Supplementary Figure for the manuscript

Longitudinal metabolic study of red blood cells from patients undergoing gender affirming testosterone therapy

Micaela Kalani Roy,[1] Rebecca Burgoyne Wilkerson, [1] Keisha Alexander,[1] Natalie J Nokoff[2,3], Melanie Cree-Green,[2,3] Angelo D'Alessandro[1]*

(1) Department of Biochemistry & Molecular Genetics, Graduate School, University of Colorado, Anschutz Medical Campus, Aurora, CO
(2) Department of Pediatrics, Division of Pediatric Endocrinology, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO
(3) Ludeman Family Center for Women's Health Research, University of Colorado, Anschutz Medical Campus, Aurora, CO

***Correspondence:**
Angelo D'Alessandro, PhD
Department of Biochemistry and Molecular Genetics
University of Colorado Anschutz Medical Campus
12801 East 17th Ave., Aurora, CO 80045
Phone # 303-724-0096
E-mail: angelo.dalessandro@ucdenver.edu

SUPPLEMENTARY MATERIAL

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Supplementary Introduction

Transgender and gender diverse (TGD) are umbrella terms that describe people whose gender identities differ from their sex assigned at birth or who are not encompassed by the gender-binary paradigm. It is estimated that up to 2% of high school students identify as TGD, a figure that has increased over the past decade.[1] TGD individuals may experience gender dysphoria, which refers to the distress experienced by the incongruence between one's gender identity and physical characteristics.[2] Gender affirming hormone therapy (GAHT), may be prescribed for eligible individuals to ameliorate gender dysphoria by producing physiological features that are more congruent with one's gender identity.[3-5] GAHT results in improved quality of life and gender dysphoria.[6-8]

The Endocrine Society and World Professional Association for Transgender Health recommend puberty blockade, typically with a gonadotropic-releasing hormone analog (GnRHa), for eligible TGD patients experiencing gender dysphoria starting at Tanner stage 2 pubertal development.[4,5] GnRHa alone reversibly pause the development of secondary sex characteristics and can provide additional time for gender identity exploration.[9,10] Little is known about the metabolic effects of GnRHa alone or with subsequent GAHT in this population.

Testosterone-based GAHT may be prescribed for eligible adolescents and adults experiencing gender dysphoria.[4,5] Prior studies have shown that testosterone therapy is associated with erythrocytosis and dyslipidemia.[11,12] Some studies suggest that testosterone may increase the risk of myocardial infarction in TGD male patients (i.e., individuals with a female sex at birth and a male gender identity) when compared to cisgender men and women, though the overall results are conflicting and inconclusive.[12-16] Because these studies rely heavily on self-reporting and cannot always account for the many external factors that affect TGD health (minority stress, socioeconomic status, healthcare disparities) additional research is needed to better understand the relationship between testosterone and cardiovascular health.[13,14] However, the mechanisms for these potential risks warrant additional investigation.

Testosterone has a significant effect on systems metabolism, a concept that has fueled the use of testosterone as an anabolic agent for doping purposes, by boosting bile acid metabolism[17] and muscle mass through its impact on amino acid metabolism [18]. Studies evaluating the effect of androgen deprivation therapy in adult men show a significant impact on the serum metabolic

profiles (notably by elevating fatty acids and bile acids, as well as reducing steroid synthesis and ketogenesis).[19,20] Among women with polycystic ovary syndrome, testosterone is associated with increases in bile acids.[17] Aside from a few studies in individuals with polycystic ovary syndrome [21] and transgender males[22], data are scarce on the effects of elevated testosterone on people assigned female at birth.[19,20] Furthermore, no studies have yet investigated the effects of testosterone treatment in TGD male patients on red blood cell (RBC) metabolism.

