm CO in air containing 5% CO_2, or air containing 5% CO_2. Cells were exposed for 6 hours and RNA extracted for RNAseq based analysis. From paired RNAseq runs in the 6 matched donor samples, bioinformatics analysis identified a series of changes which may relate directly to CO exposure. This analysis showed several findings:

1) A reduction in platelet coagulation. This gene expression pathway regulates lymphocyte adherence to vascular endothelium in response to cell stress and elevation of cellular reactive oxygen species. NADPH Oxidase (NOX), which produces cellular superoxide via a haem prosthetic group in the cytochrome b subunit of the complex, may be directly inhibited by CO. This in turn leads to altered expression of matrix metalloproteases and vascular adherence.
2) Activation of the cell energy response system. CO is known to directly inhibit haem-containing enzymes of the mitochondrial respiratory chain and this is likely to cause a reduction in cellular ATP and elevated AMP. This in turn activates the cellular AMP-kinase which responds to elevated AMP levels in order to reverse cellular energy depletion.
3) A reduction in lymphocyte inflammatory response. CO is known to inhibit the haem containing NOS3 enzyme which activates various lymphocyte inflammatory pathways. The haem containing cytochrome P450 enzymes CYP2S1 and CYP4F2, involved in prostaglandin and leukotriene production, are key mediators of inflammation and are also reduced following CO exposure. These alterations are likely to be associated with decreased lymphocyte activation and altered immune responses.
4) Cell proliferation and differentiation. Cytoskeletal changes including stress responses (RBM42) and direct changes in microtubule elements (MAP1B) are evident which may relate to DLL3 expression and the notch/Wnt/GSK3β signalling pathway which would typically allow leukocytes to migrate through vascular endothelia. Such changes may be related to specific findings with carbon monoxide releasing molecules (CORMs) which appear to have similar effects.

The changes in PBMC gene expression following CO exposure appear to be related to direct or downstream effects of CO's known interaction with haem-containing proteins. These gene expression changes will require further targeted validation in a larger experimental cohort to determine the reproducibility of these changes and how long lasting these changes are following CO withdrawal. If the gene expression changes are robust, then a trial of the markers using peripheral blood samples from people who are known to have been poisoned by CO and healthy volunteers will be required to demonstrate if the CO gene expression changes may be of use in a clinical setting.

Background

Estimates suggest that up to 4,000 people attend hospital emergency departments each year due to accidental CO exposure, presenting with a variety of symptoms, with over 200 requiring hospitalisation and potentially 30 preventable deaths in the UK [2]. In many instances, malfunctioning or improperly used fuel appliances appear to be a leading source of CO exposure although other sources are known [3]. For exposed individuals there is 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 fall rapidly in either blood or in breath [4]. The reaction of CO with haemoglobin to produce carboxyhaemoglobin (HbCO) is well known and is thought to underlie the toxicity of CO in causing tissue hypoxia by displacing oxygen from haemoglobin. HbCO can be easily measured in blood and has been used extensively to monitor CO exposure. Typically, healthy normal individuals have HbCO levels of below 1% however 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 [5, 6]. Blood HbCO levels may not be a reliable indicator of the source of environmental CO exposures and blood HbCO levels of above 10% may only reliably indicate toxic CO exposure [7] given the findings of high levels of CO and HbCO found in heavy smokers [6]. Blood levels of HbCO can rapidly fall in an individual when the person is removed from the source of CO exposure and within a few hours blood HbCO levels can return to normal making clinical investigation difficult unless CO exposure is suspected in the patient. 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 [8]. A suitable marker of CO exposure is therefore required for identifying exposed individuals following their removal from a CO contaminated environment and 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 sequelae.

The Role of Endogenous CO in Normal Physiology

CO is produced by the body in tissues and cells as an elimination product of haem metabolism. It is produced from the breakdown of haem along with biliverdin in the liver, through the activity of haem oxygenase (HO) enzymes that act both as a substrate and as a catalyst in the breakdown process [9]. Numerous studies have shown that due to the activity of haem oxygenase, CO acts as a potent signalling molecule which affects multiple intracellular pathways causing changes in cell physiology and responses [10]. A major target of CO signalling within cells is through regulation of cellular cyclic guanosine monophosphate (cGMP) production by activation of soluble guanylate cyclase β (sGCSβ, *GUCY1B3*) enzyme which produces cGMP [11]. When CO binds to the haem containing enzyme sGCSβ, a conformational change occurs in the enzyme leading to enhanced cGMP production [12]. Since a major target of CO is interaction with haem within haem containing proteins, it can be expected that regulation of various enzymes that have a haem active site may play a role in the normal physiological effects of CO, and may also underlie some of the symptoms observed during CO exposure. CO can for example interact with the haem moiety found in large conductance Ca^{2+}-dependent K^{+} channels (KCNMA1) which are expressed at high levels in cerebrovascular arterioles. The interaction of CO with KCNMA1 causes calcium influx into cells and results in acute vasodilation of cerebral vessels [13, 14]. As headache due to changes

in cerebral blood flow through the effects of CO on the vasculature is one of the early features of CO poisoning, changes to haem proteins that interact with CO provide important indicators of CO effects. Identifying those proteins and enzymes which CO can interact with would therefore provide potential biomarkers for determining CO effects and also for monitoring the activity of CO.

