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Adipocyte-specific Nrf2 deletion negates nitro-oleic acid benefits on glucose tolerance in diet-induced obesity

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ABSTRACT

Obesity is commonly linked with white adipose tissue (WAT) dysfunction, setting off inflammation and oxidative stress, both key contributors to the cardiometabolic complications associated with obesity. To improve metabolic and cardiovascular health, countering these inflammatory and oxidative signaling processes is crucial. Offering potential in this context, the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) by nitro-fatty acids (NO_2-FA) promote diverse anti-inflammatory signaling and counteract oxidative stress. Additionally, we previously highlighted that nitro-oleic acid (NO_2-OA) preferentially accumulates in WAT and provides protection against already established high fat diet (HFD)-mediated impaired glucose tolerance. The precise mechanism accounting for these protective effects remained largely unexplored until now. Herein, we reveal that protective effects of improved glucose tolerance by NO_2-OA is absent when Nrf2 is specifically ablated in adipocytes (ANKO mice). NO_2-OA treatment did not alter body weight between ANKO and littermate controls ($Nrf2^{fl/fl}$) mice on both the HFD and low-fat diet (LFD). As expected, at day 76 (before NO_2-OA treatment) and notably at day 125 (daily treatment of 15 mg/kg NO_2-OA for 48 days), both HFD-fed $Nrf2^{fl/fl}$ and ANKO mice exhibited increased fat mass and reduced lean mass compared to LFD controls. However, throughout the NO_2-OA treatment, no distinction was observed between $Nrf2^{fl/fl}$ and ANKO in the HFD-fed mice as well as in the $Nrf2^{fl/fl}$ mice fed a LFD. Glucose tolerance tests revealed impaired glucose tolerance in HFD-fed $Nrf2^{fl/fl}$ and ANKO compared to LFD-fed $Nrf2^{fl/fl}$ mice. Notably, NO_2-OA treatment improved glucose tolerance in HFD-fed $Nrf2^{fl/fl}$ but did not yield the same improvement in ANKO mice at days 15, 30, and 55 of treatment. Unraveling the pathways linked to NO_2-OA's protective effects in obesity-mediated impairment in glucose tolerance is pivotal within the realm of precision medicine, crucially propelling future applications and refining novel drug-based strategies.

1. Introduction

More than two-thirds of American adults grapple with overweight or obesity according to the National Health and Nutrition Examination Survey (NHANES) conducted by the Centers for Disease Control and Prevention in 2020. Excess weight is associated with the development of cardiometabolic comorbidities including insulin resistance and type 2 diabetes. In normal physiology, white adipose tissue (WAT) serves as a crucial signaling hub influencing energy regulation and glucose homeostasis. However, obesity goes beyond the mere expansion of adipose tissue; it is also marked by dysfunction in adipose tissue. This dysfunction is evident through persistent inflammation, abnormal cytokine production, and increased reactive species (RS) derived from oxygen and nitrogen oxide (ROS and RNS, respectively) [1–4].

Over the past decade, several studies have revealed the positive effects of electrophilic nitro-fatty acids (NO_2-FA) on metabolism and inflammation (reviewed in Refs. [5–8]). These compounds hinder RS generation and the activation of inflammatory pathways by regulating multiple transcription factors and enzymes related to inflammation through post-translational modifications [9–11]. These effects have been observed in various preclinical models, including atherosclerosis, diabetic kidney disease, vascular inflammation, hypertension, insulin resistance, and pulmonary arterial hypertension [12–21]. Notably,

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Abbreviations

ANKO	Adipocyte-specific Nrf2 knockout mice
Nrf2fl/fl	Cre-negative mice; control genotype
WAT	white adipose tissue
Nrf2	nuclear factor erythroid 2-related factor 2
NO2-FA	nitro-fatty acids
NO2-OA	nitro-oleic acid
HFD	high fat diet
DIO	diet-induced obesity
RS	reactive species derived from oxygen and nitrogen oxide
GTT	glucose tolerance test
Keap1	kelch-like ECH-associated protein 1
PB	peanut butter

10-nitro-octadec-9-enoic acid (NO_2-OA) has completed phase I clinical studies (NCT: 02127190, 02248051, 02460146, 02313064, 02547402) and is currently being evaluated in a phase II trial for treating subjects with obesity-related asthma.

As adipose tissue mass, inflammation, and systematic ROS levels are all increased in obese subjects [3], the nuclear factor erythroid 2-related factor 2 (Nrf2)/kelch-like ECH-associated protein 1 (Keap1) pathway has also attracted research interest in the field of obesity and diabetes [22–24]. Under basal conditions, the transcription factor Nrf2 is sequestered in the cytoplasm by Keap1. The cysteine-rich protein Keap1 facilitates the ubiquitination of Nrf2, and ultimately leads to its degradation by the proteasome. Upon exposure to ROS, the sulfhydryl groups of Keap1 cysteines undergo oxidation, inducing allosteric conformational changes in Keap1. These changes prevent its binding to Nrf2 molecules. Consequently, newly transcribed Nrf2 accumulates, undergoes post-translational modifications, and enters the nucleus. There, it binds to the promoters of target genes, which encompass antioxidant and cytoprotective enzymes, among others [25].

Obesity, recognized as a multifactorial disease, demonstrates an intricate association with insulin resistance, diabetes, and other related pathologies. This connection encompasses a diverse range of signaling pathways, including those related to inflammation and oxidative stress [26,27]. In this context, a treatment capable of improving glucose tolerance while simultaneously decreasing inflammation and reactive species generation could prove beneficial in managing obesity, impaired glucose tolerance, insulin resistance and possibly other related comorbidities. We previously demonstrated that NO_2-OA effectively improves glucose tolerance and mitigates hepatic steatosis in mice fed a HFD [11,12]. The precise mechanisms behind these effects have not been explored. Previous work revealed that NO_2-OA preferentially accumulates in WAT, suggesting that adipocytes serve as both a reservoir and a buffering system with the capacity to regulate and maintain NO_2-OA levels [28,29]. Additionally, *in vivo* and *in vitro* treatment with NO_2-OA potently induces Nrf2-dependent gene signaling, and thereby impacting the expression of numerous antioxidant and cytoprotective enzymes, such as heme-oxygenase–1 [5,10,30]. With this knowledge, we hypothesized that Nrf2 signaling in adipocytes could mediate the beneficial effects of NO_2-OA in ameliorating glucose homeostasis in a diet-induced obesity (DIO) setting. Herein, we show that the NO_2-OA-induced improvement in glucose tolerance is lost following the targeted deletion of Nrf2 in adipocytes. This reveals that the Nrf2 pathway in adipose tissue plays a pivotal role in mediating specific protective effects associated with NO_2-OA. In the era of precision medicine, discerning the pathways implicated in NO_2-OA's protective effects is crucial for advancing future applications and refining drug design strategies.

