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# Fatty acid desaturase activity in mature red blood cells and implications for blood storage quality

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## Abstract

**Background—**Increases in the erythrocyte degree of fatty acid desaturation is reported in response to exercise, aging or diseases associated with systemic oxidant stress. However, no studies have focused on the presence and activity of fatty acid desaturases (FADS) in the mature erythrocyte.

**Study Design and Methods—**Steady state metabolomics and isotope-labeled tracing experiments, immunofluorescence approaches and pharmacological interventions were used to

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**Authors' contributions** FC, EJM, TN, MHA, AK, IW, MPB, SK, PWB, SLS, TT, XF, JCZ collected and stored the samples. MPB, SK, XF and JCZ contributed to the design, execution and finalization of RBC storage studies in the REDS III RBC Omics cohort. FG performed immunofluorescence studies. JB, EJM, FC, FG, ADA performed metabolomics analyses (steady state and tracing experiments). AH, KHD, JCZ performed mouse studies. ADA prepared all figures and tables and wrote the first draft of the manuscript, which was revised and finally approved by all the authors.

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determine the degree of fatty acid unsaturation, FADS activity as a function of storage, oxidant stress and G6PD deficiency in human and mouse erythrocytes.

**Results—**In 250 blood units from the REDS III RBC Omics recalled donor population, we report a storage-dependent accumulation of free mono-, poly-(PUFAs) and highly unsaturated fatty acids (HUFAs), which occurs at a faster rate than saturated fatty acid accumulation. Through a combination of immunofluorescence, pharmacological inhibition, tracing experiments with stable isotope-labeled fatty acids and oxidant challenge with hydrogen peroxide, we demonstrate the presence and redox sensitive-activity of FADS2, FADS1 and FADS5 in the mature erythrocyte. Increases in PUFAs and HUFAs in human and mouse erythrocytes correlates negatively with storage hemolysis and positively with post-transfusion recovery. Inhibition of these enzymes decreases accumulation of free PUFAs and HUFAs in stored erythrocytes, concomitant to increases in pyruvate/lactate ratios. Alterations of this ratio in G6PD deficient patients or units supplemented with pyruvate-rich rejuvenation solutions corresponded to decreased PUFA and HUFA accumulation.

**Conclusion—**Fatty acid desaturases are present and active in mature erythrocytes. Their activity is sensitive to oxidant stress, storage duration and alterations of the pyruvate/lactate ratio.

### Keywords

PUFA/HUFA; pyruvate/lactate; oxidant stress; erythrocyte; transfusion medicine

## Introduction

Red blood cells (RBCs) provide an easily accessible window on systems physiology, owing to their sheer numbers (~84% of total human cells) and capillary perfusion of the whole body.[1] Preservation of RBC morphology is essential for RBCs to squeeze through capillaries to perform gas exchange in the peripheral circulation. However, RBC morphology and membrane flexibility are impacted by factors such as membrane lipid composition as well as the degree of lipid unsaturation and oxidation.[2] Historically, RBCs have been reported to take up fatty acids from circulation and, as such, the fatty acyl composition of RBC membrane lipids closely mirrors that of plasma and is significantly modulated by dietary intervention (e.g., supplementation of n-3 fatty acids).[3,4] Factors such as exercise[5–7], aging,[8–10] nutrition,[11,12] and diseases – from neurological disease[13] to cancer[14,15] – have all been associated with alterations of RBC membrane fatty acyl composition. Because RBCs are incapable of de novo synthesis of long-chain fatty acids such as palmitate,[16] they do rely on an acyl-carnitine system in equilibrium with high-energy acyl-CoAs for the repairing of damaged fatty acyl groups,[17] a pathway that is referred to as the Lands cycle. The Lands cycle is up-regulated under pathological conditions that increase RBC oxidant stress (e.g., sickle cell disease[18]).

Studies have identified the role of RBC membrane lipid composition and oxidation as a critical modifier of stored RBC capacity to circulate following transfusion.[19] Approximately 100 million units of packed RBCs are stored yearly in blood banks for up to 42 days with the potential for oxidant stress during refrigeration.[20] Storage-induced oxidant stress promotes a series of biochemical and morphological changes,[21] such as decreases in high

energy phosphate compounds, altered ion homeostasis – including increases in intracellular calcium, as well as the migration to the membrane of antioxidant enzymes with moonlighting phospholipase A2 function (e.g., peroxiredoxin 6 [22]). Activation of such enzymes ultimately targets membrane lipids by promoting fatty acid release from complex membrane lipids as a potential damage repair mechanism.[21] Under physiological conditions, these phenomena are mitigated by the RBC antioxidant systems, including NADH and NADPH-dependent antioxidant enzymes.[23] However, such systems are dysregulated in blood collected from some donor volunteers, such as those with deficient activity of glucose 6-phosphate dehydrogenase (G6PD). G6PD is the rate limiting enzyme of the pentose phosphate pathway (PPP), which generates the NADPH required to reduce oxidized glutathione and several NADPH-dependent redox systems (e.g., glutathione peroxidase, catalase, peroxiredoxins, glutaredoxins, thioredoxin reductase system, biliverdin reductase B, and the ascorbate-tocopherol axis[23]). G6PD deficiency – a series of mutations that result in a varying degree of residual G6PD activity from < 10% to < 1% - is the most common enzymopathy in humans, affecting ~400 million people and up to 13% of the African American donor population. Refrigerator stored RBCs from G6PD deficient donors demonstrated decreased 24-hour post-transfusion recovery in autologous recipients.[24] RBCs from G6PD deficient donors are characterized by a compensatory activation of glycolysis – owing to the ablation of fluxes through the hexose monophosphate shunt – and by a characteristic increase in pyruvate/lactate ratios.[9] This effect is explained by the consumption of NADH by methemoglobin reductase to counteract the accumulation of ferric iron and increased susceptibility to oxidant stress-induced hemolysis in these erythrocytes. [9,25]

Evidence in cancer cells suggests a role of fatty acid desaturase (FADS) activity as a means to promote glycolysis by recycling NADH back to NAD+, as a compensatory mechanisms to the Warburg phenotype- i.e., reliance on aerobic glycolysis - in highly proliferating cells.[26] Altered FADS activity is linked to impaired hematopoiesis in the context of aging,[10] however, it remains unclear if FADS could play a role in mature RBCs. Of note, RBC dependency on glycolysis as the sole source of energy production in the form of Adenosine Triphosphate (ATP) has helped to understand parallelisms between the Warburg phenotype of cancer cells and RBC metabolism.[27] Observation of FADS activity in cancer cells suggests that delta-5 desaturase (D5D) and delta-6 desaturase (D6D), which are encoded by FADS1 and FADS2, respectively, are required for the synthesis of highly unsaturated fatty acids (HUFAs).[26] Additional studies suggest a role for FADS2 and FADS5 (delta-9 desaturase – D9D or stearoyl-CoA desaturase) in mediating cancer cell plasticity in hepatocellular carcinoma[28] and leukemia[29]. While FADS5 participates in saturated fatty acid (SFA) conversion from stearoyl-CoA to oleate, and other delta-7 desaturases (D7D) catalyze palmitate conversion to palmitoleate, FADS1 and 2 introduce double bonds at fixed positions from the carboxyl end during the successive elongation and desaturation of the essential fatty acid linoleic acid (18:2n-6) to arachidonic acid (20:4n-6), and α-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3). Of note, these fatty acids are reported to increase in refrigerator stored RBCs in all currently licensed additives,[30–34] no study to date has focused on the potential presence and activity of FADS in RBCs.

## Methods

Since all the methods used in this study have been described in prior work, extensive analytical details and related references to methodological papers and their application to recent RBC storage studies are provided in the Supplementary File–Materials and Methods extended.