Whilst CO is known to have acute effects at physiological levels, the fact that CO is a gaseous molecule and can dissipate rapidly means the effects of CO are generally short lived if exposure to CO ceases. The longer term effects of prolonged or subacute CO exposure are only poorly defined but may relate to the more severe symptoms experienced by individuals exposed to CO. For example, CO exposure is frequently associated with severe headache, a symptom which would be at odds with the cerebral vasodilation observed as a direct response to CO exposure. However, the vasoconstriction seen with subacute CO exposure may be due to downstream effects of CO on cellular signalling, whereby elevated levels of CO inhibits the haem site within nitric oxide synthase, causing reduced nitric oxide production and the vasoconstriction and headache observed [13-15]. CO may also interact with other cellular systems since elevated cGMP due to sGCSβ activation causes inactivation of cGMP-dependent protein kinase PKG1α which in turn regulates several intracellular signalling pathways including mitogen-activated protein kinase (MAPK) [10, 16]. Given the absence of haem containing proteins in MAPK pathways, there is the direct possibility that CO can modulate both haem and non-haem containing cellular signalling pathways [17, 18]. Subacute exposure to CO may therefore cause changes to different cell signalling systems and this may be evidenced by our finding of elevated p38MAPK and reduced sGCSβ levels in brain tissue from CO exposed individuals (Tsefou et al, Gas Safety Trust report 2017; Figure 1). The reduced

levels of sGCSβ may be a physiological attempt to reduce the levels of cGMP back to the normal basal levels by reducing transcription and translation of sGCSβ. Similarly, the elevated levels of p38MAPK suggest the downstream effectors of CO are elevated in response to CO as part of the normal tissue response. These findings suggest that CO may have longer term effects by causing changes to the homeostatic mechanisms that normally respond to CO exposure. Identifying what these specific changes are in response to CO would provide an indication of both the long term cellular effects of CO, but also provide biological markers of CO effects which could be used to define CO exposure.

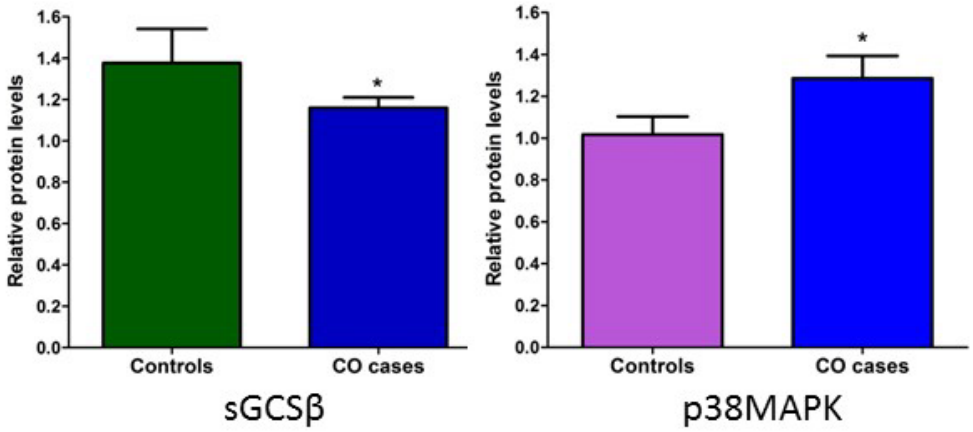

Figure 1: Altered Protein Expression in the Frontal Cortex of CO Exposed Individuals.

Frontal cortex tissue was obtained at post mortem from individuals known to have died from the consequences of acute CO exposure and probed using Western blotting for the CO responsive enzyme soluble guanylate cyclase (sGCSβ) or the downstream stress response protein p38 mitogen activated protein kinase (p38MAPK). Significant (*, $p<0.05$) changes in both proteins were observed suggesting that CO has an effect on protein expression.

Aims

Our main aim is guided by the central hypothesis that physiological systems exist which respond to CO which in the presence of pathological levels of CO, respond by regulating gene

and protein expression to limit toxicity of CO. This specifically relates to the reduction of sGCSβ and increase of p38MAPK as observed in post mortem brain tissue from individuals acutely exposed to CO. Determining if these changes can be identified in peripheral blood would provide a readily accessible means to determine CO exposure in exposed individuals.

The specific aims arising from the central hypothesis are:

1. To identify changes in gene expression in peripheral blood lymphocytes following CO exposure.
2. To determine if these gene expression changes are stable during CO exposure.
3. Establish if the gene expression changes translate to appropriate protein changes in lymphocytes exposed to CO. (NB: This aspect of the study will form the basis of future work to identify suitable biomarkers at the protein level).

The key outcomes relating to the specific aims are:

1. To assess changes in lymphocyte gene expression due to CO;
2. To determine if accompanying protein changes can be observed in lymphocytes following CO exposure. As previously, this will form the basis of a future study based on outcome 1.

Plan of Investigation

To align with potential clinical investigations, we have utilised human peripheral blood lymphocytes exposed to CO as a model system. Lymphocytes express soluble guanylate cyclase (sGCSβ) mRNA (http://www.gtexportal.org/home/gene/GUCY1B3) and also express high levels of sGCSβ protein [19] indicating a system for responding to CO is present under normal circumstances and that lymphocytes may respond acutely and also chronically to

environmental CO. Peripheral blood lymphocytes will therefore be exposed to an acutely elevated level of CO which will cause both short and long term changes in gene expression as a response to CO. These changes in expression will be identified using RNAseq and bioinformatic analyses with future investigations culminating in determination of protein changes in lymphocytes. These protein changes have the potential to be translated into a clinical test for the detection of CO exposure in the absence of changes in HbCO or detectable CO in blood.

Methods

Ethical approval for the study was provided by the Newcastle University Research Ethics Committee under the Newcastle University Human Tissue Authority research sector license. Following open advert, volunteers provided fully informed consent to provide an anonymised blood sample which was taken by fully trained staff within the Newcastle upon Tyne Hospitals NHS foundation Trust Clinical Research Facility. Age, sex, and demographic data including smoking history were obtained from donors.