2. Materials and methods

2.1. Mouse model

All animal studies adhered to the University of Pittsburgh Institutional Animal Care and Use Committee's approval (protocol numbers 19116506 and 20016708). Mice were housed under conditions of 22 °C temperature, 50 % humidity, and a 12-h light/dark cycle. $Nrf2^{flox/flox}$ ($Nrf2^{fl/fl}$) and $Nrf2^{fl/fl}$:Adipoq-Cre (adipocyte-specific Nrf2 knockout mice, ANKO) mice were described previously [31] with all of the mice on the albino C57Bl/6J background, B6(Cg)-Tyrc-2J/J. Our breeding colonies were maintained with $Nrf2^{flox/+}$ and $Nrf2^{flox/+}$:Adipoq-Cre mice. The validation of ANKO mice had been established in our prior research [31]. Briefly, ANKO mice display impaired Nrf2 signaling at baseline, and the upregulation of Nrf2 target genes is attenuated following treatment with an Nrf2 inducer, consistent with findings reported in our previous studies [31]. The Cre-negative ($Nrf2^{fl/fl}$) mice were used as the control genotype. Male $Nrf2^{fl/fl}$ and ANKO mice were fed either a HFD (D12492, with 60 % of the adjusted calories derived from fat, 20 % protein, and 20 % carbohydrate) or a low fat (10 %), 70 % carbohydrate (with matching sucrose to D12492), and 20 % protein diet (LFD) for 20 wk beginning at age 5–7 wk. Mouse diets were purchased from Research Diets Inc. (New Brunswick, NJ). Diet and water were supplied *ad libitum* for the entire study. Mouse weights were recorded weekly.

2.2. Treatment protocol

NO_2-OA or vehicle was delivered using peanut butter (PB) pellets. The method of administering drugs through PB pellets has been scientifically validated as an alternative to oral gavage, offering a less stressful means for dosing mice [32,33]. Briefly, peanut butter (smooth Jif™) was heated to 55 °C in a water bath. NO_2-OA or DMSO (vehicle, 0.01 %) was added and mixed by hand with a spatula for 10 min establishing a homogenous suspension. The warm PB mixture with vehicle or NO_2-OA was dripped into a pellet mold (Corticosterone Pellet Mold, Prod No. 106A, Ted Pella, Inc., Redding, CA). This was done using a spatula, with care taken to avoid creating air pockets. Each mold consisted of 15 square wells. Quality control experiments showed we could consistently make PB pellets that weighed 100 ± 2 mg. PB pellets were made weekly to adjust for the mean body weight for each treatment group. The target amount provided a specific dose of NO_2-OA at 15 mg/kg per 100 mg PB pellet. After each mold was cooled to room temperature (less than 3 min), placed on dry ice, and stored at −80 °C. Quality control experiments were conducted weekly to verify the NO_2-OA concentration present in the PB pellet (details regarding measurements are provided below). These weekly measurements consistently indicated the absence of breakdown and/or oxidation products of the NO_2-OA in the PB pellet (data not shown).

Prior to the treatment period, mice underwent a 10-day acclimation period to the PB pellets (with only vehicle). During this time, all mice were dosed daily by placing a single PB pellet (with DMSO) in an empty cage and carefully placing the mouse in the cage. By the conclusion of the acclimation period, the average pellet consumption time for all mice was less than 1 min. During the treatment phase, frozen pellets of PB with NO_2-OA or vehicle were removed from the mold, placed in separate containers, and then placed on dry ice to keep the pellets frozen. Among all mice, there was no difference in PB pellet consumption time between the vehicle control (DMSO) or NO_2-OA treatments. Throughout the treatment phase, there were fewer than 10 instances of incomplete PB pellet consumption, all of which were recorded.

2.3. Glucose tolerance tests

Mice were fasted for 5 h and a glucose tolerance test (GTT) was performed as previously described [21].

2.4. Body composition

Body weight was measured using a precision scale. Body composition analysis of mice, including the measurements of fat- and lean-mass, was conducted using the EchoMRI system (EchoMRI, Houston, TX). This system employs an NMR-MRI-based technology. EchoMRIs were conducted on day 72 (just before treatment initiation) and day 124 (during the treatment phase, 16 days before the endpoint).

2.5. Blood and tissue collection

At week 20, mice were weighed, euthanized, and tissues and blood were collected. Tissues were immediately snap frozen in liquid nitrogen and blood was collected by cardiac puncture as previously described [34].

2.6. NO_2-OA measurements

The quality control assessment of NO_2-OA levels in PB pellets involved the following steps. Frozen PB pellets were extracted by adding methanol in the presence of isotopically labeled standard d4-NO_2-OA. This was followed by vortexing and then centrifugation at 12,000 g for 5 min. The supernatant was collected and subsequently analyzed using HPLC-MS/MS as previously described [35]. For the quantification of NO_2-OA, NO_2-16:1, and NO_2-14:1 *in vivo*, plasma was obtained from the terminal blood draw at week 20. The plasma levels of NO_2-OA and its metabolites were extracted using acetonitrile, analyzed by LC-MS/MS, and quantified using the isotopically labeled d4-NO_2-OA standard as previously described [35–37].

