

# Accepted Manuscript

Hypoxia causes increased monocyte nitric oxide synthesis which is mediated by changes in dimethylarginine dimethylaminohydrolase 2 expression in animal and human models of normobaric hypoxia

S. Lambden, D. Martin, K. Vanezis, B. Lee, James Tomlinson, S. Piper, O. Boruc, M. Mythen, J. Leiper

PII: S1089-8603(16)30074-X

DOI: [10.1016/j.niox.2016.06.003](https://doi.org/10.1016/j.niox.2016.06.003)

Reference: YNIOX 1577

To appear in: *Nitric Oxide*

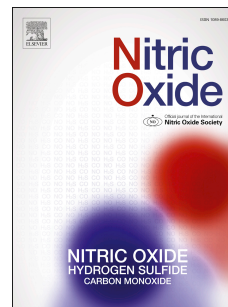
Received Date: 2 February 2016

Revised Date: 2 June 2016

Accepted Date: 15 June 2016

Please cite this article as: S. Lambden, D. Martin, K. Vanezis, B. Lee, J. Tomlinson, S. Piper, O. Boruc, M. Mythen, J. Leiper, Hypoxia causes increased monocyte nitric oxide synthesis which is mediated by changes in dimethylarginine dimethylaminohydrolase 2 expression in animal and human models of normobaric hypoxia, *Nitric Oxide* (2016), doi: 10.1016/j.niox.2016.06.003.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



**Hypoxia causes increased monocyte nitric oxide synthesis which is mediated by changes in Dimethylarginine Dimethylaminohydrolase 2 expression in animal and human models of normobaric hypoxia**

S Lambden<sup>#</sup>, D Martin<sup>\*</sup>, K Vanezis<sup>#</sup>, B Lee<sup>#</sup>, James Tomlinson<sup>#</sup>, S Piper<sup>#</sup>, O Boruc<sup>#</sup>, M Mythen<sup>\*</sup>, J Leiper<sup>#</sup>

<sup>#</sup>Nitric Oxide Signalling Group, Clinical Sciences Centre, MRC, Hammersmith Hospital, London, UK

<sup>\*</sup>Centre for Altitude, Space and Extreme Environment Medicine, UCL, London, UK

**Corresponding Author:**

Dr J Leiper

Nitric Oxide Signalling Group, Clinical Sciences Centre, Medical Research Council, Hammersmith Hospital, London, W120NN, UK

james.leiper@csc.mrc.ac.uk

Telephone: 0208 3833419

Fax: 0208 3838577

**Abstract**

**Background** Tissue hypoxia is a cardinal feature of inflammatory diseases and modulates monocyte function. Nitric oxide is a crucial component of the immune cell response. This study explored the metabolism of the endogenous inhibitor of nitric oxide production asymmetric dimethylarginine (ADMA) by monocyte dimethylarginine dimethylaminohydrolase 2 (DDAH2), and the role of this pathway in the regulation of the cellular response and the local environment during hypoxia.

**Methods** Peritoneal macrophages were isolated from a macrophage-specific DDAH2 knockout mouse that we developed and compared with appropriate controls. Cells were exposed to 3% oxygen followed by reoxygenation at 21%. Healthy volunteers underwent an 8h exposure to normobaric hypoxia with an inspired oxygen percentage of 12%. Peripheral blood mononuclear cells were isolated from blood samples taken before and at the end of this exposure.

**Results** Intracellular nitrate plus nitrite (NO<sub>x</sub>) concentration was higher in wild-type murine monocytes after hypoxia and reoxygenation than in normoxia-treated cells (mean(SD) 13.2(2.4) vs 8.1(1.7) pmols/mg protein,  $p=0.009$ ). DDAH2 protein was 4.5-fold (SD 1.3) higher than in control cells ( $p=0.03$ ). This increase led to a 24% reduction in ADMA concentration, 0.33(0.04) pmols/mg to 0.24(0.03),  $p=0.002$ ). DDAH2-deficient murine monocytes demonstrated no increase in nitric oxide production after hypoxic challenge. These findings were recapitulated in a human observational study. Mean plasma NO<sub>x</sub> concentration was elevated after hypoxic exposure (3.6(1.8)  $\mu$ M vs 6.4(3.2),  $p=0.01$ ), which was associated with a reduction in intracellular ADMA in paired samples from 3.6(0.27) pmols/mg protein to 3.15(0.3) ( $p<0.01$ ). This finding was associated with a 1.9-fold(0.6) increase in DDAH2 expression over baseline ( $p=0.03$ ).

**Discussion** This study shows that in both human and murine models of acute hypoxia, increased DDAH2 expression mediates a reduction in intracellular ADMA concentration which in turn leads to

elevated nitric oxide concentrations both within the cell and in the local environment. Cells deficient in DDAH2 were unable to mount this response.

### Short Title

DDAH2 regulates immune cell nitric oxide synthesis in acute hypoxia.

### Key words

Nitric Oxide

Hypoxia

Dimethylarginine Dimethylaminohydrolase

Asymmetric Dimethylarginine

### Abbreviations

Ddah2 - Dimethylarginine Dimethylaminohydrolase 2 gene

DDAH2 - Dimethylarginine Dimethylaminohydrolase 2 protein

Ddah2<sup>flox/flox</sup> – LoxP positive Cre negative litter mate controls

Ddah2<sup>M $\Phi$</sup>  - Ddah2flox/flox LysM-cre Monocyte specific DDAH2 knockout animals

PBMC - peripheral blood mononuclear cells

PRMT - Protein Arginine Methyltransferases

ADMA- asymmetric dimethylarginine

SDMA- symmetric dimethylarginine

L-NMMA- monomethyl-L-arginine

DDAH- dimethylarginine dimethylaminohydrolase

NO- nitric oxide

NOS- nitric oxide synthase

eNOS – endothelial nitric oxide synthase

iNOS- inducible nitric oxide synthase

LC-MS/MS - liquid chromatographic assay with tandem mass spectrometric detection

NOx- nitrate and nitrite

FiO<sub>2</sub> - Fraction of inspired oxygen

## 1. Introduction

Hypoxia is a cardinal feature of critical illness of many aetiologies<sup>1</sup>. It arises as a consequence of both increased metabolic demand<sup>2</sup> and also changes in the microcirculation that impair delivery of oxygen to the tissues<sup>3</sup>. Pro- and anti-inflammatory activation is also a major component of the response to critical illness<sup>4,5</sup>. Mediated in large part by immune cells<sup>6</sup>, the interaction between hypoxia and monocytes has been shown to play a role in the immune response<sup>7</sup> and may give insights to the pathological responses seen in some patients in whom exaggerated systemic inflammation leads to organ failure and death.

Nitric oxide (NO) is an important regulator of a broad range of physiological processes<sup>8</sup>. In addition, it plays an important role in the response to infection<sup>9-12</sup>. NO synthesised in response to infection has diverse functions including bactericidal and phagocytic function by monocytes<sup>13</sup> and the regulation of the macro<sup>14</sup> and microcirculation<sup>15,16</sup>. The interaction between nitric oxide signalling and hypoxia is critically important in regulating the immune response to infection<sup>17</sup>.

Synthesised by the two constitutive and one inducible isoforms of nitric oxide synthase (NOS)<sup>18</sup>, NO production is regulated in part by the methylarginines asymmetric dimethylarginine (ADMA) and Monomethyl-L-arginine (L-NMMA)<sup>19</sup>.

Methylarginines are produced by post translational methylation of certain arginine residues in proteins by the family of Protein Arginine Methyltransferases (PRMTs). In mammals there are three methylarginine species, ADMA, symmetrical dimethylarginine (SDMA) and L-NMMA. ADMA and L-NMMA competitively inhibit arginine binding to NOS and reduce NO production<sup>20,21</sup>. SDMA does not inhibit the activity of the NOS enzymes<sup>22</sup>. Elevated circulating concentrations of ADMA have been associated with poor outcomes in a variety of conditions including cardiovascular disease<sup>23,24</sup>, metabolic disorders<sup>25</sup> and sepsis<sup>26</sup>.

