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# Unique plasma metabolite signature for adolescents with Klinefelter syndrome reveals altered fatty acid metabolism

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## Abstract

Conditions related to cardiometabolic disease, including metabolic syndrome and type 2 diabetes, are common among men with Klinefelter syndrome (KS). The molecular mechanisms underlying this aberrant metabolism in KS are largely unknown, although there is an assumption that chronic testosterone deficiency plays a role. This cross-sectional study compared plasma metabolites in 31 pubertal adolescent males with KS to 32 controls of similar age (14 ± 2 years), pubertal stage, and body mass index *z*-score of 0.1 ± 1.2 and then between testosterone-treated ($n = 16$) and untreated males with KS. The plasma metabolome in males with KS was distinctly different from that in controls, with 22% of measured metabolites having a differential abundance and seven metabolites nearly completely separating KS from controls (area under the curve > 0.9, $P < 0.0001$). Multiple saturated free fatty acids were higher in KS, while mono- and polyunsaturated fatty acids were lower, and the top significantly enriched pathway was mitochondrial β-oxidation of long-chain saturated fatty acids (enrichment ratio 16, $P < 0.0001$). In contrast, there were no observed differences in metabolite concentrations between testosterone-treated and untreated individuals with KS. In conclusion, the plasma metabolome profile in adolescent males with KS is distinctly different from that in males without KS independent of age, obesity, pubertal development, or testosterone treatment status and is suggestive of differences in mitochondrial β-oxidation.

**Keywords**

- metabolomics
- Klinefelter syndrome
- sex chromosome aneuploidy
- fatty acid metabolism
- mitochondrial function

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## Introduction

Klinefelter syndrome (KS), or 47,XXY, is strongly associated with a high risk for cardiometabolic comorbidities, including dyslipidemia, type 2 diabetes, and cardiovascular disease (1). Historically, chronic untreated hypogonadism, a nearly universal finding in adult men with KS, has been implicated as the underlying pathologic mechanism leading to this increased risk (2). As an anabolic steroid, testosterone has effects on body composition and mitochondrial metabolism, which are widely accepted contributors to insulin resistance (3). The hypogonadal pathology assumption remains challenged, given cardiometabolic disease risk factors are identified in younger cohorts of boys with KS naïve to years of hypogonadism, as well as findings that suggest testosterone replacement does little to improve cardiometabolic disease in adults (4, 5). Therefore, there is a need to deepen our understanding of the molecular mechanisms underlying the high cardiometabolic risk in KS.

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Metabolomics, or the study of small molecules in biological samples, is a powerful tool to inform metabolic processes in disease states. Metabolomics is a comprehensive approach to assessing systems biology, encompassing both endogenous metabolic processes and exogenous exposures. It can be useful for disease diagnosis, prognosis, risk prediction, therapeutic development and assessment of response, and understanding pathophysiology, particularly when the underlying molecular mechanisms of a disease state are unknown (6). Of particular relevance to KS, metabolomics has been extensively used in characterizing cardiometabolic-related disease states, including metabolic syndrome and type 2 diabetes (7), as well as in the setting of male hypogonadism with testosterone-replacement therapy (8). Analysis of circulating levels of substrates and intermediate metabolites from intra-mitochondrial pathways, particularly β-oxidation of fatty acids, offers a non-invasive assessment of mitochondrial metabolism – a viable strategy to predict intracellular metabolic states from extracellular metabolomics data (9). When fatty acid oxidation is impaired, as in the case with multiple cardiometabolic disorders, the reliance on protein catabolism to provide tricarboxylic acid (TCA) cycle intermediates increases. As a result, the metabolite profile includes an increase in intermediates of fatty acid oxidation (classically long-chain acylcarnitine species) as well as branched-chain amino acids (10). These metabolites not only are suggested to be biomarkers of disrupted mitochondrial metabolism but also may contribute to the progression of insulin resistance.

A few studies have investigated specific metabolites in KS, such as steroids, melatonin, and markers of bone metabolism (11, 12, 13); however, profiling central energy and redox metabolites presents a unique opportunity to better understand aberrant metabolism observed in this population. The aim of this study was to compare the plasma metabolic signatures of adolescents with KS to controls as well as between testosterone-treated and untreated males with KS. We hypothesized that we would observe a pattern suggestive of aberrant mitochondrial metabolism and that testosterone would improve this metabolic profile.