2.7. Statistical analysis

All statistical analyses were performed using Prism 10.1.2 (GraphPad, San Diego, CA). Data were expressed as mean ± SEM and all distributions were tested for normality and homoscedasticity. Data were analyzed by one-way or two-way analysis of variance (ANOVA) with Dunnett's multiple comparison post hoc comparisons unless otherwise specified. Differences between groups with $p < 0.05$ were deemed significant. In the first cohort, prior to treatment, each genotype comprised $n = 15$ mice on the HFD (total of 30 mice). Additionally, there were $n = 5$ per genotype for LFD (total of 10 mice). Following treatment, there were $n = 7$ mice per vehicle group and $n = 8$ mice per NO_2-OA group for HFD-fed $Nrf2^{fl/fl}$ and ANKO mice, respectively. In the second cohort, prior to treatment, each genotype comprised $n = 8$ mice on the LFD (total of 16 mice). As a control to demonstrate the induction of obesity, two male $Nrf2^{fl/fl}$ mice were fed the HFD for 20 weeks. Post treatment, mice were divided into 4 groups: $n = 4$ mice per vehicle group and $n = 4$ mice per NO_2-OA group for HFD-fed $Nrf2^{fl/fl}$ and ANKO mice, respectively.

3. Results

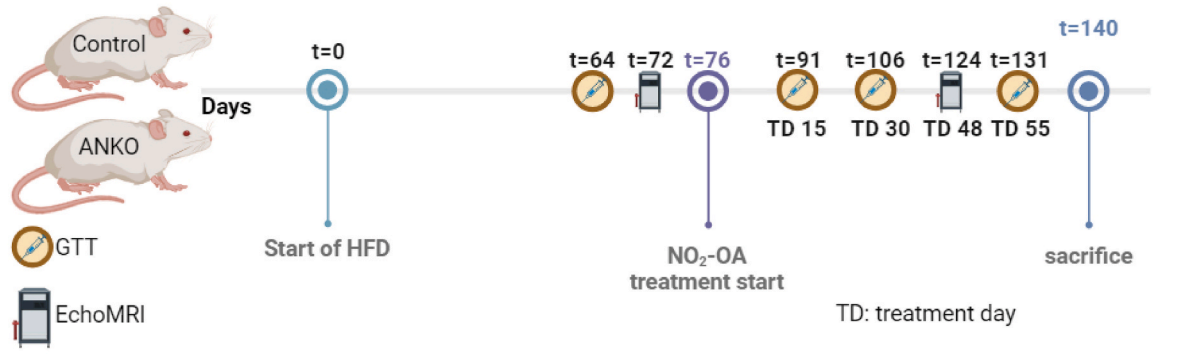
3.1. NO_2-OA treatment did not affect the weight gain in HFD-fed $Nrf2^{fl/fl}$ and ANKO mice

In previous murine model studies, NO_2-OA was administered by gavage using olive oil or triolein as carrier, or delivered via osmotic mini pump, which required minor surgery and anesthesia. To mitigate unnecessary stress and potentially confounding effects, we opted to use PB pellets in this study. PB pellets have been successfully utilized in prior studies and validated as a reliable alternative to oral gavage for dosing mice [32,33]. Each 100 mg PB pellet delivered a dose of 15 mg/kg of NO_2-OA. Scheme 1 provides a visual representation of the timeline for the entire study.

Both $Nrf2^{fl/fl}$ and ANKO mice fed a HFD gained weight rapidly compared to $Nrf2^{fl/fl}$ mice on the LFD. By day 20, a statistically significant difference in body weight emerged between the $Nrf2^{fl/fl}$ and ANKO mice fed a HFD compared to the LFD-fed mice. This difference in weight not only persisted but also notably increased as the study progressed, culminating by day 140 when the study concluded (Fig. 1). The same data was re-plotted showing the body weight before treatment (Fig. 1B) and after treatment with NO_2-OA (Fig. 1C). At day 75, the mice were randomized to groups of equal weight and treatment with vehicle or NO_2-OA started at week 11. The body weight curves are illustrated in Fig. 1C. There is no statistically significant difference between NO_2-OA and vehicle between $Nrf2^{fl/fl}$ and ANKO mice fed the HFD. Additionally, there is no difference in weight with the treatment of NO_2-OA in the $Nrf2^{fl/fl}$ mice fed the LFD. In this original study, we did not have enough ANKO mice to put on the LFD. A subsequent study was performed with an emphasis of $Nrf2^{fl/fl}$ and ANKO mice fed the LFD (Supp Fig. 1). There was no statistically significant difference in body weight between $Nrf2^{fl/fl}$ and ANKO mice on the LFD before or after NO_2-OA treatment. In this cohort, as expected, there was a gain in body weight by $Nrf2^{fl/fl}$ mice fed the HFD.

3.2. The administration of NO_2-OA did not alter the fat mass ratio in HFD-fed mice

$Nrf2^{fl/fl}$ and ANKO mice fed the HFD had similar increases in fat mass and decreases in lean mass percentage compared to LFD-fed $Nrf2^{fl/fl}$ mice at day 72 (immediately before treatment started). Both fat and lean mass are reported as a ratio of body weight (Fig. 2A). On day 48 of treatment (day 124 of HFD/LFD feeding), EchoMRI was performed. As expected, the body composition differences between the mice on the HFD and those on the LFD remained consistent (Fig. 2B). Moreover, no disparity was observed in fat mass and lean mass ratios, and overall body



Scheme 1.