ADMA is metabolised by dimethylarginine dimethylaminohydrolase (DDAH) to dimethylamine and citrulline<sup>27</sup>. The two isoforms of DDAH have different tissue distributions<sup>27,28</sup> which lead to differing roles in both basal and pathological states. DDAH1 knockout or pharmacological inhibition leads to a hypertensive phenotype<sup>29</sup> and is protective in animal models of septic shock<sup>30</sup> whereas knockout of DDAH2 leads to minimal cardiovascular disturbance but profound immune dysfunction and excess mortality in sepsis<sup>31</sup>.

Recently we have demonstrated that in pulmonary endothelial cells hypoxia induces miRNA-mediated reduction in DDAH1 expression that results in increased ADMA concentration and reduced nitric oxide synthesis that is associated with pulmonary hypertension<sup>32</sup>. The role of DDAH2 – the only isoform found in immune cells – in regulating the synthesis of NO in response to acute hypoxia has not been elucidated. Here we examine for the first time the impact of normobaric hypoxia on NO synthesis, ADMA level and DDAH2 expression in murine models and human healthy volunteers. Our data provide novel insights into the pathways by which hypoxia regulates NO synthesis following acute hypoxic stress.

## 2. Materials and Methods

### Animal Models

#### *Husbandry*

Animals were housed in accordance with Home Office guidelines and procedures were performed under Project Licence (70/7049) and Personal License (76/26000). Throughout the care and

experimental phases animals were kept in standard environmental conditions with free access to food and water.

#### *Development of Genetically modified animals*

DDAH2<sup>flox/flox</sup> LysMCre animals employed the LoxP Cre recombinase model with tissue specificity delivered via Cre expression at the Murine M Lysozyme promoter using a previously established technique<sup>33</sup>. We have previously shown that immune cells express only DDAH2 but not DDAH1<sup>28, 31</sup>. See supplementary material for further details.

#### *Isolation of resident peritoneal macrophages*

Isolation of primary macrophages was undertaken using a peritoneal washout technique. Further details can be found in the supplementary materials.

#### *Hypoxic Chamber incubation*

To determine the impact of subacute hypoxia on isolated primary macrophages Cells were incubated for varied amounts of time in a sealed hypoxic incubator at 92% nitrogen, 3% oxygen and 5% CO<sub>2</sub> at 37°C. Culture medium (High Glucose DMEM with Glutamine) was placed in the chamber at least 12 hours prior to experiment in order to equilibrate medium partial pressure of oxygen with that of the hypoxic atmosphere.

#### *Human Normobaric Hypoxia Study*

Ethical Approval was received from the University College London Ethical review panel on 4th March 2014 ref: 2416.001 for conduct of a prospective observational study into the effects of acute normobaric hypoxia on endogenous regulators of nitric oxide synthesis on healthy volunteers.

#### *Normobaric Hypoxia*

In order to study the relationship between acute hypoxia and methylarginine regulation, a healthy volunteer study was designed that explored the effect of moderate normobaric hypoxia on plasma methylarginine concentrations, monocyte DDAH2 expression and indices of haemodynamic function. In brief, healthy male volunteers aged between 18 and 60 were recruited and consent obtained. Following baseline haemodynamic and clinical observations, patients underwent phlebotomy and samples of blood were taken for plasma separation and isolation of peripheral blood mononuclear cells (PBMCs). Cardiovascular assessment was undertaken before entry to the hypoxic chamber.

Participants then underwent an 8 hour exposure to 12.0% oxygen in a hypoxic chamber with continuous observation of patient and environmental conditions. At 20 minutes after chamber entry and after each successive hour of hypoxic exposure, volunteers underwent haemodynamic and oxygenation assessments and completed a Lake Louise acute mountain sickness assessment modified to exclude the sleep assessment. This ensured that features of acute mountain sickness could be detected early and participants removed from the hypoxic chamber in this eventuality. Details of the hypoxic chamber, monitoring and safety can be found in the supplementary materials.

Following completion of the eight hour exposure period, participants underwent a repeat cycle of testing including blood sampling, observations and cardiovascular assessment. Samples from both the pre and post exposure phases were prepared and stored immediately upon collection at -80°C.

#### *Isolation of peripheral blood mononuclear cells*

Blood collected from the patient was diluted with twice the volume of balanced salt solution and layered carefully over an equal volume of Ficoll-Paque Premium (GE Life Sciences, UK) separation medium to avoid mixing of the two liquids.

The sample was centrifuged at 400g at 18-20°C for 40mins in a swinging bucket centrifuge without break to facilitate separation of the sample into plasma/platelets, monocyte and erythrocyte/granulocyte layers

Following separation, the plasma portion of the separated blood was removed using manual pipetting and stored for later analysis. The mononuclear cell layer was removed without disruption of the Ficoll Medium and resuspended in RLT buffer for subsequent mRNA analysis or phosphate buffered saline with protease inhibitor for protein quantification and western blotting

#### *Plasma sample preparation for analysis*

Whole blood was collected in EDTA at 1.5mg/ml for analysis and stored on ice for subsequent preparation. Within 60mins of collection, the cells were removed from plasma by centrifugation for 10 minutes at 1,000-2,000g. Centrifugation for 15 minutes at 2,000g depletes platelets in the plasma sample. The separated plasma was stored separately at -80 °C pending subsequent analysis.

#### Sample preparation and analysis

*Measurement of nitric oxide concentration in biological tissues:* The Sievers NOA 280i (GE Analytical Instruments) was used to measure nitrate + nitrite (NO<sub>x</sub>) content of biological samples using a chemiluminescent technique. Further details can be found in the supplementary materials. Nitric oxide was re-derived from nitrites and nitrates (stable end-products of NO activity) by reduction in heated vanadium chloride. NO was detected and quantified in a gas-phase chemiluminescent reaction with ozone which emits in the red/infra-red spectrum. Tissue homogenate or plasma samples underwent protein extraction using methanol precipitation. The supernatant from the samples were run in triplicate, averaged and NO quantified by calculation against a standard curve of sodium nitrate (0-200µM).

#### *Preparation of samples for Mass Spectrometric analysis*

Deuterium<sup>7</sup> labelled ADMA was used as an internal labelled control. A 50µL sample of supernatant from cell lysis or conditioned medium was collected and a known concentration of labelled deuterium standard was added. Following protein extraction with methanol the solution underwent centrifugation at 16000g for 10minutes. The sample was then dried and the residue re-suspended in mobile phase (0.1% formic acid) for analysis. A standard curve of ADMA samples of 10 known concentrations was prepared (0 to 100µM).

#### Statistics

Statistical analysis was performed using the Prism software package (GraphPad Inc, CA, USA). Normally distributed data was analysed using a t test or Analysis of Variance (ANOVA) with Bonferroni post-test comparison of groups as appropriate. Non parametric data was analysed using a Mann Whitney U test. In human studies, pre and post intervention samples are compared using paired analyses. All values were expressed as mean ± (SD). Significance was accepted for values of p < 0.05.