RBC are the most abundant cell type and are critical in the exchange of gases and nutrients throughout the body. To maintain optimal tissue perfusion, RBCs must be deformable, and capable of passing through narrow capillaries. Numerous studies have uncovered various sex-dependent hematological and metabolic characteristics of RBCs.[23-26] Notably, previous studies have shown that increased testosterone affects RBC deformability and erythropoiesis.[27] This observation potentially underlies the superiority in metabolic (e.g., energy and redox metabolism) and deformability traits observed in blood from healthy female blood donors compared to male counterparts.[24] RBC deformability affects post-transfusion performances of blood, which is impacted by donor and recipient sex dimorphisms (e.g., hemoglobin increments).[28] As testosterone stimulates erythropoiesis by inducing erythropoietin and suppressing hepcidin,[29-32] testosterone-replacement therapy in hypogonadal men has been observed to induced erythrocytosis. [33] Blood donation is indicated as a therapy to counteract excess RBC count following testosterone supplementation-induced erythrocytosis, though the osmotic and metabolic fragility of these RBCs are significantly altered, with a potential impact on the quality of blood in the supply chain. Indeed, testosterone-stimulate erythropoiesis results in erythrocytes carrying alterations in acyl-carnitine metabolism and free fatty acid levels in red blood cells, with orchiectomy in male mice having opposite metabolic effects.[27] In TGD people, increases in hematocrit and blood viscosity that can accompany testosterone therapy may underly the cardiovascular and cerebral risk potentially associated with testosterone.[12-16,34,35] However, there are no data on the impact of testosterone on the metabolome of RBCs in TGD individuals. Because RBC are devoid of organelles and heavily dependent on metabolic processes to cope with stress and damage, understanding the RBC metabolome is critical to fully understanding the mechanisms through which testosterone affects cardiovascular health. To address this gap in the research, we performed an exploratory study on the metabolomics on RBC samples from TGD adolescents before and after testosterone therapy.

Supplementary materials and methods – extended

Participants

Fifteen adolescent TGD participants assigned female at birth between 13-16 years of age were enrolled in a longitudinal, observational study, evaluating the relationship between testosterone and changes in metabolic profile. Study visits occurred prior to, and one and 12 months after exogenous testosterone (NCT03557268). Seven participants were receiving GnRHa treatment. Youth were recruited from 6/2018 to 8/2019 from the TRUE (Trust, Understand, Respect, Emerge) Center for Gender Diversity at Children's Hospital Colorado (CHCO). Participants were excluded if they had cognitive, psychiatric, or physical impairment resulting in inability to tolerate study procedures, diabetes, weight >181 kg, hypertension ($\geq$140/90 mmHg), were taking antipsychotic medications, or receiving exogenous estrogen and/or progesterone. All participants were clinically prescribed subcutaneous testosterone cypionate with a dose escalation schedule over the course of the 12 months

Research Visits

All participants had a research visit in the morning in the CHCO Clinical Translational Research Center (CTRC) after an overnight fast. The study visit occurred in the follicular phase of menstrual cycle (for those having menses) and at a testosterone trough (for 1- and 12-month time points). Blood was drawn for fasting laboratory evaluation. Pubertal staging was performed by a pediatric endocrinologist using the standards of Tanner and Marshall.[36] Height was measured on a Harpenden stadiometer and weight on a digital electronic scale. Height and weight were recorded to the nearest 0.1 centimeter and kilogram, respectively. Blood pressure was measured after sitting for at least 5 minutes, with an age-appropriate manual cuff. BMI was calculated by weight in kilograms divided by height in meters squared.

Body composition was measured by total body dual energy x-ray absorptiometry (DXA, Hologic Horizon W, Apex 5.6.05) at baseline and 12 months after testosterone therapy. VO_2 peak was assessed at all timepoints. Participants pedaled an upright stationary bicycle (Lode, Groningen, Netherlands) with a graded increase in pedal resistance and breathed air into a metabolic cart, as previously described.[37]

Laboratory Analysis

Hematocrit was measured by standard assays. Low-density lipoprotein cholesterol (LDLc) was directly measured. SHBG was measured at the CU-AMC CTRC lab and free and total testosterone by liquid chromatography with tandem mass spectrometry (LC-MS/MS) at the Brigham Research Assay Core lab (Boston, MA).

