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Prospective study of changes in the metabolomic profiles of men during their first three months of androgen deprivation therapy for prostate cancer

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Abstract

Background—Androgen deprivation therapy (ADT) for prostate cancer causes a rise in fasting insulin and adverse changes in body composition and serum lipid profile. It is unknown what other metabolic alterations are caused by ADT. In order to better characterize the metabolic effects of ADT, we measured changes in plasma metabolomic profile at baseline and after the first three months of therapy.

Methods—Fasting plasma samples were drawn from 36 subjects at baseline and after three months of gonadotropin releasing hormone (GnRH) agonist therapy. Extracted samples were split into equal parts for analysis on the gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry platforms.

Results—Of the 292 identified metabolites, 56 changed significantly ($p<0.05$) from baseline to three months. Notable changes were grouped as follows: (a) Multiple steroids were lower at three months, consistent with the effect of therapy on gonadal androgen synthesis. (b) Most bile acids and their metabolites were higher during treatment. Cholesterol levels changed very little. (c) Markers of lipid beta-oxidation (acetyl-carnitines, ketone bodies) and omega-oxidation were lower at three months. (d) Two previously-identified biomarkers of insulin resistance (2-hydroxybutyrate, branch chain keto-acid dehydrogenase complex products) were stable to lower at three months.

Conclusions—Unbiased metabolomic analyses revealed expected, novel, and unexpected results. Steroid levels fell, consistent with the effects of ADT. Most bile acids and their metabolites increased during ADT, a novel finding. Biomarkers of lipid metabolism and insulin resistance fell, unexpected given that ADT has been shown to increase fasting insulin.

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Keywords

prostate cancer; androgen deprivation therapy; GnRH agonist; metabolomics; diabetes; bile acids

Introduction

Androgen deprivation therapy (ADT) is the foundational systemic therapy for men with prostate cancer. It can be accomplished with a gonadotropin releasing hormone (GnRH) agonist, a GnRH antagonist, or bilateral orchiectomy. ADT is associated with improved survival in combination with external beam radiation for intermediate or high risk localized prostate cancer[1-7] and as monotherapy for metastatic disease[8, 9]. Despite these benefits, ADT has been reproducibly shown to cause a number of adverse metabolic effects and may negatively impact the overall health of prostate cancer survivors.

GnRH agonist therapy most prominently causes adverse effects on body composition, serum lipid profile, and insulin . Prospective studies have shown that ADT causes men to gain fat mass and lose lean muscle mass.[10-13] ADT has also been shown to result in metabolic changes typical of obesity including increased serum cholesterol and triglycerides and increased fasting insulin.[10, 14-16] Further, population-based analyses have shown that GnRH agonist use is associated with higher incidence of diabetes and of coronary artery disease.[17-23] These hypothesis-driven prior studies have focused on metabolic outcomes associated with obesity (e.g. hyperlipidemia, insulin resistance). As a result of this limited scope, it is unknown what broader alterations are induced by ADT.

Metabolomics is a technique that allows for the unbiased study of small-molecule metabolites present in fluids or tissues. Current metabolomic methods can be used to screen broadly for changes in plasma hormones and metabolites of potential biological significance in a variety of clinical settings. For example, this technique has previously been used to identify biomarkers of insulin resistance[24, 25] as well as a potential biomarker of prostate cancer progression[26]. The metabolomic platform used in these studies incorporates mass spectrometry coupled with liquid and/or gas chromatography and bioinformatics software for compound identification.[27]

We hypothesized that metabolomic analyses following initiation of a GnRH agonist would reveal treatment-induced perturbations in biochemical pathways that had not previously been associated with ADT. In order to more broadly characterize the metabolic effects of ADT, we measured fasting plasma metabolomic profiles at baseline and after the first three months of ADT in men with prostate cancer. This time-frame was chosen because body composition[28], lipid profile[16], and fasting insulin[14] all change within months of ADT initiation; changes in relative risks for diabetes and coronary artery disease with just three months of ADT can not be assessed with currently-available data. Characterization of the metabolomic signature of patients receiving ADT has the potential to generate new insights that would not be possible within more narrowly focused testing. It may facilitate discovery of biomarkers of host metabolic changes. Further, identification of such biomarkers has the potential to produce clinically relevant mechanistic insights.

