Published in final edited form as:

J Proteome Res. 2020 February 07; 19(2): 667–676. doi:10.1021/acs.jproteome.9b00558.

Using Multiple Analytical Platforms to Investigate the Androgen Depletion Effects on Fecal Metabolites in a Mouse Model of Systemic Lupus Erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by circulating autoantibodies that deposit in target organs (e.g., kidneys), resulting in chronic inflammation and eventual destruction of the organ. SLE is much more prevalent in females than males in both humans and spontaneous mouse models of lupus, such as NZBxNZW F1 (BWF1) mice. Depleting androgens by castration dramatically increases the susceptibility of BWF1 male to lupus. We compared fecal metabolite profiles of castrated BWF1 (androgen-depleted) male, intact (androgen-replete) male, and female mice. Four analytical platforms were employed to study the profiles of polar metabolites in mouse feces collected from adult BWF1 mice, and a total of 435 metabolites was identified. Of these, the abundance levels of 72 metabolites were significantly different between castrated and intact male groups, and 63 metabolites were different between female and male groups. Pathway analysis indicated that the pathway differences between castrated and intact male mice closely resembled the pathway differences between female and intact male mice, suggesting that low levels of androgens, whether due to depletion (castrated

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.9b00558>.

PLS-DA results of data from (A) GC×GC–MS with MSTFA derivatization; (B) GC×GC–MS with MTBSTFA derivatization; and (C) 2DLC–MS/MS (–) and (D) 2DLC–MS/MS (+) (Figure S1); mass spectrometer parameters for 2DLC–MS analysis (Table S1); ChromaTOF software parameters used for analysis of GC×GC–MS data (Table S2); metabolites with significant abundance change between castrated and intact male groups (Table S3); metabolites with significant abundance change between female and male groups (Table S4); pathways affected by the metabolic differences between castrated and intact male groups (Table S5); pathways affected by the metabolic differences between female and male groups (Table S6); metabolites with significant abundance change between female and castrated male groups (Table S7); pathways affected by the metabolic differences between female and castrated male groups (Table S8) (PDF)

The authors declare no competing financial interest.

male) or endogenous (female), are associated with multiple fecal metabolomic alterations, which could potentially affect SLE progression. Our findings demonstrate that analyzing fecal metabolites using multiple analytical platforms holds great promise for detecting metabolomic alterations in complex disease model systems.

Graphical Abstract

Keywords

metabolomics; mass spectrometry; mouse feces; androgen depletion; systemic lupus erythematosus

1. INTRODUCTION

Metabolomics is the study of all metabolites in an organism. Metabolites can be polar or nonpolar, as well as organic or inorganic compounds. The diverse chemical characteristics and a huge number of entities make metabolite separation and quantification challenging in metabolomics. Currently, gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) are widely used in metabolomics.¹ To obtain better separation and higher peak capacity, multidimensional separation methods have been developed for metabolomics study, including comprehensive two-dimensional gas chromatography–mass spectrometry (GC×GC–MS)^{2–6} and two-dimensional liquid chromatography–mass spectrometry (2DLC–MS).^{7–9} Our group analyzed polar metabolites extracted from biological samples by GC×GC–MS and parallel 2DLC–MS.^{10,11} Our data demonstrated that these platforms detected different types of metabolites in the biological samples. Analyzing each biological sample by GC×GC–MS and parallel 2DLC–MS and then integrating the experimental data can not only increase metabolite coverage but also achieve high confidence in metabolite identification, metabolite quantification, and metabolic pathway assignment.

The improvements in metabolomic identification offered by these platforms have opened up new avenues of study into how the metabolome affects many biological systems, including those involved in disease. One such disease is systemic lupus erythematosus (SLE). SLE is an autoimmune disease characterized by the loss of immune tolerance to the body's nuclear antigens and the initiation of immune responses against these antigens. This leads to the production of autoantibodies, the deposition of immune complexes in target organs (e.g., kidneys), and chronic inflammation that eventually results in organ failure. SLE has a strong sex bias, occurring much more often in females than males in both humans and spontaneous mouse models of lupus, such as NZB×NZW F1 (BWF1) mice.^{12,13} Although the

mechanisms of action behind this sex difference are currently unknown, considerable evidence indicates androgens, male sex steroids that are normally 20 times higher in males than in females, play a protective role in male mice and humans with lupus.^{14–16} In humans, females with SLE have lower serum levels of androgen than healthy female controls, and higher disease activity correlates with lower levels of androgen.^{17–19} Males with SLE have also been found to have lower androgen levels than healthy males.^{20,21} Treatment with androgens has been shown to lower autoantibody production and disease severity in both male and female lupus patients.^{22–24} These effects of androgens are also seen in the BWF1 mouse model. Androgen depletion by castration increases disease incidence, severity, and mortality in BWF1 male mice, and female mice are protected from disease by androgen treatment.^{15,25,26} The BWF1 mouse model is therefore well suited to investigating the mechanisms of sex differences in lupus.