Metabolomics Analyses

Metabolomics analyses were performed as previously described, and detailed in **Supplementary materials and methods.**[24,38] After RBC samples were received at the CU Anschutz School of Medicine Metabolomics Core, they were thawed at 4°C. As previously described, ice-cold lysis buffer (5:3:2 ACN:MeOH:H_2O) was added to each sample at a 10:1 ratio. Samples were vortexed at 4°C for 30 minutes, then centrifuged at 4 ºC, 30,130g for 10 minutes to separate out the remaining solids. The pellets were discarded, and the supernatants were transferred to pre-cooled autosampler vials for UHPLC-MS analysis. Extracts were randomized and resolved at a flow rate of 450uL/min on a Kinetex C18 column (150 x 2.1 mm, 1.7 μm, Phenomenex, Torrance, CA, USA) using 5-minute gradients in positive and negative ion polarity modes.[39,40] Samples were then introduced to the mass spectrometer via electrospray ionization with the MS scanning in full MS mode (2 μscans) and ddMS2 (top15) over the range of 65-950 m/z. After analysis, compounds were integrated by hand in Maven (Princeton University) with reference to the KEGG database. Data for clinical variables were tested for normality. A time series analysis (repeated measure ANOVA) of the metabolomics data was performed, and a principal component analysis (PCA) and variable importance in projection (VIP) were calculated through MetaboAnalyst 5.0. Additionally, a hierarchical clustering analysis (HCA) was performed on the metabolomics data as well as Spearman's rank order correlations. A pathway analysis was performed on the metabolomics data using the 25 metabolites with the highest VIP loading weights. A linear model controlling for testosterone as a covariate revealed was performed to reveal metabolic effects of GnRHa status. Statistical analyses were performed using Metaboanalyst 5.0 and Graphpad Prism.

Supplementary Results – extended

Metabolomics data indicate two patterns following testosterone treatment independent from GnRHa status

Random forest analyses were performed to cluster subjects on the basis of their metabolic phenotypes following the testosterone (**Supplementary Figure 3A)**; an analysis that identified two sub-groups of 10 and 5 subjects each, not explained by GnRH treatment status. A linear model analysis controlling for testosterone as a covariate revealed significant differences between the two groups in estrone and progesterone, as shown by the line plots in **Supplementary Figure 3B** and the PCA in **Supplementary Figure 3C**. Group 2 displayed lower overall levels of estrone, and higher levels of progesterone—previously described effects of testosterone supplementation. An HCA of the top 30 significant features by ANOVA between the groups further asserted that group 2 responded more dramatically to T than group 1 as defined by group 2 increases in glycolysis, PPP, and purine metabolism compounds with little change in group 1. (**Supplementary Figure 3C)**. Group 2 showed increases in metabolites of glycolysis, the PPP, purine metabolism, and carboxylic acids over time, while group 1 showed little change over the duration of the study.

Effect of Testosterone on glycolysis and the PPP differs between group 1 and group 2 in RBC

A linear model analysis controlling for testosterone as a covariate revealed differences between the effects of testosterone on glycolysis between group 1 and group 2 (**Supplementary Figure 4**). group 2 showed striking increases in numerous metabolites of glycolysis and the PPP and a decrease in glucose. Conversely, group 1 showed slight decreases in most glycolytic metabolites and little change in the PPP.

A linear model controlling for testosterone as a covariate revealed various features that were significantly impacted by GnRHa status (**Figure 2D**). Notably, estradiol, FSH, and bone mineral density significantly differed based on GnRHa status. Metabolomics analyses of glycolysis (**Supplementary Figure 3**) revealed time-dependent effects of testosterone on RBCs, but little effect of GnRHa. Notably, GSH, DPG, and PEP increased significantly over time. Increases in GSH may suggest a higher oxidative burden in the RBCs.