Blood collection, cell extraction and seeding

On the day of the experiment, a total volume of 50 ml whole blood was collected from the participant via venepuncture into BD Vacutainer® CPT™ tubes containing Sodium Heparin (Becton, Dickinson and Company, New Jersey, USA). Within 30 minutes after blood collection, cell extraction was commenced following the manufacturer's instructions for the separation of mononuclear cells from whole blood (REF 362753, Becton, Dickinson and Company). Briefly, collection tubes were gently inverted 10 times and centrifuged at 1,700 x g for 25 minutes at room temperature (RT). After centrifugation, the cloudy layer containing peripheral blood mononuclear cells (PBMCs) was collected from each tube and transferred into a single 50 ml collection tube. The cell suspension was diluted to a total volume of 45 ml with calcium and magnesium free phosphate buffered saline (PBS) (Thermo Fisher Scientific, Paisley UK) and centrifuged at 300 x g for 15 minutes at RT. Supernatant was discarded and the cell pellet gently suspended in 10 mL PBS using a 10 ml serological pipette. The cell suspension was diluted to a total volume of 25 ml with PBS, inverted 5 times and centrifuged for 10 minutes at 300 x g at RT. The supernatant was discarded and the cell pellet gently suspended in 10 ml warm AIM V medium (Thermo Fisher Scientific) using a 10 ml serological

pipette. The cell suspension was diluted to a total volume of 45 ml, inverted 7 times and seeded at 1 ml/well in pre-warmed 6-well plates for suspension cell cultures (Greiner Bio-One, Kremsmuenster, Austria). Each well had previously been filled with 1 ml warm AIM V medium per well so that a 1:2 dilution of the cell suspension was achieved in a total volume of 2 ml per well. Seven plates were seeded for each donor and placed back into the humidified incubator at 37°C. After seeding, cell count and viability in the remaining cell stock suspension was determined in duplicate using disposable Countess™ counting slides and the automatic cell counter Countess™ II FL (both Thermo Fisher Scientific) according to the manufacturer's instructions for trypan blue exclusion.

Carbon monoxide exposure

For the exposure experiments, 6-well plates containing PBMCs were placed in customised venting chambers situated in a humidified incubator at 37°C. The chambers had been pre-warmed for at least 2 hours and equilibrated with air containing 5% CO_2. One chamber at a time was closed and vented at 1.5 psi with a customised gas mix containing 200 ppm CO, 5% CO_2, 20% O_2 and 75% N_2 (BOC). Venting was performed under a class III extractor, monitored continuously with a combustion gas analyser (Kane 506) and fans assisted to speed-up the venting process and avoid dead-space. One box was filled with 200ppm CO within ~2 min. When 200ppm was reached, valves were closed, the chamber disconnected from the cylinder and placed into the incubator at 37°C. In order to avoid overpressure due to the increase of temperature, excess gas was allowed to overflow from the chamber into an attached impermeable foil-balloon during the warming-up process (Figure 2). The battery-operated fan inside the chamber supported warming up the CO-gas mix to 37°C, which was achieved within ~15 minutes. Control boxes were closed one at a time just before the referring CO-box was

placed in the incubator to maintain 5% CO_2 in the control gas mix (5% CO_2, 20% O_2 and 75% N_2). Cells were exposed to CO and control gas, respectively, for 1, 6 and 24 hours before harvesting.

Cell extraction

After each exposure time point, cells were briefly examined under a light-microscope and cells in suspension transferred into 2 ml reaction tubes on ice. Warm PBS was added immediately to each well of the 6 well culture plate, which contained the remaining adherent cells (monocytes and macrophages). PBS was quickly and thoroughly removed from 6-well plates and the plates placed on ice. Two hundred and fifty (250) microliters of chilled RNA-lysis buffer (Promega, Wisconsin, USA) containing 4 M guanidine thiocyanate, 0.01 M tris (pH 7.5) and 2% 1-thioglycerol was added to the wells of the 6-well plate. Adherent cells were scraped and thoroughly homogenised in RNA lysis buffer using sterile cell scrapers (Greiner Bio-One). Plates with lysates were left on ice until the harvest of the suspension cells was finalised: Suspension cells (lymphocytes) were centrifuged for 5 min at 300 x g, at 4°C in a pre-cooled centrifuge. One ml of supernatant was carefully removed from the tubes, and transferred into cryo-vials (Alpha-laboratories, Hampshire, U.K.) and stored at -80°C for miRNA analysis. The remaining supernatant was carefully removed, 1 ml ice-cold PBS added to the cell pellets and tubes centrifuged again for 5 min at 300 x g, at 4°C. After the second centrifugation step, PBS was carefully and thoroughly removed from the reaction tubes. RNA lysates obtained from adherent cells were transferred from the 6-well plate into the respective tubes containing pelleted suspension cells. Through this, RNA and protein lysates from suspension and adherent cell fractions were combined in a single tube. (Figure 3). RNA lysates were stored immediately at -80°C.