### 3. Results

#### *Animal models of hypoxia*

The synthesis of nitric oxide by isolated wild type primary murine monocytes exposed to hypoxic and normoxic conditions was determined. Intracellular nitrate plus nitrite concentration was higher in wild-type murine monocytes after hypoxia and reoxygenation than in normoxia-treated cells (mean(SD)) 13.2(2.4) pmols/mg protein vs 8.1(1.7) (p=0.009)(Fig 1A), and accumulation of extracellular nitric oxide (Nitrate+Nitrite) increased after hypoxic challenge (13.7(3.1) $\mu$ M vs 1.9(0.18), p=0.002)(Fig 1B). This was associated with induction of the inducible form of nitric oxide synthase in monocytes, (mean (SD)) 4.0 (1.3) fold increase in iNOS mRNA (p=0.01) (Fig 1C). DDAH2 protein was 4.5-fold (SD 1.3) higher than in control cells (p=0.03) (Fig 1D) and Ddah2 mRNA was also increased by (mean(SD)) 3.6(0.12) fold over control cells (Fig 1E). This increase was associated with a 24% reduction in ADMA concentration (mean(SD) 0.33(0.04)pmols/mg to 0.24(0.03), p=0.002)(Fig 1F). There was no significant difference observed in intracellular L-arginine (Fig S1A, p=0.597) or L-NMMA (Fig S1B, p=0.74) concentrations following hypoxic incubation.

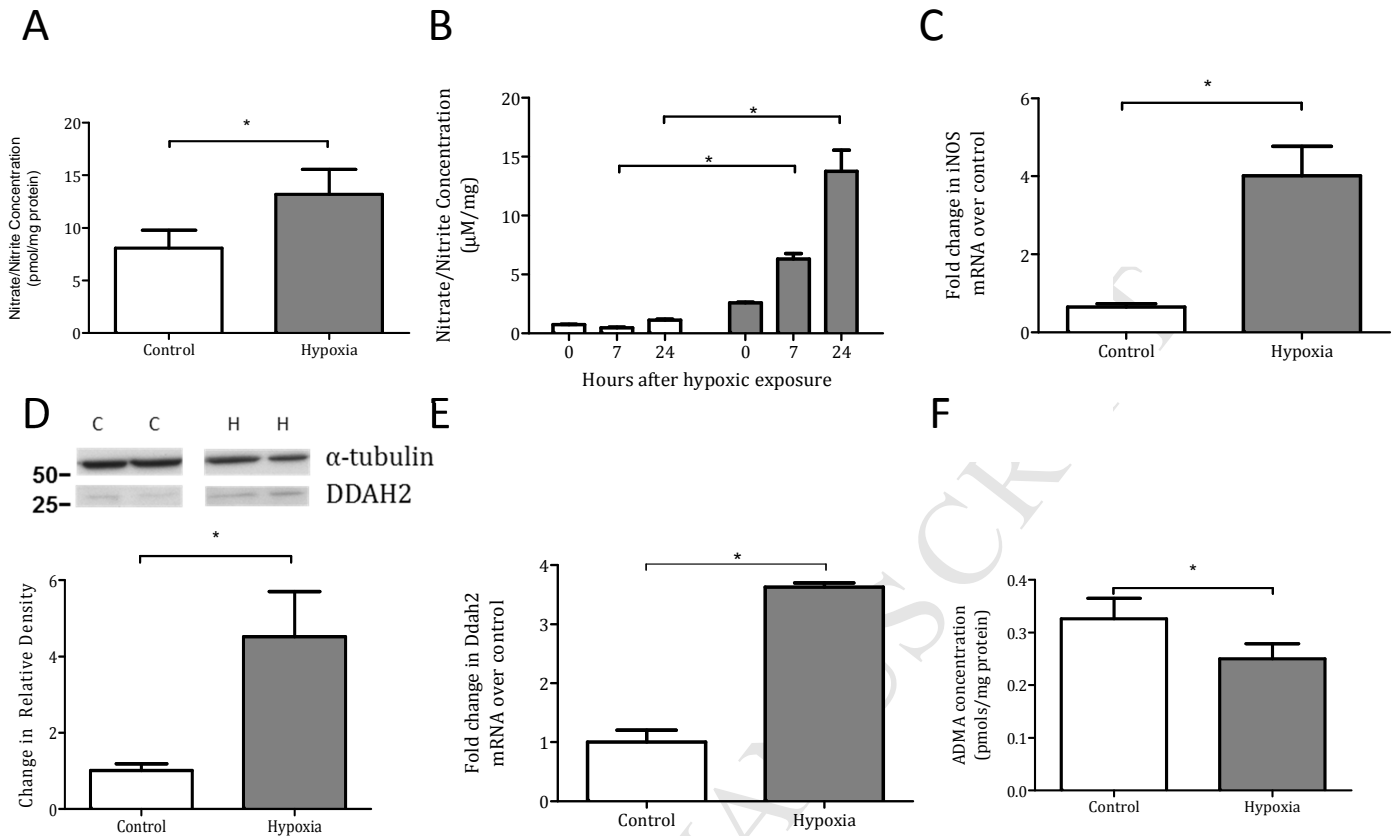


Figure 1: The impact of hypoxia on endogenous regulators of nitric oxide (NO) synthesis in primary peritoneal murine macrophages. A: Cell lysate NO<sub>x</sub> concentration at 24 hours of reoxygenation following 12 hours of hypoxic exposure (White bars: control cells not exposed to hypoxia, grey bars: hypoxia-treated cells, n=6 per group, \* p<0.05, NO<sub>x</sub> concentration in culture medium corrected to cell lysate protein concentration at the termination of the experiment). B: Serial measurements of the accumulation of nitrate/nitrite (NO<sub>x</sub>) in culture medium during reoxygenation at a FiO<sub>2</sub> of 21% following hypoxic exposure for 12 hours at a FiO<sub>2</sub> of 3%. (White bars: control cells not exposed to hypoxia, grey bars: hypoxia-treated cells, n=6 per group, \* p<0.05, NO<sub>x</sub> concentration in culture medium corrected to cell lysate protein concentration at the termination of the experiment). C: Quantitative PCR of inducible nitric oxide synthase mRNA expression in resident peritoneal macrophages from wild type (C57BL/6) mice: from control cells (white bar) and those exposed to 12 hours of hypoxia (grey bar). (n=6 per group, \* p<0.05). D: Change in DDAH2 protein expression following 12 hour hypoxic exposure. Representative image of control (C) vs hypoxia (H) treated cells and quantification of n=6 per group, control (white) and hypoxia-treated (grey bar) (\* p<0.05). All bars represent mean (+SEM). E: Quantitative PCR of *Ddah2* mRNA expression in resident peritoneal macrophages from wild type (C57BL/6) mice: from control cells (white bar) and those exposed to 12 hours of hypoxia. (n=6 per group, \* p<0.05). F: Change in cell lysate asymmetric dimethylarginine (ADMA) concentration in murine primary peritoneal macrophages in control (white) and hypoxia-treated (grey) cells, corrected for lysate protein concentration (n=6 per group, \* p<0.05).

Successful knockout of DDAH2 from primary peritoneal macrophages isolated from monocyte-specific *Ddah2* knockout mice (*Ddah2*<sup>flox/flox</sup> *LysM-cre*; *Ddah2*<sup>MΦ</sup>) was demonstrated by western blot and qPCR (Fig 2A). Comparison of NO<sub>x</sub> synthesis before and after hypoxic exposure was undertaken in peritoneal monocytes from the *Ddah2*<sup>MΦ</sup> mice and their *Ddah2*<sup>flox/flox</sup> litter mate controls. Cells deficient in DDAH2 displayed reduced intracellular NO<sub>x</sub> concentrations at baseline compared to controls, mean (SD) 5.15(0.61) vs 7.7(0.87) µM/mg protein (p=0.014) (Fig 2B). Following hypoxic exposure, *Ddah2*<sup>flox/flox</sup> cells displayed significant induction of NO<sub>x</sub> synthesis (11.6(0.94) µM/mg



protein ( $p < 0.01$ ). By contrast, peritoneal macrophages from  $Ddah2^{M\Phi-}$  mice displayed no significant increase in intracellular NOx following hypoxic exposure ( $p = 0.10$ ) (Fig 2B). This was associated with changes in intracellular ADMA in  $Ddah2^{flox/flox}$  mice; cells showed a similar decrease in ADMA concentration to that seen in wild type peritoneal macrophages ( $p = 0.01$ ) (Fig 2C). In contrast to this,  $Ddah2^{M\Phi-}$  mice displayed a trend to increased ADMA level following hypoxia with concentrations of  $0.13(0.04) \mu\text{M}/\text{mg}$  protein prior to exposure and of  $0.28(0.03) \mu\text{M}/\text{mg}$  protein following hypoxic treatment ( $p = 0.06$ ) (Fig 2C). No significant differences were observed in L-arginine (Fig 2D) or SDMA (Fig 2E) concentrations between the monocytes derived from  $Ddah2^{M\Phi-}$  mice and their  $Ddah2^{flox/flox}$  litter mate controls in their response to hypoxia.