Accumulating evidence suggests that the resident gut microbiota (i.e., intestinal bacteria) can have a major influence on lupus pathogenesis in both humans and mouse models of SLE.^{27,28} This influence is probably mediated, at least partially, by the metabolites the bacteria produce.²⁹ Interestingly, recent studies have shown that the gut microbiota can influence and are influenced by androgen levels.^{30–33} Although androgens can affect the immune response via a direct effect on immune cells and lymphoid tissues, recent evidence suggests that androgens also can alter the composition of the microbiota and may affect the bacterial metabolomic activities, resulting in changes in the metabolites produced by both the intestinal bacteria and the host.^{32,33} Many microbiota-associated metabolites have been identified that have potent immunomodulatory properties and could potentially influence the progression of lupus.²⁹

In the BWF1 mouse strain, females and castrated (androgen-depleted) males develop lupus spontaneously while intact (androgen-replete) males do not. The objective of this pilot study was to identify changes in metabolites resulting from androgen depletion and correlate those changes with increased susceptibility to disease by comparing the metabolomic profiles of healthy androgen-replete males with the disease-prone androgen-depleted males and females. We collected fecal samples from 21 week old intact male, castrated male, and female BWF1 mice. We chose to analyze the metabolite profiles at the 21 week time point because at that age in female BWF1 mice immune complex deposition typically has occurred and kidney disease is normally beginning to develop. We extracted polar metabolites from mouse feces and analyzed them by GC×GC–MS and parallel 2DLC–MS/MS. To further increase metabolite coverage, each fecal sample was respectively derivatized using two derivatization reagents, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and *N*-methyl-*N*-tert-butyltrimethylsilyltrifluoroacetamide-(MTBSTFA), for GC×GC–MS analysis. And for parallel 2DLC–MS/MS analysis, each fecal sample was analyzed in both negative and positive ionization modes. Then, we integrated the data from multiple platforms and assessed the androgen depletion effects in the metabolic profile of mouse feces.

2. EXPERIMENTAL METHODS

2.1. Animals, Surgical Procedures, Disease Assessment, and Feces Collection

New Zealand Black (NZB) female and New Zealand White (NZW) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The NZB and NZW mice were bred in our animal facility to produce the NZBxNZW F1 (BWF1) mice. Mice were maintained under specific pathogen-free conditions in our animal facilities following the guidelines stipulated by the University of Louisville Institutional Animal Care and Use Committee. Prepubertal (24 day old) male BWF1 mice ($n = 8$) were either untreated or castrated. For castration, the scrotum was incised, the vas deferens and spermatic vessels were ligated, the testes and epididymis were removed, and the skin was sutured. Age-matched BWF1 female mice were untreated ($n = 10$). The mice were monitored for disease/incidence progression by tracking autoantibody levels and kidney disease. Starting at 16 weeks of age, serum anti-dsDNA autoantibody levels were measured biweekly by enzyme-linked immunosorbent assay (ELISA). The quantity of protein in the urine, proteinuria, is an indication of kidney disease severity. Starting at 20 weeks of age, proteinuria was measured biweekly by urinalysis strips (Albustix, Siemens Diagnostics, Los Angeles, CA) to track kidney disease. Proteinuria was scored as trace (0 mg/dL protein), 1 (>30 mg/dL), 2 (>100 mg/dL), 3 (>300 mg/dL), or 4 (>2000 mg/dL) based on the colorimetric urinalysis strips as described by the manufacturer. Mice that died were given a score of 5. Mice receiving a proteinuria score of ≥ 3 for two consecutive biweekly readings were considered to have full-blown kidney disease, and kidney disease incidence indicates the percentage of mice within a group that fell into this category at a given time point. Statistical analysis of anti-dsDNA antibody levels was performed using two-tailed Student's *t*-tests. Statistical analysis of disease scores was performed using a two-way analysis of variance and two-tailed Student's *t*-tests. Statistical analyses of incidence and survival curves were performed using the log-rank (Mantel-Cox) test.