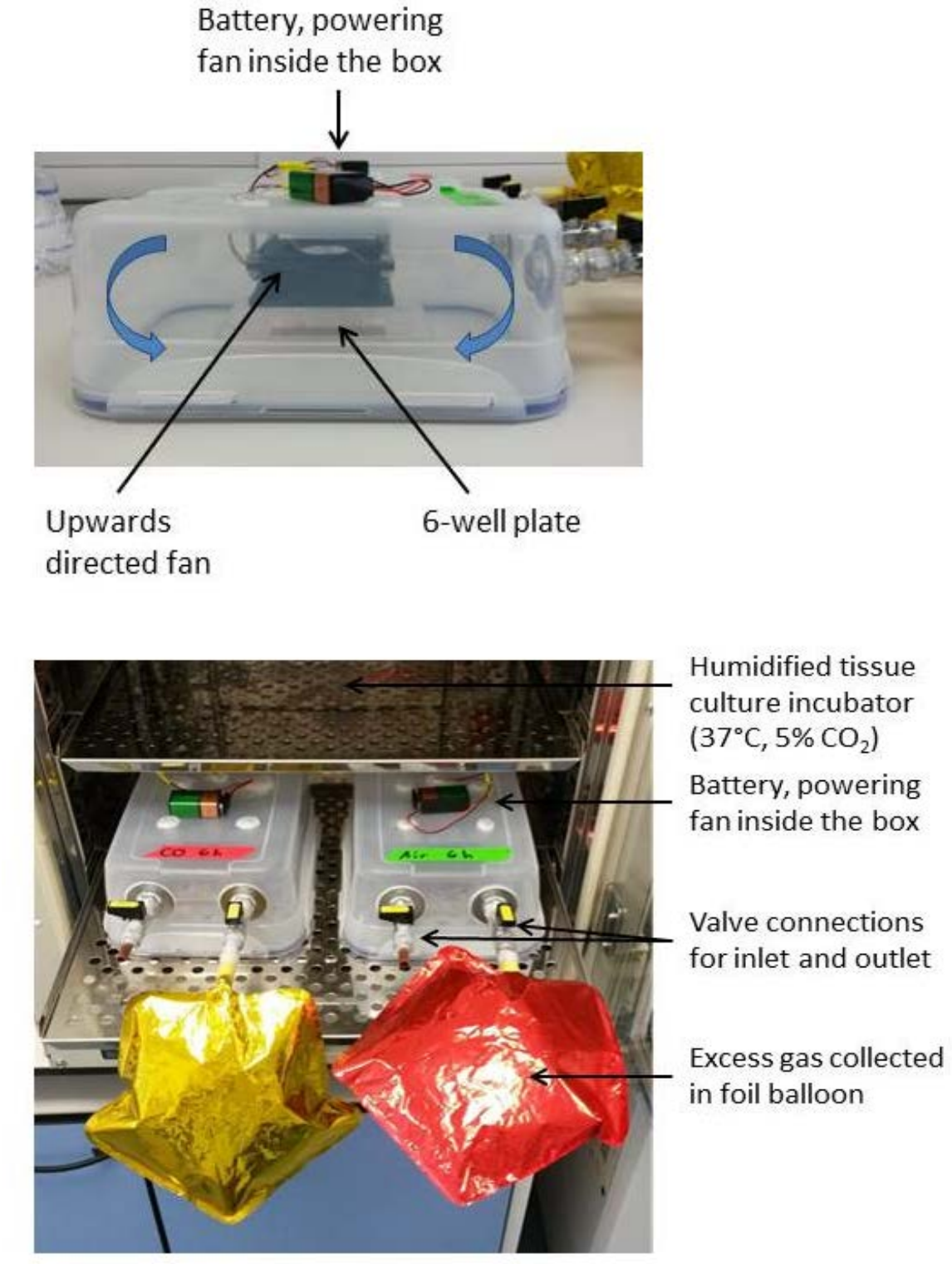

Figure 2: Equipment set-up using customised venting boxes
5-litre air-tight plastic boxes (Lakeland) were modified: Two valve connections were inserted allowing connection to CO gas cylinder for inlet and outlet of gas. Upwards directed battery-operated fans were installed inside the boxes to improve circulation of CO-mix (blue arrows). Excess gas could overflow into attached foil balloons in order to avoid overpressure inside the boxes. Boxes were situated in standard humidified tissue culture incubators at 37°C and 5% CO_2.

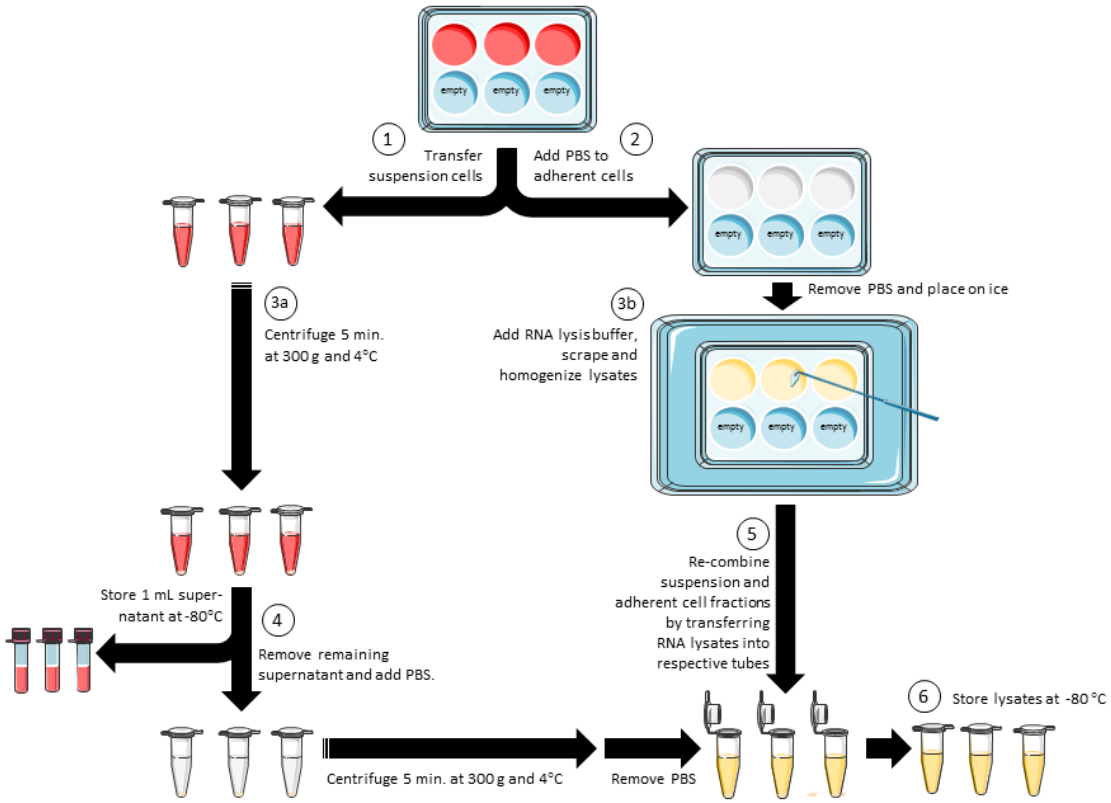

Figure 3: Workflow for simultaneous harvest of suspension and adherent cells from 6-well plates after CO exposure.