Supplementary Discussion – extended

Testosterone treatment has a well-established impact on erythropoiesis, by promoting erythropoietin stimulation and inducing erythrocytosis.[29,31,41,42] Hypogonadal men on testosterone supplementation may be encouraged to donate blood to counteract testosterone-induced erythrocytosis.[32] However, recent studies indicate an increased susceptibility to hemolysis following storage, osmotic or oxidant stress of RBCs donated from these subjects. Despite this body of literature, little is known about the impact of testosterone regimens on RBC biology and metabolism, which regulates RBC function and survival in vivo.[43] To bridge this gap, here we performed metabolomics analyses of longitudinal samples from TGD patients undergoing testosterone therapy for 12 months. Results revealed strong signatures associated with testosterone treatment, especially with respect to energy, redox and membrane lipid metabolism.

Glycolysis – the main only energy-generating pathway in RBCs - ranked amongst the most significantly impacted pathways following testosterone therapy. Our results showed increases in glucose, DPG and phosphoglycerate isomers, and decreases in lactate and pyruvate over time. These contrasting trends for metabolites in the same pathway are suggestive that (i) the observed increases in DPG, ATP and ADP levels are not merely attributable to increased RBC mass (testosterone-induced increases in hematocrit); and (ii) testosterone may induce rewiring in late glycolysis with modulation of the activity of enzymes downstream to phosphoglycerate, such as redox sensitive[44] pyruvate kinase.[45] On the other hand, increases in high-energy phosphate compounds are consistent with testosterone treatment positively impacting the RBC capacity to off-load oxygen, given the role of these metabolites in stabilizing the tense deoxygenated state of hemoglobin.[46] These observations are consistent with an increase in VO_2 peak in TGD individuals following testosterone, especially in group 2. Moreover, sphingosine 1-phosphate – another contributor to deoxyhemoglobin allostery at DPG saturation – was found to increase over the duration of treatment, possibly suggesting a stimulation of cell proliferation and survival pathways.[47] Because testosterone treatment has been previously shown to stimulate erythropoiesis, it may also be possible that increases in some metabolites reflect an increased proportion of reticulocytes or other nucleated erythroid precursors in the blood.[41,42] However, carboxylic acids decreased over time, suggesting that an increased retention of mitochondria-containing reticulocytes or erythroid precursors in circulation following testosterone is unlikely. Of note, the regimen also impacted RBC levels of creatinine, a marker of dysregulated nitrogen metabolism

and RBC-mediated kidney dysfunction[48,49] along with sphingosine 1-phosphate[50] and acyl-carnitines.[51] This is relevant in light of the role of kidneys in erythropoiesis and the role of testosterone on the stimulation of renal erythropoietin.[29] Notably, decreases in all carboxylic acids following testosterone were accompanied by increases in 2-oxoglutarate (also known as alpha-ketoglutarate), a negative regulator of hypoxia inducible factor 1alpha (HIF1a) by mechanism of prolyl hydroxylase-dependent post-translational modification of HIF1a.[52,53] This observation is suggestive of potential compensatory mechanisms counteracting testosterone-induced erythropoiesis by antagonizing HIF1a signaling.

The polyamines spermine and spermidine, which are protective against oxidative stress, increased in RBC samples over time.[54] These compounds also stabilize RBC membranes and could give insight into the mechanism by which testosterone impacts RBC deformability.[55] Prior studies on testosterone-supplemented hypogonadal men[56,57] and mice with orchiectomy and/or testosterone supplementation showed that RBC deformability was decreased in response to testosterone treatment and negatively correlated with the levels of acyl-carnitines in RBCs.[58] Interestingly, in our study testosterone was associated with decreases in short-chain acyl-carnitines, but also with increases in long chain ones, suggesting that some of the observations reported in testosterone-supplemented males may not apply to TGD individuals. The increases in acyl-carnitines seen in this study may imply an effect of testosterone on membrane stability and increased membrane remodeling through the carnitine-dependent Lands cycle.[59,60]Testosterone therapy has been previously correlated with susceptibility to hemolysis, suggesting that the androgen impacts membrane damage and repair and may generate a higher demand for acyl-carnitines.[26,27] An alternative or complementary explanation comes from the appreciation that testosterone treatment may have a differential effect on males compared to people with a female sex at birth, in that carrying two copies of chromosome X in the latter group could result in increased dosage of enzymes coded by genes on this chromosome, despite chromosome X inactivation. Examples of such genes are the rate limiting enzymes of the pentose phosphate pathway – glucose 6-phosphate dehydrogenase (G6PD), hypoxanthine guanosine phosphoribosyl transferase, creatine transporters or ATP-dependent phosphatidylserine flippases – all enzymes relevant to RBC redox biology and in vivo clearance in the spleen.

