Title: A NO way to BOLD?: Dietary nitrate alters the hemodynamic response to visual stimulation.

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Abstract
Neurovascular coupling links neuronal activity to vasodilation. Nitric oxide (NO) is a potent vasodilator, and in neurovascular coupling NO production from NO synthases plays an important role. However, another pathway for NO production also exists, namely the nitrate-nitrite-NO pathway. On this basis, we hypothesized that dietary nitrate (NO\textsubscript{3}) could influence the brain’s hemodynamic response to neuronal stimulation. In the present study, 20 healthy male participants were given either sodium nitrate (NaNO\textsubscript{3}) or sodium chloride (NaCl) (saline placebo) in a crossover study and were shown visual stimuli based on the retinotopic characteristics of the visual cortex. Our primary measure of the hemodynamic response was the blood oxygenation level dependent (BOLD) response measured with high-resolution functional magnetic resonance imaging (0.64 × 0.64 × 1.8 mm) in the visual cortex. From this response, we made a direct estimate of key parameters characterizing the shape of the BOLD response (i.e. lag and amplitude). During elevated nitrate intake, corresponding to the nitrate content of a large plate of salad, both the hemodynamic lag and the BOLD amplitude decreased significantly (7.0 +/- 2 \% and 7.9 +/- 4 \%, respectively), and the variation across activated voxels of both measures decreased (12.3 +/- 4 \% and 15.3 +/- 7 \%, respectively). The baseline cerebral blood flow was not affected by nitrate.
Our experiments demonstrate, for the first time, that dietary nitrate may modulate the local cerebral hemodynamic response to stimuli. A faster and smaller BOLD response, with less variation across local cortex, is consistent with an enhanced hemodynamic coupling during elevated nitrate intake. These findings suggest that dietary patterns, via the nitrate-nitrite-NO pathway, may be a potential way to affect key properties of neurovascular coupling. This could have major clinical implications, which remain to be explored.

**Keywords**
Nitrate, hemodynamic lag, BOLD amplitude, cerebral blood flow, nitric oxide.

**Abbreviations**

1. **Introduction**
Nitric oxide (NO) is a potent vasodilator playing an important role in establishing neurovascular coupling in the brain (Attwell et al., 2010). In this context, NO is normally considered to originate from NO synthases (NOSs), but the human body also possesses another pathway for NO production that has received much attention recently. This pathway is known as the nitrate-nitrite-NO pathway (Lundberg et al., 2008). However, the impact of this pathway on the brain's hemodynamic responses has so far attracted little attention. Nitrate is often considered a toxin (Avery, 1999), but via its reduction to nitrite and NO, it is known to subserve several important physiological functions (Kevil et al., 2011). E.g. nitrate intake can increase exercise performance (Larsen et al., 2007) and decrease blood pressure (Larsen et al., 2006), and increased levels of nitrite in the blood can help protect against ischemia reperfusion injury (Jung et al., 2006). Most of the nitrate consumed originate from green leafy vegetables (Alexander et al., 2008), and after ingestion, nitrate is reduced to nitrite via symbiotic salivary bacteria (Goaz and Biswell, 1961). Subsequently, nitrite can be converted into NO through an enzyme (van Faassen et al., 2009) and in a pH-dependent manner (Aamand et al., 2009; Li et al., 2008; Li et al., 2009; Zweier et al., 2010). As NO readily crosses cell membranes (Garthwaite, 2008), this means that the blood-brain-barrier is also readily crossed. Within the brain, the conversion
of nitrite to NO could be facilitated by hemoglobin (Huang et al., 2005), xanthine oxidase (Zhang et al., 1997), endothelial NOS (Gautier et al., 2006), or carbonic anhydrase (CA) (Aamand et al., 2009). As an increase in pCO₂ leads to a decrease in pH by CA (Lindskog, 1997), the NO production from the nitrate-nitrite-NO pathway is inherently linked to the energy metabolism of a tissue. Since NO is a potent vasodilator (Ignarro et al., 1987), this could afford a direct coupling between neuronal activity and increases in blood flow.

To study whether the nitrate-nitrite-NO pathway is of physiological importance to the hemodynamic response to neuronal activation, we increased or minimized the dietary intake of nitrate in two groups of young healthy male participants in a randomized, double-blinded, placebo-controlled, crossover design. A 3-day intervention period was chosen as the effects of prolonged nitrate intake is not necessarily mimicked by acute interventions (Larsen et al., 2011). Participants ingested weight-adjusted dosages of sodium chloride (NaCl = saline placebo) or sodium nitrate (NaNO₃) corresponding to the amount of nitrate in 500 mL [R2.3] beetroot juice or a large plate of salad (Alexander et al., 2008; Larsen et al., 2007).

In human participants, the blood oxygenation level dependent (BOLD) response used in functional magnetic resonance imaging (fMRI) (Ogawa et al., 1990) is a highly sensitive way of studying the cerebral vasculature’s response to neuronal activity. The BOLD response depends on local changes in the deoxyhemoglobin (deoxyHb) content modulated by cerebral blood flow (CBF), cerebral blood volume (CBV), and the cerebral O₂ consumption (CMRO₂). Upon neuronal stimulation, the concerted changes in these parameters cause the local deoxyHb content to decrease, and as a consequence the BOLD signal increases (Ogawa et al., 1990).