<u>RNA extraction and determination of RNA concentration</u>

RNA extractions were performed using the ReliaPrep™ RNA Miniprep system (Promega). RNA samples that had been stored in BL+TG buffer at -80°C were allowed to thaw on ice and briefly centrifuged to collect residual buffer from the sides of the tubes. Using a p200 micropipette, DNA was sheared by pipetting the lysate up and down 10-15 times on ice. While on ice, 85µL 100% isopropanol was added to each sample and tubes were vortexed for 5 seconds. After a brief spin to collect residual RNA lysate from the side of the tubes, the whole lysate was transferred onto a minicolumn and RNA extraction carried out according to the manufacturer's instructions (Promega), including an on column DNAse I treatment. RNA was eluted in 30µL RNAse free water and concentration measured using a NanoDrop microvolume spectrophotometer.

RNA sequencing

RNA quality control and RNA-sequencing was performed by LGC Genomics Ltd (Middlesex, U.K.) on twelve selected samples (six samples exposed to CO for 6 hours and six matching controls). RNA quality was assessed using the Eukaryote Total RNA Nano kit on an Agilent Bioanalyzer 2100 (both Agilent Technologies Inc., Santa Clara, USA). NEBNext Ultra II RNA Library Prep Kits for Illumina were used for mRNA isolation and cDNA synthesis (New England Biolabs Inc., Massachusetts, USA). Following this, the Ovation Rapid DR Multiplex system was used for fragmentation, end repair, and adaptor ligation (NuGen Technologies Inc., California, USA). RNA-sequencing was performed with 75 bp single reads (Illumina NextSeq 500 V2) and with greater than 20 million reads per sample. Demultiplexing of all libraries was performed using the Illumina bclfastq 2.17.1.14 software and all raw reads were adapter trimmed. Quality control FastQC reports were included with the data.

Bioinformatics analysis and statistics

Data analysis was undertaken in conjunction with the Bioinformatics Support Unit, Newcastle University.

In summary, FastQC files were passed to MultiQC to aggregate the results for quality control (see appendix: MultiQC report). Reads were then quantified against transcripts using Salmon, a program for quantifying expression of transcripts from RNA-Seq data [20]. Differential gene expression analysis was carried out with the R package DESeq2 [21]. DESeq2 provides methods to test for differential expression by use of negative binomial generalised linear models. Differential expression was calculated between samples exposed to CO and samples exposed to normal air.

Following calculation of fold changes and p-values, data was imported into the software program Ingenuity® Pathway Analysis (IPA®) (Qiagen, Venlo, Netherlands). IPA was used to identify altered pathways which may have a role in CO exposure responses (see appendix: IPA® summary report and figures of affected canonical pathways). Since the dataset showed strong variation between participants, but lower effects by treatment (Figure 4 and 5), we additionally extracted only the protein coding transcripts from the datasets of each participant and compared the individual log fold changes (logFC) to search for joint variability. Combining these two approaches, we identified 22 potential biomarkers (Table 1).

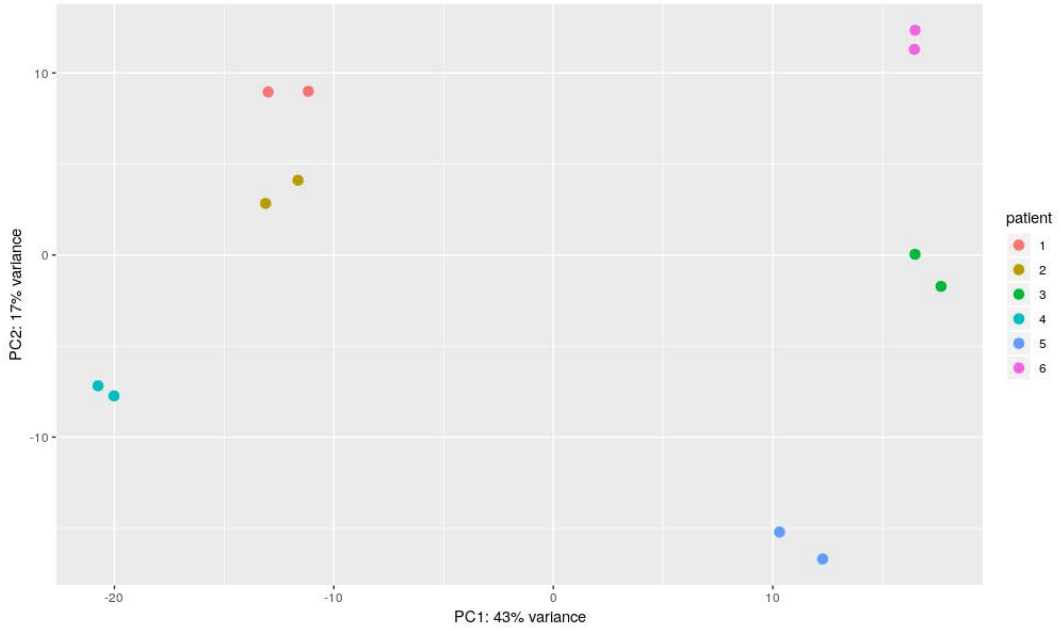

Figure 4: Overall variability by patient

RNA sequencing data of six CO and six corresponding air-treated samples shows high overall variability by participants.