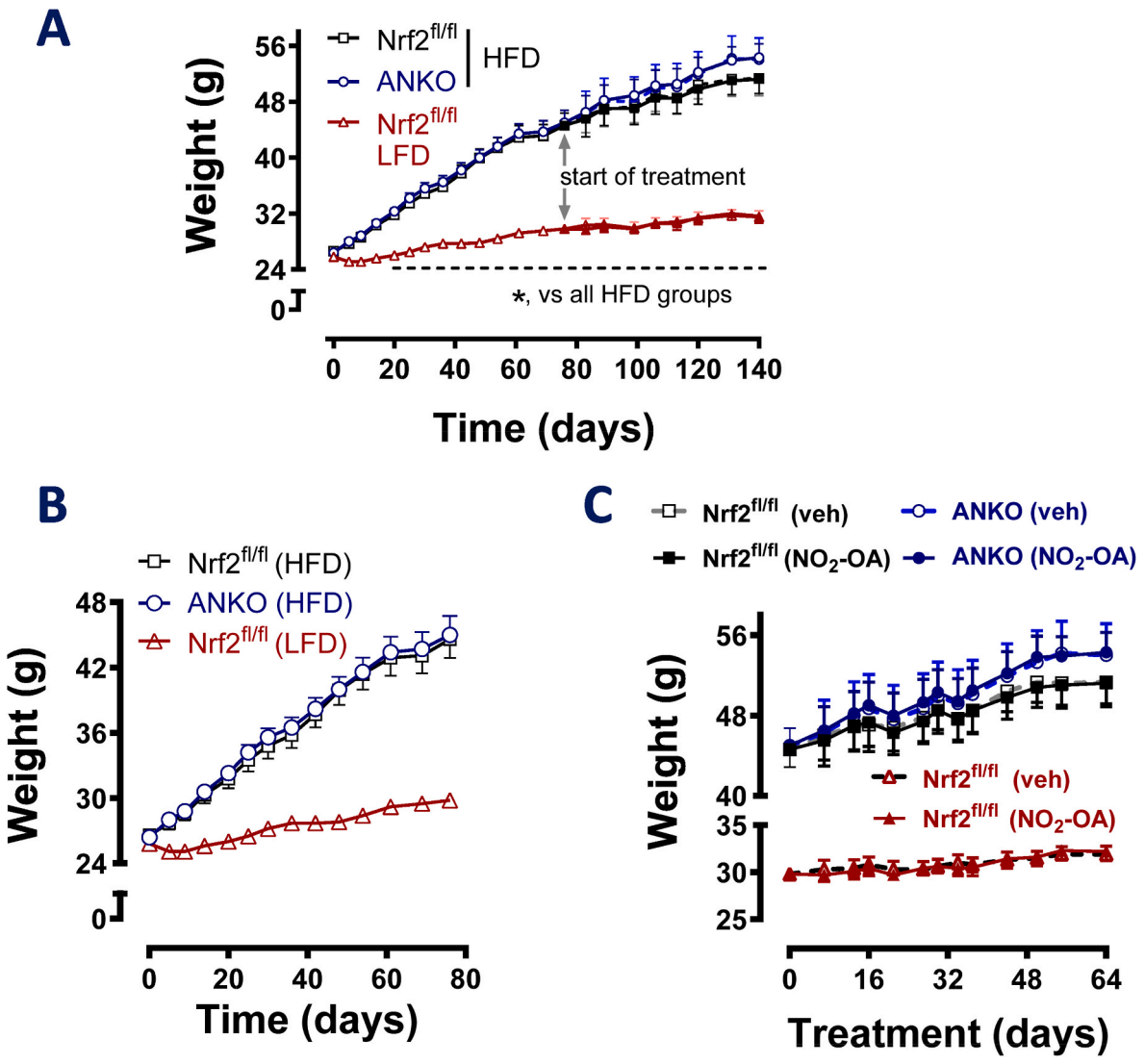


Fig. 1. Body weight curves of mice fed LFD or HFD for 20 weeks (**A**). Mice were randomized to groups of equal weight and treatment with vehicle or NO_2-OA started at week 11. Re-plotted data showing the weights of ANKO mice on HFD as well as $Nrf2^{fl/fl}$ mice on LFD and HFD before treatment (**B**) and during treatment with vehicle of NO_2-OA (C). Values shown are mean ± SEM (n = 7–8 mice/group for HFD and n = 5 mice/group for LFD). For body weight curves, 2-way ANOVA was performed using Šídák's multiple comparisons test. Starting at day 20, both $Nrf2^{fl/fl}$ and ANKO mice on the HFD were significantly different from the $Nrf2^{fl/fl}$ mice on the LFD. There was no significant difference between NO_2-OA and vehicle for any of the groups throughout the treatment course.

weight between the HFD-fed $Nrf2^{fl/fl}$ and ANKO mice treated with NO_2-OA compared to those treated with the vehicle (Fig. 2B). Additionally, no difference was observed between LFD-fed $Nrf2^{fl/fl}$ and ANKO mice before and after treatment with NO_2-OA (Suppl Fig. 2).

3.3. HFD-fed $Nrf2^{fl/fl}$ and ANKO mice have impaired glucose tolerance at day 64

A glucose tolerance test (GTT) was performed on day 64. As expected after a glucose load (1.4 mg/g), the mice fed the HFD were not able to dispose of their circulating glucose as quickly as the LFD-fed $Nrf2^{fl/fl}$ mice (Fig. 3). In terms of glucose curves, there was no difference between the HFD-fed $Nrf2^{fl/fl}$ and ANKO mice. The mice were fasted for 5 h before the GTT experiment was performed. On the day of the GTT, fasting blood glucose levels were recorded at t = 0 (Fig. 3B) and mice were weighed (Fig. 3C). Both genotypes on the HFD had significantly increased fasting blood glucose levels and body weight compared to the LFD-fed $Nrf2^{fl/fl}$ mice. No significant difference was observed between the two genotypes.

3.4. NO_2-OA treatment improves glucose homeostasis in HFD-fed $Nrf2^{fl/fl}$ mice but not in ANKO mice

GTTs were performed at day 15, 30, and 55 of treatment with NO_2-OA or vehicle. $Nrf2^{fl/fl}$ mice treated with NO_2-OA showed an improvement in HFD-induced impaired glucose tolerance as early as 15 days after the initiation of the treatment, whereas the same treatment failed to yield similar improvements in ANKO mice (Fig. 4A). The area under the curve (AUC) was calculated for each time point (Fig. 4B) as well as the fasting blood glucose levels at t = 0 of each GTT at days 15, 30, and 55 (Fig. 4C). The improved glucose disposal observed in $Nrf2^{fl/fl}$ mice treated with NO_2-OA was sustained throughout the entire treatment period of 15, 30, and 55 days. However, no significant differences were observed in fasting blood glucose levels (t = 0) between Nrf2 and ANKO, irrespective of NO_2-OA treatment (Fig. 4C). Additionally, on day 55, a GTT conducted on the control LFD-fed $Nrf2^{fl/fl}$ and ANKO mice did not reveal significant differences in glucose tolerance, fasting blood glucose, and body weight (Suppl Fig. 3). To confirm similar circulating NO_2-OA levels between the two genotypes, we quantified both the native form and the two most prevalent β-oxidation products of NO_2-OA. No