### REDS-III RBC-Omics study participants and samples:

Donor selection and recruitment for the RBC-Omics study under approved protocols (BioLINCC Study: HLB02071919a) were previously detailed.[35–37] Samples from 13,403 RBC units were stored for ~39–42 days, prior to evaluation for osmotic[38,39] and oxidative hemolysis[38] (12,799 and 10,476, respectively). A subset (n=250) of extreme hemolyzers in this cohort (5$^{th}$ and 95$^{th}$ percentile) donated a second unit, which was sampled at storage day 10, 23 and 42. A subset of these samples (599 total samples) were made available for fatty acid analysis via ultra-high-pressure liquid chromatography coupled to high resolution mass spectrometry (UHPLC-MS – Vanquish-QExactive, Thermo Fisher, San Jose, CA, USA)[38,39] and targeted Multiple-Reaction Monitoring (MRM)-based quantitation, as described.[40]

### Blood collection, processing and storage:

Blood was donated by healthy donor volunteers at Vitalant Research Institute, Denver (n=11) or the University of Athens, Greece (n=6 G6PD deficient and pool of 4 G6PD sufficient matching control donors). Blood was collected into acid citrate dextrose, leukofiltered (i.e., undergoing log2.5 platelet and log4 white blood cell removal by filtration), and stored in AS-3 (studies in the USA) or CPD-SAGM (Greece). RBCs were stored at 4–6°C for 42 days. RBCs and supernatants were separated via centrifugation upon sterile sampling of each unit on days 0, 7, 14, 21, 28, 35, and 42.

### Tracing experiments with $^{13}C_{16}$ -Palmitate and $^{13}C_{18}$-Linoleate, inhibitors and hydrogen peroxide challenge

RBC units (fresh AS-3 leukocyte-filtered units) were supplemented with 5 mM $^{13}C_{16}$-palmitate (product no: CLM-409–0.5, Cambridge Isotopes). FADS inhibitors CP 24,879 (product no: sc-205269A) or SC26196 (product no: sc-361350 - ChemCruz) or PluriSln1 (product no: 484710 – Fisher Scientific) were spiked in RBC units at day 0 at 5 μM concentration in 0.5% DMSO. For $H_2O_2$ challenge, day 0 leukocyte-filtered RBCs (n=3) were incubated with 0.1% hydrogen peroxide for 30 min at room temperature. Alternatively, fresh leukofiltered packed RBC units (n=11, 6 males and 5 females) were spiked with 5 mM $^{13}C_{18}$-linoleate prior to incubation at 37°C for 24h.

### RBC cold rejuvenation:

Conventional or phosphate/inosine/pyruvate/adenine (PIPA)-treated units were processed at Duke University, under approved proteocols, as previously described[41]. Four units of commercially available CPD/AS-1 PRBCs (leukocyte filtered, group A+) were purchased after 3 days of storage to perform the metabolomics analysis. Each unit was split into a pediatric bag aliquot system (Charter Medical, Winston-Salem, NC), and then stored at 1–6 °C in accordance with AABB regulations. For the cold rejuvenation aliquot, rejuvenation

solution (Rejuvesol® red blood cell processing solution, Zimmer Biomet, Warsaw, IN) was added in a 1:7 volume ratio at day 3 of storage. All bags were stored for 15 days from the date of collection prior to either (i) no treatment (control); (ii) cold rejuvenation with Rejuvesol® added to the RBCs in a 1:7 volume ratio at 4°C, without incubation at 37 °C for one hour[42] (Plasmatherm, Barkey/Genesis, Ramsey, NJ) then washed with a CATS cell saver (Terumo BCT, Lakewood, CO) on day 15 prior to sampling at the end of storage for fatty acid measurements.

### Mouse RBC storage and post-transfusion recoveries

All the animal studies described in this manuscript were reviewed and approved by the BloodworksNW Institutional Animal Care and Use Committee (protocol n: 105–02). The use of thirteen different mouse strains, as well as Ubi-GFP and HOD mice have been previously described in prior work from our group.[43] Whole blood was drawn by cardiac puncture as a terminal procedure for the mice, prior to storage under conditions simulating human blood banking and post-transfusion recovery studies, as described.

### RBC storage and sampling:

RBCs were stored at 4–6°C for 42 days. Sampling was performed in a biosafety cabinet, by obtaining 500 μL from each unit using a 16G/1-inch needle and 1 ml syringe after thorough mixing of each bag. Collections were performed on days 0, 7, 14, 21, 28, 35, and 42. Samples were centrifuged at 2,500 rpm for 10 minutes at 4°C, separated into supernatant and RBCs for each time point, and stored at −80°C until analysis.

### Ultra-High-Pressure Liquid Chromatography-Mass Spectrometry (MS) metabolomics:

The analytical platform employs a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). UHPLC-MS metabolomics analyses were performed as described,[44–46] and as detailed in the Supplementary Methods–Extended file.

### Fluorescence microscopy:

Media were aspirated and cells rinsed twice in 100 μl PBS, fixed and permeabilized in 70% acetone and 30%methanol at −20 °C for 10 min, air-dried, and incubated in blocking solution (10% Normal Donkey Serum in PBS) for 1 h at room temperature. Primary antibodies against FADS1 (ab126706), FADS2 (ab232898)) and FADS5 (ab39969) were purchased from Abcam (Cambridge, MA, USA). Antibodies against the specific proteins, and respective isotype controls, were applied at concentrations of 4 μg/mL overnight at 4 °C. Antispecies anti-rabbit secondary antibodies conjugated to Alexa Fluor 555 were purchased from Molecular Probes and Thermo Fisher Scientific. Slides were washed three times for 10 min each in PBS to eliminate excess antibody, and secondary antibodies were applied at a 1:100 dilution, for 1 h at room temperature. Confocal images were collected using a Zeiss Observer.Z1 inverted microscope equipped with Semrock wheel filters with ec/em spectra for the Alexa Fluor probes used. Images were acquired and analyzed using Slidebook 6.0 (Intelligent Imaging Innovations, Denver, CO).

### Statistical Analyses:

Graphs and statistical analyses (either t-test or repeated measures ANOVA) were prepared with GraphPad Prism 5.0 (GraphPad Software, Inc, La Jolla, CA).[46]

## Results

### Fatty acid desaturation increases in stored RBCs or fresh RBCs exposed to oxidant insults

As part of the REDS III – RBC Omics study (Figure 1.A), free fatty acids were measured with a targeted quantiative mass spectrometry-based assay on a total of 250 leukocyte-filtered units from healthy donor volunteers at storage day 10, 23 and 42. While all free fatty acids quantified in this study were found to increase, polyunsaturated fatty acids (PUFAs) were found to accumulate at a higher rate at later storage time (Figure 1.B). Indeed, significant increases in the degree of free fatty acid desaturation were observed as a function of storage duration (e.g., Dihomo-γ-linolenic acid – DGLA to linoleic acid – LA ratios; arachidonic acid – AA to DGLA – Figure 1.C). Since storage in the blood bank is associated with increases in oxidant stress,[21] we hypothesized that the observed increases in fatty acid desaturation in stored RBCs could be at least in part explained as a response to oxidant insults. To test this hypothesis, we incubated fresh (day 0) leukocyte-filtered RBCs with hydrogen peroxide (0.1% for 30 min) prior to characterization of fatty acid content and desaturation (Figure 2). Results showed a significant increase in free highly-unsaturated fatty acids (HUFAs) in RBCs upon incubation with hydrogen peroxide (Figure 2).

Free fatty acids in stored RBCs units from an independent cohort of 21 subjects suggested that higher levels of free HUFAs (AA, EPA, DPA and DHA) correlate with decreases in spontaneous hemolysis (Figure 3), one of the gold standards to determine quality of end of storage RBCs. Similarly, levels of saturated fatty acids (16:0 and 18:0) in stored RBCs correlated negatively with post-transfusion recovery – the second gold standard for RBC storage quality - in thirteen different mouse strains (Figure 4.A–B). Vice versa, stored RBC levels of total HUFAs and PUFAs compared to total SFA (or even total PUFA divided by SFA of the C18 series) are positively correlated to post-transfusion recovery (Figure 4.C).