Materials and Methods

Study participants were recruited and gave written informed consent at Massachusetts General Hospital (MGH) with Institutional Review Board approval. All subjects had locally advanced or recurrent adenocarcinoma of the prostate and were planned for ≥ three months of ADT with a GnRH agonist. Exclusion criteria included scan-detectable bone metastases,

Karnofsky performance status less than 90, history of diabetes or glucose intolerance, treatment with medications known to alter glucose or insulin levels, and history of prior hormonal therapy for prostate cancer within the past year.

A total of 36 subjects met eligibility criteria and took part. Subjects were evaluated at the MGH Clinical Research Center at baseline and after three months of ADT (range: 71-112 days). All participants were receiving ongoing ADT with a GnRH agonist at the time of repeat testing. Fasting blood samples were collected on the morning of each visit and were stored at -70°C for subsequent batch measurements. After the baseline visit, subjects received leuprolide 3-month depot (Lupron depot; TAP Pharmaceuticals Inc., Deerfield, IL; 22.5 mg IM). Subjects also received bicalutamide (Casodex; AstraZeneca PLC, London, UK; 50 mg by mouth daily) for 4 weeks to prevent effects of the androgen flare associated with initiation of a GnRH agonist.

Metabolomic analyses were performed by Metabolon, Inc. (Durham, NC) as reported previously.[29]

Sample Preparation

Each sample is accessioned into a Laboratory Information Management System (LIMS) system, assigned a unique identifier, and stored at -70 °C. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins are precipitated with methanol, with vigorous shaking for 2 minutes (Glen Mills Genogrinder 2000). The sample is then centrifuged, supernatant removed (MicroLab STAR® robotics), and split into four equal volumes. All biological samples were then randomized across a 2 day run and analyzed in this order across each of the LC+, LC-, and GC platforms; one aliquot is retained for backup analysis, if needed.

Liquid Chromatography/Mass Spectrometry (LC/MS/MS) and Gas Chromatography/Mass Spectrometry (GC/MS)

The LC/MS portion of the platform incorporates a Waters Acquity UPLC system and a Thermo-Finnigan LTQ mass spectrometer, including an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. Aliquots of the vacuum-dried sample are reconstituted, one each in acidic or basic LC-compatible solvents containing 8 or more injection standards at fixed concentrations (to both ensure injection and chromatographic consistency). The acidic extracts were monitored for positive ions (LC+) and basic extracts were monitored for negative ions (LC-). Extracts are loaded onto columns (Waters UPLC BEH C18-2.1 × 100 mm, 1.7 μm) and gradient-eluted with water and 95% methanol containing 0.1% formic acid (acidic extracts) or 6.5 mM ammonium bicarbonate (basic extracts). Samples for GC/MS analysis are dried under vacuum desiccation for a minimum of 18 hours prior to being derivatized under nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). Injection standards at fixed concentrations to monitor the injection and chromatographic consistency were also included. The GC column is 5% phenyl dimethyl silicone and the temperature ramp is from 60° to 340° C in a 17 minute period. All samples are then analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument is tuned and calibrated for mass resolution and mass accuracy daily.

Quality Control

All columns and reagents are purchased in bulk from a single lot to complete all related experiments. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to

injection into the mass spectrometers. Also included were several technical replicate samples created from a homogeneous pool containing a small amount of all study samples ("Client Matrix"). Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e. non-instrument standards) present in 100% of the Client Matrix samples, which are technical replicates of pooled client samples. Values for instrument and process variability in association with these samples meet Metabolon's acceptance criteria. Specifically, instrument variability for the internal standards was found to have a median RSD of 7%. Total process variability for the endogenous biochemicals was found to have a median RSD of 18%.

Bioinformatics

The LIMS system encompasses sample accessioning, preparation, instrument analysis and reporting, and advanced data analysis. Additional informatics components include data extraction into a relational database and peak-identification software; proprietary data processing tools for QC and compound identification; and a collection of interpretation and visualization tools for use by data analysts. The hardware and software systems are built on a web-service platform utilizing Microsoft's .NET technologies which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing.