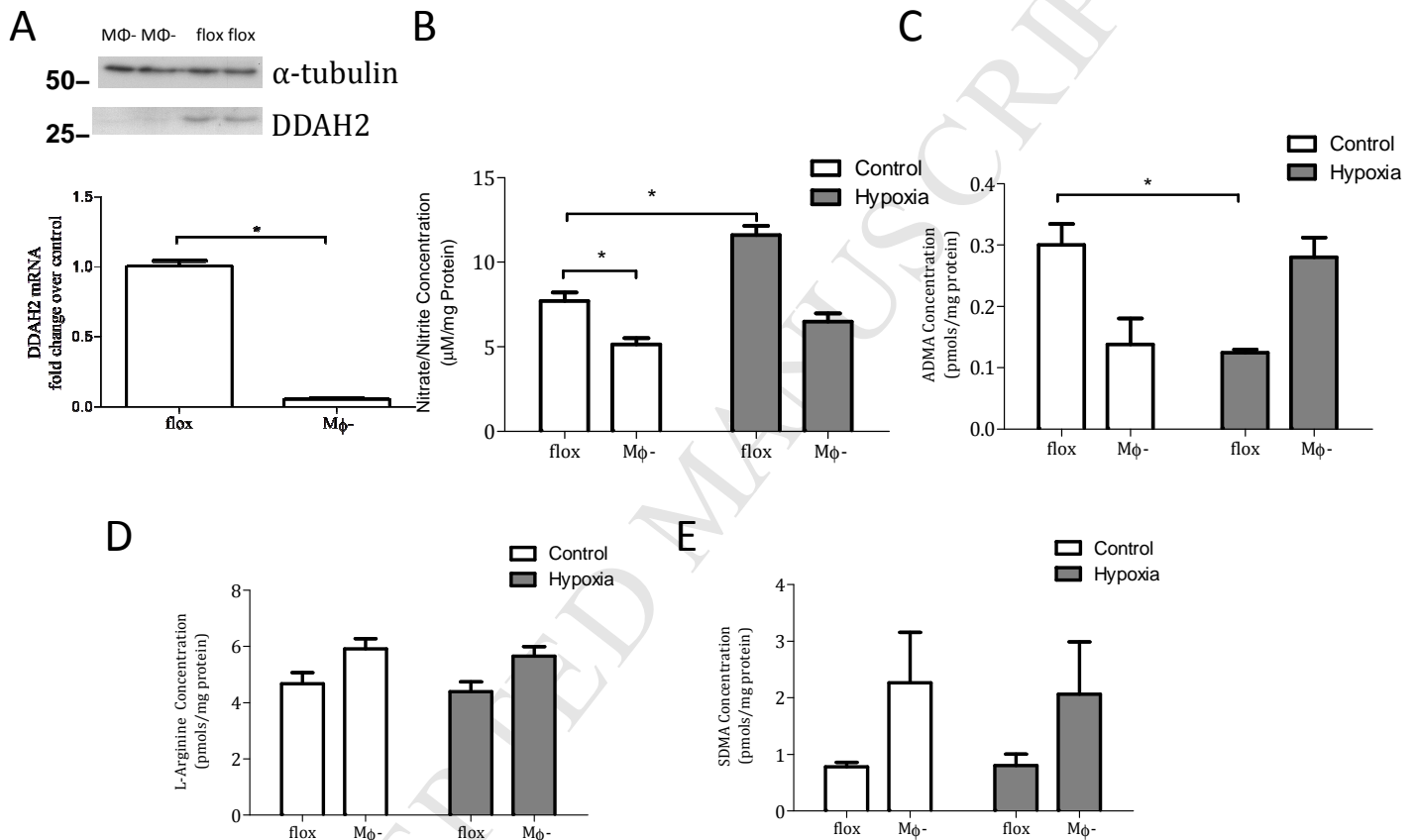


Figure 2: The impact of *Ddah2* knockout on endogenous regulators of NO synthesis in response to hypoxic exposure. A: Demonstration of *Ddah2* mRNA and protein knockout in peritoneal macrophages derived from *Ddah2* macrophage-specific knockout mice ( $Ddah2^{M\Phi-}$ ) or littermate flox/flox controls ( $Ddah2^{flox/flox}$ ). Representative western blot and quantitative PCR analysis of *Ddah2* mRNA expression ( $n = 6$ ,  $* p < 0.01$ ). Panels B to E: analysis of  $Ddah2^{M\Phi-}$  or  $Ddah2^{flox/flox}$ -derived macrophages following 12 hour hypoxic exposure and 24 hour reoxygenation. White bar: control cells, grey bar: hypoxia-treated cells. B: Change in intracellular NOx concentration (corrected for cell lysate protein concentration) ( $n = 6$  per group,  $* p < 0.05$ ). C: Change in intracellular ADMA concentration (corrected for cell lysate protein concentration) ( $n = 6$  per group,  $* p < 0.05$ ). D: Change in intracellular L-arginine concentration (corrected for cell lysate protein concentration) ( $n = 6$  per group). E: Change in intracellular SDMA concentration (corrected for cell lysate protein concentration) ( $n = 6$  per group). All bars represent mean (+SEM).

*Human Hypoxia studies*

Of fifteen participants, four exhibited subjective symptoms of acute mountain sickness with modified Lake Louise score of between 1 and 3 without significant objective symptoms. One volunteer developed significant nausea during the second half of the experimental period and was withdrawn from the hypoxic chamber. After a period of observation following extraction from the chamber there were no subjective or objective sequelae in this volunteer and follow up at 24 hours revealed no residual symptoms. The participant was excluded from the study and further analysis. Of the participants that completed the study, nine physiological, plasma and paired monocyte samples collection and analysis, a further three underwent plasma and physiological analysis only.

Hypoxic exposure in the fifteen participants led to an immediate reduction in arterial oxygenation to a mean(SD) value of 86(2.0)% which was sustained throughout the period of hypoxic chamber exposure (Fig 3A). No significant differences in indices of cardiovascular function were detected by non-invasive assessment of cardiovascular function, although a trend towards reduced systemic vascular resistance with compensatory increase in cardiac output was observed in the post prandial period (Fig 3B-F). Following completion of the hypoxia study protocol, participants were removed from the hypoxic chamber and arterial oxygen saturations assessed after 5mins of exposure to the normoxic environment. At this time, mean(SD) oxygen saturations were 97(1.0)% and heart rate was 71(10) bpm.