## Methods

### Overall study design

This was a cross-sectional study of untargeted metabolite concentrations in the plasma of 31 males with KS compared to 33 controls selected for similar age, pubertal stage, and body mass index (BMI). The study was approved by the Colorado Institutional Review Board (COMIRB #16-0248); written consent was obtained from a parent and assent from each participant.

### Study participants and procedures

Pubertal adolescents 12–17 years of age with non-mosaic KS were recruited to participate in a study on cardiometabolic health, which was powered to detect differences in cardiorespiratory function not included here. Prepubertal exam, hypertension, diabetes, or exercise restrictions were exclusionary. Additionally, boys who were being treated with testosterone had to be on treatment for at least a year. Participants with KS were recruited from the eXtraordinarY Kids Clinic and Research Program and advertisement through AXYS (Association for X and Y Variations) advocacy organization. All participants provided written assent, and a parent provided written consent prior to any study procedure. All 31 participants had a fasting venous blood draw with blood collected in a sodium heparin tube. Tubes were centrifuged at 2000 ***g*** for 15 min, and plasma was aliquoted into cryovials and stored at –80°C until batch analysis.

Plasma samples that had been obtained and processed in an identical manner from fasting healthy males participating in the Health Influences of Puberty Study (NCT01775813) and the Glycemic Monitoring in Cystic Fibrosis Study (NCT02211235) were used as a comparison group. Out of a pool of >100 participants in these studies with permission to use these samples for additional research, 37 pubertal males aged 12–17 years were selected based on age and BMI similar to the KS cohort. Of those, 33 had usable plasma, and one was later excluded due to outliers for most metabolite concentrations, resulting in the analytic cohort in Table 1.

### Metabolomics analysis

Plasma samples were analyzed in the University of Colorado School of Medicine Metabolomics Core, as previously described (14). In brief, plasma metabolites were extracted with a solution of methanol, acetonitrile, and water (5:3:2 v), and 10 µL were injected for analysis via ultra-high-pressure liquid chromatography coupled to mass spectrometry on a Thermo Vanquish UHPLC–Thermo Q Exactive MS system. Compound identification

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**Table 1** Characteristics of the analytic sample.

| | KS ($n = 31$) | Controls ($n = 32$) | *P*-value |
|---|---|---|---|
| Age (years) | 14.5 ± 1.7 | 14.8 ± 1.7 | 0.49 |
| BMI *z*-score | 0.24 ± 1.51 | −0.05 ± 0.93 | 0.36 |
| Pubertal stage | | | 0.14 |
| Tanner 2 | 2 (6.5%) | 4 (12.5%) | |
| Tanner 3 | 0 (0%) | 4 (12.5%) | |
| Tanner 4 | 17 (54.8%) | 16 (50%) | |
| Tanner 5 | 12 (38.7%) | 8 (25%) | |
| Testosterone, ng/dL | 366 (271, 454) | 304 (112, 471) | 0.45 |
| Untreated ($n = 15$) | 338 (226, 438) | | |
| Treated ($n = 16$) | 380 (298, 513) | | |

Data are represented as mean ± S.D., median (25th, 75th percentiles), or *n* (%), where appropriate. BMI, body mass index; KS, Klinefelter syndrome.

was based on intact mass, $^{13}C$ isotope pattern, and retention times from an in-house reference of over 5000 analytical standards. Relative metabolite abundance for each sample was reported. Analytical details are extensively provided in technical notes (15) and previous applications to similar matrices (16, 17).