At 21 weeks of age, feces were collected directly from the mice and then transferred to a -80°C freezer. They were maintained at -80°C until metabolomic analysis.

2.2. Metabolite Sample Preparation

Each fecal sample was weighed and ground on ice in a 1.5 mL Eppendorf tube after adding cold 80% methanol at a ratio of 1 mg feces/20 μL 80% methanol. After 2 min of vigorously vortex-mixing, the mixture was sonicated for 20 min followed by centrifugation at 4°C , 14 000 rpm for 20 min. The supernatant was aliquoted into three 200 μL aliquots. Each aliquot was dried by speed vacuum at 4°C to remove methanol followed by freeze-dry to remove water. Dried aliquots were stored at -80°C until use.

One dried aliquot was redissolved in 100 μL of 20% acetonitrile for 2DLC-MS analysis, while the other two dried aliquots were respectively used for GC $\times$ GC-MS analysis after derivatization by MSTFA or MTBSTFA. For derivatization, each of the two dried aliquots was dissolved in 30 μL of methoxyamine hydrochloride solution (20 mg/mL in pyridine) and vortex-mixed for 2 min. Methoxylation was carried out at 60°C for 1 h. Then, 20 μL of MSTFA with 1% trimethylchlorosilane or 20 μL of MTBSTFA with 1% *tert*-

butyldimethylchlorosilane was added to the sample. Derivatization was carried out at 60 °C for 1 h. After centrifugation at 20 °C, 3000 rpm for 10 min, the supernatant of each sample was transferred into a GC vial. Methoxylation and derivatization were conducted immediately before the GC×GC–MS analysis.

For purpose of quality control in 2DLC–MS and acquisition of a MS/MS spectrum for each metabolite by 2DLC–MS/MS, a group-based pooled sample was prepared by mixing a small amount of each sample in the same group. For the GC×GC–MS analyses, each QC sample was derivatized by MSTFA or MTBSTFA, respectively. A mixture of C7–C26 *n*-alkanes was also prepared and analyzed by GC×GC–MS. The GC×GC–MS data of *n*-alkanes were used to convert retention time into retention index.

2.3. GC×GC–MS Analysis

GC×GC–MS analysis was performed on a LECO Pegasus 4D GC×GC-TOF MS system (LECO Corporation, Saint Joseph, MI) consisting of a Gerstel MPS2 autosampler (GERSTEL Inc., Linthicum, MD), an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA), a LECO two-stage cryogenic modulator, and a LECO Pegasus Time-of-Flight mass spectrometer with an electron ionization (EI) ion source. The first dimension column was a J&W DB-5ms Ultra Inert column (60 m × 0.25 mm × 0.25 μm), and the second dimension column was a J&W DB-17 column (1 m × 0.1 mm × 0.1 μm). Both columns were purchased from Agilent Technologies.

For GC×GC–MS analysis, the sample injection volume was 1 μL with a split ratio of 10. The carrier gas was helium (99.999% purity), and the flow rate was 2.0 mL/min. The temperatures of the inlet and transfer lines were all set to 280 °C. The primary oven temperature was set as 60 °C for 0.5 min, then increased at a rate of 5 °C/min to 270 °C, and held at 270 °C for 15 min. The secondary oven temperature was set as 10 °C higher than the temperature of the primary oven and moved in parallel with the first primary oven. The modulator temperature was 15 °C warmer than the secondary oven, and the modulation time was set to 2 s. The mass acquisition range was 29–800 Da at an acquisition rate of 200 spectra/s. The ion source temperature was 230 °C, the detector voltage was 1521 V, and the electron energy of EI source was 70 eV. The acceleration voltage was turned on after a solvent delay of 540 s for MSTFA or 645 s for MTBSTFA. To avoid systematic bias, all samples were analyzed by GC×GC–MS in a random order. The QC sample was analyzed after analysis of every 6 biological samples.