As neuronal activity causes the extracellular pH to decrease (Chesler, 2003), and as more NO is produced from nitrite at lower levels of pH, we expected that a higher intake of nitrate would cause more NO to be produced via the nitrate-nitrite-NO pathway during neuronal activity. This could cause the local vasculature to dilate more readily when the neuronal activity increases, with little or no impact globally. We thus hypothesized that a decrease in the hemodynamic lag, in this case a decreased lag of the BOLD response, could be expected upon increased intake of nitrate. With regards to the amplitude of the BOLD response we were less certain of what to expect. Following the same logic, a larger BOLD amplitude could be expected. However, nitrate also increases the oxidative phosphorylation efficiency (P/O ratio) of the mitochondria (Larsen et al., 2011), and thus potentially
decreases the need for $O_2$ and glucose. Hence, if the BOLD amplitude has any relation to neuronal metabolism, one could expect a decrease in BOLD amplitude upon heightened nitrate intake. Despite early acknowledgement of its importance (Bandettini et al., 1993; Henson et al., 2002; Menon et al., 1998), the hemodynamic lag is rarely explicitly estimated and studied in BOLD fMRI studies – perhaps because studies have to be specifically designed with this in mind for it to be feasible. An exception to this is the field of retinotopic mapping where the hemodynamic lag is estimated routinely in order to reliably delineate the borders of the visual cortex (Sereno et al., 1995). However, in this context the hemodynamic lag itself is rarely a topic of scrutiny, but rather a confounder, which needs to be estimated in order in order to construct reliable retinotopic maps. In the present study, we turned this priority around. Three features of the visual cortex makes it an optimal area of choice for estimating hemodynamic lag: 1) Its relatively large size allows for a robustly sized dataset; 2) the existence of ocular dominance columns makes it possible to minimize vascular smearing by using a visual stimulus matching the extent of such columns (Turner, 2002); and 3) it is possible, in an fMRI setting, to reverse the stimulation order due to the retinotopic organization of the visual cortex. Together these three features allow for a reliable and time-efficient estimation of the hemodynamic lag in a large number of voxels, with minimal contribution from large vessels. This is done by considering the difference between the phase angles obtained for opposite stimulus directions (i.e. contraction versus expansion) (Sereno et al., 1995). This also means that the BOLD response is easily parameterized in terms of phase, amplitude, and frequency.

2. Material and Methods

2.1 Participants

20 healthy male participants (25 +/- 0.9 years, 77 +/- 1.5 kg) were recruited. Males were chosen in order to minimize the variations within the study group. The Central Denmark Region Committee on Health Research Ethics approved the protocol (27934), and all participants gave their informed written consent prior to participation.

2.2 Modulation of Dietary Nitrate Intake

Each participant’s intake of nitrate was increased or minimized in two consecutive rounds in a randomized, double-blinded, placebo-controlled, crossover design. For three consecutive days prior to each examination, participants drank a saline solution of either NaNO₃ or NaCl (Sigma Aldrich) amounting to 0.1 mmol/kg/day. On the first day, the solution was consumed when handed out,
whereas on the second day the participants managed the intake themselves. On the third day, the solution was ingested 45 minutes prior to scanning. Participants in Group A received NaNO$_3$ in the first examination and NaCl in the second examination. This order was reversed in Group B. During the two rounds of saline intake, participants were told to stay away from vegetables, cured meats, nuts, alcohol, and nicotine. The two examinations were separated by a washout period of 9-11 days. We note that the two saline solutions do not taste the same, and as no masking agent was used, we cannot exclude the possibility that some participants may have identified the group to which they belonged. As a consequence, the participants may only be considered partly blinded, even though they were not informed on any specific hypotheses regarding nitrate intake prior to examination.

### 2.3 Blood Samples

Venous blood samples were drawn: (a) prior to each three-day period of dietary nitrate modulation (autonomous diet) to test baseline/pretreatment nitrate and nitrite levels; and (b) on the third day of dietary modulation, immediately after the arterial spin labeling (ASL) scan (~ 90 min. after ingestion and 10 min. after BOLD image acquisition). Lithium-Heparin glasses with no other additives were used to collect the blood samples. The blood samples were immediately placed in an ice-bath, and centrifuged at 4 °C and 13,000g for 10 minutes. After centrifugation the plasma samples were transferred to a -80 °C freezer.

Nitrite formation and nitrate levels in plasma are reported to be fairly stable from 30 min. to at least 120 min. after nitrate ingestion (Cortas and Wakid, 1991). Hence, the nitrate levels measured directly after the experiment was completed are a good estimation of the nitrate levels in plasma during the acquisition of both sets of images.

### 2.4 Nitrate and Nitrite Measurements

Nitrate measurements were carried out in a two-step analysis – enzymatic reduction to nitrite followed by chemiluminescence detection. Chemiluminescence detection was carried out in a reaction vessel containing I$_3^-$ reagent preequilibrated and purged with helium at room temperature (21°C) using a Sievers (Boulder, CO, USA) Nitric Oxide Analyzer (NOA 280i) to detect NO in the gas phase at a sampling rate of 4 s$^{-1}$ (Yang et al., 2003). For analysis of nitrate in plasma, plasma samples were incubated with nitrate reductase (0.1 U/ml), flavine adenine dinucleotide (FAD, 5 μM), and reduced β-nicotinamide dinucleotide phosphate (NADPH, 30 μM) (Sigma Aldrich) at 37°C for 15 min. to reduce plasma nitrate to nitrite (Bories and Bories, 1995). Thereafter, plasma samples were injected into the reaction vessel containing the I$_3^-$ reagent, and the amount of NO from the reduction of nitrite was
measured using the Sievers Nitric Oxide Analyzer (NOA 280i) software. Calibration was done by fully reducing known amounts of nitrite to NO with the \( \text{I}_3^- \) reagent in the reaction vessel. Nitrite measurements were carried out in the same way, but without the enzymatic reduction step.

### 2.5 Auxiliary Recordings

A MEDRAD (Warrendale, USA) Veris MR Vital Signs Monitor was used to measure and record blood pressure, pulse oximetry, and expired CO\(_2\). Respiratory frequency was measured and recorded using the standard Siemens respiratory belt. Blood pressure was measured in the scanner before and after the BOLD scans while the other parameters were recorded continually and used as nuisance regressors in the fMRI analysis. The participants’ fixations upon the stimuli was monitored using an eye tracking system (Eyelink 1000, SR research, Mississauga, Canada).

### 2.6 Stimulus Delivery

Visual stimulation was provided by means of a DILA video projector (JVC DLA-HD950). A Navitar SST300 3.00X ScreenStar Telephoto Converter with a custom made lens-holder was placed in a wave-guide behind the scanner and projected the image onto a 500 x 280 mm semi-transparent screen (Novadisplay) located behind the participant’s head. The screen covered 48 x 28 degrees of the visual field and was visible to the participants through an infrared compatible mirror (SR-research) mounted on the head coil. Participants wore glasses with a red left lens and a green right lens. This allowed us to stimulate each eye separately with a red and green stimulus. We did so in order to take advantage of the ocular dominance columns (parallel stripes 500-1000 µm apart) in the human visual cortex and thereby increase the separation of cortical activation based on stimulus eccentricity. Thus we ensured small activation volumes, which reduced the large draining vein contribution (Turner, 2002), and hence a more accurate estimation of lag time could be obtained. The stimuli [R2.5] were programmed and presented using Matlab (MathWorks, Massachusetts, USA) with the Psychophysics Toolbox extensions.