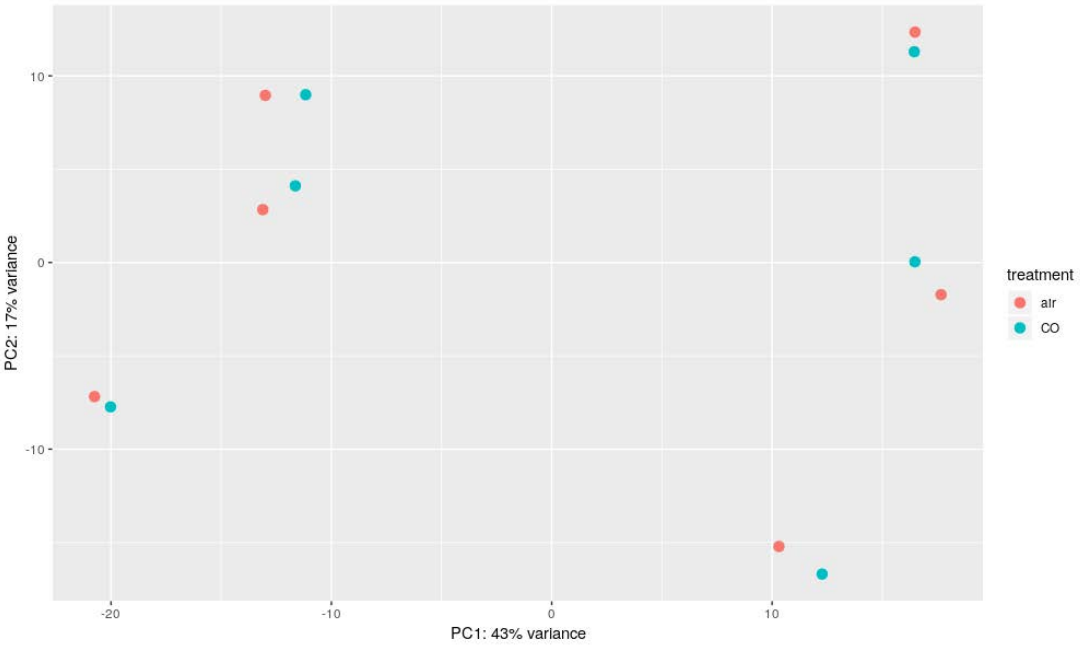

Figure 5: Overall variability by treatment

RNA sequencing data of six CO and six corresponding air-treated samples shows relatively small overall variability by treatment.

Table 1: List of potential biomarkers for low-level (200ppm) CO exposure. Joint variation was defined here as positive (yes) if at least 4 of 6 patients showed the same effect to carbon monoxide treatment, indicated by similar individual fold changes of each participant (logFC).

Transcript	Name	Function	logFC*	p-value*	Joint variation	IPA pathway
Mitochondria/ energy household						
ENST0000 0568517	cytochrome c oxidase subunit 5A (COX5A)	Subunit of mitochondrial complex IV, involved in energy production. Contains haem.	4.34	0.0131	no	yes
ENST0000 0313408	NADH ubiquinone oxidoreductase core subunit S7 (NDUFS7)	Subunit of mitochondrial complex I, involved in energy production.	4.90	0.0217	no	yes
ENST0000 0418900	hypoxia inducible domain family member 1A (HIGD1A)	Proposed subunit of mitochondrial complex IV, regulates transcription in response to hypoxia.	0.96	0.0932	yes	no
ENST0000 0548065	protein kinase AMP-activated non-catalytic subunit gamma 1 (PRKAG1)	AMP/ATP-binding subunit of AMP-activated protein kinase (AMPK), AMPK is a cellular energy sensor.	1.67	0.1601	yes	yes
Platelet activation/ coagulation						
ENST0000 0297494	nitric oxide synthase 3 (NOS3)	Produces nitric oxide (NO), regulates cGMP mediated vascular smooth muscle relaxation, mediates vascular endothelial growth factor (VEGF)-induced angiogenesis, promotes blood clotting through activation of platelets. Contains haem.	-0.49	0.2625	no	yes
ENST0000 0264424	guanylate cyclase 1 soluble subunit beta 1 (GUCY1B1)	Mediates responses to nitric oxide (NO) by catalysing the biosynthesis of the signalling molecule cGMP. Contains haem.	0.32	0.2574	yes	no
ENST0000 0334047	coagulation factor III (F3)	Cell surface glycoprotein that enables cells to initiate the blood coagulation cascades.	0.52	0.1986	yes	no
Inflammation						
ENST0000 0392351	homer scaffold protein 3 (HOMER3)	Negatively regulates T cell activation through negative regulation of IL2 expression.	0.72	0.0923	yes	no
ENST0000 0442510	Protein tyrosine-protein phosphatase (PTPRC)	Regulator of T- and B-cell antigen receptor signalling, suppresses JAK kinases and functions as a regulator of cytokine receptor signalling.	0.69	0.0012	yes	no
ENST0000 0011989	cytochrome P450 family 4 subfamily F member 2 (CYP4F2)	Plays a key role in vitamin K catabolism and production of 20-hydroxyeicosatetraenoic acid (20-HETE), thereby regulating vascular tone,	-1.20	0.0020	yes	no

		vascular inflammation and remodelling. Contains haem.				
ENST0000 0310054	cytochrome P450 family 2 subfamily S member 1 (CYP2S1)	May play an important role in inflammatory processes. Contains haem.	-0.56	0.0334	yes	no
Cell proliferation/ cell differentiation						
ENST0000 0262633	RNA binding motif protein 42 (RBM42)	Protects CDKN1A (=p21) mRNA from degradation during stress.	0.13	0.4397	yes	yes
ENST0000 0244741	cyclin dependent kinase inhibitor 1A (CDKN1A)	Involved in p53/TP53 mediated inhibition of cellular proliferation in response to DNA damage.	0.17	0.3305	no	no
ENST0000 0296755	microtubule associated protein 1B (MAP1B)	Binds to and stabilises microtubules. Involved in cytoskeletal changes and neurite extension, positive cofactor in DAPK1-mediated autophagic vesicle formation.	0.76	0.1875	yes	no
ENST0000 0356433	delta like canonical Notch ligand 3 (DLL3)	Activates Notch signalling pathways. Inhibits primary neurogenesis, may be required to divert neurons along a specific differentiation pathway.	0.89	0.0496	yes	no
Leukocyte extravasation						
ENST0000 0397501	protein tyrosine kinase 2 beta (PTK2B), also known as FAK kinase	Non-receptor protein-tyrosine kinase that regulates reorganization of the actin cytoskeleton, cell polarization, cell migration, adhesion and more. Promotes activation of NOS3, regulates cGMP production, promotes activation of MAPK signalling cascade, promotes activation of RhoGTPases, regulates p53 activity, regulates NMDA receptor activity and intracellular calcium levels and increases SRC kinase activity.	0.76	0.4732	no	yes
ENST0000 0248899	neutrophil cytosolic factor 4 (NCF4)	Component of the NADPH-oxidase, responsible for electron transport from NADPH to molecular oxygen thereby generating reactive oxidant species.	-0.56	0.0141	yes	yes
ENST0000 0372330	matrix metallopeptidase 9 (MMP9)	Breaks down extracellular matrix proteins and is involved in leukocyte migration. Downregulation of MMP9 is associated with CO-releasing molecules treatment (CORMs).	-0.64	0.0069	yes	yes