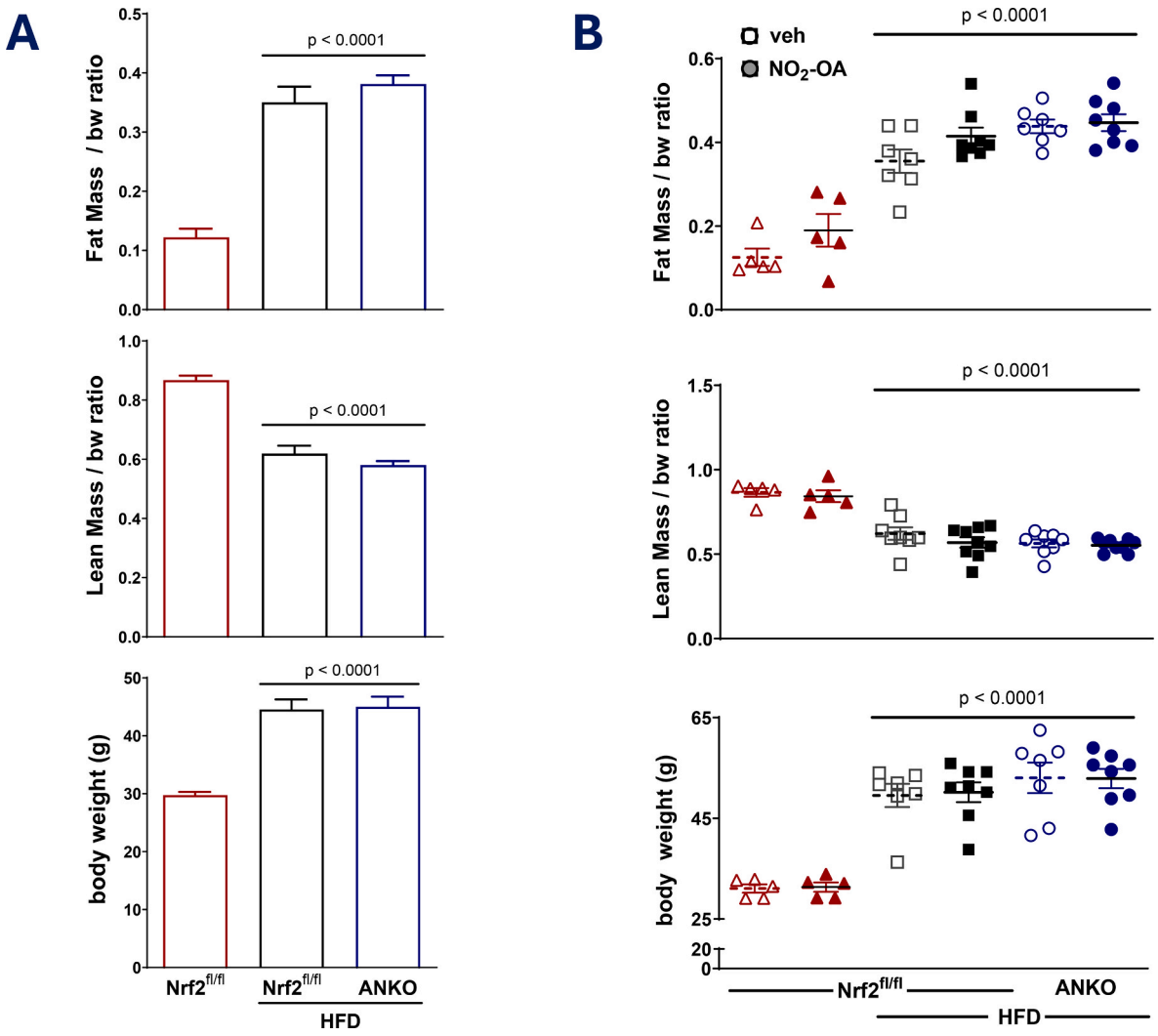


Fig. 2. Body weight and body composition of $Nrf2^{fl/fl}$ and ANKO mice fed LFD or HFD at day 72 (before treatment, **A**). Body composition of $Nrf2^{fl/fl}$ and ANKO mice treated with vehicle or NO_2-OA at day 48 of treatment (day 124 of HFD/LFD, **B**). Fat and lean mass are expressed as a ratio to body weight. Values shown are mean ± SEM (n = 7–8 mice/group for HFD and n = 5 mice/group for LFD). Results of one-way ANOVA with Dunnett's multiple comparison test was used to determine significance. The p values are indicated in the graphs and compared to the $Nrf2^{fl/fl}$ mice on the LFD.

significant differences were observed in plasma NO_2-OA, as well as its electrophilic β-oxidation products, the dinor (C16) and tetranor (C14), across all treatment groups (Supp Fig. 4). These results highlight the consistent levels of plasma NO_2-OA and metabolite concentrations across both genotype and dietary conditions. This reinforces the notion that the observed effects in glucose homeostasis are unrelated to variations in NO_2-OA levels or metabolism between the genotypes and diet.

3.5. The adminstration of NO_2-OA did not result in any changes in the weight of liver, visceral-, subcutaneous-, and peri-renal-adipose tissue depots

As anticipated, there was a significant increase in epididymal, inguinal, and peri-renal absolute fat weight (and as a ratio to body weight) in the HFD-fed compared to LFD-fed mice. Conversely, the LFD-fed mice showed significantly lower kidney-to-BW and heart-to-BW ratios in comparison to their HFD-fed counterparts. Despite improved glucose tolerance in the NO_2-OA treated HFD-fed $Nrf2^{fl/fl}$ mice, there was no difference in any of the organ weights compared to the HFD-fed mice treated with vehicle or ANKO mice with or without NO_2-OA treatment (Fig. 5). In the second cohort, the gross weight of liver, kidney, heart, epididymal, inguinal, and peri-renal fat remained unchanged in both the LFD-fed $Nrf2^{fl/fl}$ and ANKO mice, regardless of whether they were treated with NO_2-OA (Suppl Fig. 5). Furthermore, preceding sacrifice, the mice underwent a 16-h fast. During this time, the fasting blood glucose levels were significantly higher in all the HFD-fed mice, irrespective of treatment, compared to the LFD-fed mice. Additionally, the changes in weight before and after fast were recorded (Suppl Fig. 6).

4. Discussion

In this study, we demonstrated that NO_2-OA improves glucose tolerance in HFD-fed mice, and this effect is dependent on adipocyte Nrf2. This marks the first study to identify a potential mediator, specifically in adipose tissue, for the protective effects of NO_2-OA against glucose intolerance.