The stimulus included a central fixation cross, which would disappear at random for short periods of time. Participants had to press a button when this happened and thus indicate that they attended to the stimulus.

**Stimulus in the BOLD Scan:** The retinotopic stimulus visualized in Supplementary Figure 1A-B, consisted of ring snippets expanding and contracting horizontally from 0-24° eccentricity, unveiling four 10° dartboard wedges positioned 2.5° above and below the horizontal midline of the screen (Supplementary Figure 1A). The stimulus was constructed in this way in order to confine cortical...
activation to each bank of the calcarine sulcus (CS) in the primary visual cortex. The dartboard pattern was interrupted at 5.3°, 10.6°, and 15.9° (Supplementary Figure 1B) with gray rings, which matched the background and had the same width as the ring snippets. The left side of the screen flickered in red/grey; the right side flickered in green/grey (10 Hz) (Supplementary Figure 1A). When a ring snippet in one side of the screen expanded above midline, it contracted below, whereas it did the opposite on the other side of the screen (Supplementary Figure 1A/B). The stimulus was constructed so in order to ensure stimulus separation. A ring-snippet completed 1 cycle of stimulation in 25 sec.; a rest period chosen to ensure an equivalent BOLD response at every stimulus. After 7 cycles of stimulation and a 25 sec. break with just the fixation-cross presented, the direction of the movement was reversed. A total of 8 runs (+ 7 fixation screens) led to the total duration of the paradigm of 8 x (7 x 25 sec.) + 7 x 25 sec. = 26 min. and 15 sec., which was tolerated well by all participants.

2.7 MRI

All MRI images were acquired on a 3T MRI Magnetom Tim Trio, Siemens (Erlangen, Germany) equipped with an anti vibration kit. Participants lay supine on the patient bed with the back of the head resting on a one cm thick TEMPUR® pad. In all experiments, only the lower part of the Siemens 32 channel head coil was used. This facilitated eye tracking and subject comfort.

2.7.1 Structural: Anatomical images were obtained using a magnetization prepared, rapid gradient echo (MPRAGE) sequence with the following parameters: echo time (TE) 2.52 ms, repetition time (TR) 1900 ms, an acquisition matrix of 256 × 256 × 176, FoV of 250 × 250 mm, flip angle (FA) of 9°, and voxel size of 0.98 × 0.98 × 1 mm. The acceleration factor was 2 using GRAPPA. Total acquisition time was 5 min. and 57 sec..

2.7.2 BOLD: T₁* weighted images were acquired continuously during the experiment using a gradient echo EPI (echo planar imaging) sequence. By carefully positioning the occiput of the participant right above a single coil element (number 22) and setting the FFT scale of all other channels to zero, it was possible to use a small FoV (50 × 50 mm) without aliasing becoming problematic. Five slices with the following parameters: TE=42 ms, TR=588 ms, acquisition matrix of 78 × 78, FA=50°, and a voxel size of 0.64 × 0.64 × 1.8 mm; were positioned sagittally to encompass the most lateral part of the left CS as well [R2.6] as possible (Figure 1A). After six initial dummy volumes, a total of 2700 volumes were acquired, and scan-nulling regressors were [R2.7] used to discard the last 22 volumes.

2.7.3 ASL: After the BOLD scan, the participants were repositioned with more comfortable padding, and now all 20 receiver coils in the lower part of the 32-channel head coil were used. CBF was
estimated with a Pulsed ASL (PASL) sequence with a PICORE (proximal inversion with a control for off-resonance effects) and Q2TIPS (quantitative imaging of perfusion with a single subtraction and thin-slice TI$_{11}$ (label saturation) periodic saturation) labeling scheme. Three slices were acquired with TE=17 ms, TR=2500 ms, FoV=460 × 460 mm, FA=90°, acquisition matrix=64 × 64, and a voxel size of 3.59 × 3.59 × 7.5 mm. Slices were again positioned along the CS to encompass the primary visual cortex as well [R2.6] as possible. TI$_{11}$ started at 500 ms and ended at 1500 ms. The inversion time was 1475 ms. The cut-off velocity of the bipolar crusher gradient was 3.2 cm/s. A 110 mm labeling slab was positioned 25 mm caudally to the imaging slab.

To control for a possible effect of nitrate intake on T$_1$ relaxation, additional acquisitions were made at inversion times of T$_{11}$=100, 200, 300, 400, 600, 900, 1500, and 4000 ms.

2.8 Image Processing and Analysis

Processing of the fMRI time series was done in SPM8 patched to release 4290 (http://www.fil.ion.ucl.ac.uk/spm).

2.8.1 BOLD: The only pre-processing step used for the BOLD series prior to modeling with the general linear model (GLM) was in-plane smoothing (FWHM=0.8 × 0.8 × 0 mm). Realignement parameters (RP) were estimated on a copy of the images, but in order not to lose data in the outermost slices the images were not resliced. Instead, movement effects were modeled as part of the Nuisance Variable Regression approach (Lund et al., 2006). In addition to the SPM8 standard discrete cosine set high pass filter (128 s cut-off), this approach includes a 24 parameter Volterra expansion of the realignment parameters at various time points, t: RP(t), RP(t)$^2$, RP(t-1) and RP(t-1)$^2$ (Friston et al., 1996), a RETROICOR (Glover et al., 2000) expansion of respiration (4$^{th}$ order), and pulse oximetry (6$^{th}$ order). In order to also model effects of fluctuations in blood CO$_2$ (Wise et al., 2004), we also included the recorded expired CO$_2$ as a regressor.