2.4. 2DLC–MS/MS Analysis

2DLC–MS and 2DLC–MS/MS analyses were performed on a Thermo DIONEX UltiMate 3000 UHPLC system coupled with a Thermo Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Inc., Germany). A reversed-phase chromatography (RPC) column and a hydrophilic interaction chromatography (HILIC) column was configured in parallel mode in the LC system. The RPC column was an ACQUITY UPLC HSS T3 column (150 × 2.1 mm, 1.8 μm, Waters Corporation, Milford, MA), and the HILIC column was a SeQuant ZIC–cHILIC column (150 × 2.1 mm, 3 μm,

Merck KGaA, Darmstadt, Germany) equipped with a SeQuant ZIC-chILIC guard column (20 × 2.1 mm, 5 μm, Merck KGaA, Darmstadt, Germany).

The parallel 2DLC conditions were as follows: mobile phase A of PRC was water with 0.1% formic acid; mobile phase A of HILIC was 10 mM ammonium acetate (pH 3.25); mobile phase B for RPC and HILIC was acetonitrile with 0.1% formic acid. The RPC gradient was 0–5 min, 5% B; 5.0–6.1 min, 5% B to 15% B; 6.1–10.0 min, 15% B to 60% B; held at 60% B for 2.0 min; 12.0–14.0 min, 60% B to 100% B; held at 100% B for 13.0 min; 27.0–27.1 min, 100% B to 5% B; held at 5% B for 12.9 min. The HILIC gradient was 0–5.0 min, 95% B to 35% B; held at 35% B for 1.0 min; 6.0–6.1 min, 35% B to 5% B; held at 5% B for 16.9 min; 23.0–23.1 min, 5% B to 95% B; and held at 95% B for 16.9 min. The flow rate was 0.4 mL/min for RPC and 0.3 mL/min for HILIC. The column temperature was 40 °C for both. The MS parameters are listed in Table S1.

All samples were analyzed in full MS scan mode (i.e., 2DLC–MS) in a randomized order to acquire retention time and parent ion *m/z* values for each metabolite. A pooled sample was analyzed after analysis of every 6 biological samples. Each group-based pooled sample was also analyzed in dd-MS² scan mode under collision energies of 10, 20, 40, and 60 eV to acquire MS/MS spectra for each metabolite. The full MS and MS/MS data were collected in both negative and positive ionization modes, respectively.

2.5. Data Analysis

GC×GC–MS data were first processed using LECO's ChromaTOF (version 4.51.6.0) for spectrum deconvolution and metabolite identification. The parameters used in ChromaTOF are listed in Table S2. The MetPP software was used for retention index matching (*p* 0.001), cross-sample peak list alignment, normalization, and statistical significance tests.^{34,35}

2DLC–MS data were first converted into mzXML format by the MSConvert (64-bit) software (Proteowizard, Palo Alto, CA).³⁶ The mzXML files were then analyzed by the MetSign software for spectrum deconvolution, metabolite initial assignment, cross-sample peak list alignment, normalization, and statistical significance tests.^{37–39} The 2DLC–MS/MS spectra of the pooled samples were used for metabolite identification as described in our previous work.^{10,11}

In both MetPP and MetSign software, a pairwise two-tail *t*-test with sample permutation was performed to determine whether a metabolite has a significant difference in abundance between two groups. Grubbs' test was employed for outlier detection before *t*-test. The *t*-test *p*-values were adjusted by up to 1000 times of sample permutation. A metabolite was considered to be significantly different in abundance between two groups if it was detected in more than 75% of samples in at least one group, had a *t*-test *p*-value < 0.05, and an abundance change > 20% (i.e., fold-change is larger than 1.2 or less than 0.8).

Quantitative pathway enrichment analysis was performed using Metaboanalyst (version 4.0). Intensities of metabolites identified with a significant difference between intact male and female mice or between intact male and castrated male mice were analyzed by the program.

The data were autoscaled and matched against the KEGG pathway library for *Mus musculus*. The “GlobalTest” and “Betweenness centrality” algorithms were respectively used for pathway enrichment and topology.⁴⁰