The transcription factor Nrf2 plays a pivotal role in various pathways affecting both regular physiology and pathophysiological responses to cellular stress. In the realm of metabolic conditions, whether by pharmacological methods or genetic manipulation, the activation of Nrf2 offers partial protection against obesity, hyperglycemia, and fatty liver disease [38–44]. Nevertheless, intriguingly, global Nrf2 knockout mice show protection against DIO [38,41,42,45]. Undoubtedly, the role of Nrf2 in obesity-related complications is a subject of considerable debate.

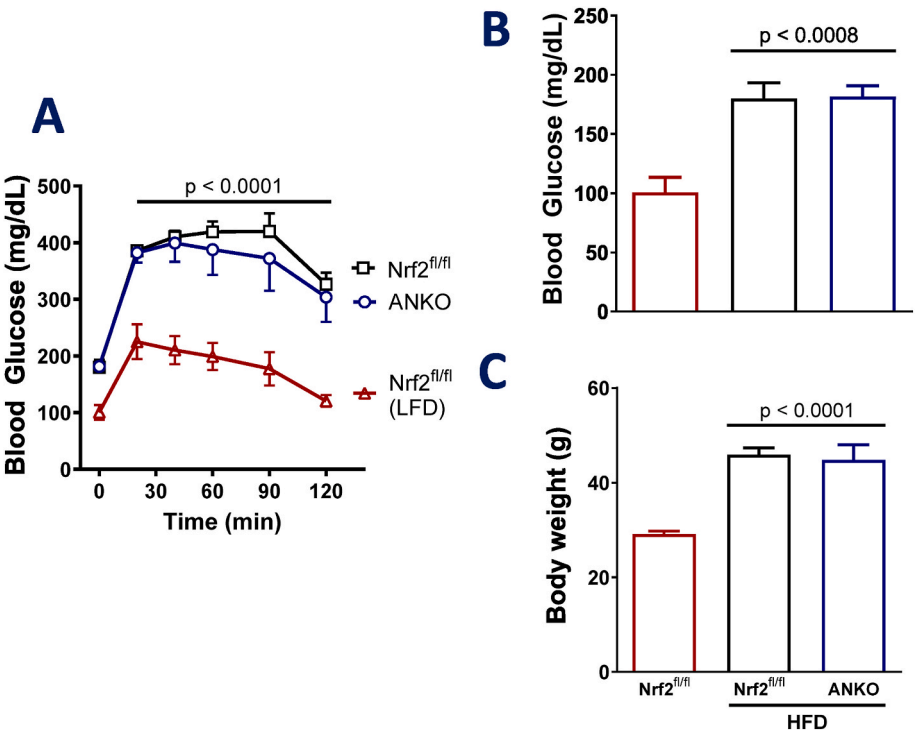


Fig. 3. Both $Nrf2^{fl/fl}$ and ANKO mice fed a HFD have impaired glucose tolerance compared to $Nrf2^{fl/fl}$ on LFD. A glucose tolerance test (GTT) was performed at week 9 of the HFD/LFD feeding (**A**). Fasting blood glucose levels were recorded at t = 0 of GTT (**B**). Weight of mice were recorded on the day of GTT (**C**). Every time-point is the mean ± SEM. For GTT, 2-way ANOVA was performed using Šídák's multiple comparisons test. For FBG and weight, significance was determined with 1-way ANOVA using the post hoc Dunnett's multiple comparison test. The p values are indicated in the graphs and compared to the $Nrf2^{fl/fl}$ mice on the LFD (n = 15 mice/genotype for HFD and n = 5 mice/genotype for LFD).

While the various mechanisms that contribute to these complications are diverse, it is not the primary focus of this article.

The activation of the Nrf2 pathway offers protection against obesity and related complications, such as fatty liver disease and hyperglycemia. This protection is typically achieved by mitigating oxidative stress and inflammation in specific tissues, while also suppressing gluconeogenic and lipogenic pathways in the liver [44,46–48]. Whole body Nrf2 deletion results in an improved phenotype marked by reduced weight gain and improved glucose tolerance after being fed a HFD. This improvement is potentially associated with increased secretion of the hepatokine FGF21 [38] and sirtuin 1 [49], as well as elevated energy expenditure [49–51]. In our previous study, we investigated the impact of adipocyte- and hepatocyte-specific deletion of Nrf2 during HFD-induced obesity. These mice were exposed for an extended duration, spanning 6 months on the HFD. It is important to note that the deletion of Nrf2 from adipocytes did not show a significant impact on glucose tolerance or body composition during the initial 4 months of HFD exposure. In contrast, hepatocyte Nrf2 deletion slightly improved insulin sensitivity without any notable differences in liver fat content [31]. In this study, we validated the results of the previous research, as the ANKO mice exhibited no differences in body weights, body composition, and glucose tolerance after a 5-month exposure to the HFD. The only distinction between the two studies was the duration of HFD exposure, with the previous study having a time point at 4 months [31].

Based on these previous observations that adipocyte-specific deletion of Nrf2 did not lead to differences in the observed phenotype after 4 months on HFD compared to their $Nrf2^{fl/fl}$ counterparts [31], we designed the present study accordingly; we introduced the NO_2-OA treatment at a timepoint (76 days on HFD) when mice were already obese but the different genotypes do not show any difference in their metabolic parameters up to a timepoint (as the last GTT was performed at day 131 on the HFD) when no genotype based differences were expected. Building on our earlier observations that adipocyte-specific deletion of Nrf2 did not result in discernible differences in the observed phenotype compared to their $Nrf2^{fl/fl}$ counterparts after 4 months on HFD, we structured the experiment accordingly. We initiated the NO_2-OA treatment at day 76 of HFD exposure, a point when mice were already obese and had impaired glucose tolerance, but there were no differences in metabolic parameters between the two genotypes. Importantly, there were no differences in weight and impaired glucose tolerance observed during all of the GTTs throughout this study between the two genotypes, a crucial point for interpreting the impact of NO_2-OA.