To match the 8 runs, the stimulus was modeled with 8 separate pairs of sine and cosine predictors with a fundamental frequency defined by the 25 sec. cycle length, sampled at intervals of TR. Similarly, a second order expansion was used to model deviations from a strictly sinusoidal behavior. Each of the 25 sec. intervening breaks was modeled using an additional set of regressors for scan nulling. An F-test was used to test for any linear combination of the fundamental oscillation, i.e. all phases. A voxel-wise estimate of phase ($\varphi$) was calculated based on the parameter estimates from the 1$^{st}$ harmonic of the reconstructed signal using the following equation:

$$\varphi = \arctan\left(\frac{\beta_c}{\beta_s}\right)$$

(1)
, where \( \arctan_2 \) is the four-quadrant inverse tangent, and \( \beta_s \) and \( \beta_c \) are [R2.8] the weights for the sine and cosine predictor, respectively. As the stimulus was [R2.9] displayed in both forward and backward runs, a voxel-wise estimate of hemodynamic lag (\( \tau \)) can be determined (Sereno et al., 1995).

\[
\tau_{i,j} = \frac{(\varphi_i - (2\pi \varphi_j)) \bmod 2\pi}{2} \times \frac{25 \text{ sec.}}{2\pi}
\] (2)

Here \( \varphi_i \) and \( \varphi_j \) are the phases estimated from forward run \( i \) and backward run \( j \) (adjusted to be in the range from 0 to \( 2\pi \), \( i \) and \( j = 1:4 \)). A graphical representation of these calculations is offered in Supplementary Figure 1C-D. With 4 forward runs and 4 backward runs, we were able to calculate 7 ascending voxel-wise lag estimates (\( \tau_{1,1}, \tau_{2,1}, \tau_{3,2}, \tau_{3,3}, \tau_{4,3}, \tau_{4,4} \)), allowing us to obtain the median \( \tau \) for each voxel. The estimated lags were subsequently corrected for the different acquisition times of the different slices, thereby eliminating the need for slice-time correction in SPM.

The voxel-wise BOLD amplitude (\( M \)) was calculated as:

\[
M = \sqrt{\sum_{i=j=1}^{4} \beta_s^2 + \beta_c^2 + \beta_c^2 + \beta_c^2} \times \frac{p2p(HRF)}{p2p(0scl)}
\] (3)

, where \( p2p(HRF) \) and \( p2p(0scl) \) designate the peak-to-peak amplitude of the canonical hemodynamic response function from SPM and the 1\(^{st} \) order oscillation, respectively. This multiplication was carried out solely in order to make our amplitude values comparable to those estimated by the canonical HRF (Supplementary Figure 1C). This had no effect on any of the following analyses.

The median \( \tau \) and mean \( M \) were obtained for all voxels within a mask defined by the F-test map, thresholded at uncorrected \( P=0.001 \)\(^1 \). As large cerebral vessels respond to stimuli within 8-14 s (Lee et al., 1995), only voxels with median \( \tau \) below 9 s went into the calculation of each participant’s overall median hemodynamic lag and mean BOLD amplitude (see Figure 1A-E for the calculation of median lag).

Note that the F- and lag thresholds are only used to calculate the per participant per examination measures (i.e. single values), which are then used to assess the treatment effects.

2.8.2 ASL: The ASL images were realigned and resliced to the mean images. Then coregistered to the anatomical image and smoothed (FWHM=6 mm isotropic). CBF was estimated using the program

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\(^1 \) We note that the family-wise error rate (FWE) correction using Gaussian random fields is not an obvious choice with data of this resolution because: 1) The estimated spatial smoothness in the residuals were 1.4 x 1.2 x 2.6 mm/2.2x1.9x1.6 voxels, i.e. only about twice as big as the voxel size, and this causes FWE to be overly conservative; 2) the 2D histogram of estimated hemodynamic lag vs. F-value has a natural noise floor (Figure 1B-C), which corresponds better to the uncorrected p-value than to the FWE corrected p-value. The false discovery rate (FDR) correction was not suitable either because: 1) The FDR correction caused a lower F-threshold than the uncorrected p-value; 2) the FDR correction included a different number of significant voxels depending on which round (high or low nitrate) the data were taken from.
ASLtbx (Wang et al., 2008) with simple subtraction, as this eliminates the need of a high-pass filter. The first image was used to correct for baseline magnetization and segmented to differentiate between white and grey matter contribution to the signal. The first and last 2 images were discarded before calculating each global average.

### 2.9 Statistical Analysis

All statistical analyses were done in Matlab. Median values were used to calculate the participant-wise lag estimates since we observed that the $\tau$ distribution had a relative large proportion of high values – i.e. it was asymmetrically heavy-tailed towards high $\tau$ values. Median values are provided with median absolute deviation (MAD). Nonetheless, the results presented on lag estimates are still valid when using means instead of medians. We believe, however, that medians offer a better description of the data given the asymmetric distributions. As the $M$ distribution (voxel-wise BOLD amplitude) was less heavy-tailed, with a low proportion of values larger than the peak of the distribution, we found it prudent to keep using the mean for the calculation of the participants’ amplitude estimates. Participant-wise mean values are provided with +/- standard error of the mean (SEM). However when the voxel-wise variation is considered the mean absolute deviation of $M$ (MeanAD($M$)) is used instead of SEM in order to facilitate comparison with MAD($\tau$). For within-participant effects of treatment a paired t-test was used. A 3-way ANOVA was used in a post hoc test for a group x scan interaction. Pearson product-moment correlation coefficient was used to test for linear correlations in the data.

### 3 Results

#### 3.1 Subjects and Auxiliary Recordings

Of the 20 subjects recruited, one participant from group B was excluded, as he did not complete the study. In addition, one participant from group A was excluded due to excessive movement during scanning. In the remaining participants, the modulation in nitrate intake caused no significant differences in blood pressure, heart rate, blood oxygen saturation, expired CO$_2$, or respiration rate (Supplementary Table 1). While nitrate intake in amounts similar to the ones used in the present study have been reported to lower the blood pressure even in young healthy participants (Kapil et al., 2010; Larsen et al., 2006), we suspect that the mild stress induced by being scanned could have masked any such effect in our study.

#### 3.2 Nitrate and Nitrite
The participants’ mean plasma concentration of nitrate prior to the dietary nitrate modulations (autonomous diet) was 23.7 +/- 1.7 μM. When the dietary intake of nitrate was minimized (NaCl), the plasma concentration of nitrate did not change significantly from this level (20.3 +/- 2.6 μM), whereas increased nitrate intake (NaNO₃) caused the mean nitrate concentration in the plasma to rise to 89.6 +/- 9.1 μM (Paired t-test: P<0.001) (Figure 2A). There was no effect of treatment order (Figure 2A).