3. RESULTS AND DISCUSSION

3.1. Disease Assessment

This study had 3 groups: intact (non-castrated) male BWF1 mice, male BWF1 mice that were androgen depleted by prepubertal castration, and female BWF1 mice that were untreated. Serum levels of anti-dsDNA autoantibodies were measured by ELISA, and kidney damage was monitored by measuring proteinuria. As expected in the BWF1 model, in as early as 16 weeks of age, the female control mice developed significantly higher levels of autoantibodies than the intact male mice (Figure 1A). The castrated male group developed autoantibody levels that were intermediate and not significantly different from either the female or intact male mice (Figure 1A). Anti-dsDNA autoantibodies are generally associated with lupus; however, they are only one of many different antinuclear autoantibodies and are not present in all lupus patients. It is standard practice to measure anti-dsDNA autoantibodies early in the disease process in lupus models, although their presence is not always predictive of kidney disease. Consequently, although high levels of anti-dsDNA autoantibodies were found in the serum of female BWF1 mice weeks before the onset of detectable kidney disease (Figure 1A–C), the presence of anti-dsDNA autoantibodies did not correlate well with the development of kidney disease in male, either intact or castrated, mice. Unlike anti-dsDNA autoantibody levels, kidney disease severity was significantly higher in both the female control and castrated male groups compared to the intact male group (Figure 1B). There was no significant difference in the incidence of kidney disease between female control and castrated male mice, but both of these groups had significantly higher disease incidence than the intact male mice (Figure 1C). Furthermore, the mortality in female control mice was accelerated by comparison to castrated male mice, and both of these groups had significantly lower survival than the intact male mice (Figure 1D). Similar to the autoantibodies, the survival phenotype of castrated male mice was in between that of the female mice and the intact male mice. These data confirm previous studies showing that androgen depletion makes male BWF1 mice significantly more susceptible to developing lupus.

3.2. Metabolite Identification

A total of 194 metabolites were identified by GC×GC–MS with MSTFA derivatization (GC×GC–MS_MSTFA), 149 by GC×GC–MS with MTBSTFA derivatization (GC×GC–MS_MTBSTFA), 141 by 2DLC–MS/MS in negative ionization mode (2DLC–MS/MS (–)), and 118 by 2DLC–MS/MS in positive ionization mode (2DLC–MS/MS (+)). Figure 2 shows the overlap of metabolites identified from the four analytical platforms. Among those identified metabolites, a total of 285 metabolites were identified by GC×GC–MS, of which 58 metabolites were identified from both MSTFA- and MTBSTFA-derivatized samples. A total of 210 metabolites were identified by 2DLC–MS/MS (–) or 2DLC–MS/MS (+) platform, and 49 metabolites were identified by both 2DLC–MS/MS (–) and 2DLC–MS/MS (+). Integrating the metabolomic data acquired by the four analytical platforms resulted in

435 metabolites identified, clearly increasing the metabolite coverage. Furthermore, identifying a metabolite from multiple analytical platforms can significantly increase the confidence of metabolite identification.

3.3. Metabolite Profiling and Quantification

Partial least square-discriminant analysis (PLS-DA) was performed to study the metabolic profile difference among groups. We merged the GC×GC-MS_MSTFA and GC×GC-MS_MTBSTFA data with the full MS data of 2DLC-MS (–) and 2DLC-MS (+) for PLS-DA clustering (Figure 3). Large R^2 and Q^2 values ($R^2 = 1.00$ and $Q^2 = 0.80$) indicate that the PLS-DA model has good discrimination and high predictability for clustering the metabolic profile data acquired in this study. Figure S1 depicts the PLS-DA results, where the data acquired by each analytical platform was used as the input for PLS-DA. It is clear that using the metabolic data acquired in the four analytical platforms improved the confidence of PLS-DA.

Statistical analysis of metabolite abundance differences was done with pairwise two-tailed t -tests. Between the castrated and intact male groups, a total of 72 metabolites had significant abundance differences (Table S3), and 22 of them were detected by multiple platforms (Table 1 and Figure 4A). In comparison, between the female and intact male groups, abundances of 63 metabolites were significantly different (Table S4), and 13 of them were detected by multiple platforms (Table 2 and Figure 4B). There were 36 metabolites that were significantly different both between castrated and intact male mice and between female and male mice. It should be noted that all significantly changed metabolites detected by multiple platforms had the same direction of regulation (i.e., upregulation or downregulation) and the magnitude of fold changes detected by those platforms were very similar (Tables S3 and S4), demonstrating the high robustness and accuracy of the sample preprocessing methods and the four analytical platforms used in this study.