### Statistical analysis

Data analysis was performed in MetaboAnalyst 5.0. Data were normalized by sum and auto-scaled (mean centered and divided by S.D. of each variable). Metabolite abundance was compared between KS and control groups (followed by testosterone treatment status) with fold change and unpaired Mann–Whitney *U*-tests. Adjustment for multiple comparisons was performed with the two-stage step-up method of Bejamini, Krieger, and Yekutieli for calculation of the false discovery rate (FDR) (18). Significance was set at $q \leq 0.05$ for definitive group differences, although comparisons resulting in $q < 0.15$ were included in exploratory pathway analyses for hypothesis-generating integration of the results. For visualization, GraphPad Prism v9.0.2 was used to generate a volcano plot identifying metabolites with a fold change >1.5 and FDR <0.05. To determine if a unique metabolome profile is present in KS, partial least squares – discriminant analysis (PLS-DA), a variant of principal component analysis that is considered the gold standard for binary classification of metabolomics datasets, was conducted (19). Receiver operating characteristic (ROC) curve analysis was conducted to identify potential biomarkers of KS, defined as metabolites with an area under the curve (AUC) >0.9. Finally, we used metabolite set enrichment analysis (MSEA) to help interpret the data with respect to normal human metabolism by identifying the metabolic pathways involved, given the observed differences in metabolite concentrations. All metabolites with $q < 0.15$ were included in the MSEA list and compared to the small-molecule pathway database (SMPD) metabolite set library to determine metabolic pathways that were enriched (up or down) between groups, with an FDR threshold of 5%.

## Results

### KS vs controls

A total of 157 metabolites were identified in the samples using a targeted data analysis focused on metabolites central to energy and redox metabolism given the phenotypic profile of insulin resistance in KS. Of these, 35 (22%) differed in abundance between KS and controls with an FDR <0.05 (Fig. 1A). Most of these were fatty acids, with nearly all saturated fatty acids higher in KS and mono- and polyunsaturated fatty acids lower (Fig. 1B). PLS-DA revealed a distinct signature for KS (Fig. 1C), with the highest predictive ability inclusive of the first three components (accuracy 0.97, $R2 = 90$, $Q2 = 0.84$). Several metabolites were identified as potential biomarkers of KS with an AUC of >0.9 (Fig. 1D), including lysophosphatidylcholine (AUC 1.0), the medium, odd-chain fatty acids such as undecanoic acid (AUC 1.0) and nonanoic acid (AUC 0.99), the long, even-chain fatty acids such as stearic acid (AUC 0.97) and palmitic acid (AUC 0.97), and prostaglandin E2 (AUC 0.90). A number of SMPD metabolic sets (31/99) were significantly enriched with an FDR up to 5%, with the top 25 shown in Figure 2. Mitochondrial β-oxidation of long-chain saturated fatty acids was the top enriched pathway with both a high enrichment ratio and strong significance. Several other enriched pathways also support aberrant mitochondrial metabolism, including fatty acid metabolism, oxidation of branched-chain fatty acids, β-oxidation of short-chain saturated fatty acids, linoleic acid metabolism, and propanoate metabolism. Additional pathways of interest include plasmalogen synthesis and β-oxidation of very long-chain fatty acids, which are both part of peroxisomal metabolism that is closely tied with mitochondrial metabolism. Catecholamine biosynthesis was the second highest enriched pathway differentiating KS from controls, which was primarily due to lower tyrosine (an amino acid essential for thyroxine and dopamine synthesis) concentrations in KS. Other amino acid metabolism pathways enriched in pathway analysis included arginine/proline, urea cycle, aspartate,