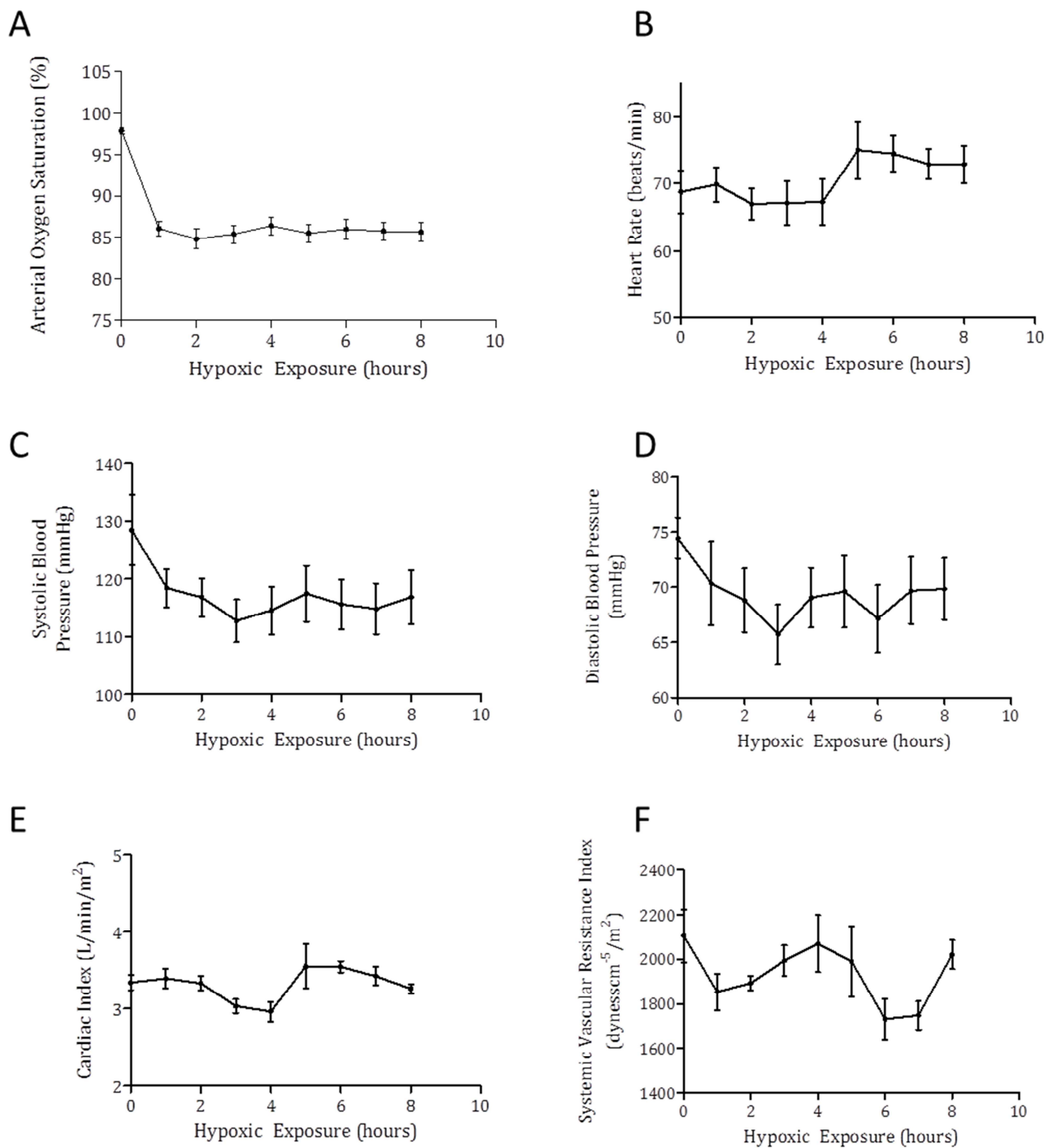


Figure 3: The impact of normobaric hypoxia on healthy volunteers exposed to an eight hour stimulus at 12% FiO<sub>2</sub>. A: The impact of exposure to 12% FiO<sub>2</sub> on healthy volunteer peripheral arterial oxygen saturations measured at rest prior to entrance (0). Subsequent regular measurements are summarised hourly. (n=12) B: The impact of exposure to 12% FiO<sub>2</sub> on healthy volunteer heart rate measured at rest prior to entrance (0). Subsequent regular measurements are summarised hourly. (n=12). C&D: The impact of exposure to 12% FiO<sub>2</sub> on healthy volunteer systolic (C) and diastolic (D) blood pressure measured at rest prior to entrance (0) and following entry to the hypoxic chamber. (n=12) E&F: Change in cardiac index (E) and systemic vascular resistance index (F) following exposure to normobaric hypoxia at a FiO<sub>2</sub> of 12% (n=12).

Eight hour hypoxic exposure led to a significant increase in plasma NO<sub>x</sub> concentration (Mean (SD)) 3.6 (1.8) μM vs 6.4 (3.2) μM p=0.01 (Fig 4A). This was associated with a significant reduction in plasma ADMA concentration from 0.42 (0.12) μM at baseline, to 0.29(0.05) μM after exposure (p<0.01) (Fig 4B). There were no significant differences in plasma arginine, SDMA or L-NMMA concentration (Fig S2A-C respectively). As a consequence of the reduction in ADMA concentration, plasma arginine:ADMA ratio was significantly reduced following hypoxia (p<0.01)(Fig 4C). Intracellular concentrations of methylarginines in peripheral blood mononuclear cells (PBMC) displayed a similar pattern with significant reduction in ADMA in paired samples from 3-6 pmols/mg

protein [0.27] to 3.15 [0.3] ( $p=0.0009$ )(Fig 4D) but no significant variation in intracellular arginine, SDMA or L-NMMA concentration (Fig S2D-F). Similarly, the intracellular Arginine:ADMA ratio was reduced ( $p<0.01$ ) (Fig 4E). Consistent with previous observations of the relationship between plasma and monocyte ADMA concentrations<sup>34</sup>, at baseline there was no apparent correlation ( $r^2=0.08$ ,  $p=0.44$ ) (Fig S2G). However following hypoxia, a positive correlation between PBMC and extracellular ADMA level was observed ( $r^2=0.72$ ,  $p=0.01$ ) (Fig 4F).