ENST0000 0279441	matrix metallopeptidase 10 (MMP10)	Breaks down extracellular matrix proteins and activates of procollagenases.	2.04	0.0144	yes	yes
ENST0000 0407086	microtubule associated cell migration factor (BCAS3)	Regulates cell polarity and directional endothelial cell migration by mediating reorganization of the actin cytoskeleton at the cell leading edge.	-3.86	0.0024	yes	no
ENST0000 0315274	matrix metallopeptidase 1 (MMP1)	Break down extracellular matrix proteins.	0.78	0.0471	yes	no
ENST0000 0523950	vascular endothelial growth factor A (VEGFA)	Induces endothelial cell proliferation and promotes cell migration, inhibits apoptosis and induces permeabilization of blood vessels.	-0.27	0.4919	no	yes

* Calculation based on pooled data from all participants for the individual transcripts.

Pathways

Based on our RNAseq data, we have identified gene expression changes of 22 transcripts in different genes that are associated with CO exposure. These changes can be categorised into five major pathways, which are partly overlapping:

1. **Mitochondrial damage/ energy household.**

CO is known to bind to haem containing proteins. Cytochrome c oxidase subunit 5A (COX5A), located in mitochondrial complex IV of the respiratory chain, contains haem. Thus, CO can bind and inhibit this subunit, resulting in ATP depletion. Inhibition of complex IV is also known to increase levels of reactive oxygen species which in themselves will contribute to mitochondrial damage [22]. The observed upregulation of COX5A, NADH ubiquinone oxidoreductase core subunit S7 (NDUFS7) and hypoxia inducible domain family member 1A (HIGD1A) may be due to the combined effects of hypoxia and ROS-mediated mitochondrial damage. This response within mitochondria, which are the energy producing organelles in cells, will result in an attempt to increase ATP production and mitochondrial function. The

lack of energy might have immediate downstream effects on the cell since we identified that AMP-activated non-catalytic subunit gamma 1 (PRKAG1), an energy sensor that mediates various responses in cells, is upregulated. CO is known to cause changes to cellular AMP levels through energy/ATP depletion and may achieve PRKAG1 upregulation directly however, CO may activate L-type voltage-gated calcium channels to indirectly activate the AMP-kinase system [23].

2. Platelet activation

CO can bind to nitric oxide synthase 3 (NOS3), a haem containing protein that produces the gaseous messenger NO [24, 25]. In contrast to COX5A, which is inhibited by CO binding, NOS3 is activated by CO [24]. As a result, strong activation of pathways affected by NO, such as activation of platelets (blood clotting), vasodilation and angiogenesis could be expected. The cell might down-regulate the over activation of NOS3 protein, by reducing its expression [24], and this is indeed observed at the gene expression level. The expression of other proteins that are involved in platelet activation and coagulation were also observed to have been affected (F3 and GUCY1B1). Of particular interest, GUCY1B1, the haem-containing subunit of soluble guanylate cyclase 1 (sGCSβ), is a well-known target of CO, causing GUCY1B1 activation [26]. GUCY1B1 activity might therefore be influenced by increasing NO levels in addition to direct CO-mediated activation.

3. Inflammation

Low levels of CO have been reported in several studies to be anti-inflammatory, reducing immune cell response to injury or pathogens [27]. We have identified several proteins involved in inflammatory systems whose expression is potentially altered after CO exposure. Gene expression of homer scaffold protein 3 (HOMER3), which negatively regulates specific T-cells, is upregulated [28, 29]. Also, protein tyrosine-protein phosphatase (PTPRC) gene

expression is upregulated following CO exposure, which is involved in signalling of specific immune cells, the B- and T-cells. How CO influences these proteins, whether directly or indirectly, is not known.

Other genes that were altered in expression following CO exposure and related to inflammation are members of the cytochrome P450 family, a large family of haem-containing proteins that are likely to be directly affected by CO. CYP4F2 is directly involved in degrading inflammatory leukotrienes, and was found to be downregulated by CO exposure suggesting a pro-inflammatory effect, which is seen *in vitro* [30] and potentially *in vivo* [31, 32] following CO exposure. CYP2S1 was found to be reduced following CO exposure but it is not known what the precise substrates for CYP2S1 are. Reduced expression of CYP2S1 is known to be associated with increased pro-inflammatory prostaglandin E2 [33] and so may also be involved in an inflammatory process.

4. Cell proliferation/ cell differentiation

It has been reported previously that renal cells exposed to CO *in vitro* show upregulated p21 protein levels [34]. The current RNAseq data indicates that PBMCs might also have higher p21 levels after CO exposure, since the gene that encodes for the RNA binding motif protein 42 (RBM42) is upregulated. RBM42 binds to p21 mRNA and prevents p21 mRNA degradation and through this increases p21 protein levels, although the actual gene expression of p21 might not be directly affected. RBM42 is also involved in the response to reduced cellular ATP levels [35] and interacts with multiple mitochondrial proteins involved in mitochondrial RNA maintenance [36], which are likely to be altered as a consequence of elevated CO. Therefore, both direct p21 mediated or indirect effects of CO exposure on mitochondria might be involved.