3.4. Metabolic Pathway Analysis and Potential Biological Significance of Findings

The increased coverage of metabolites resulting from multiple platform analysis also enhances the ability to identify differentially regulated metabolic pathways that could be relevant to the development and/or progression of lupus. Recent reports suggest that the resident gut microbiota (i.e., intestinal bacteria) can affect lupus pathogenesis in both humans and mouse models of SLE.^{27,28} The gut microbiota very likely influence the host, at least in part, via the metabolites they produce.²⁹ Lupus is a sex-biased disease in both humans and the BWF1 mouse model. Female mice develop lupus, whereas male mice do not unless androgen is depleted via castration. One possible mechanism, in addition to the direct effect on immune cells, for this protective effect of androgens is the alteration of microbiota metabolite production. Androgens influence and are influenced by the gut microbiota,^{30–33} and, as mentioned above, an increasing number of studies have found links between lupus and alterations in the gut microbiota.^{41–43} Changes in the gut microbiota directly and indirectly affect the production of intestinal metabolites that can significantly affect a variety of host, including immune activities.²⁹

We used the metabolites with significant differences in abundance between groups for pathway analysis. The metabolic pathway analysis showed that the differences in pathways between castrated and male mice closely resembled the differences between female and male mice. Of the 41 pathways differentially regulated in castrated vs intact male mice, 27 were also differentially regulated in female vs male mice (Tables S5 and S6). Thus, the data showed that depleting androgens in castrated male mice causes shifts in intestinal metabolites toward a female-like phenotype that correlates with an increase in susceptibility of male mice to lupus.

Androgen does not account for all biological differences between male and female mice. The changes in the 27 common pathways differentially regulated in both the castrated vs intact male analysis and the female vs male analysis could be due to androgen depletion or due to other sources of sex differences (i.e., Y chromosome effects). This is evident from the fact that there were 43 metabolites and 33 pathways that were significantly different between castrated male mice and female mice (Table S7 and S8). To narrow our pathway analysis to identify metabolic pathways that are dependent on androgen, we performed a pathway analysis on the set of metabolites that were significantly different between both castrated and intact male mice as well as between female and male mice but were not significantly different between female and castrated male mice (i.e., metabolites present in both Tables S3 and S4 but not in Table S7). We found that after removing the metabolites that were significantly different between female and castrated male mice, 18 of the 27 common pathways remained (indicated with an “a” in Tables S5 and S6). These pathways are the most likely to be different as a result of androgen depletion.

The significance of the pathway analysis is that it identifies potential areas of study for how the differences in intestinal metabolite production could affect lupus progression. One of the pathways differentially affected in both female and castrated male mice compared with the intact male mice was histidine metabolism. Histidine is a precursor to histamine, an effector molecule that can mediate either inflammatory or regulatory responses depending on the target cell and type(s) of histamine receptor expressed (i.e., H1R, H2R, H3R, and H4R). Recent studies have found that probiotic and endogenous bacterial species could produce histamine and affect intestinal immune responses.^{44–46} Our study showed both castrated and female mice had lower levels of histamine and higher levels of the precursor, histidine than intact male mice. Additionally, castrated and female mice had lower levels of methylimidazoleacetic acid, which is the end-product of histamine breakdown and is used as an indicator of histamine production (Tables S3 and S4).⁴⁷ Therefore, intact male mice have a higher production of histamine in the gut that correlates with their lower susceptibility to lupus. However, whether altered histamine metabolism is simply a consequence of lupus or if it actually plays a role in the progression of disease has not been established. It is uncertain how gut-generated histamine could affect the development or progression of lupus, but it could be at the local, i.e., intestinal, level. While histamine is classically associated with allergic reactions, microbe-produced histamine has been shown to directly inhibit inflammatory factors in the gut.^{48–50} Since “leaky gut” and, consequently, low-grade intestinal inflammation are associated with a number of autoimmune diseases, including lupus,^{41,51} inhibition of gut inflammation in lupus-prone mice (or humans) via microbe-produced histamine could potentially affect the development and/or progression of the