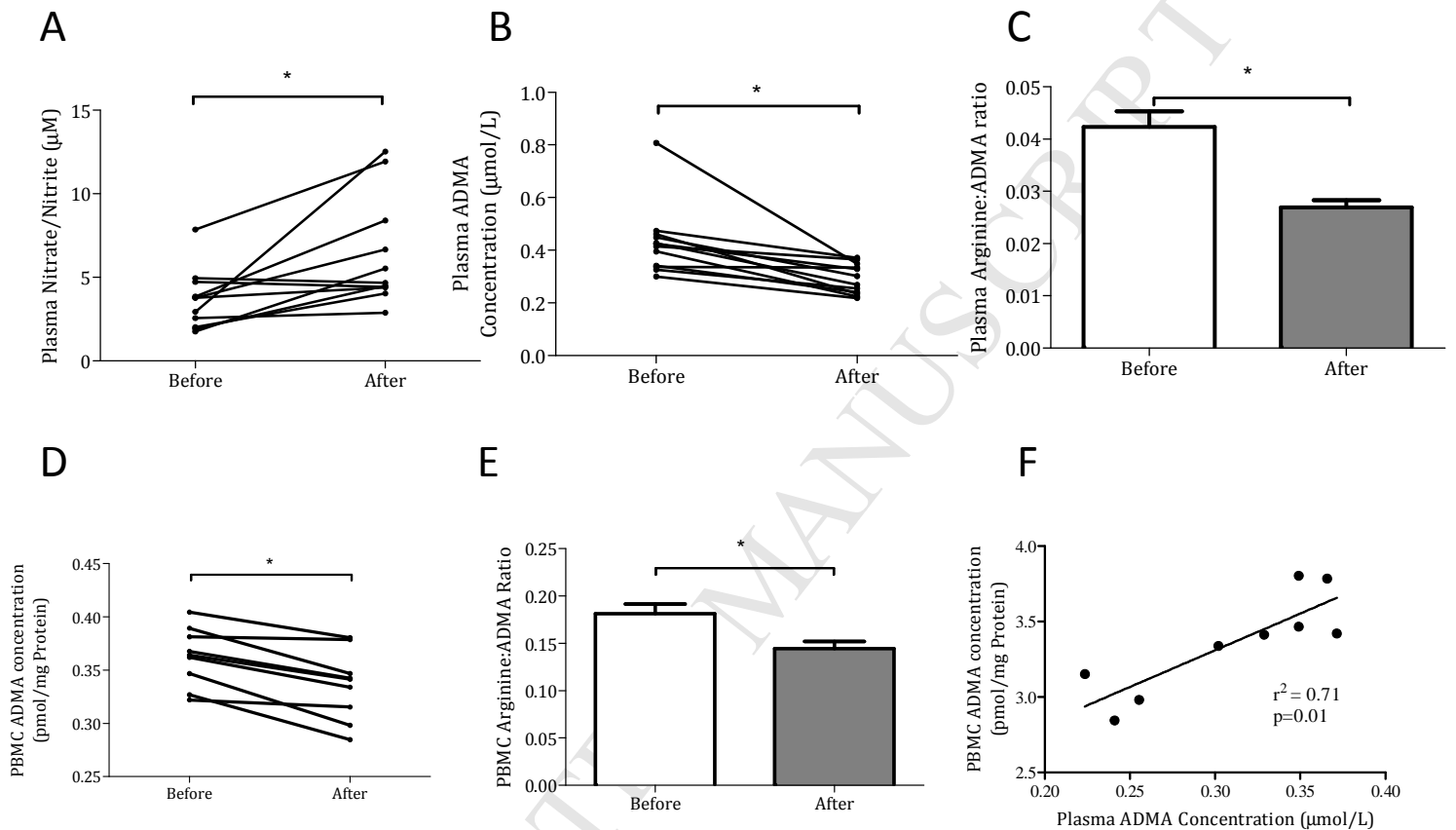


Figure 4: The impact of normobaric hypoxia at a  $FiO_2$  of 12% on nitric oxide synthesis and methylarginine concentrations in plasma and peripheral blood mononuclear cells (PBMC) from a prospective observational healthy volunteer study. A: Change in plasma Nitrate/Nitrite (NOx) following 8 hour hypoxic exposure, each line represents plasma NOx concentration before and at the end of the exposure period, ( $n=12$ , \*  $p<0.05$ ). B: Change in plasma ADMA following 8 hour hypoxic exposure, each line represents plasma concentration before and at the end of the exposure period, ( $n=9$ , \*  $p<0.05$ ). C: Change in plasma L-arginine:ADMA ratio following 8 hour hypoxic exposure ( $n=9$ , \*  $p<0.05$ ). D: Change in PBMC ADMA concentration following 8 hour hypoxic exposure, each line represents intracellular concentration, corrected for cell lysate protein level before and at the end of the exposure period, ( $n=9$ , \*  $p<0.05$ ). E: Change in PBMC L-arginine:ADMA ratio following 8 hour hypoxic exposure, corrected for cell lysate protein level before and at the end of the exposure period, ( $n=9$ , \*  $p<0.05$ ). F: The relationship between PBMC (corrected for PBMC lysate protein) and plasma ADMA concentrations following 8 hour hypoxic exposure ( $n=9$ ). All bars represent mean (+SEM).

In human subjects, the increase in NO<sub>x</sub> synthesis was not mediated by increased eNOS expression in PBMCs (Mean (SD)) change over baseline 0.68(0.28) fold ( $p=0.09$ )(Fig 5A). There was no discernible iNOS mRNA and iNOS protein was not detectable in human PBMCs (data not shown). Reduction in monocyte ADMA was associated with an increase in DDAH2 mRNA expression (Mean (SD) increase of 1.9(0.6) fold over baseline ( $p=0.03$ ) (Fig 5B) DDAH2 protein (Mean (SD) fold increase 2.5(0.94,  $p=0.034$ )(Fig 5C).

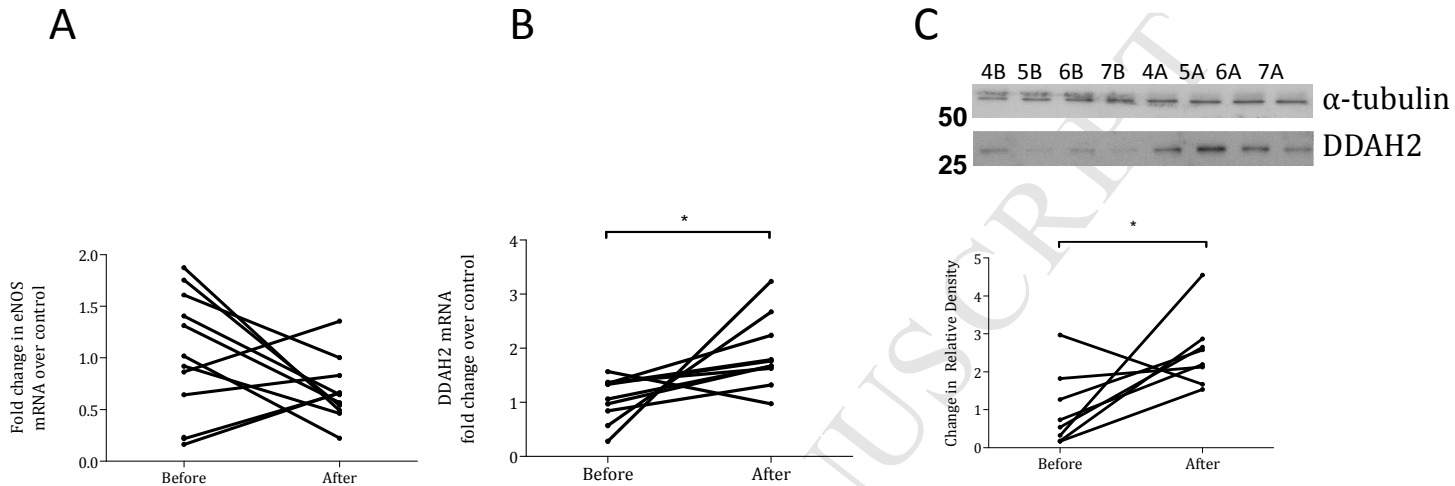


Figure 5: Impact of normobaric hypoxic exposure on regulators of nitric oxide synthesis in human peripheral blood mononuclear cells. A: Change in eNOS mRNA following 8 hour hypoxic exposure, each line represents eNOS mRNA expression before and at the end of the exposure period ( $n=10$ , \*  $p<0.05$ ). B: Change in DDAH2 mRNA following 8 hour hypoxic exposure, each line represents DDAH2 mRNA expression before and at the end of the exposure period, ( $n=10$ , \*  $p<0.05$ ). C: Representative western blot and quantification of DDAH2 protein expression following hypoxic challenge. Representative image: participant number (x) followed by B, Before or A, After hypoxic challenge. Quantification: Fold change in DDAH2 expression over the course of the study period ( $n=8$ , \*  $p<0.05$ ). Bars represent mean (+SEM).

#### 4. Discussion

Inflammation either at the site of infection or systemically leads to an environment in which monocytes are exposed to grossly deranged conditions including hypoxia. It has been shown that acute hypoxia leads to delay in constitutive apoptosis in monocytes<sup>35</sup>. Hypoxia also has organism specific effects on bactericidal activity and the microenvironment itself<sup>7</sup>. The impact of acute hypoxic stress on regulators of NO synthesis by circulating immune cells has not been well elucidated and potentially has an impact both on immune cell function and also on the local microenvironment.

This study deepens our understanding of this process by exploring the impact of hypoxia on monocyte DDAH2 expression and regulation of NO synthesis. By using primary cells isolated from macrophage specific DDAH2 deficient mice and their relevant controls we have been able to elucidate the role this enzyme plays in regulating NO synthesis in hypoxia. Translating this work into humans has allowed us to demonstrate that this mechanism is preserved in humans following a clinically relevant degree of systemic hypoxia. It has previously been shown that in endothelial cells, DDAH2 is downregulated in Hypoxia. This suggests that DDAH2 regulation in hypoxia may be tissue specific and reflect differing adaptive responses to hypoxic stress.