The plasma concentrations of nitrite were modulated similarly according to dietary nitrate intake (autonomous diet: 0.12 +/- 0.07 μM, NaCl: 0.11 +/- 0.1 μM, NaNO₃: 0.17 +/- 0.09 μM – median values with MAD) (Figure 2B), but this did not reach statistical significance (Paired t-test: P=0.08). This observation caused us to use the plasma concentrations of nitrate, instead of nitrite, as indicator of the efficiency of the dietary treatment in the following analyses. This choice will be further explained in the discussion.

### 3.3 Hemodynamic Lag

We found that increased nitrate intake significantly decreased the participants’ estimated hemodynamic lag (Paired t-test: P<0.005) (Figure 3A): When the participants were given NaCl, their mean estimated hemodynamic lag was 4.35 +/- 0.11 sec., whereas the mean lag decreased to 4.05 +/- 0.09 sec. when they where given NaNO₃. The main effect of the increased nitrate intake was thus a mean decrease in the hemodynamic lag of 7.0 +/- 2%. Substantiating these results, we also observed a linear and negative correlation between the estimated hemodynamic lag and the plasma concentrations of nitrate of the participants (Pearson: rho=-0.346, P<0.05) (Figure 3C).

An inspection of a single participant’s τ (voxel-wise estimate of hemodynamic lag) histograms (Figure 1D-E) led us to wonder whether the lowered mean hemodynamic lag estimates during high nitrate intake could be related to a lowered variation in the τ estimates. Therefore we performed a post hoc test on the group level in order to study whether the variation in the τ estimates was reduced during elevated dietary nitrate intake. Doing so, we found that the mean MAD (median absolute deviation) of the τ estimates (MAD(τ)) was significantly lower during NaNO₃ intake (1.00 +/- 0.04 sec.) than during NaCl intake (1.15 +/- 0.05 sec.) (Paired t-test: P<0.01). This is shown in Figure 4F. Across all participants, this amounted to a mean difference in MAD(τ) of 12.3 +/- 4%. Furthermore, but still post hoc, the MAD(τ) was significantly negatively correlated with the participants’ plasma concentrations of nitrate (Pearson: rho=-0.4114, P<0.05) (data not shown).

In addition to this observed difference in the variation of the τ estimates, we also observed that a greater proportion of voxels responded faster when the participants had an increased nitrate intake.
This unexpected phenomenon is illustrated across participants in Figure 4G. In total, we calculated that 7.3% of the voxels responded faster with high nitrate intake than with minimized nitrate intake. Studying the difference between the NaNO$_3$ and the NaCl $\tau$ histograms (Red circles in Figure 4G), we noticed that the proportion of voxels with a $\tau$ faster than 5.1 sec. increased during NaNO$_3$ intake, leading to a lower proportion of voxels responding with a $\tau$ above 5.1 sec. (i.e. 5.1 sec. is where the red circles crosses from one side to the other in Figure 4G). Using 5.1 sec. as a divide, we calculated that 20% of the voxels with a $\tau$ above 5.1 sec. during minimized nitrate intake, had lags faster than 5.1 sec. during high nitrate intake.

### 3.4 BOLD Amplitude

The effect of nitrate intake on the BOLD amplitude across examinations did not reach our chosen significance threshold (Paired t-test: $P=0.06$). However, doing a post hoc test for a group x scan interaction across examinations, we observed a significant difference in the BOLD amplitude between the two groups (3-way ANOVA: $P<0.05$) (Figure 3B). This translated into a mean decrease in the BOLD amplitude of 7.9 +/- 4% at high nitrate intake compared to when nitrate intake was minimized. We found no general correlation between the plasma concentration of nitrate and the estimated BOLD amplitude (Supplementary Figure 2A). However similar to the change in the variation of the $\tau$ estimates, a post hoc test showed that the MeanAD($M$) (mean absolute deviation of the voxel-wise estimate of BOLD amplitude) was also significantly reduced (Paired t-test: $P<0.05$) when the nitrate intake was increased compared to when it was minimized (from 0.67 +/- 0.04 % to 0.56 +/- 0.03 %). This is visualized in Figure 4E. This amounted to a mean decrease in the MeanAD($M$) of 15.3 +/- 7% during NaNO$_3$ intake compared to during NaCl intake. However, this decrease did not correlate significantly with the participants’ plasma concentrations of nitrate (data not shown). Inspecting the $M$ histogram in Figure 4C, we note that the increased nitrate intake in general increased the proportion of low amplitude voxels.

Regarding both the amplitude and the lag we note that the MAD($\tau$) and the MeanAD($M$) correlate significantly with the median lag and mean amplitude estimates, respectively ($p<0.005$ and $p<0.0001$, respectively – data not shown). However, we are unable to characterize the causality in this relationship in more detail.

### 3.5 Amplitude and Lag Relations

In the present study, the mean BOLD amplitude of the participants was significantly positively and linearly correlated with their median hemodynamic lag (Pearson: $\rho=0.465$, $P<0.005$) (Supplementary
Figure 2B). This could in theory be due to a coupling between our estimates of lag and amplitude. However, if this was the case, then a similar correlation between lag and amplitude should also be visible across voxels. To study this we constructed 2D histograms of mean $M$ vs. median $\tau$ incorporating the data from all participants. Doing so, we observed no correlation between the $\tau$ and $M$ estimates due to any of the two modulations of nitrate intake (Figure 4A-B). However, the 2D histograms did help visualize that increased nitrate intake markedly increased the frequency of voxels with low $M$ estimates and $\tau$ estimates around the mode of the $\tau$ distribution (Figure 4B vs. Figure 4A). This is emphasized in the 2D difference histogram shown in Figure 4D, where the histogram in Figure 4A have been subtracted from the histogram in Figure 4B.

3.6 Cortical Depth Histograms
To investigate whether the effect of nitrate was consistent across cortical depth, we created histograms similar to those in Figure 4 for voxels within three gray matter masks corresponding to different cortical depths. The effect of nitrate on $\tau$ across cortical depth was similar to that observed in the main $\tau$ histogram (Figure 4G vs. 5G-I). However, it is worth noting the large proportion of high lags seen in the most superficial/pial layer (Figure 5G).