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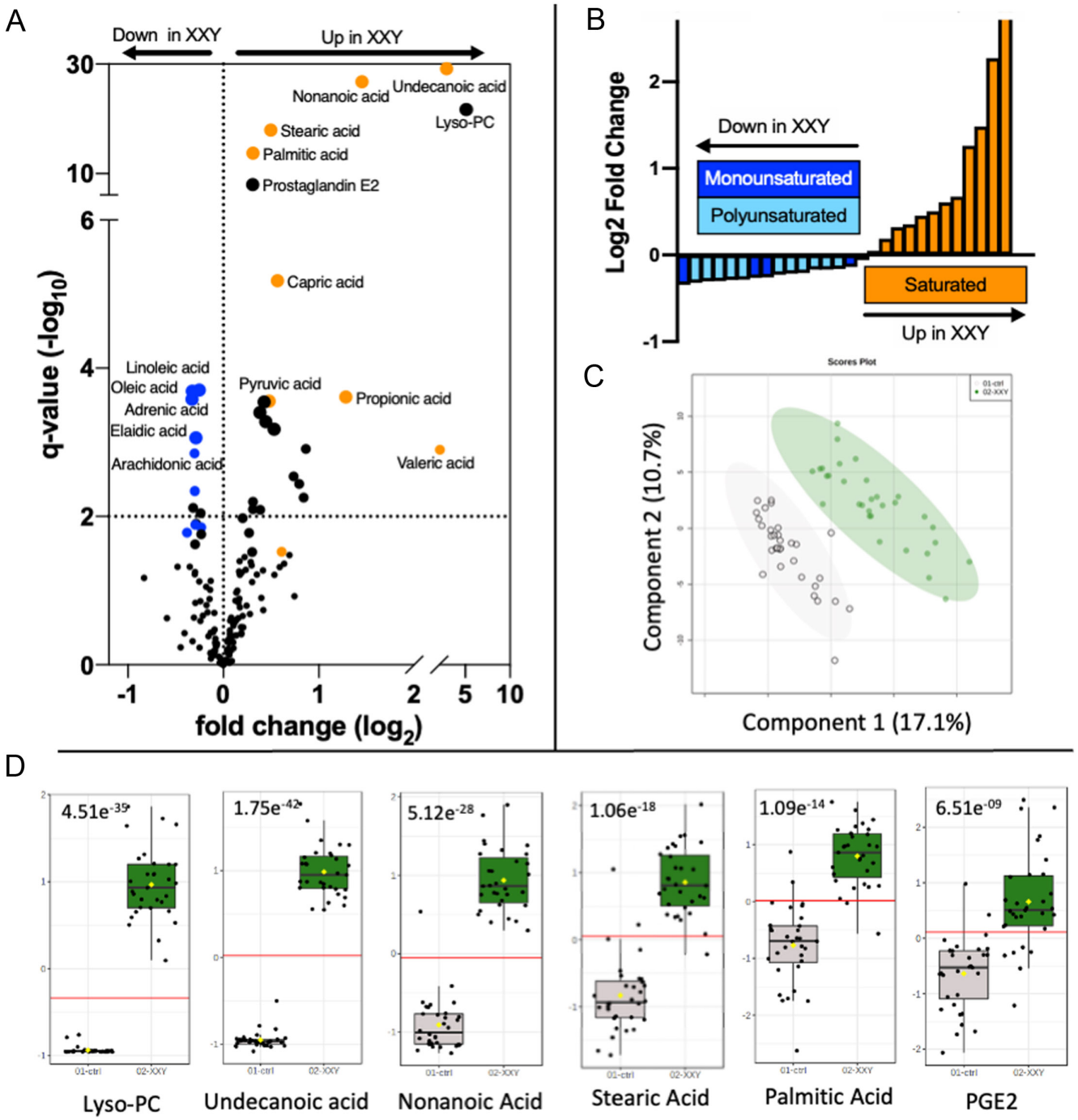


**Figure 1**

(A) Volcano plot of metabolites that are higher (right) or lower (left) in KS compared to controls. Horizontal dashed line is set at $q = 0.05$, indicating *a priori* significance. Orange points represent saturated fatty acids, blue points represent unsaturated fatty acids, and black points are all other metabolites. (B) Bar graph of fatty acids. A positive log2fold change indicates that metabolite values are higher in KS compared to controls. (C) PLS-DA plot of the first two components, clearly differentiating KS (green) from controls (gray). (D) Individual plots of the top six metabolites loading into PLS-DA analysis as well as most significant as biomarkers of KS, with an AUC of >0.9 in ROC analysis. All metabolites are higher in KS (green) compared to controls (gray), with *P* values shown in the top left corner for every metabolite. Lyso-PC, lysophosphatidylcholine; PGE2, prostaglandin E2.