### Fatty acid desaturases are present in the mature RBCs and can be inhibited by drugs

After observing progressive increases in free fatty acid degree of unsaturation and in the ratios of free mono-/saturated fatty acids (MUFA/SFA) and polyunsaturated (PUFA)/SFA in stored RBCs, we questioned whether these observations could be justified by enzymatic activity of fatty acid desaturases (FADS), which have not been reported to be present and active in the mature erythrocyte. To investigate this further, imunofluorescence experiments were carried out to look for the presence of FADS1, FADS2 and FADS5 in the freshly-drawn mature erythrocytes from healthy blood donors (Figure 5.A–D). As a result, all three enzymes were detected - though at significantly lower levels than in white blood cells or platelets as internal positive controls in non-leukofiltered samples. In RBCs, these enzymes appeared to localize on RBC membranes, especially FADS2 – the most abundant FADS detected in RBCs, followed by FADS1 and traces of FADS5. Since these enzymes catalyze different steps in the fatty acid desaturation pathway (detailed in green in the scheme in Figure 5), we incubated leukocyte-filtered RBC concentrates with three distinct inhibitors

for FADS2 (CP 24,879), FADS1/2 (SC26196) and FADS5 (PluriSln1) prior to storage for up to 6 weeks and weekly sampling for fatty acid analysis. Results indicate an accumulation of SFA (e.g., palmitate) and decrease in MFA/SFA (oleate/stearate) or PUFAs (AA/DGLA – not significant in this analysis) in RBCs incubated with FADS5 and FADS2 inhibitors, respectively (Figure 5.E). These results suggest that free MUFA and PUFA accumulation in stored units can be targeted by FADS inhibitors.

Since the generation of FADS products could be decreased by pharmacological inhibitors of FADS activity, we performed tracing experiments with $^{13}C_{16}$-palmitate to determine whether FADS activity could be directly observed through the incorporation of heavy carbon atoms into fatty acids downstream to palmitate in the desaturation and elongation pathway (Figure 6.A), the latter reportedly minimally active in the mature erythrocyte.[16] Leukocyte-filtered packed RBCs from 7 donor volunteers (3 females and 4 males) were supplemented with palmitate labeled at each one of its 16 carbon atoms. As a result, we could positively detect $^{13}C_{16}$ isotopologues of palmitoleic acid, stearic acid and oleic acid (Figure 6.A reports extract ion chromatograms, retention times and isotopic pattern for the labeled metabolites monitored here). However, while labeled palmitoleic and stearic acid were detected in all units after storage day 21 and 7, respectively, 18 carbon mono-unsaturated fatty acids were only detected after storage day 28 in one of the seven units (female donor), consistent with a negligible residual elongase activity and potential preference for linoleate-derived fatty acids for unsaturation of 18 carbon MUFAs. To follow up on these observations, we incubated fresh RBCs from 11 healthy volunteers (6 male and 5 females) with $^{13}C_{18}$-linoleate for up to 24h at 37°C, which resulted in detectable levels of $^{13}C_{18}$-linolenate with minor (10%) yet appreciable increases during the time course of the analysis (Figure 6.B).

## Inhibition of FADS2 in stored RBCs decreases the accumulation of oxidant stress markers of the metabolic storage lesion, including pyruvate to lactate ratios

FADS inhibitors were used to test the hypothesis that inhibition of FADS activity in stored RBCs would impact RBC storage quality and redox metabolism. Notably, FADS1/2 or FADS2 specific inhibitors – but not FADS5 inhibitors - had similar beneficial effects on the levels of several metabolic mediators of redox homeostasis, including redox sensitive thiols (reduced glutathione – GSH, methionine), methyl-group donors involved in oxidant stress-induced isoaspartyl-damage repair[47,48] (S-Adenosylmethionine – SAM - Figure 7.A), purine oxidation (decreased levels of inosine monophosphate – IMP, hypoxanthine and xanthine – Figure 7.B), pyruvate and pyruvate to lactate ratios (Figure 7.C - markers of methemoglobin reductase activity[9,25]).

## Decreases in FADS activity are observed in parallel to increases in pyruvate/lactate ratios in G6PD deficient donors or units undergoing cold rejuvenation

Since FADS activity requires NADH (Figure 7.D), and pyruvate to lactate ratios are inversely proportional to NADH/NAD+ ratios,[49] in light of the results above, it is interesting to hypothesize that FADS activation is a potentially beneficial phenomenon that is triggered in response to energy/redox metabolism-dependent modulation of NADH/NAD+ ratios. This hypothesis would predict that conditions that increase pyruvate/lactate ratios in stored RBCs

would result in decreased levels of free unsaturated fatty acids, in like fashion to what observed in stored RBCs incubated with pharmacological inhibitors of the enzymes. To test this hypothesis, we performed free fatty acid characterization in stored RBCs from G6PD deficient donors. RBCs from these donors are characterized by increased oxidant stress secondary to an impaired capacity to generate NADPH, the main intracellular reducing equivalent required for the reduction of oxidized glutathione as well as in the regeneration of several antioxidant enzymes. Consistent with the hypothesis, RBCs from G6PD deficient donors showed lower levels of several PUFAs compared to a pool of G6PD sufficient controls (Figure 8). In this view, it is interesting to note that RBCs from G6PD deficient donors are characterized by lower post-transfusion recoveries, comparably to our observations in mice, where stored RBCs with the highest levels of HUFA/PUFA showed higher post-transfusion recoveries.

Exogenous supplementation of pyruvate to refrigerator stored RBCs is proposed as part of the metabolic intervention strategy to foster rejuvenation of RBC units.[41] Notably, exogenous pyruvate supplementation to fresh RBCs increases pyruvate/lactate ratios and decreases several PUFAs without changing SFAs and MUFAs (Supplementary Figure 1).

## Discussion

In the present study we report for the first time that FADS2, FADS1 and FADS5 are present and active in the mature erythrocyte. This observation suggests a mechanism for increases in PUFA and HUFA in stored RBCs from healthy donor volunteers found here to occur at a higher rate than SFAs and MUFAs, a phenomenon that can not be rationalized by phospholipase activity onto complex lipids alone. Storage in presence of FADS inhibitors – especially FADS2 – significantly mitigated PUFA and HUFA accumulation in stored RBCs. In addition, we show that fatty acid desaturation is not only triggered by iatrogenic interventions such as blood storage, but more generally by oxidant stress – such as incubation with hydrogen peroxide. Oxidant stress-induced increases in free unsaturated fatty acids may represent a compensatory mechanism to counteract stress to the RBC lipids, as suggested by the association between the levels of PUFAs and HUFAs and end of storage hemolysis and post-transfusion recoveries, negatively and positively correlated, respectively. Borrowing from observations in cancer cells,[26] we thus hypothesized that FADS activity is dependent on/participates in regulating RBC NADH/NAD+ ratios – which are both increased by ongoing glycolysis and fuel methemoglobin reductase activity. While we did not directly measure NADH/NAD+ ratios, here we show that inhibition of FADS activity increases pyruvate/lactate ratios (inversely proportional to NADH/NAD+ ratios by law of mass action). The impaired capacity to generate NADPH as a reducing equivalent through the PPP, enzymopathies like G6PD deficiency are characterized by the up-regulation of glycolysis and concomitant increase in oxidant stress.[9,24,25] Further, we demonstrate that storage of RBCs from G6PD deficient donors – which are characterized by increases in pyruvate/lactate ratios other than lower post-transfusion recoveries – phenocopies FADS inhibition and results in decreases in PUFAs and HUFAs compared to normal G6PD counterparts. Storage of RBCs in presence of pyruvate-enriched rejuvenation solutions also decreases the rate of PUFA and HUFA accumulation, despite decreases in oxidant stress in these units, suggesting that FADS activity might be regulated by metabolic constraints (e.g.,

NADH/NAD+ ratios) rather than oxidant stress per se. Indeed, inhibition of FADS1/2 in stored units also corresponded to improved redox markers of the hydrophilic fraction (e.g., methionine, SAM, xanthine and hypoxanthine) in human RBCs, despite higher post-transfusion recoveries in mice with the highest levels of PUFA and HUFA to SFA ratios. Altogether, these almost paradoxical findings in part contribute to explaining the disconnect between the observed decreases in post-transfusion recovery observed in G6PD deficient subjects, despite apparent improved morphology and decreased membrane alterations in stored RBCs from these subjects.