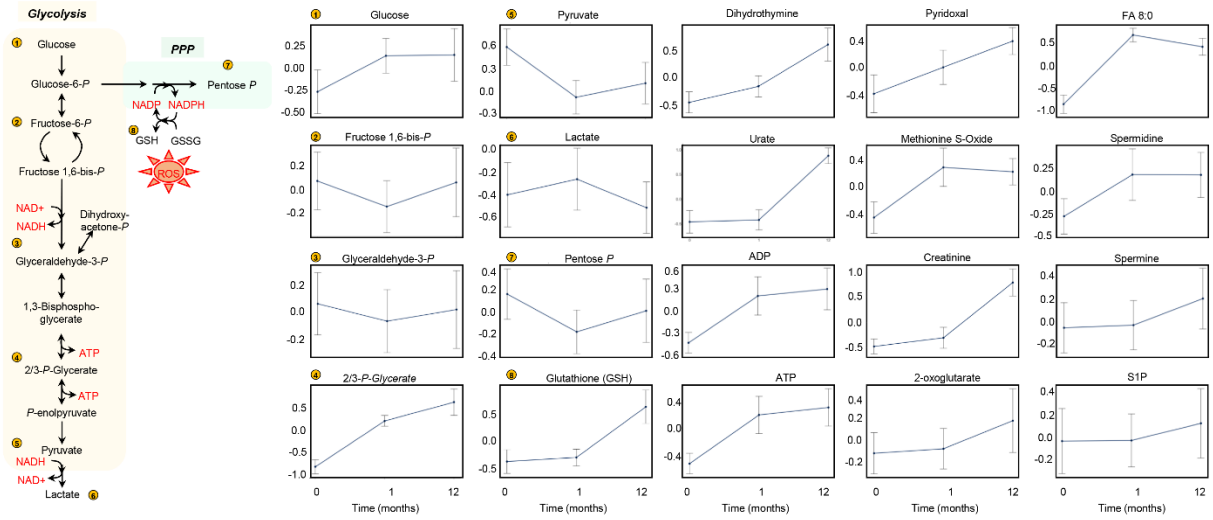
Here testosterone treatment was found to modulate the PPP and glutathione homeostasis in a way consistent with increased oxidant stress in these subjects. Individuals with G6PD deficiency, the most common enzymopathy in humans (affecting ~600 million people),[61] are more susceptible to oxidant stress due to the failure of the main NADPH-generating pentose phosphate pathway (PPP) and a reduced capacity to regenerate oxidized glutathione and related enzymes.[62] Subsequently, this study prompts the question as to whether caveats should apply in the administration of testosterone treatment to G6PD hemizygous or deficient TGD patients assigned female at birth. Of note, statistical analyses revealed two groups based on metabolic profiles after testosterone treatment, one of which (here arbitrarily referred to as group 2) showed striking increases in numerous metabolites of glycolysis and the PPP and a decrease in glucose. These increases corroborate previous studies, which suggest testosterone can upregulate glycolysis and the PPP in various cell types.[27,63-65] Testosterone-related decreases in membrane deformability could cause an accrual of oxidative damage - e.g., increases in purine oxidation markers - and a greater demand for reactive oxygen species scavengers like GSH.[27,66]

Indeed, increases in the levels of purine oxidation markers (hypoxanthine, urate and hydroxy-isourate) were observed following testosterone treatment, especially in group 2. The X chromosome-linked enzyme hypoxanthine guanosine phosphoribosyl transferase salvages deaminated purines by converting hypoxanthine to guanosine – a marker of oxidatively damaged-RBC capacity to circulate in vivo,[67] suggesting that while this pathway may be regulated by testosterone, the extent to which purine degradation occurs in males undergoing testosterone-replacement[58] may diverge from that observed in TGD individuals on testosterone.