The effect of nitrate on the mean $M$ histograms was also similar across all cortical depths (Figure 5A-C). One thing to note, though, is the apparent decrease in the mode of the $M$ distributions the deeper the cortical layer.

With respect to the 2D difference histograms of $M$ vs. $\tau$ the effect of nitrate was again similar across cortical depths (Figure 5D-F). However, more variation was noted.

3.7 CBF
To control for possible baseline effects of nitrate intake on CBF, we estimated this parameter with ASL during rest. The mean global CBF during minimized nitrate intake was 58.3 +/- 1.8 ml/100g/min. whereas it was 57.4 +/- 2.1 ml/100g/min. with increased nitrate intake. Nitrate thus caused no significant difference in resting global CBF.

3.8 Nitrate and $T_1$
Nitrite can oxidize hemoglobin into methemoglobin (metHb). This could potentially impact both the BOLD and ASL measurements as metHb causes a higher $T_2^*$ (Duewell et al., 1996) and $T_1$ (Leung and Moody, 2010) relaxivity. To control for this, we estimated the $T_1$ relaxation times measured as part of the ASL sequence. However, we found no impact of nitrate intake on the $T_1$ relaxation. This would not be expected, either, as the nitrate intake used in the present study was not beyond what can be
encountered in a normal healthy diet, and as dietary levels of nitrate are not known to alter the metHb concentration in adults (Manassaram et al., 2010).

4 Discussion

Previously, dietary nitrate has been reported to increase resting CBF in older people (Presley et al., 2011). However, this is the first study to show that dietary nitrate intake, in amounts comparable to that of a large plate of salad, is a modulator of the hemodynamic response to visual stimuli in the human visual cortex. In this study, we report that dietary nitrate decreases the hemodynamic lag, the BOLD amplitude, and the voxel-wise variation of both measures, with the latter resulting in a more homogenous hemodynamic response to the stimulus across the local cortex.

4.1 Nitrate and Nitrite

The plasma concentrations of nitrate and nitrite are normally observed to rise in unison upon intake of nitrate in amounts similar to those used in the present study (Larsen et al., 2011; Larsen et al., 2007). However, we observed only a strong correlation between the plasma concentrations of nitrate and nitrite in the blood samples taken prior to the nitrate modulation and when the participants were given NaCl (Pearson: rho=0.423, \( P<0.001 \)) (data not shown). As nitrite reacts with oxyHb to produce metHb with a half-life of ~10 min. (Dejam et al., 2005), the lack of a heme-oxidizing solution in the vacutainers could influence the blood samples with high nitrite content relatively more than blood samples with low nitrite concentrations. Therefore we have used the plasma concentrations of nitrate, rather than the plasma concentrations of nitrite, as the measure for the effect of treatment. We note however, that in the body, nitrate most likely acts via its conversion to nitrite. Nitrate is a rather inert anion, and it is not known to act directly on cerebrovascular circulation. On the other hand, cortical nitrite superfusion is known to act on the brain and is capable of rescuing the CBF response to neuronal stimulation when the NOS system is blocked pharmacologically (Piknova et al., 2011).

4.2 The Parameter Estimates and the BOLD Response

The BOLD response can be described in terms of amplitude, phase and frequency. In our analyses, both the amplitude and the phase estimates were used, with the latter forming the basis for our lag estimates. The frequency was simply denoted by the stimulus. Supplementary Figure 1C shows that this approach captures the nature of SPM’s canonical HRF fairly well. However, as true for all parametric models, the quality of our estimates is influenced by how well the model fits reality. In other
words, if nitrate changes the composition of the fit, it will influence the estimates. A finite impulse response analysis of the BOLD response would afford a detailed insight into possible changes in the shape of the BOLD response. Our data, however, does not allow us to conduct such an analysis, as our experiment was not constructed with this in mind. Nonetheless, we tested the median variance of the error (“goodness of fit”) in the activated voxels, and nitrate did not influence this measure significantly.

### 4.3 Nitrate in the Brain

A higher nitrate intake would allow for more NO to be produced locally from nitrite upon neuronal activity. This would facilitate local vasodilation and thus allow the local vasculature to be more responsive to neuronal activity. Our finding of a decreased hemodynamic lag upon nitrate intake thus fits our initial hypothesis. The only other thing that could cause this would be faster responding neurons. However, this we consider unlikely [R1.2]/[R2.12], as neurons under normal conditions already respond within a few ms after being stimulated.

A decrease in BOLD amplitude is not as straightforward in terms of interpretation. In this study, we could not distinguish between possible contributions from nitrate-altered CBF-, CBV- and OEF-responses that could have caused the decreased BOLD amplitude, given a lack of similarly high-resolution data of these measures. It can be postulated that several combinations of increased CBV- and CMRO2-responses and/or decreased CBF- and oxygen extraction fraction (OEF)-responses could theoretically result in a decreased BOLD amplitude. Of these four parameters, we regard an increased CMRO2-response as rather unlikely, since dietary nitrate has been reported to increase the oxidative phosphorylation efficiency (P/O ratio) of the mitochondria (Larsen et al., 2011), which would decrease the need for O2.

### 4.4 $\text{MAD}(\tau)$ and $\text{MeanAD}(M)$ vs. Lag and Amplitude.

The correlations observed between the participants’ [R2.13] voxel-wise variation and the magnitude of the participants’ lag and amplitude estimates could have several explanations. One could argue that the magnitude of the estimates would necessarily dictate a larger spread of the voxel distributions. However, as the variance of a distribution is based on the difference between a realized variable and the expected value (mean/median/mode), this is not a mathematically inherent property of a distribution. Rather, the correlation between the expected value and the variation is based on the underlying physiology. We therefore propose that the correlation is driven by a preferential decrease in the number of voxels responding with high $M$ and/or long $\tau$. This would decrease both the variation...
and the magnitude of the overall estimates. This could be the main cause of the observed correlation, though we cannot afford any conclusion in the matter.