The observations reported in this study are relevant in that they provide additional evidence of the unappreciated complexity of the RBC proteome and metabolome.[50] Appreciation of FADS presence and activity in the mature erythrocyte paves the way for interventional strategies aimed at targeting membrane lipid fatty acyl degree of unsaturation as a strategy to impact RBC membrane fluidity and resistance to osmotic and mechanical insults. Storage-induced activation of RBC FADS may represent a compensatory mechanism to preserve membrane fluidity in the face of oxidant challenge to structural proteins and membrane lipids.[21] Indeed, increases in the degree of unsaturation of conjugated fatty acids in membrane lipids is associated with decreases in membrane rigidity, lower hemolysis and increased post-transfusion recoveries in humans and mice. However, it must be noted that in the present study we exclusively focused on free fatty acids, which are represntative of conjugated fatty acid species.[51]

Our data suggest that oxidant stress-induced alterations of NADH/NAD+ homeostasis as a function of storage or other oxidant insult (e.g., hydrogen peroxide, evaluated herein) may indirectly regulate the activation of NADH-dependent FADS. Since double bonds are more susceptible to attack by radicals (e.g., reactive oxygen species), FADS-dependent accumulation of unsaturated fatty acids would provide a sink for radical species at the membrane lipid level. Similarly, our model would predict that strategies aimed at increasing glycolytic metabolism as a means to counteract the depletion of high-energy phosphate compounds in stored RBCs (e.g., alkaline additives or hypoxic storage)[30,32,52–55] and/or interfere with redox chemistry (by constraining O2 availability in the case of hypoxic storage or by promoting activation of glucose 6-phosphate dehydrogenase, in the case of alkaline additives) may indirectly affect RBC membrane fatty acyl composition and properties by activating FADS.

Observations made here may also help explain why several systemic oxidant stressors that regularly occur during ongoing physiological changes from exercise[5] to aging[8] and in progressive pathophysiological process from neurological disease[13] to cancer[14,15] are all associated with increases in the degree of unsaturation of RBC lipids. This consideration warrants attention in that it would suggest that findings relevant to RBC biology could also contribute to advancing knowledge of cancer biology, where FADS are being increasingly tied to metabolic reprogramming, a driver of malignant transformation.[28,56,57] Side-by-side understanding of these processes could uniquely advance blood storage and cancer treatment strategies. As a corollary to our model, dietary interventions that alter fatty acyl-membrane lipid composition[58] (e.g., with HUFA-rich fish oil diets) would influence not only RBC lipid homeostasis, but also energy metabolism and other critical metabolic pathways that impact

FADS activation to balance NADH/NAD+ ratios. In addition, because differences in donor RBCs storage is known to occur, further work to understand how FADS activity and lipid oxidations are mechanistically associated in refrigerator stored RBCs as a function of donor demographics and their impact on RBC storage quality, such as donor sex, age, ethnicity,[9,59,60] processing strategies[31] or donor habits (e.g., nicotine,[61,62] alcohol[63], caffeine[64] or taurine consumption).[65] These hypothesis could be amenable to direct testing in tractable murine models of blood storage and transfusion. For example, prior work in such models has demonstrated a strain-specific heterogeneity in storability,[66] lipid peroxidation[67] and post-transfusion recoveries.[43] Genetic components such as the NADPH-dependent ferrireductase STEAP3 have been identified as an etiological contributor to this phenomenon, at least between some strains [43]. However, it is interesting to speculate whether the role of STEAP3 in lipid peroxidation – e.g., via its contribution to Fenton chemistry by providing ferrous iron (FADS are iron-dependent enzymes) – would be constrained by a decrease in the degree of fatty acid unsaturation (i.e., a decrease in the total number of double bonds amenable to radical attack).

In conclusion, we provide evidence of FADS presence and residual activity in mature RBCs. We show that FADS activation following oxidant insults or storage seems to benefit RBCs with respect to their capacity to counteract hemolysis and circulate after transfusion in the recipient. We provide data associating inhibition of FADS activity to increases in pyruvate/lactate ratios and show that genetic (G6PD deficiency) or iatrogenic (rejuvenation) manipulation of this ratio prevents the accumulation of HUFAs and PUFAs.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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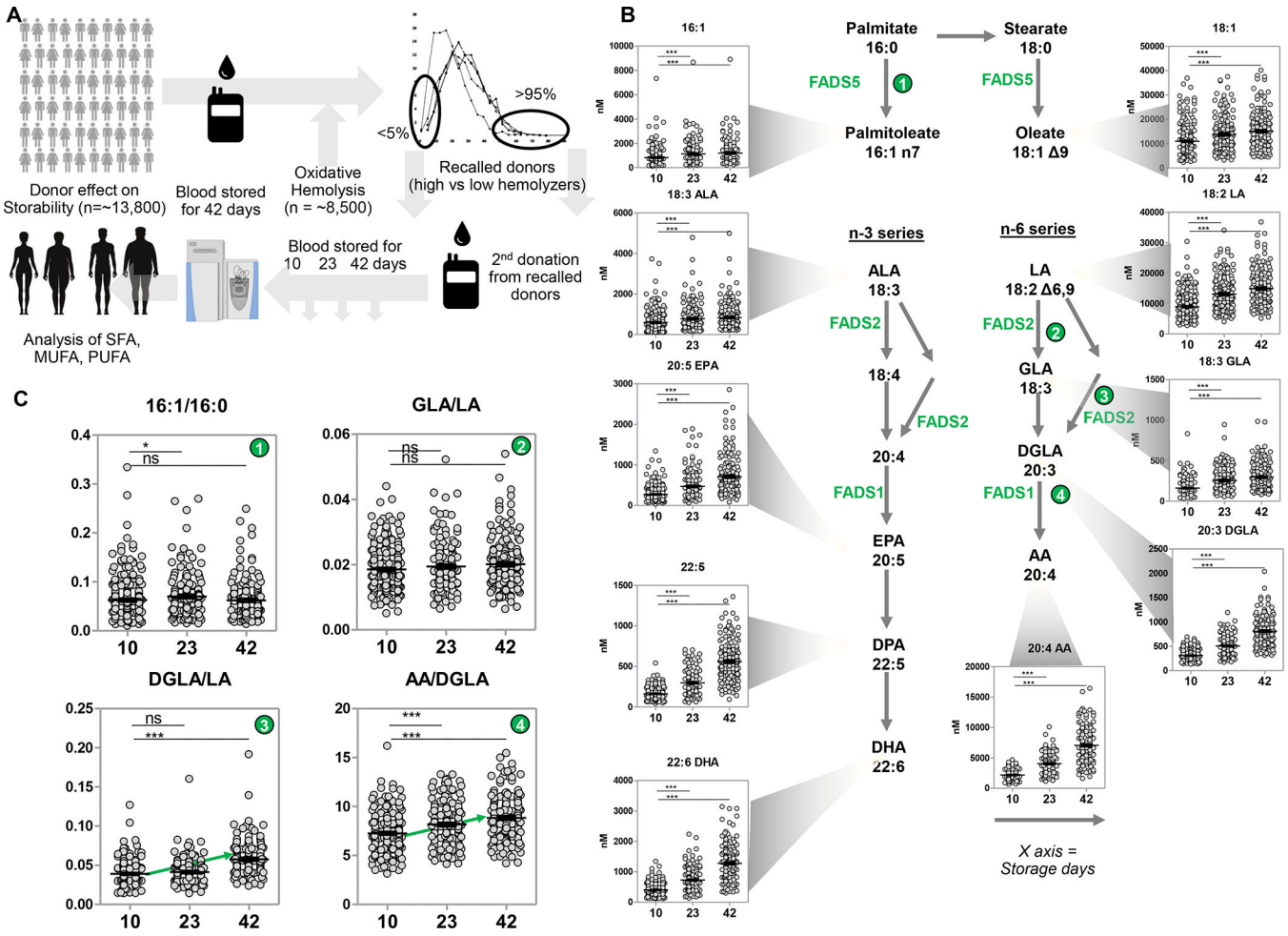