We have previously shown that global and macrophage specific knockout of DDAH2 in murine models of severe sepsis leads to a significant excess mortality which is mediated by impaired macrophage phagocytosis, bactericidal ability and NO synthesis<sup>31</sup>. Sepsis is a complex physiological insult in which multiple processes contribute to organ dysfunction and death. This study shows that a cardinal feature of critical illness – hypoxia - leads to a significant increase in monocyte NO synthesis mediated by an increase in DDAH2 expression that reduces ADMA concentrations and facilitates increased NOS activity. The mechanism of hypoxia mediated induction of DDAH2 requires further elucidation however recent work has shown that NRF2 induces DDAH2 expression<sup>36</sup> which in turn, has been shown to be induced by hypoxia<sup>37</sup>.

In macrophage specific knockout cells exposed to a hypoxic challenge, basal NO<sub>x</sub> is reduced and the increase in intracellular NO<sub>x</sub> seen in the floxed control cells upon exposure to hypoxia is not observed in the knockout cells. We postulate that this is one of the mechanisms by which animals deficient in DDAH2 demonstrate impaired monocyte function and elevated mortality in animal models of sepsis<sup>31</sup> and may in part explain the association between polymorphisms of the human *DDAH2* gene and outcome in patients with septic shock<sup>38</sup>

This study also highlights differing mechanisms in the synthesis of NO across species. In our murine studies, elevated iNOS was observed in isolated hypoxic primary murine macrophages consistent with previous studies of the impact of hypoxia on the inducible isoform of the enzyme<sup>39</sup>. By contrast, in the human samples, only a trend towards increased eNOS expression was observed. This is consistent with studies showing that in the absence of a pro inflammatory cytokine, iNOS induction is not observed in human hypoxia<sup>40</sup>. A number of studies have demonstrated previously that eNOS is present in human monocytes. There is limited evidence regarding the impact of hypoxia on immune cell eNOS expression, however a number of differing stimuli have been shown to regulate eNOS in isolated human monocytes<sup>41</sup>. Our data presented here suggest that regulation of eNOS activity in human macrophages by modulation of the concentration of competitive inhibitors contributes significantly to hypoxia-induced NO synthesis by these cells.

In summary, this study demonstrates that DDAH2 regulates ADMA mediated inhibition of NO synthesis in isolated murine primary monocytes and translates this observation into humans exposed to a clinically relevant model of normobaric hypoxia. We postulate that we have identified a novel mechanism, conserved between mice and humans that contributes to the monocyte response to hypoxia. It may also give insights into the mechanism by which polymorphisms in the human *DDAH2* gene functionally impact NO synthesis and lead to clinically relevant outcomes in humans with septic shock.

## 5. Conclusions

- The regulation of nitric oxide synthesis by immune cells is a key component of the immune response to pathophysiological stress and regulates both cell function and the microenvironment
- Here we show that acute hypoxia – a cardinal feature of pathological stress – leads to increased immune cell nitric oxide synthesis and that this is mediated by asymmetric dimethylarginine and the enzyme that metabolises it in monocytes, dimethylarginine Dimethylaminohydrolase 2.
- This translational study gives insights into the mechanism through which tissue hypoxia leads to local increases in nitric oxide level and offers avenues for further investigation of how this response may become pathological in some patients. Furthermore, DDAH2

modulation may possess therapeutic potential as a modulator of immune cell nitric oxide synthesis in response to pathological stress.

## 6. References

1. Taccone FS, Su F, De Deyne C, Abdellhai A, Pierrakos C, He X, Donadello K, Dewitte O, Vincent J-L and De Backer D. Sepsis Is Associated With Altered Cerebral Microcirculation and Tissue Hypoxia in Experimental Peritonitis\*. *Critical Care Medicine*. 2014;42:e114-e122.
2. Sair M, Etherington PJ, Winlove CP and Evans TW. Tissue oxygenation and perfusion in patients with systemic sepsis. *Critical care medicine*. 2001;29:1343-1349.
3. De Backer D, Creteur J, Preiser J-C, Dubois M-J and Vincent J-L. Microvascular blood flow is altered in patients with sepsis. *American journal of respiratory and critical care medicine*. 2002;166:98-104.
4. Novotny AR, Reim D, Assfalg V, Altmayr F, Friess HM, Emmanuel K and Holzmann B. Mixed antagonist response and sepsis severity-dependent dysbalance of pro-and anti-inflammatory responses at the onset of postoperative sepsis. *Immunobiology*. 2012;217:616-621.
5. Salomao R, Brunialti MKC, Rapozo MM, Baggio-Zappia GL, Galanos C and Freudenberg M. Bacterial sensing, cell signaling, and modulation of the immune response during sepsis. *Shock*. 2012;38:227-242.
6. Wiersinga WJ, Leopold SJ, Cranendonk DR and van der Poll T. Host innate immune responses to sepsis. *Virulence*. 2013;5:36-44.
7. McGovern NN, Cowburn AS, Porter L, Walmsley SR, Summers C, Thompson AAR, Anwar S, Willcocks LC, Whyte MKB, Condliffe AM and Chilvers ER. Hypoxia Selectively Inhibits Respiratory Burst Activity and Killing of Staphylococcus aureus in Human Neutrophils. *Journal of immunology (Baltimore, Md : 1950)*. 2011;186:453-463.
8. Hirst DG and Robson T. Nitric oxide physiology and pathology *Nitric Oxide*: Springer; 2011: 1-13.
9. Thiemermann C. Nitric oxide and septic shock. *General Pharmacology: The Vascular System*. 1997;29:159-166.
10. Feihl F, Waeber B and Liaudet L. Is nitric oxide overproduction the target of choice for the management of septic shock? *Pharmacology & Therapeutics*. 2001;91:179-213.
11. Cauwels A. Nitric oxide in shock. *Kidney Int*. 2007;72:557-565.
12. Wong HR, Carcillo JA, Burckart G and Kaplan SS. Nitric oxide production in critically ill patients. *Archives of Disease in Childhood*. 1996;74:482-489.
13. MacMicking J, Xie Q-w and Nathan C. NITRIC OXIDE AND MACROPHAGE FUNCTION. *Annual Review of Immunology*. 1997;15:323-350.
14. Schulz R, Rassaf T, Massion P, Kelm M and Balligand J. Recent advances in the understanding of the role of nitric oxide in cardiovascular homeostasis. *Pharmacol Ther*. 2005;108:225 - 256.
15. Seddon M, Chowienzyk P, Brett S, Casadei B and Shah A. Neuronal nitric oxide synthase regulates basal microvascular tone in humans in vivo. *Circulation*. 2008;117:1991 - 1996.
16. Broussard M, Parrillo J, Prancan A and Hollenberg S. Inducible nitric oxide synthase (iNOS) deficient septic mice show improved microvascular responsiveness to endothelin-1 [abstract]. *FASEB J*. 2000;14:A404.
17. Bogdan C. Nitric oxide synthase in innate and adaptive immunity: an update. *Trends in Immunology*. 2015;36:161-178.
18. Alderton W, Cooper C and Knowles R. Nitric oxide synthases: structure, function and inhibition. *Biochem J*. 2001;357:593 - 615.
19. Tran CTL, Leiper JM and Vallance P. The DDAH/ADMA/NOS pathway. *Atherosclerosis Supplements*. 2003;4:33-40.