P21 mediates cell cycle arrest and may therefore have different effects outside of energy metabolism [37]. P21 is anti-apoptotic and facilitates cellular DNA repair, but is also involved in cellular differentiation, enabling cells to cease cell division and alter morphology and function. The current RNAseq data indicates the differentiation system (the cytoskeleton) that allows cells to change their shape, is activated after CO treatment. There is the potential for upregulation of the microtubule associated protein 1B (MAP1B) and the canonical delta like Notch ligand 3 (DLL3) occurs. DLL3 can activate Notch signalling pathways, and Notch signalling is involved in dendritic cell differentiation [38], with dendritic cells being intimately involved in immune system signalling. Furthermore, DLL3, via Notch, Wnt and GSK3β promote cytoskeletal rearrangements through MAP1B [39] linking this system. Aspects of the differentiation system do overlap with the system involved in leukocyte extravasation and therefore changes may also be directly related to CO effects.

5. Leukocyte extravasation

The most significantly affected pathway following CO exposure identified through IPA gene expression analysis and the second most significantly altered pathway using only protein coding transcripts involved leukocyte migration across blood vessels [40]. Expression of neutrophil cytosolic factor 4 (NCF4) transcripts was downregulated after CO treatment (Table 1). NCF4 is part of the NADPH oxidase enzyme complex that interacts with NCF2 (p67-PHOX) and CYBB (p91-PHOX), a haem containing protein that is directly influenced by CO [41, 42]. NADPH oxidase produces reactive oxygen species (ROS) which activate matrix-metallopeptidases (MMPs) [43] resulting in extracellular matrix degradation. As the extracellular matrix of the endothelial cells break down, lymphocytes migrate to tissues through the leukocyte extravasation process, where immune cells from the blood enter the tissue [40]. Previous data obtained in chondrocytes suggest, that CO inhibits NADPH oxidase,

leading to downregulation of matrix-metallopeptidase 1 and matrix-metallopeptidase 9 (MMP1, MMP9) [43, 44]. This is similar to the findings in the current study. Whilst downregulation of genes encoding MMP-9 and MMP-1 occurs following CO exposure, upregulation of MMP-10 also occurs. The changes in MMP- and NOX-associated genes was also accompanied by downregulation of the microtubule associated cell migration factor (BCAS3), which regulates endothelial cell migration by mediating reorganization of the actin cytoskeleton. This would normally permit movement of leukocytes through capillary endothelia into tissues, however CO exposure appears to reduce this. This would correlate with the use of CORMs which have been shown to reduce leukocyte and endothelial cell interaction [45, 46]. Last, the expression of the vascular endothelial growth factor A (VEGFA) was seen to be altered after CO treatment. However, half of our participants responded with strong upregulation while the other half had a strong downregulation. VEGFA expression is known to be altered by CORM and CO treatment and whilst changes may relate to cellular anoxia [23, 47] and be cell type specific [48], this would have to be confirmed at the protein level.

Discussion

The results using RNAseq analysis point to CO having several interrelated effects on gene expression in PBMC. Using the in vitro approach, 22 changes in gene expression were identified which relate to CO exposure, including targets which could directly interact with CO, and indicate the potential for future biomarker identification. CO appears to cause cell energy depletion, probably by affecting the mitochondrial respiratory chain and specifically complex IV. This energy depletion leads to activation of cellular energy sensors such as AMPK. This CO effect on haem proteins also causes reductions in nitric oxide (NO) signalling and

reactive oxygen species (ROS) signalling. Together, this combined NO and ROS effect reduces vascular reactivity and also the ability of leukocytes to adhere to blood vessels through alterations in leukocyte shape and reduced expression of extracellular matrix degrading enzymes. This predominantly appears to be through CO affecting haem containing enzyme systems within lymphocytes which is in line with the known effects of CO.

In this first phase, we have determined the gene expression changes that occur in lymphocytes following 200ppm exposure to CO. As a component of subsequent studies, we would need to validate these gene expression changes in a larger series of lymphocyte samples and further investigate protein changes.

What is unclear is how long these identified gene expression changes are likely to last following removal of CO exposure, the implications this poses to: the strength of an identified new blood biomarker, and the CO exposed patient's successful treatment, recovery and long-term health. Initially, this will require exposure and recovery experiments where cells are exposed to CO at different levels. Gene expression will then be determined at set time periods once CO exposure ceases.

A longer-term plan will be to use the data to identify specific protein changes as a marker of CO exposure in a future pilot clinical study where CO exposure is suspected. A large study between exposed and non-exposed tissues and correlation between measured CO, HbCO and other variables (such as estimated time of exposure, exposure duration etc.) would be used to determine if the proposed biomarkers are suitable for clinical use.

For individuals attending Accident and Emergency Departments, a pilot study such as this would aim to evaluate the expression of the genes or proteins found in patients' routine blood samples. Such a study could be undertaken at a single hospital site and over a prolonged period (1-2 years), for people attending with non-specific symptoms, where determination of

lymphocyte protein levels (using an ELISA type assay) and blood CO, HbCO, pH, pO_2/pCO_2, along with clinical parameters (Glasgow Coma Score, Heart Rate, Blood Pressure etc.) would be established following a standardised triage protocol. These protein marker measures could be related to clinical outcome and longer term health measures. We will aim to determine if such a study is possible locally, or if collaborative studies using individuals with known CO exposure could be organised.

Conclusion

Exposure of normal peripheral blood lymphocytes to 200ppm carbon monoxide is associated with changes in gene expression.

Changes in gene expression can be mediated through the known interaction of CO with haem containing proteins.

These gene expression changes and any associated protein changes are suitable for further development as CO exposure biomarkers.

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