4.5 Effects of nitrate modulation in relation to modulation of the NOS system

The NOS system has been more extensively studied with respect to the cerebral hemodynamic response than the nitrate-nitrite-NO pathway. Inhibiting the NOS system decreases the amplitude of the hemodynamic response, while the hemodynamic lag, though not studied in detail, seems to be unaffected (Liu et al., 2008; Piknova et al., 2011). A modulation of the NOS system in a way more similar to what we have done would be l-arginine administration, as l-arginine is the precursor of NO in the NOS system. L-arginine supplementation is known to improve endothelial function (Bode-Böger et al., 2007), and thus the diminished variation in the $M$ and $\tau$ estimates upon increased nitrate intake would be in line with this. However we have not been able to find any studies regarding the impact of l-arginine on the hemodynamic response to neuronal stimulation in healthy subjects.

4.6 Nitrate and CBF

Nitrate ingestion had no impact on resting CBF estimates in the present study. This differs from another study on nitrate’s effect on CBF in humans (Presley et al., 2011) where an increased CBF in frontal white matter was found. Our study focused on the visual cortex, for which the experimental setup necessitated using only the lower part of the 23 channel headcoil. This did not allow comparable signal-to-noise-ratio in the frontal areas, and thus it would be hard to replicate Presley et al.’s results for the frontal white matter. Further differences include the fact that our participants were considerably younger than the participant group in the Presley et al. study. Furthermore, the plasma levels of nitrate reported in that study were substantially higher than ours, and the nitrate intake was modulated via alternate diets instead of saline solutions.

4.7 Perspectives

The fact that the local hemodynamic response to neuronal activity, and not the resting CBF, is changed by nitrate intake in young healthy adults, indicates that nitrate, in this setting, may act more as a facilitator of the communication between neurons and the nearby microvasculature than as a simple plasma-contained vasodilator. Such an effect could, for example, be based on a nitrite-related facilitation of the vasculature’s response to neuronal metabolism in the form of pH shifts, as hypothesized in the introduction. It could perhaps also be based on an improvement in the oxygen efficiency of the neuronal metabolism, which could reduce any potential $O_2$ limitations on neuronal activity. But based on the present data, it is not possible for us to address this matter in any further
detail. However, if nitrate intake could be proven to facilitate the communication between neurons and
the microvasculature, this could be an important finding with respect to diseases relating to
cerebrovascular dysfunction, as well as affordable treatment options.

With respect to neuroimaging, knowing or controlling the nitrate levels of the participants studied could
help account for some of the variance in the BOLD responses and thus help in discerning the actual
effects one wishes to measure. Even though few studies look specifically at the hemodynamic lag or
the amplitude of the BOLD response, changes in these parameters can still affect the statistical
parametric maps of cerebral activity upon cognitive tasks. This could be of particular importance in
studies involving patient groups where inflammatory responses can cause inducible NOSs to produce
large amounts of NO, thus increasing the plasma concentration of nitrate to the same extent as the
nitrate intake in the current study did (Hersch et al., 2005): median plasma NO$_x$ [R1.3]: 110 μM with a
interquartile range of 39-250 μM reported).

4.8 Future Directions

To exclude the potentially confounding effects of metHb on the neurovascular response, metHb should
be measured in future studies to ensure that nitrate intake does not significantly impact the metHb
level. Furthermore, the immediate addition of a heme-oxidizing solution to the blood samples would
aid the quality of the nitrite measurements.

A faster but smaller BOLD response is reminiscent of an enhanced metabolic coupling in the brain.

Therefore, high resolution animal studies on the physiological effects of nitrate/nitrite on the CBF-,
CBV- and hemoglobin/oxygenation responses to neuronal activation would certainly aid the
understanding of these anions’ role in the cerebrovascular system.

Finally, studies characterizing the impact of nitrate on the shape of the BOLD response would be a
natural way to go from this study.

5 Conclusions

Dietary intake of nitrate decreases the hemodynamic lag, the BOLD amplitude, and the voxel-wise
variation of both these measures.

Together, the results presented here hint at the potential importance of nitrate in cerebrovascular
physiology, and for the first time reveal that even modest variations in dietary nitrate intake can
modulate the local cerebral hemodynamic response to visual stimuli in human visual cortex. This
suggests a role for dietary nitrate in neurovascular coupling.
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Disclosure of Interests/Conflicts Statement
The authors declare no conflicts of interest.

References


Figure A shows a series of brain images with color-coded regions indicating different median lag estimates in seconds (s).

In Figure B, the data for NaCl is displayed with a scatter plot showing the relationship between F and Median τ (s), along with a graph showing the median lag estimates. The FWE (Family Wise Error) and FDR (False Discovery Rate) thresholds are indicated with dashed lines.

Figure C presents similar data for NaNO₃, with additional labels for PWE and Unc.

Figures D and E illustrate the same data for NaCl and NaNO₃, respectively, focusing on the distribution of Median τ (s) with bars indicating the median lag estimates and the MAD (Median Absolute Deviation).
Group A: NaCl  NaNO\(_3\)  NaCl  NaNO\(_3\)

**Median Lag (s)**

**Mean Amplitude (%)**

---

**Plasma NO\(_3\) in uM**

**Median Lag (s)**

Group A: ○ NaCl ● NaNO\(_3\)
Group B: □ NaCl ■ NaNO\(_3\)
A  NaCl

B  NaNO₃

C

D

E

F

G

Median (s)

Median (s)

Median (s)

Median (s)

Median (s)

Median (s)

Median (s)

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

difference(NaNO₃, NaCl)

mean(NaNO₃, NaCl)

lag +/- sem

Mean (M) (%)

Mean (M) (%)

Mean (M) (%)

Mean (M) (%)

Mean (M) (%)

Mean (M) (%)

Mean (M) (%)

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

NaCl  NaNO₃

*  **
Figure Captions

Figure 1.
Lag estimates. A: Exemplary slices of the BOLD images with median $\tau$ estimates visualized in voxels with uncorrected $P < 0.001$ and lag-cut-off at 9 s superimposed on structural image. B and C: Exemplary voxel-wise median $\tau$ vs. F-plots from one participant. B is with minimized dietary intake of nitrate; C is with high nitrate intake. Median lag estimates with F-cut-offs at FWE=0.05, uncorrected $P=0.001$ (Unc) and FDR=0.05 is shown. Note that both Unc and FDR roughly correspond to the noise floor in the figure, and that FDR is less conservative than Unc. The lag-cut-off at 9 s is also shown. D and E: Exemplary excised voxel-wise $\tau$ histograms for the same participant as in B and C. D is with minimized dietary intake of nitrate; E is with high nitrate intake. The white bar indicates MAD. Median lag estimates with F-cut-offs at uncorrected $P=0.001$ and lag-cut-off at 9 s is also shown.