disease. Many studies have found that the intestinal environment and the production of metabolites greatly affects how intestinal dendritic cells (DCs) regulate immune responses after migrating to the mesenteric lymph nodes (mLN),^{52–54} and DCs that migrate to the mLN can affect systemic immune responses through the induction and activation of regulatory T cells.^{55–58} Multiple studies have found links between lupus and decreases in regulatory T cell frequency or function in both humans and mouse models.^{59–65} An alteration in the induction of regulatory T cells in the mLN could clearly impact the progression of lupus in BWF1 mice. It has been shown that in an inflamed, colitic intestine, for example, the intestinal DCs lose their ability to induce regulatory T cells and instead drive inflammatory immune responses in the intestine and mLN.⁶⁶ Bacterial histamine can also directly inhibit the intestinal DC production of TNF α ,⁶⁷ an inflammatory cytokine that has been linked to lupus in both humans and mouse models.^{68–71} Inhibiting intestinal DC production of TNF α would not only reduce intestinal inflammation but also alter the mLN environment where intestinal DCs induce regulatory T cells.⁶⁶ TNF α signaling has been shown to directly block the induction of regulatory T cells, so inhibiting DC TNF α production could increase their generation of regulatory T cells.⁷² In summary, the higher levels of histamine in the intact male intestine could affect the development and/or progression of lupus by promoting DC induction of regulatory T cells indirectly by reducing intestinal inflammation and/or directly by suppressing DC production of TNF α .

Another pathway, tryptophan metabolism, was also significantly different in the intact male mice compared with castrated and female mice. Tryptophan can be broken down into many different metabolites, including the neurotransmitter serotonin. Like histamine, serotonin metabolism is regulated by the gut microbiota.^{73–76} In comparison to intact male mice, castrated male and female mice had lower levels of the serotonin precursor 5-hydroxytryptophan and higher levels of the serotonin breakdown product 5-hydroxyindole-3-acetic acid, suggesting that lower levels of androgen may lead to increased conversion of tryptophan into serotonin (Tables S5 and S6). This fits previous studies that found serotonin concentrations are elevated in the plasma and urine of SLE patients.^{77–80} Additionally, higher levels of intestinal serotonin production and signaling are associated with intestinal inflammation.^{81,82} This could affect lupus progression because, as described above, intestinal inflammation can compromise the ability of intestinal DCs to induce regulatory T cells.^{52–54,66}

3.5. Limitations of the Study

The current study is an early-stage development with several limitations. First, we analyzed fecal samples from 21 week old female, and castrated and intact male BWF1 mice ($n = 6–8$). While this sample size provides a satisfied statistical power, it is possible that a certain degree of variations may be introduced for metabolite quantification owing to the relatively small sample size. However, this is less of a concern in mouse compared to human studies, because the mice are genetically identical and are housed under identical conditions (including same food, water, bedding, environment, etc). Second, we only extracted polar metabolites from mouse feces using 80% methanol. Most of hydrophobic molecules such as hydrophobic lipids were not collected and therefore excluded in the current study. For analysis of the polar metabolites, we derivatized each metabolite extract using two

derivatized reagents and analyzed the derivatized metabolites by GC×GC–MS. The same metabolite extract was also analyzed by parallel 2DLC–MS in both positive and negative modes. While applying multiple analytical platforms significantly increases the metabolite coverage, the existing analytical platforms still cannot resolve all polar metabolites so that a certain number of low abundance metabolites were not detected by these platforms, resulting in some biomarkers potentially not being detected in the current study.

4. CONCLUSIONS

Four analytical platforms were employed to study polar metabolites in adult BWF1 mouse feces collected from castrated and intact male, and female mice. A total of 435 metabolites were identified, and statistical tests showed that 72 metabolites had significantly different abundances between castrated and intact male mice, and 63 metabolites were different between female and male mice. Pathway analysis of these significantly different metabolites indicated that the differences in pathways between castrated and intact male mice closely resembled the differences between female and male mice. Overall, our analysis discovered that low levels of androgen, whether due to depletion (castrated male) or endogenous (female), are associated with multiple fecal metabolic alterations, including lower histamine levels and increased tryptophan metabolism to serotonin, that could potentially affect lupus progression. Integrating the data from the four analytical platforms improved metabolite coverage and confidence in metabolite identification and quantification, leading to higher confidence in these metabolic pathway assignments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (1R01AR067188 to M.M.K.; S10OD020106 to X.Z.) and a grant from the Alliance for Lupus Research (to M.M.K.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1.

Effect of androgen depletion on anti-dsDNA antibody levels and kidney disease in castrated (Cast) male mice compared to intact male (M) and female (F) control mice. (A) Serum anti-dsDNA antibody levels were measured by ELISA and represented by optical density. *M* vs *F* *p*-values: 16 weeks = 0.032, 24 weeks = 0.047, 26 weeks = 0.022. (B) Kidney disease score as measured by proteinuria (scale = 1–5; see Methods and Materials for description of scoring strategy). *M* vs *F* *p*-value < 0.0001; *M* vs Cast *p*-value = 0.0004. (C) Incidence of kidney disease (i.e., % mice with a proteinuria score of 3 for two consecutive biweekly readings). *M* vs *F* *p*-value < 0.0001; *M* vs Cast *p*-value = 0.002. (D) Survival curve. *M* vs *F* *p*-value = 0.0002; *M* vs Cast *p*-value = 0.0495. **p* < 0.05, ****p* < 0.001.