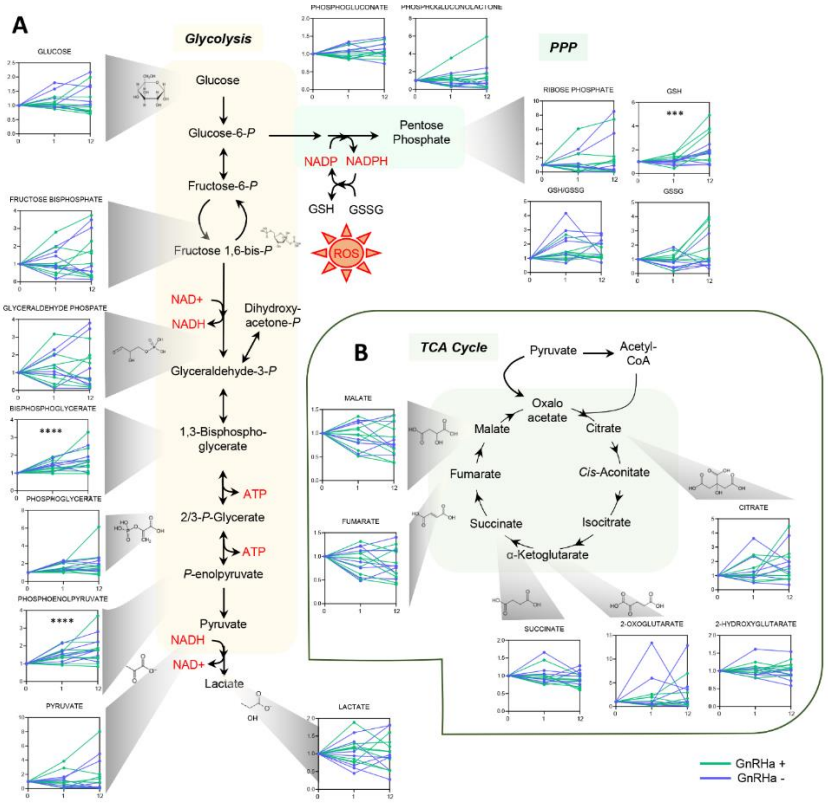
Despite clear trends observed in RBC metabolism as a function of the duration of testosterone treatment, there was inter-subject variability, which was not attributable to GnRHa status. Several descriptive and metabolic features significantly correlated with measured serum testosterone concentrations. Increases in free testosterone across all timepoints were negatively correlated with LDL-direct and cholesterol, markers of body mass index and fat mass, which in turn all strongly correlated with the RBC levels of free fatty acid and acyl-carnitines, consistent with recent reports on the impact of blood donor BMI on RBC metabolism and storage quality.[68]

In summary, here we provide the first comprehensive characterization of the RBC metabolome in TGD males undergoing testosterone treatment. We report a significant positive impact of testosterone on hematocrit, RBC levels of high-energy phosphate compounds DPG and ATP, consistent with increased VO_2 peak. On the other hand, we report an impact of testosterone resulting in increases in the RBC levels of acyl-carnitines and free fatty acids, especially polyunsaturated ones, oxidized purines, glutathione (reduced and oxidized), methionine sulfoxide[69] and PPP metabolites – markers of oxidant stress to the RBC. We commented on similarities and divergent trends upon testosterone supplementation in cisgender males[58] and TGD subject enrolled in the present study, especially with respect to acyl-carnitine metabolism. Since testosterone was here found to impact RBC PPP, further studies should determine the impact of X-linked common inborn errors of metabolism (e.g., G6PD deficiency) on individuals undergoing T. Since factors like GnRHa status were found to impact clinically-relevant covariates, but not RBC metabolism, it is unclear from our study whether other organ or cell autonomous responses mediate the clinically observed alterations in hormone concentrations, VO_2 peak, BMI following GnRHa treatment upon testosterone supplementation.