Figure 2.
Plasma concentrations of nitrate and nitrite: Color- and symbol coding as in inset. Aut = autonomous dietary control. A: Aut1 = Autonomous intake 1, S1 = examination 1, Aut2 = Autonomous intake 2, and S2 = examination 2. A: Dietary intake of nitrate predictively increases plasma concentrations of nitrate and reveals the crossover design of the experiment. B: In the plasma concentrations of nitrite the crossover design is not obvious (median values).

Figure 3.
Hemodynamic lag and BOLD amplitude in relation nitrate intake. Color and symbols are coded as in Figure 2. Red boxes indicates mean. A: High intake of nitrate significantly decreases hemodynamic lag. B: High intake of dietary nitrate significantly decreases the mean BOLD amplitude. C: Hemodynamic lag is significantly correlated with plasma concentrations of nitrate. ***: $P<0.005$, *: $P<0.05$. [R1.5r]

Figure 4.
Treatment-wise lag histograms pooled across participants. Symbol and color-coding as indicated in the figure. About 40,000 voxels contribute to each histogram. Neither with low (A) nor with high nitrate intake (B) is there any correlation between $\tau$ and $M$. C: High dietary nitrate intake (black histogram) causes more voxels to respond with low amplitude (the red circles indicating the difference between the two histograms then lie in the black histogram). D: The low nitrate 2D histogram (A) subtracted from the high nitrate 2D histogram (B). Here it is clear that nitrate causes more voxels to respond with
low amplitude and a lag around the mode. E: Nitrate causes the variation in the \( M \) estimates to decrease significantly. The same is true for the \( \tau \) estimates (F). G: More voxels respond faster during high nitrate intake than during minimized nitrate intake. This is perhaps easiest to discern when studying the difference between the 2 lag histograms indicated by the red circles. Red circles located within the black histogram indicate more voxels responding during high nitrate intake than during minimized nitrate intake. Conversely, red circles located within the white histogram indicate more voxels responding during minimized nitrate intake.

\( **: P<0.01. *: P<0.05. \)

**Figure 5.**

Treatment-wise \( \tau \), \( M \) and 2D histograms masked according to cortical depth. The green and red masks indicate voxels where the probability of being in gray matter (P(GM)) was >30 \%, with the green mask also fulfilling the condition of having a higher probability of being in white matter (P(WM)) than in CSF (P(CSF)). The red mask fulfilled the condition of P(CSF)>P(WM). The superficial GM surface mask (blue) of the outermost layer fulfilled P(GM)<40 \% and P(CSF)>P(WM). Overlap between the red and blue mask is shown in purple. A-C: Mean \( M \) histograms for high (black) and low (white) nitrate intake. Nitrate intake decreases the amplitude of the responding layers at all cortical depths. D-F: 2D histograms of \( \tau \) vs \( M \) where the low nitrate 2D histogram has been subtracted from the high nitrate 2D histogram. Again a general decrease in amplitude and a gathering of the lags around the mode are noticeable across the cortical layers, though some degree of variation is obvious. G-I: Nitrate in general causes the lags to gather around the mode of the histogram across all layers, however note the expansion of the characteristic “tail” of voxels with long lags in the more superficial mask (I).
Group A: NaCl, NaNO<sub>3</sub>
Group B: NaCl, NaNO<sub>3</sub>
**Supplementary Figure 1.**

Stimulus used in the BOLD experiment and the data analysis used in the BOLD experiment. A: The visual stimulus was presented to the participants as ring snippets unveiling the dartboard pattern shown in B. B: Right and left hemifields of the basic dartboard used shown in polar coordinates. Color indicates where one ring snippet visualized in A is situated during a single stimulation cycle. C/D: A graphical representation of the calculations done to estimate $\tau$ and $M$. C: The fit to the canonical hemodynamic response function (HRF) used by SPM and calculation of amplitude. The stimulus onset is shown as a red stick. The HRF is shown in lilac. The fitted response incorporating both the 1st and 2nd harmonic is shown in blue. The fitted response with only the 1st harmonic is shown in black. The peak to peak amplitude (p2p) of the HRF is larger than the p2p of the 1st harmonic (the p2p of a sinusoidal oscillation equals 2 times its amplitude). To estimate the amplitude of the HRF from the oscillation we have thus calculated the ratio between the two and multiplied the p2p of the oscillation with this number. D: To estimate $\tau$, the phase estimate from a forward run is subtracted from the inverse phase from a backward run. This amounts to 2 times $\tau$, and $\tau$ is easily obtained by division with 2.

**Supplementary Figure 2.**

BOLD amplitude in relation to plasma levels of nitrate and hemodynamic lag. A: The mean BOLD amplitude is not correlated with the concentration of nitrate in the participants’ plasma. B: BOLD amplitude and hemodynamic lag is positively correlated. ***: $P<0.005$. [R1.5r]
<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>NaNO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Systolic Blood Pressure</strong></td>
<td>127.6 +/- 2.4 mmHg</td>
<td>126.4 +/- 2.4 mmHg</td>
</tr>
<tr>
<td><strong>Diastolic Blood Pressure</strong></td>
<td>93.7 +/- 1.7 mmHg</td>
<td>92.6 +/- 2.2 mmHg</td>
</tr>
<tr>
<td><strong>Heart Rate</strong></td>
<td>60.0 +/- 2.1 beats/sec.</td>
<td>59.9 +/- 1.9 beats/sec.</td>
</tr>
<tr>
<td><strong>SpO₂</strong></td>
<td>98.2 +/- 0.1 %</td>
<td>98.2 +/- 0.1 %</td>
</tr>
<tr>
<td><strong>Respiratory Rate</strong></td>
<td>15.6 +/- 0.7 breaths / min.</td>
<td>16.4 +/- 0.5 breaths / min.</td>
</tr>
<tr>
<td><strong>Expired CO₂</strong></td>
<td>42.5 +/- 0.6 mmHg</td>
<td>42.2 +/- 0.7 mmHg</td>
</tr>
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*SpO₂ = Blood oxygen saturation measured at the fingertip. Mean +/- SEM. All non-significant*