This current study emphasizes that the protective impact of improved glucose tolerance in the HFD-fed mice treated with NO_2-OA is specific for only the $Nrf2^{fl/fl}$ mice. Deleting Nrf2 in adipocytes blocks the protective effects of NO_2-OA, highlighting the pivotal role of adipose tissue Nrf2 in mediating at least part of NO_2-OA's protective effect. There was no change in body weight throughout the course of the treatment of 9 weeks between the $Nrf2^{fl/fl}$ and ANKO mice on the HFD (Fig. 1). Nor any change in body composition at day 48 of treatment with NO_2-OA or vehicle between the $Nrf2^{fl/fl}$ and ANKO mice on the HFD (Fig. 2). These findings align with our previous study [34], showing no change in body weight, fat mass, or lean mass between the HFD-fed mice treated with NO_2-OA or vehicle. It is important to highlight the experimental differences between the aforementioned work and this current study, most notably in the method of drug delivery (osmotic mini pumps versus daily PB pellet consumption) and the treatment duration (42- versus 64-days). Th present study administered a daily dose of NO_2-OA (via PB), reflecting the clinical relevance of taking medicine orally daily and the subsequent first-pass metabolism in the liver. Additionally, there's a slight difference in the background of the mice employed in this study. While both studies used mice on a C57BL/6J background, in the present study, these mice carried a homozygous mutation in the tyrosinase gene (Tyr^{c-2J}), resulting in the absence of pigment in the skin,

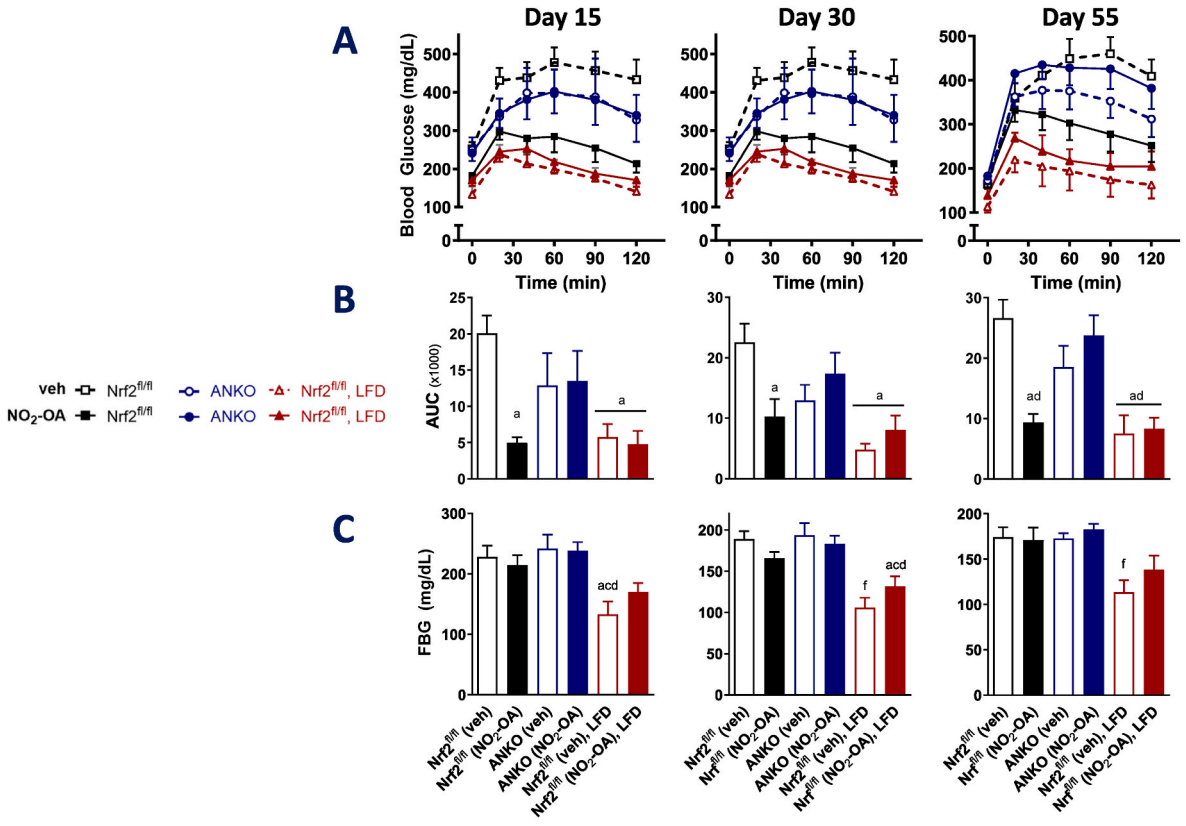


Fig. 4. NO_2-OA treatment improved HFD-mediated impaired glucose tolerance in $Nrf2^{fl/fl}$ - but not ANKO-mice. GTTs were performed at day 15, 30, and 55 of treatment (**A**). Area under the curve (AUC) analysis for each time point is plotted (**B**). Fasting blood glucose levels were recorded at t = 0 of GTT at each day (**C**). Every time-point is the mean ± SEM. For GTT, 2-way ANOVA was performed using Šídák's multiple comparisons test. For AUC and FBG, 1-way ANOVA with Dunnett's multiple comparison was used test to determine significance. $p < 0.05$ determined significance for the following groups: a, vs $Nrf2^{fl/fl}$ (veh); c, ANKO (veh); d, ANKO (NO_2-OA); f, vs all mice on the HFD (n = 7–8 mice/group for HFD and n = 5 mice/group for LFD).