**Figure 1 –. Increases in fatty acid desaturation in stored RBCs from the REDS III – RBC Omics study.**

A total of 13,800 healthy donor volunteers were enrolled in the Recipient Epidemiology and Donor Evaluation Study – REDS-III RBC-Omics study. A unit of packed RBCs from each donor was stored for 42 days. At the end of the shelf-life, units were tested for the RBC susceptibility to hemolyze following oxidant and osmotic insults. The units showing the highest and lowest hemolysis values (5$^{th}$ and 95$^{th}$ percentiles) were selected as criterion to recall donors to donate a second unit of blood. A total of 250 units from recalled donors were tested for fatty acid levels at storage day 10, 23 and 42 (x axis for all the panels in this figure; y axis are absolute quantitative measurements – nM), as reported in the dot plots in this figure. Significant increases in the degree of fatty acid unsaturation were noted as a function of storage duration (e.g., Dihomo-γ-linolenic acid – DGLA to linoleic acid – LA ratios; arachidonic acid – AA to DGLA). Significance was determined by One-Way ANOVA with Dunn's multiple column comparisons (* *p<0.05; ** p<0.01; *** p<0.001).*

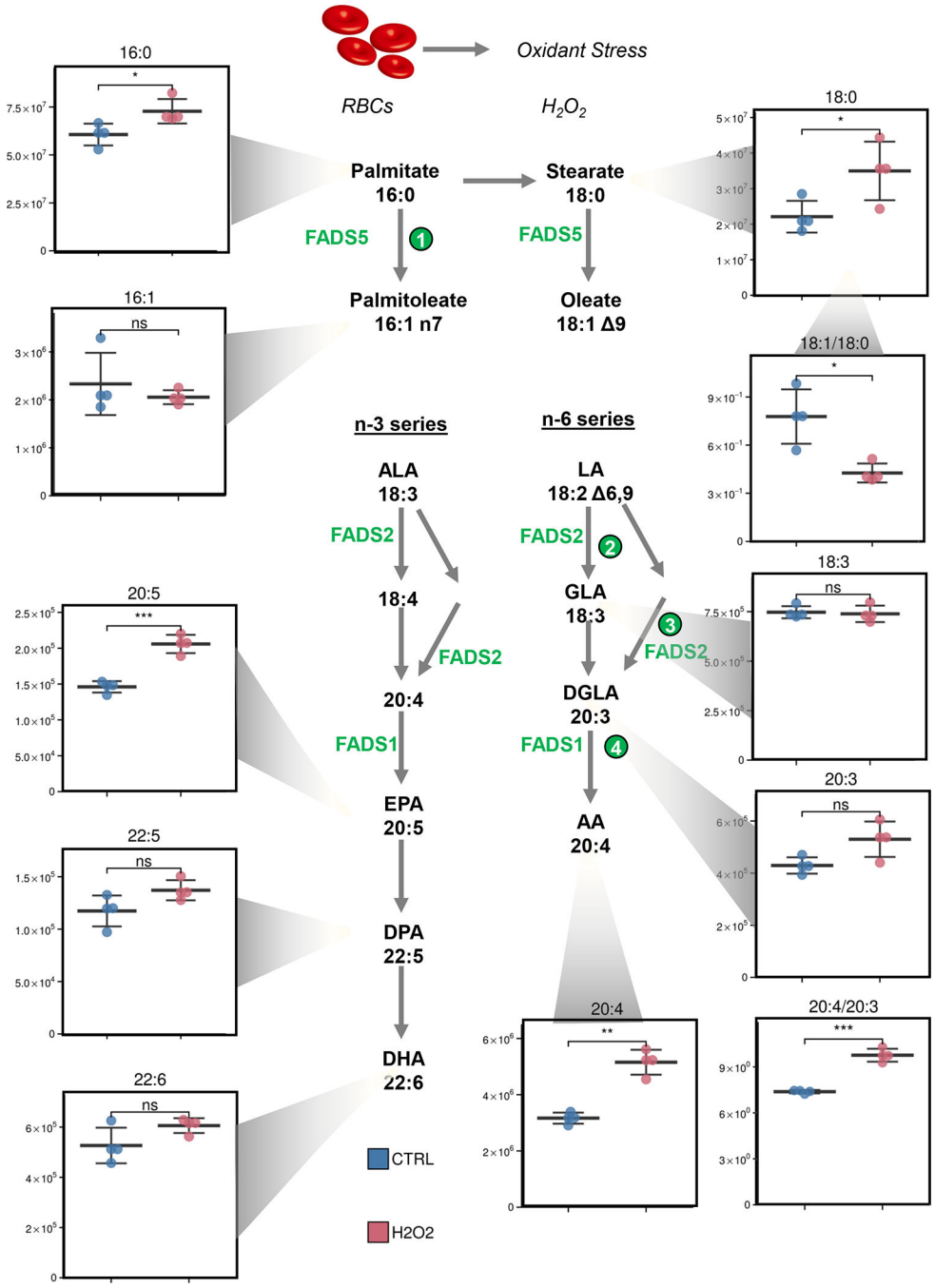


**Figure 2 –. Oxidant stress promotes increases in desaturated fatty acids in freshly drawn human red blood cells.**

Leukocyte depleted RBCs from three healthy volunteers were incubated with hydrogen peroxide (0.1% for 30 min) prior to mass spectrometry-based analysis of free fatty acids. Oxidant stress promoted significant increases (paired T-test) in polyunsaturated fatty acids. Y axis indicate relative concentrations of each free fatty acid in AU – *arbitrary units.* (*ns* = not significant; * *p<0.05; ** p<0.01; *** p<0.001).*