Supplementary Figures

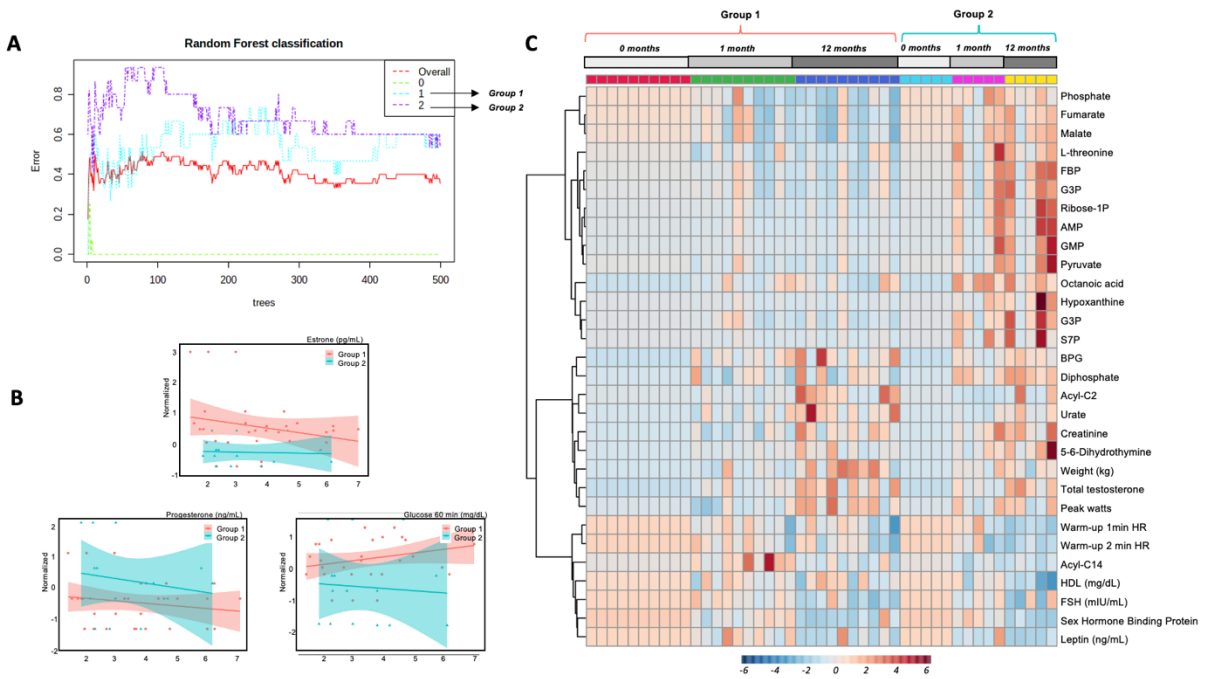


Supplementary Figure 1. Testosterone impacts RBC energy metabolism and damage/repair pathways. Line plots of the data normalized to baseline uncovered metabolite-specific trends in glycolysis, the PPP, and in several oxidative stress markers over treatment duration (mean ±SEM).