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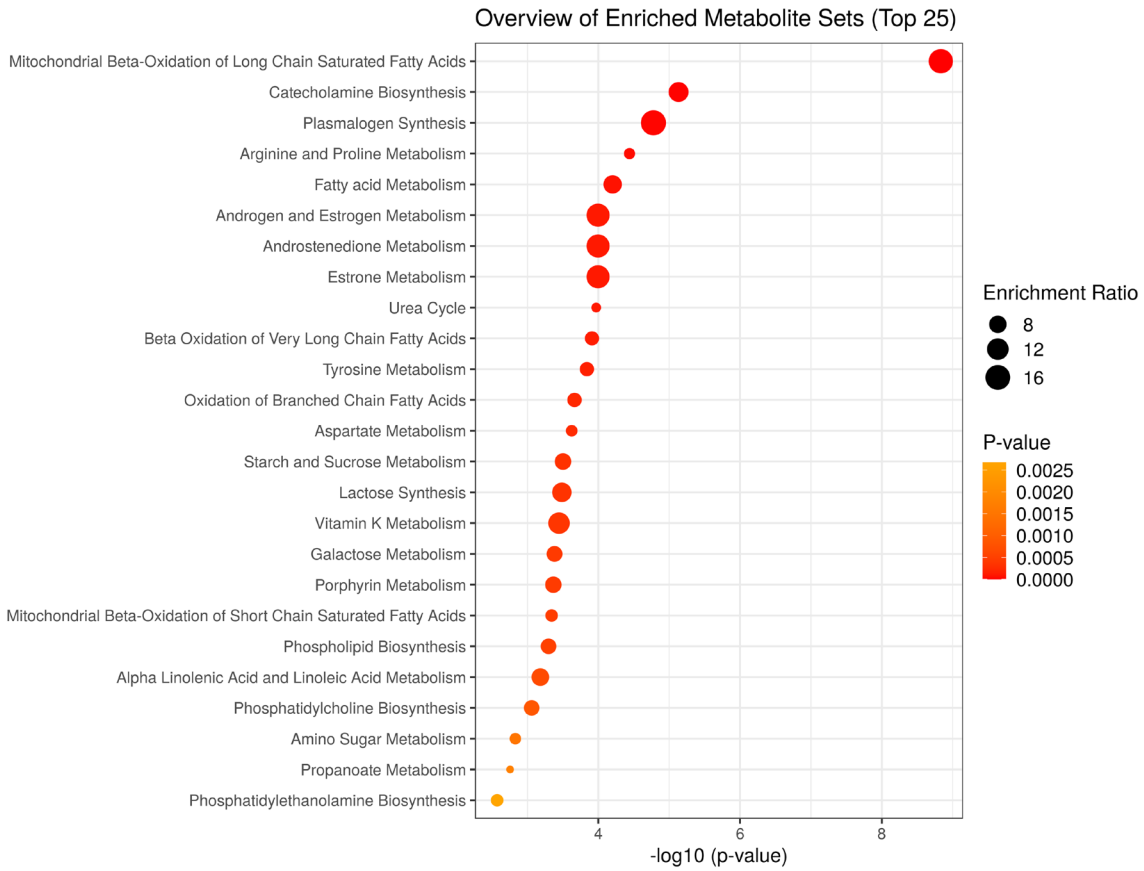


**Figure 2**
Top 25 significantly enriched metabolic sets in KS compared to controls from the SMDPB library of human metabolites. These enriched pathways suggest dysregulation of four more general ontologies of metabolism: mitochondrial, peroxisomal, amino acids, and steroid hormones.

and propionate metabolism. The methods utilized herein chemically target the polar metabolome and were not optimized for steroid metabolites. Future research should specifically evaluate gonadal and adrenal metabolites in KS, both with and without exogenous testosterone.

### Testosterone treated vs untreated

Approximately half of the KS group was treated with exogenous testosterone on a clinical basis, and there were no statistical differences in age ($P=0.94$), BMI *z*-score ($P=0.51$), or systemic testosterone concentrations (median (interquartile range): 380 (298-513) vs 338 (226-438) ng/dL, $P=0.59$) between treated and untreated participants. Unlike the analysis between KS and controls, no metabolites differed between testosterone-treated and untreated males with KS at an FDR of 15% (Fig. 3). For exploratory purposes, we proceeded to evaluate metabolites that were significantly different at a nominal *P* value of <0.05. Stearidonic acid ($P=4.0e^{-3}$) and eicosapentaenoic acid ($P=3.7e^{-2}$) were lower in the testosterone-treated group, while mercaptopurine ($P=1.9e^{-2}$), threonine ($P=2.4e^{-2}$), carnitine ($P=3.3e^{-2}$), 2-oxo-7-methylthioheptanoic acid ($P=3.3e^{-2}$), lysine ($P=4.1e^{-2}$), and phenylalanine ($P=4.5e^{-2}$) were all higher in the testosterone-treated group.