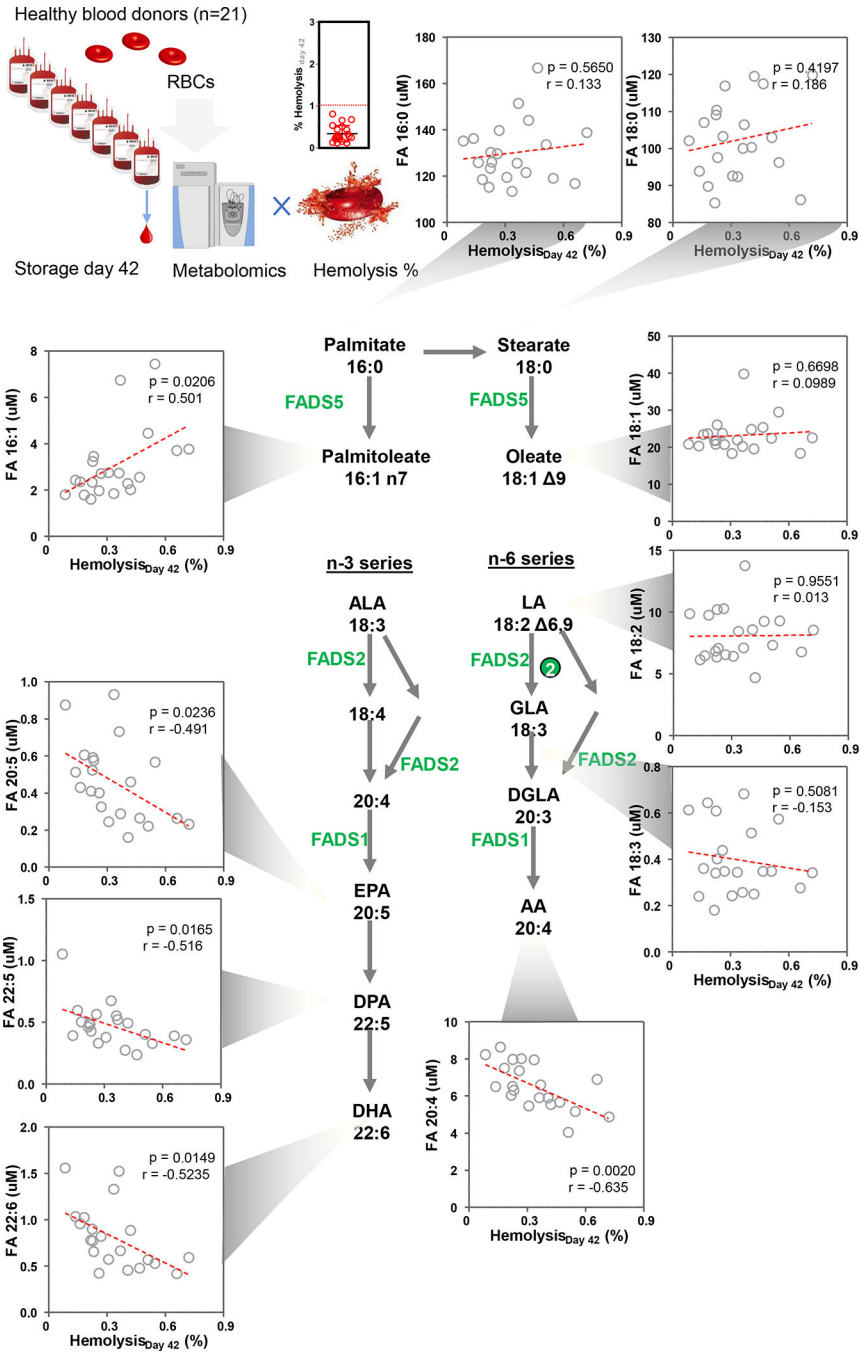


**Figure 3 –.**

Increases in the levels of free very long-chain unsaturated fatty acids negatively correlate with storage hemolysis in humans (n=21) in a separate cohort.

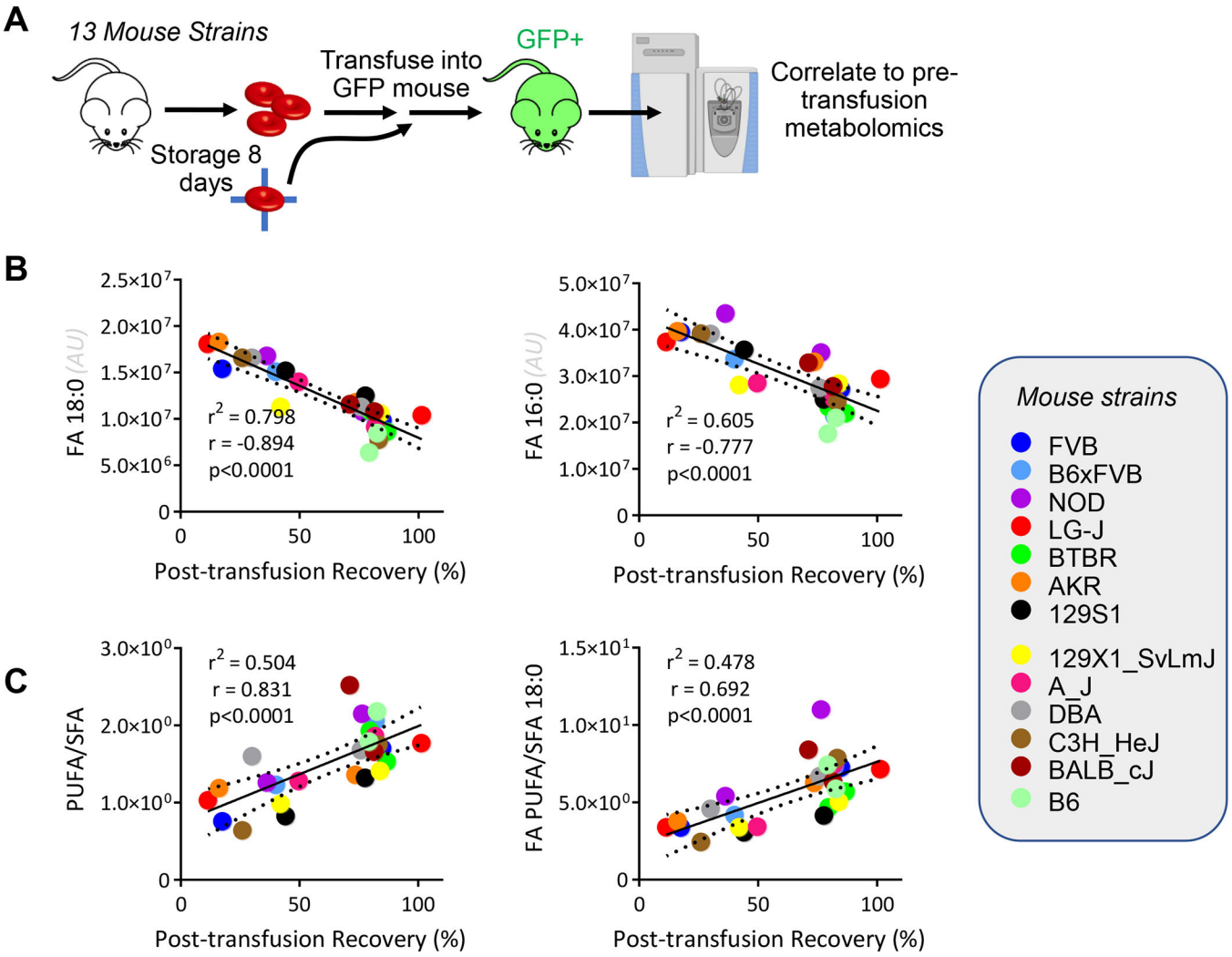


**Figure 4 –. Fatty acid desaturation and post-transfusion recovery in mice.**

Post-transfusion recovery studies were performed on stored RBCs from 13 different mouse strains stored for 8 days under conditions simulating human blood banking (**A**). The levels of palmitate (FA 16:0) and stearate (FA 18:0) negatively correlated with end of storage post-transfusion recovery in mice (**B**). On the other hand, the ratios of total PUFA to SFA or PUFA to SFA of 18 carbon fatty acid series were positively correlated to post-transfusion recovery (**C**).