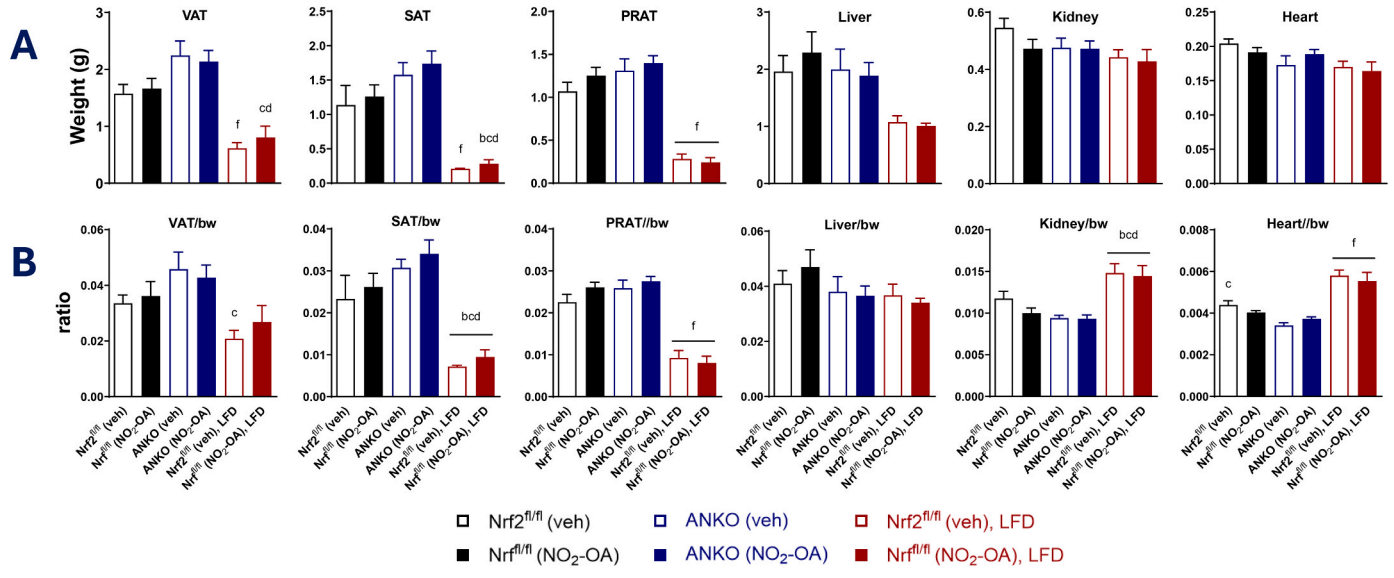


Fig. 5. The weights of epididymal fat pad (visceral adipose tissue, VAT), inguinal fat pad (subcutaneous adipose tissue, SAT), peri-renal adipose tissue (PRAT), liver, kidney, and heart were recorded at sacrifice (**A**). The organ mass is normalized to body weight (**B**). Values shown are mean ± SEM (n = 7–8 mice/group for HFD and n = 5 mice/group for LFD). Results of one-way ANOVA with Dunnett's multiple comparison was used test to determine significance. $p < 0.05$ determined significance for the following: a, vs $Nrf2^{fl/fl}$ (veh); b, vs $Nrf2^{fl/fl}$ (NO_2-OA), c, ANKO (veh); d, ANKO (NO_2-OA); f, vs all mice on the HFD.

hair, and eyes. This study also validated our previous findings regarding improved glucose control without weight change. Despite the minor variations between these two studies, the net results support that compounds such as NO_2- OA may provide an efficient treatment for obesity without adverse effects, specifically that adipocyte Nrf2 is essential for transducing the beneficial actions of NO_2- OA on glucose homeostasis.

We centered this study on adipose tissue, building on previous findings [28,29] demonstrating the accumulation of NO_2-OA in adipose

tissue. Additionally, we already knew the effects of adipocyte-specific deletion of Nrf2 in the long-term outcomes of HFD-induced obesity. The implemented experimental strategy precisely targets adipocytes with *Adipoq* promoter-driven Cre recombinase expression (Adipoq-Cre mice). An alternative approach could involve utilizing the AdipoqCreER mice, allowing for the induction of Nrf2 deletion by tamoxifen when necessary. However, the introduction of tamoxifen into the treatment would have required additional controls. Since we were already aware that Nrf2 deletion in adipocytes does not affect the metabolic phenotype of mice after 4 months on HFD, we opted for the model presented in this work. Additionally, exploring the potential involvement of Nrf2 in macrophages in mediating the protective effects of NO_2-OA would be intriguing. This is particularly pertinent, considering that NO_2-OA is widely acknowledged for its potent anti-inflammatory effects [5,52–54]. Nrf2 suppresses macrophage inflammatory responses [55], and myeloid-specific Nrf2 deletion results in a deteriorated metabolic phenotype, characterized by increased NASH, worsened glucose tolerance, and elevated inflammatory markers following HFD exposure [56].

With the recent advent and growing popularity of incretin-based therapies for obesity, including glucagon-like-peptide-1 (GLP-1) and GLP1/glucose-dependent insulinotropic polypeptide (GIP) receptor agonists, which were originally designed for diabetes treatment, the landscape of medical approaches to obesity has shifted in everyday practice [57]. It is no longer limited to lifestyle modifications such as diet and exercise. These drug categories can act as companions to these measures and aid patients in losing 15–20 % of their original body weight [58–60]. Even though these drugs have pleiotropic effects [61] and can reduce the overall cardiovascular risk (20 % in the case of semaglutide) [62], their main effect for the weight loss lies on their action on various hypothalamic nuclei by increasing the sensation of satiety and partially reducing the appetite [63]. There is growing evidence suggesting that the long-term administration of these drugs is required to sustain weight loss over an extended period [64,65]. Moreover, considering that the effects of GLP-1/GIP analogs on adipose tissue are relatively limited and their impact on the liver is mainly indirect, the simultaneous introduction of another drug alongside these analogs, ideally as a dietary supplement, could be of benefit. It's important to note that there are patients for whom this treatment is not efficient due to reasons not entirely understood, and there are also relative contraindications, such as in patients with pancreatitis, to the use of these incretin-based treatments [66–68]. The utilization of NO_2-OA and related small molecule nitroalkenes could function as a complementary drug or dietary supplement, offering an alternative treatment option. Additionally, the exploration of strategies for tissue-specific delivery of nitroalkenes has the potential to minimize any potential side effects.

NO_2-OA could serve as a safe drug that can be easily administered and has the potential to improve glucose metabolism. The development of methods for specific delivery to adipocytes could potentially facilitate cell-specific activation of the Nrf2 pathway, thereby limiting potential side effects associated with systemic Nrf2 activation. The exact mechanisms of adipocyte Nrf2-dependent ameliorated glucose tolerance after treatment with NO_2-OA warrants further investigation. Targeted drugs managing glucose levels in obesity are crucial in preventing diabetes and its complications. If used on a large scale, they could offer hope in curbing the diabetes epidemic and enhancing overall metabolic well-being.

CRediT authorship contribution statement

D.V. Chartoumpekis: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **I. Chen:** Writing – review & editing, Investigation. **S.R. Salvatore:** Writing – review & editing, Methodology, Investigation, Formal analysis. **F.J. Schopfer:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization. **B.A. Freeman:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **N.K.H. Khoo:** Writing – review & editing, Writing – original draft, Validation, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2024.06.002.

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