20. Leiper J and Nandi M. The therapeutic potential of targeting endogenous inhibitors of nitric oxide synthesis. *Nat Rev Drug Discov.* 2011;10:277-91.
21. Vallance P and Leiper J. Cardiovascular biology of the asymmetric dimethylarginine: dimethylarginine dimethylaminohydrolase pathway. *Arterioscler Thromb Vasc Biol.* 2004;24:1023 - 1030.
22. Bode-Böger SM, Scalera F, Kielstein JT, Martens-Lobenhoffer J, Breithardt G, Fobker M and Reinecke H. Symmetrical Dimethylarginine: A New Combined Parameter for Renal Function and Extent of Coronary Artery Disease. *Journal of the American Society of Nephrology.* 2006;17:1128-1134.
23. De Gennaro Colonna V, Bianchi M, Pascale V, Ferrario P, Morelli F, Pascale W, Tomasoni L and Turiel M. Asymmetric dimethylarginine (ADMA): an endogenous inhibitor of nitric oxide synthase and a novel cardiovascular risk molecule. *Medical science monitor : international medical journal of experimental and clinical research.* 2009;15:RA91-101.
24. Wanby P, Teerlink T, Brudin L, Brattström L, Nilsson I, Palmqvist P and Carlsson M. Asymmetric dimethylarginine (ADMA) as a risk marker for stroke and TIA in a Swedish population. *Atherosclerosis.* 2006;185:271-277.
25. Garcia RG, Perez M, Maas R, Schwedhelm E, Böger RH and López-Jaramillo P. Plasma concentrations of asymmetric dimethylarginine (ADMA) in metabolic syndrome. *International journal of cardiology.* 2007;122:176-178.
26. Nijveldt R. Asymmetrical dimethylarginine (ADMA) in critically ill patients: high plasma ADMA concentration is an independent risk factor of ICU mortality. *Clinical Nutrition.* 2003;22:23-30.
27. Leiper JM. The DDAH-ADMA-NOS Pathway. *Therapeutic Drug Monitoring.* 2005;27:744-746.
28. Tran CT, Fox MF, Vallance P and Leiper JM. Chromosomal localization, gene structure, and expression pattern of DDAH1: comparison with DDAH2 and implications for evolutionary origins. *Genomics.* 2000;68:101-5.
29. Leiper J, Nandi M, Torondel B, Murray-Rust J, Malaki M, O'Hara B, Rossiter S, Anthony S, Madhani M, Selwood D, Smith C, Wojciak-Stothard B, Rudiger A, Stidwill R, McDonald NQ and Vallance P. Disruption of methylarginine metabolism impairs vascular homeostasis. *Nat Med.* 2007;13:198-203.
30. Zhen Wang SL, Valerie Taylor, Elizabeth Sujkovic, Manasi Nandi, James Tomlinson, Alex Dyson, Neil McDonald, Stephen Caddick, Mervyn Singer and James Leiper. Pharmacological inhibition of DDAH1 improves survival, hemodynamics and organ function in experimental septic shock. *Biochemical Journal.* 2014;460:309-316.
31. Lambden S, Kelly P, Ahmetaj-Shala B, Wang Z, Lee B, Nandi M, Torondel B, Delahaye M, Dowsett L, Piper S, Tomlinson J, Caplin B, Colman L, Boruc O, Slaviero A, Zhao L, Oliver E, Khadayate S, Singer M, Arrigoni F and Leiper J. Dimethylarginine Dimethylaminohydrolase 2 Regulates Nitric Oxide Synthesis and Hemodynamics and Determines Outcome in Polymicrobial Sepsis. *Arteriosclerosis, Thrombosis, and Vascular Biology.* 2015;35:1382-92.
32. Lucio Iannone LZ, Olivier Dubois, Lucie Duluc, Christopher J. Rhodes, John Wharton, Martin R. Wilkins, James Leiper, Beata Wojciak-Stothard and miR-21/DDAH1 pathway regulates pulmonary vascular responses to hypoxia. *Biochemical Journal.* 2014;462:103-112.
33. Clausen BE, Burkhardt C, Reith W, Renkawitz R and Förster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Research.* 1999;8:265-277.
34. Davids M and Teerlink T. Plasma concentrations of arginine and asymmetric dimethylarginine do not reflect their intracellular concentrations in peripheral blood mononuclear cells. *Metabolism.* 2013;62:1455-61.
35. Walmsley SR, Print C, Farahi N, Peyssonnaud C, Johnson RS, Cramer T, Sobolewski A, Condliffe AM, Cowburn AS, Johnson N and Chilvers ER. Hypoxia-induced neutrophil survival is mediated by HIF-1 $\alpha$ -dependent NF- $\kappa$ B activity. *The Journal of Experimental Medicine.* 2005;201:105-115.



36. Luo Z, Aslam S, Welch WJ and Wilcox CS. Activation of Nuclear Factor Erythroid 2–Related Factor 2 Coordinates Dimethylarginine Dimethylaminohydrolase/PPAR- $\gamma$ /Endothelial Nitric Oxide Synthase Pathways That Enhance Nitric Oxide Generation in Human Glomerular Endothelial Cells. *Hypertension*. 2015;65:896-902.
37. Malec V, Gottschald OR, Li S, Rose F, Seeger W and Hänze J. HIF-1 $\alpha$  signaling is augmented during intermittent hypoxia by induction of the Nrf2 pathway in NOX1-expressing adenocarcinoma A549 cells. *Free Radical Biology and Medicine*. 2010;48:1626-1635.
38. O'Dwyer MJ, Dempsey F, Crowley V, Kelleher DP, McManus R and Ryan T. Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydrolase II gene: a prospective observational study. *Crit Care*. 2006;10:R139.
39. Nagai H, Kuwahira I, Schwenke DO, Tsuchimochi H, Nara A, Ogura S, Sonobe T, Inagaki T, Fujii Y, Yamaguchi R, Wingenfeld L, Umetani K, Shimosawa T, Yoshida K-i, Uemura K, Pearson JT and Shirai M. Pulmonary Macrophages Attenuate Hypoxic Pulmonary Vasoconstriction via  $\beta(3)$ AR/iNOS Pathway in Rats Exposed to Chronic Intermittent Hypoxia. *PLoS ONE*. 2015;10:e0131923.
40. Ho JJD, Man HSJ and Marsden P. Nitric oxide signaling in hypoxia. *J Mol Med*. 2012;90:217-231.
41. Jung J-Y, Madan-Lala R, Georgieva M, Rengarajan J, Sohaskey CD, Bange F-C and Robinson CM. The Intracellular Environment of Human Macrophages That Produce Nitric Oxide Promotes Growth of Mycobacteria. *Infection and Immunity*. 2013;81:3198-3209.

### Acknowledgements

The authors would like to thank the facility management team at the Institute for Sports and Exercise Health for their support in conducting the human study, Mr Matthew Delahaye for his support in the husbandry of the animals and Edwards Life Sciences for the donation of the ClearSight™ system for use in this study.

### Declaration of Interest

The authors declare no competing interests relating to the conduct of this study.

### Funding

This study was supported in part by a project grant from the Royal College of Anaesthetists awarded to SL and by MRC intramural funding (JL). Edwards Life Sciences donated the ClearSight™ system for unrestricted use in this study.

### Presentation

This work has been submitted in part for presentation at the Academy of Medical Sciences Spring meeting

### Author Contribution

Experimental Design – SL, DM, MM, OB, JL.

Experimental Conduct – SL, BL, SP, JT, KV, OB, DM

Analysis – SL, DM, JL, KV, JT, SP

Preparation of Manuscript – SL, DM, BL, KV, JT, SP, MM, DM, JL

**Highlights**

- The regulation of nitric oxide synthesis by immune cells is a key component of the immune response to pathophysiological stress and regulates both cell function and the microenvironment
- Here we show that acute hypoxia – a cardinal feature of pathological stress – leads to increased immune cell nitric oxide synthesis and that this is mediated by asymmetric dimethylarginine and the enzyme that metabolises it in monocytes, dimethylarginine Dimethylaminohydrolase 2.
- This translational study gives insights into the mechanism through which tissue hypoxia leads to local increases in nitric oxide level and offers avenues for further investigation of how this response may become pathological in some patients. Furthermore, DDAH2 modulation may possess therapeutic potential as a modulator of immune cell nitric oxide synthesis in response to pathological stress.