## Discussion

In this exploratory study, we found a unique plasma metabolic signature in adolescent males with KS compared to male controls of similar age, BMI, and pubertal stage. The fatty acid profile was the most disparate between KS and controls, with the KS group having higher saturated fatty acids and lower unsaturated fatty acids. Mitochondrial β-oxidation of long-chain saturated fatty acids was the top enriched metabolic pathway in KS vs controls. KS individuals on testosterone treatment were not protected from these metabolic differences and, in comparison to untreated individuals, did not result in a distinct metabolite signature. These results suggest an underlying

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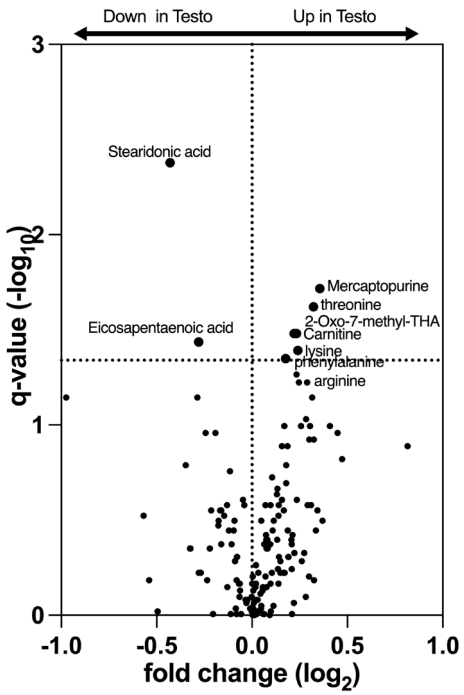


**Figure 3**
Volcano plot of metabolites that are higher (right side of *X*-axis) or lower (left) in testosterone-treated boys with KS compared to untreated boys. The horizontal dashed line indicates a raw $P = 0.05$ due to the lack of differences at $q = 0.05$. Note the difference in both axes compared to Figure 1A.

difference in metabolism in individuals with KS that is likely independent of androgen exposure, in addition to the known contribution of androgen deficiency that has been demonstrated in adults with KS. Whether this profile is simply reflective of aberrant metabolism in KS (biomarker) or actually contributes to the pathophysiology of the KS phenotype requires further study.

Fatty acid metabolism through oxidation in the mitochondria is critical for cellular energy production. The overall metabolite pattern, both by manual analysis and by pathway enrichment analysis, suggests males with KS have impaired ability to oxidize fatty acids. Unlike mitochondrial disorders that result from a single enzyme deficiency, our results suggest that KS is associated with a more global impairment in mitochondrial metabolism. Many studies have identified elevated acylcarnitine esters and branched-chain amino acids as markers of impaired mitochondrial function and potential contributors to insulin resistance, which was our original hypothesis for this study (10, 20, 21, 22). A recent study in girls with Turner syndrome found lower branched-chain amino acids compared to controls, and no correlation of these metabolites with BMI *z*-score despite strong correlations in the control group, suggesting that obesity in Turner syndrome was not associated with altered amino acids (23). While we also did not observe distinct abnormalities in the metabolites typically associated with obesity and insulin resistance, the accumulation of precursors of both β-oxidation and the TCA cycle implies relative impaired mitochondrial metabolism in KS. This profile may be unique to the type of mitochondrial dysfunction in KS, and these results support the need for additional investigation of *in vivo* mitochondrial function in males with KS, as this may be an important contributor to cardiometabolic pathology.

The finding of lower mono- and polyunsaturated fatty acid species in KS is also notable, as these are often considered 'healthier' fatty acids with cardio- and neuroprotective properties. Kim *et al.* found a similar profile in men with overweight/obesity compared to men with healthy weight (24). While our KS and control groups had a similar BMI, our work and that of others has shown that adiposity and other cardiometabolic risk factors are greater in KS despite normal BMI. This raises the possibility that this specific finding we observed in KS is secondary to differences in body composition among this cohort rather than a unique genetic effect from the extra X chromosome specifically. We also did not assess or standardize dietary intake in this cohort; therefore, differences in diet composition may contribute to these findings, although to our knowledge participants were not taking supplements that would explain these results. Given the fatty acid profile we observed, dietary supplementation of polyunsaturated fatty acids may be a potential therapeutic avenue to further investigate in future studies in KS. In particular, boys with KS treated with testosterone had lower omega-3 fatty acids, which have been inversely associated with testosterone concentrations in men (25). Although we cannot determine causality in this cross-sectional study, supplementation of omega-3 fatty acids has also been shown to increase endogenous testosterone production in men (26) and could be a promising intervention to study KS, particularly given the cardio- and neuroprotective features of omega-3s.