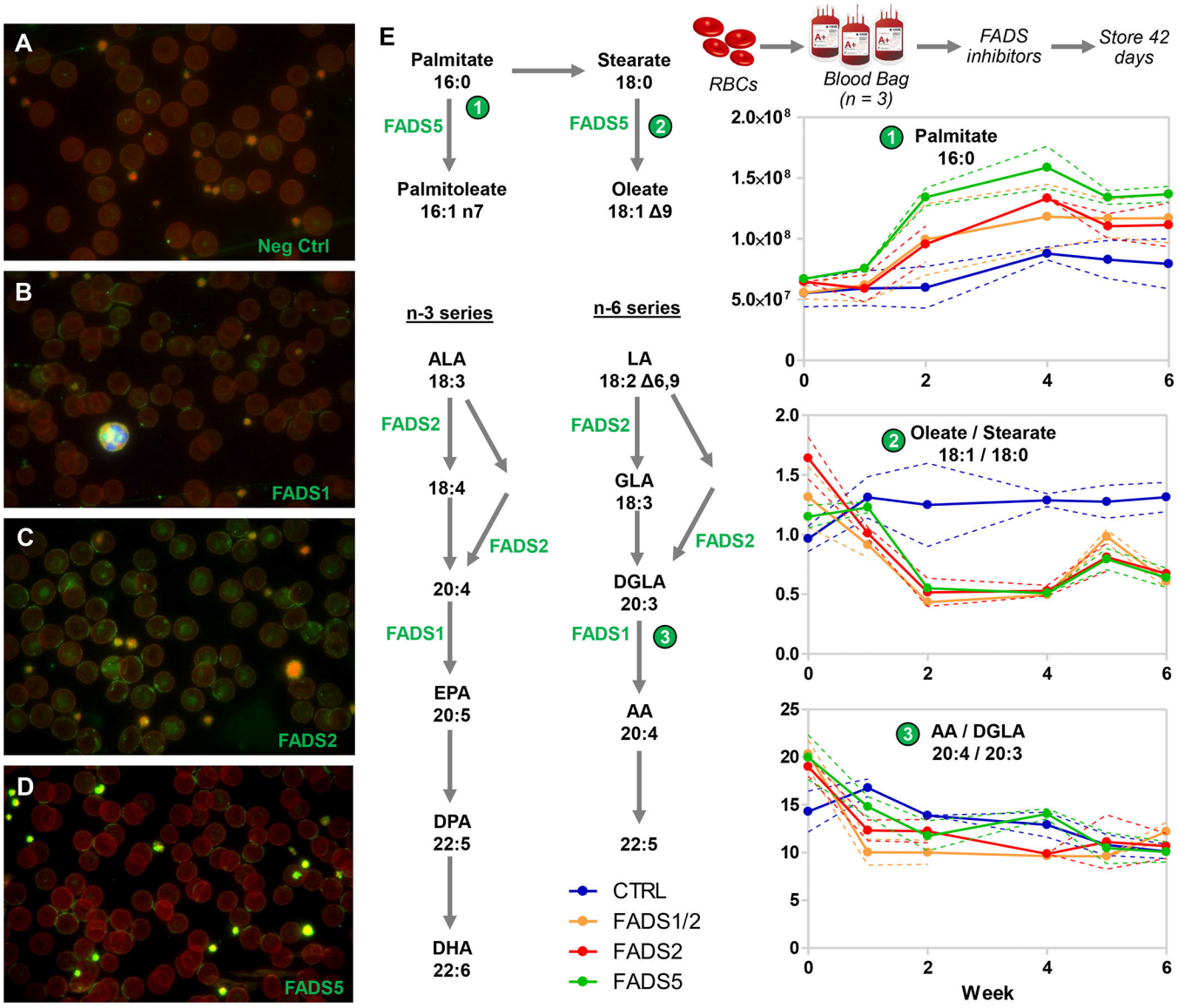


**Figure 5 –. Fatty acid desaturases are present and active in red blood cells.**

Immunofluorescence microscopy experiments were performed to detect fatty acid desaturase 1, 2 and 5 in mature RBCs from whole blood against negative controls (i.e., secondary Ab only – **A-D**). Three units of log4 leukocyte- and log2.5 platelet-depleted packed red blood cells were incubated at day 0 with inhibitors for FADS1/2 (orange), FADS2 (red) and FADS5 (green) prior to refrigerated storage for 42 days. Line plots show median ± SD (solid ± dotted line) for each group for palmitate (fatty acid – FA 16:0), oleic acid to stearic acid ratios (18:1/18:0) and arachidonic acid to di-homo-gamma-linoleic acid ratios (20:4/20:3). For each panel, y axis represents peak areas (arbitrary units; palmitate) or ratios, while x axis indicate storage weeks.

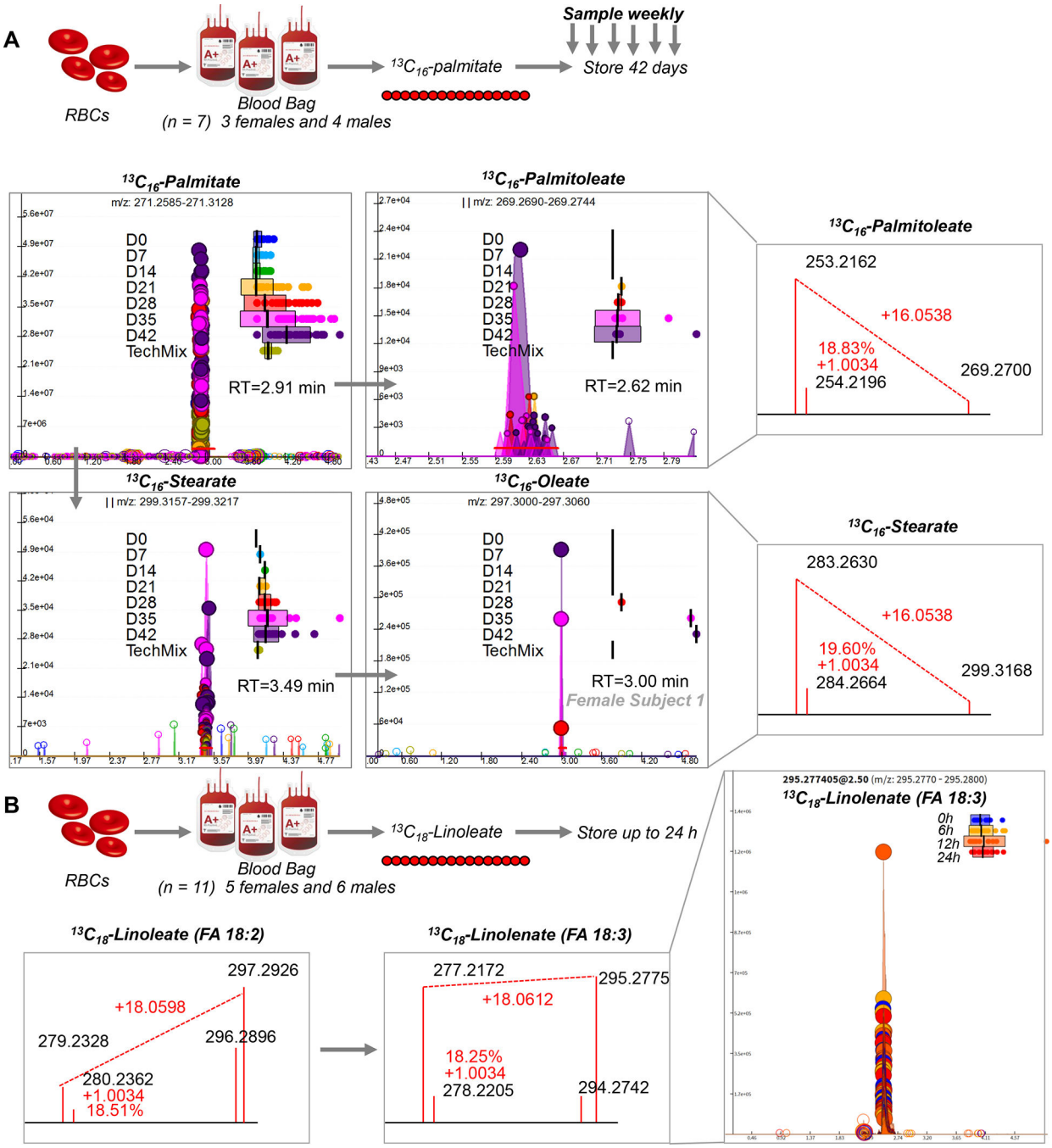


**Figure 6 –. Tracing experiments with $^{13}C_{16}$-palmitate and $^{13}C_{18}$-linoleate confirm residual fatty acid desaturase activity in the mature red blood cells of healthy donor volunteers.**

Seven healthy volunteers (three females, four males) donated red blood cells for refrigerated storage experiments in presence of uniformly labeled $^{13}C$ palmitate in all 16 carbon atoms (**A**). Panels report $^{13}C_{16}$ isotopologues for downstream metabolic products of fatty acid desaturase activity. Retention times and spectra are provided for representative metabolites in the pathway. RBCs from one of the female donors had significant accumulation of labeled 18 carbon atom fatty acids derived from labeled palmitate at storage day 28 through 42,

suggesting activation of elongase and desaturase over storage. To follow up on these observations, we incubated fresh RBCs from 11 healthy volunteers (6 male and 5 females) with $^{13}C_{18}$-linoleate for up to 24h at 37°C, which resulted in detectable levels of $^{13}C_{18}$-linolenate with minor (10%) yet appreciable increases during the time course of the analysis. X axes for extract ion chromatograms in **A** and **B** indicate the metabolite retention time (RT – in minutes), while the y axes indicate arbitrary units (AU).