Figure 2.
Overlap of metabolites identified from four analytical platforms.

Figure 3.
PLS-DA results of integrated data from four analytical platforms.

Figure 4.

Overlap of significantly changed metabolites from four platforms. (A) Castrated male group compared with the intact male group. (B) Female group compared with the intact male group.

Table 1.
Metabolites with Significant Abundance Change between Castrated and Intact Male Groups

platform name	p-value				fold change (castration/male)							
	P-1 ^a	P-2 ^a	P-3 ^a	P-4 ^a	P-1 ^a	P-2 ^a	P-3 ^a	P-4 ^a	P-1 ^a	P-2 ^a	P-3 ^a	P-4 ^a
3-hydroxybutyric acid	0.048		0.031		0.38		0.08					
4-hydroxy-proline			0.0033	0.026			1.85	1.63				
azelaic acid	0.040		0.040		1.31		1.38					
carnosine			0.00033	0.0040			0.44	0.58				
citrulline			0.034	0.027			1.99	2.12				
creatinine			0.0020	0.000097			1.51	1.48				
glutamine			0.0061	0.014			0.69	0.49				
glutaric acid	0.00099		0.043		1.89		1.45					
histidine			0.00015	0.013			1.80	1.33				
isoleucine	0.00067	0.000085			0.19	0.23						
leucine	0.00031	0.000044			0.27	0.24		0.20				
lysine			0.0054	0.00031			2.00	1.93				
malate	0.00044	0.000911	0.000010		5.08	4.39	5.24					
methionine	0.0094	0.0032	0.022	0.0033	0.47	0.62	0.61	0.64				
phenylalanine		0.00045	0.016	0.0024		0.26	0.42	0.44				
phytol	0	0			0.37	0.18						
pipecolic acid	0.0030	0.00073			1.60	1.57						
serine		0.0017	0.015			0.54	0.73					
succinate	0.011		0.00054		1.86		2.35					
tyrosine	0.025		0.010		0.53		0.43					
urocanic acid		0.000041		0.00076		2.29		1.70				
valine	0.00052	0.000042		0.00043	0.21	0.23		0.34				

^a P-1: GC×GC-MS with MSTFA derivatization; P-2: GC×GC-MS with MTBSTFA derivatization; P-3: 2DLC-MS/MS (-); P-4: 2DLC-MS/MS (+).

Table 2.
Metabolites with Significant Abundance Changes between Female and Intact Male Groups

platform name	<i>p</i> -value				fold change (female/male)							
	<i>P</i> -1 ^a	<i>P</i> -2 ^a	<i>P</i> -3 ^a	<i>P</i> -4 ^a	<i>P</i> -1 ^a	<i>P</i> -2 ^a	<i>P</i> -3 ^a	<i>P</i> -4 ^a	<i>P</i> -1 ^a	<i>P</i> -2 ^a	<i>P</i> -3 ^a	<i>P</i> -4 ^a
adenine			0.047	0.013			0.40	0.25				
alanine	0.026	0.0029		0.034	1.47	1.43		1.41				
histidine			0.00027	0.0085			1.65	1.31				
isoleucine	0.017	0.0023			0.61	0.57						
lysine			0.0061	0.0015			2.02	1.71				
malate		0.0025	0.042			2.14	2.63					
methionine		0.0078	0.046	0.026		1.23	1.41	1.91				
myristic acid	0.00065	0.0033			0.55	0.59						
phytol	0.00036	0.0026			0.45	0.47						
proline	0.0062	0.010			1.65	1.77						
threonine			0.016	0.043			1.39	1.56				
tryptophan			0.018	0.011			1.65	1.60				
valine	0.00041	0.00034			0.56	0.50						

^a *P*-1: GC×GC-MS with MSTFA derivatization; *P*-2: GC×GC-MS with MTBSTFA derivatization; *P*-3: 2DLC-MS/MS (-); *P*-4: 2DLC-MS/MS (+).