Our results yielded a few other intriguing findings beyond differences in fatty acid metabolism. First, lysophosphatidylcholine (lyso-PC) was strikingly more abundant in KS, with no overlap between groups. Lyso-PC is a normal component of blood plasma but has been associated with metabolic and neurological disease, including hyperlipidemia, atherosclerosis, ischemia, and demyelination (27, 28, 29). It has also been identified as a potential biomarker of obesity in men (24).

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Although this is intriguing, lyso-PC was below the limit of detection in many of our controls and thus warrants further investigation. Additionally, our approach was not optimized for lipidomics; therefore, we do not have lyso-PC species which is an important future direction. Higher levels of the inflammatory mediator prostaglandin E2 is another finding of this study that warrants further investigation, considering the cardiometabolic, neurologic, and immune system dysregulation in KS. Finally, catecholamine biosynthesis was the second highest enriched pathway differentiating KS from controls, which was primarily due to lower tyrosine (an amino acid essential for thyroxine and dopamine synthesis) concentrations in KS. Future directions include investigating the relationship of disparate metabolites with the clinical phenotype.

The differences in metabolism based on testosterone treatment status were unimpressive. No metabolites were different based on our initial significance threshold, although when this was relaxed we observed that several amino acids and carnitine were higher in the testosterone-treated group, while two omega-3 polyunsaturated fatty acids were lower. The largely null results were surprising, given the suspected effects hormones have on metabolism. In direct comparison, red blood cell metabolites from blood donors on testosterone replacement therapy analyzed in the same laboratory used in this study revealed significant differences compared to blood from male donors not on testosterone, suggesting increased activation of antioxidant pathways and higher acylcarnitines (8). In addition, a large NHANES (National Health and Nutrition Examination Survey) study found that many fatty acids inversely correlate with testosterone concentrations in men, including alpha-linolenic acid, the parent compound for the two omega-3 fatty acids that were lower in our study (25). These authors proposed that fatty acids reduced endogenous testosterone production; however, our results suggest that testosterone may instead impact fatty acid metabolism and perhaps males treated with testosterone would benefit from supplementation with omega-3 fatty acids. Indeed, the influence of testosterone on unsaturated fatty acid biosynthesis has been reported previously both *in vivo* (rodent models) and *in vitro* (Sertoli cell cultures) (30, 31), although longitudinal studies in humans will be needed to elucidate the relationship between systemic testosterone and fatty acid concentrations and therapeutic implications. Overall, our results may underestimate the impact of testosterone treatment on the metabolome due to our small sample size, significant heterogeneity in the testosterone-treated group due to diverse clinical management, and no differences in serum testosterone concentrations between treated and untreated groups (suggesting all were eugonadal either endogenously or with treatment). Nonetheless, exogenous testosterone in adolescent males with KS seems to have a minimal influence on the plasma metabolome and does not normalize the striking metabolic disparities observed between KS and controls.

To our knowledge, this is the first investigation of the plasma metabolome in individuals with KS. This study yielded several important findings for furthering our understanding of the unique metabolism in KS and speculation toward the association with clinical pathology. However, there are several important limitations. First, we utilized a convenience sample of available plasma specimens that may have introduced artificial group differences. However, we are unaware of any pre-analytical factors that would result in the profile we observed here, and most of the known factors that would alter the metabolomic profile including fasting status, morning collection time, processing procedures, and lack of repeated freeze/thaw cycles were identical between groups (32). While the plasma metabolome has been used in many studies, it does not necessarily represent tissue-level metabolic processes. Finally, although our underlying hypothesis was that the metabolite profile would reflect differences in mitochondrial metabolism, which our results support, the approach was largely exploratory with a relatively small sample size; therefore, the inferences should be considered preliminary.

In conclusion, the plasma metabolomic profile in adolescent males with KS is distinctly different from their peers of similar BMI and pubertal stage. This presents an opportunity to further our understanding of the underlying molecular pathology involved in KS, particularly with regard to mitochondrial metabolism.

**Declaration of interest**

No authors have conflicts of interest to report.

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