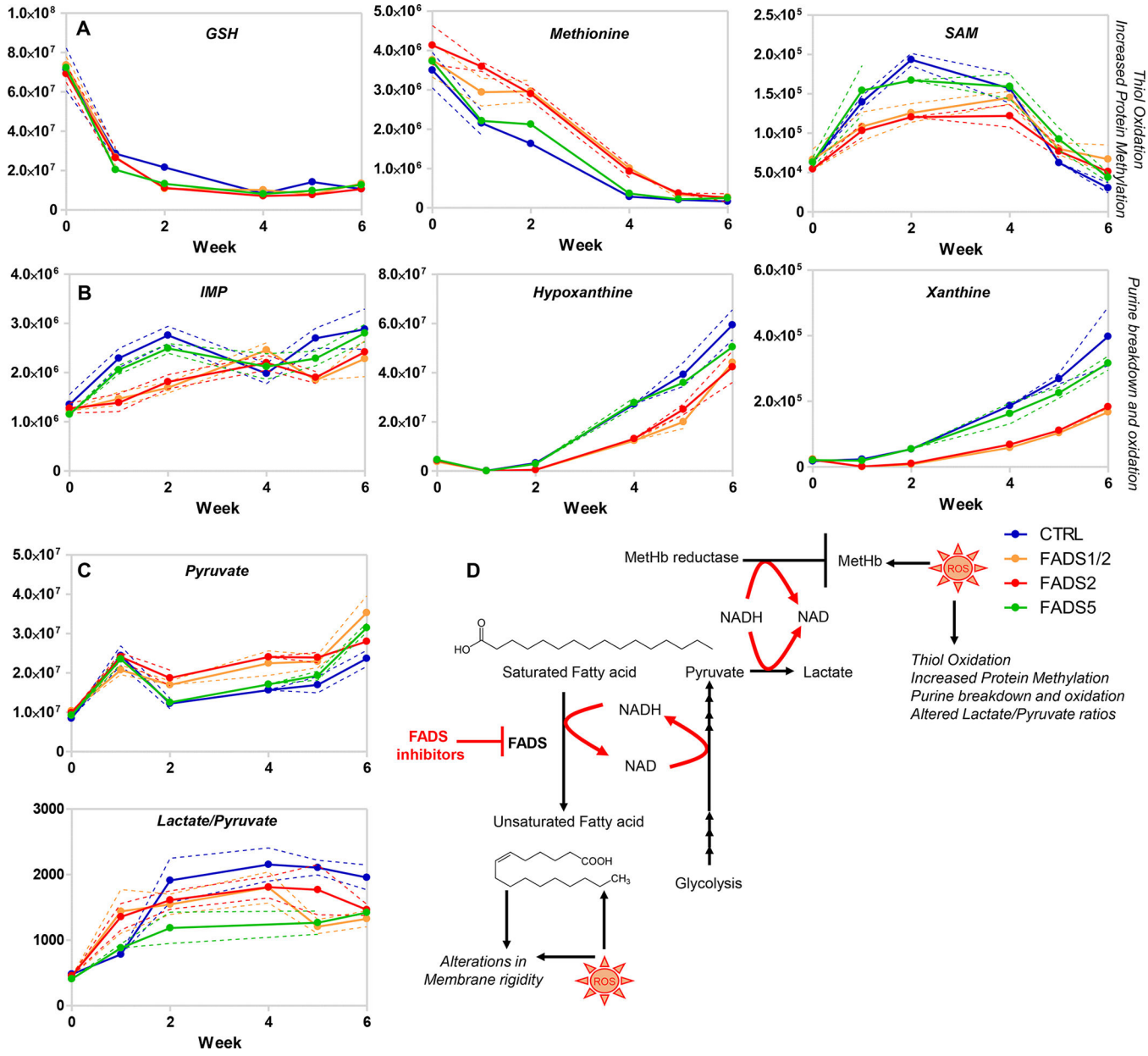


**Figure 7 –. RBC storage in presence of fatty acid desaturase inhibitors decreases oxidant stress to thiols and purines and increases in pyruvate to lactate ratios.**

Three units of log4 leukocyte- and log2.5 platelet-depleted packed red blood cells were incubated at day 0 with inhibitors for FADS1/2 (orange), FADS2 (red) and FADS5 (green) prior to refrigerated storage for 42 days. Line plots show median ± SD (solid ± dotted line) for each group for metabolites involved in redox reactions and oxidant stress-induced protein isoaspartyl-damage repair (**A**), purine oxidation (**B**) and pyruvate levels and ratios to lactate (**C**). For each panel, y axis represents peak areas (arbitrary units) or ratios, while x axis indicate storage weeks. A summary overview of these pathways is provided in **D**.

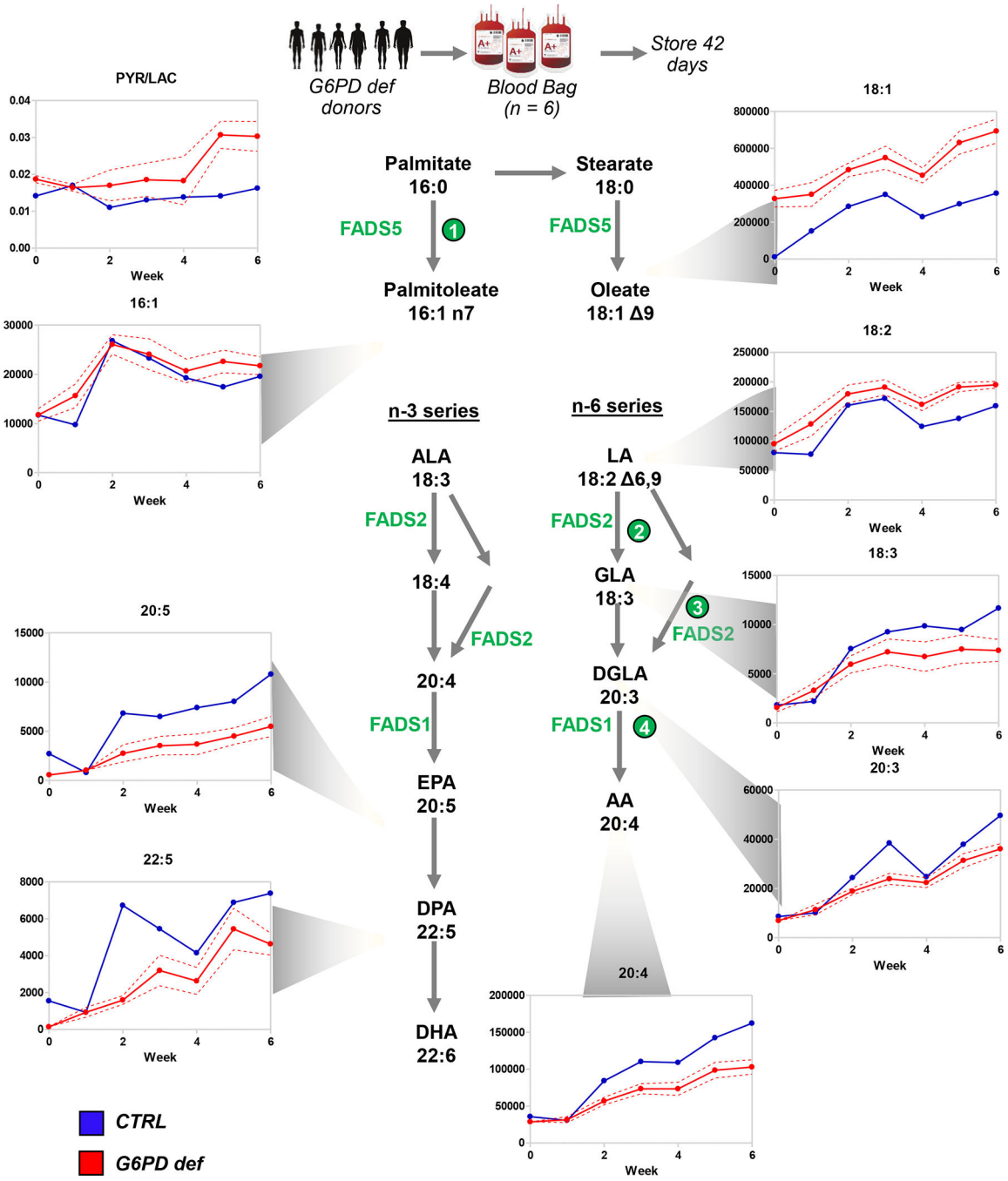


**Figure 8 –. Very long chain fatty acid desaturation is decreased in stored red blood cells from G6PD deficient donors, who are characterized by higher pyruvate/lactate ratios in the face of increased glycolysis and ablated pentose phosphate shunt.**

Units were collected from 6 G6PD deficient donors (red line) and healthy donor volunteers (blue line shows results from a pool of samples from this group). Continuous and dotted lines indicate median and SD for the peak areas of any given fatty acid per each group, consistent with the pathway map in the center of the figure.