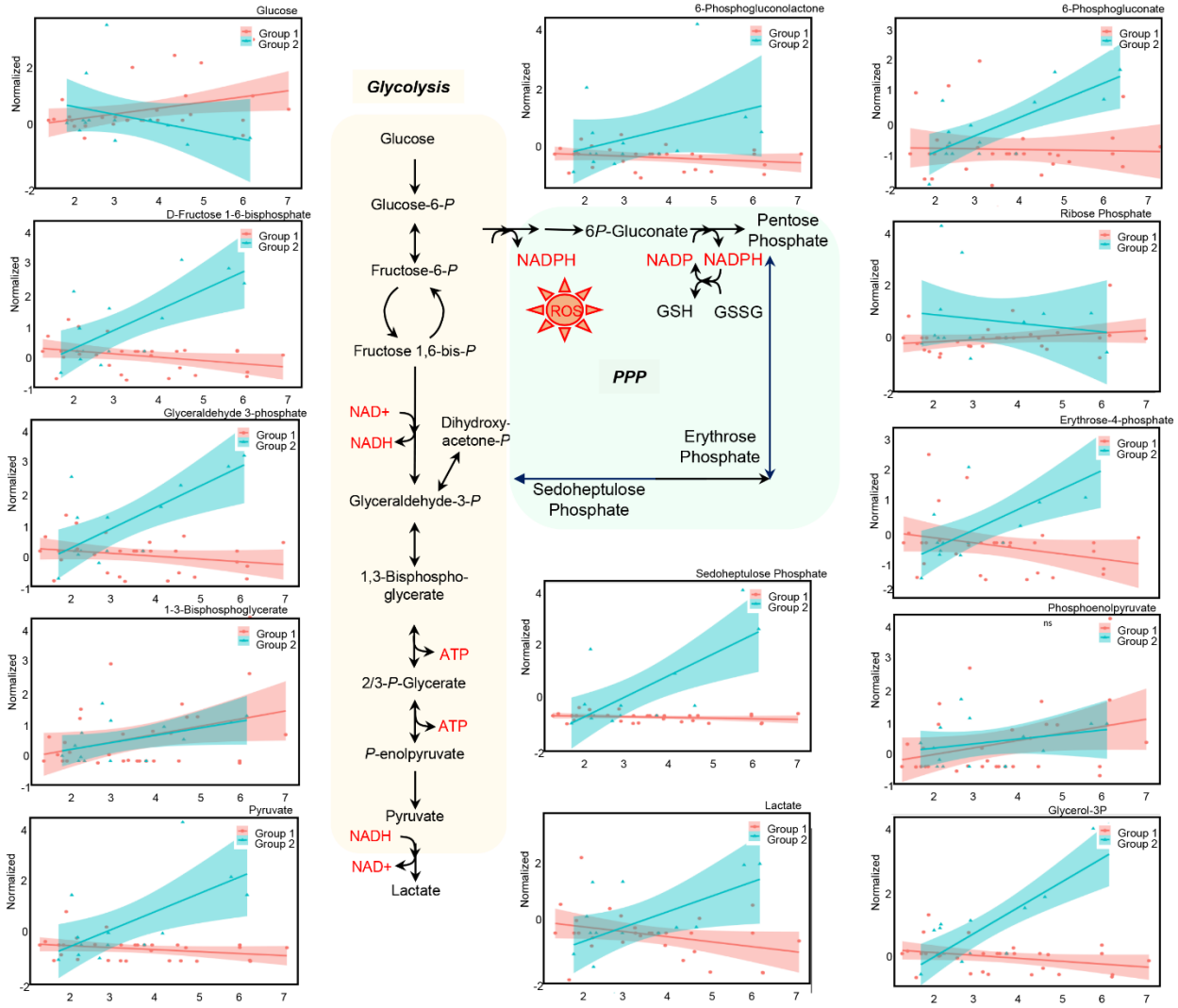


Supplementary Figure 2. Focused analyses of energy metabolism suggest negligible effect of GnRHa status. (A) An analysis of glycolysis and the PPP was performed to determine effect of

GnRHa but some significance testosterone over time (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). (B) Line plots of TCA metabolites uncovered no effect of GnRHa status.



Supplementary Figure 3. Metabolomics analyses reveal two distinct groups. (A) A random forest analysis revealed two groups. (B) A linear model of the data controlling for testosterone as a covariate revealed differences between group 2 and group 1 in estrone, and progesterone. (C) A hierarchal clustering analysis between group 1 and group 2 indicated that metabolites of glycolysis, the TCA, and purine metabolism changed over time in group 2, but showed little change in group 1.



Supplementary Figure 4. Energy metabolism shows dramatic changes in Group 2 but negligible change in Group 1. A linear model controlling for testosterone as a covariate indicated increases in many metabolites of glycolysis and the PPP in group 2 samples, but little to no change in Group 1 samples.

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