Compound Identification, Quantification, and Data Curation

Biochemicals are identified by comparison to library entries of purified standards. At present more than 2400 commercially available purified standards are registered into LIMS for distribution to both the LC and GC platforms for determination of their analytical characteristics. Chromatographic properties and mass spectra allow matching to the specific compound or an isobaric entity using proprietary visualization and interpretation software. Additional recurring entities may be identified as needed via acquisition of a matching purified standard or by classical structural analysis. Peaks are quantified using area under the curve. Subsequent QC and curation processes are designed to ensure accurate, consistent identification, and to minimize system artifacts, mis-assignments, and background noise. Library matches for each compound are verified for each sample.

Statistical Analysis

Missing values (if any) are assumed to be below the level of detection. However, biochemicals that were detected in all samples from one or more groups but not in samples from other groups were assumed to be near the lower limit of detection in the groups in which they were not detected. In this case, the lowest detected level of these biochemicals was imputed for samples in which that biochemical was not detected. Based on this, please note that our method of imputation does generate fold change values that may be conservative in nature.

Biochemicals were mapped to pathways based on KEGG given the multiple comparisons inherent in analysis of metabolites. Values for each compound were normalized by calculating the median of the values that were generated on each individual run day ("block normalization"). This normalization step was performed to correct variation resulting from instrument inter-day tuning differences. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately. Missing values were assumed to be below the level of detection for that compound with the instrumentation used. Missing values were imputed with the observed minimum for that compound and added after block normalization. Biochemical fold differences were generated based on group means after imputation with ratios greater than one representing fold increases while ratios less than one show fold decreases. Matched

pairs t-tests were performed to identify biochemicals that differed between baseline and after three months of ADT when comparing the metabolic profiles of participant plasma samples. P-values < 0.05 were considered statistically significant.

Results

A total of 504 metabolites were observed. Of these, 292 were identified as named metabolites within the Metabolon library. The remaining 212 metabolites were classified as unidentified, reflecting expected limitations of the platform's compound library. Plasma levels of 88 biochemicals changed significantly (14 rose, 74 fell; P < 0.05 for each) from baseline to three months. Of those 88 biochemicals, 56 were identified compounds (9 rose, 47 fell) and 32 were unidentified compounds (5 rose, 27 fell). Consistent changes in identified biochemicals were grouped thematically based on investigator judgment into the following four categories:

a. Steroid levels

Levels of multiple steroids were significantly lower at three months compared with baseline (see Table 1a). Cholesterol levels changed very little with treatment (1.06 fold change, P = 0.029). Significant decreases were observed for the following sulfated sterols: DHEAS, epiandrosterone sulfate, androsterone sulfate, cortisol, 4-androsten-3β,17β-diol disulfates 1 & 2, 5α-androstan-3β,17β-diol disulfate, pregnen-diol disulfate, pregn steroid monosulfate, and andro steroid monosulfates 1 & 2. Testosterone was not detected at either timepoint.

b. Bile acids and intermediates of bile acid metabolism increased

Most bile acids and their metabolites that were identified by this analysis were higher during treatment (see Table 1b), though most were not statistically significant. Increases were detected in cholate, glycocholate, taurocholate, chenodeoxycholate, taurochenodeoxycholate, ursodeoxycholate, hyodeoxycholate, deoxycholate, taurodeoxycholate glycodeoxycholate, glycochenodeoxycholate, 7-ketodeoxycholate, glycochenodeoxycholate, glycolithocholate sulfate, and taurolithocholate 3-sulfate. The rise in cholate was statistically significant. Glycocholenate sulfate and taurocholenate sulfate each fell slightly.

c. Markers of lipid oxidation

Several indications of lipid metabolism were lower at three months compared with baseline (see Table 1c):

- Carnitine metabolism: Acyl carnitines function as chaperones for fatty-acyl Co-A transport into the mitochondrion where β-oxidation takes place. Several acyl carnitines were significantly lower following ADT, including deoxycarnitine, acetylcarnitine, hexanoylcarnitine, octanoylcarnitine, decanoylcarnitine, laurylcarnitine, palmitoylcarnitine, stearoylcarnitine and oleoylcarnitine ($p<0.05$ for each compound).
- Ketone bodies: β-oxidation of fatty acids produces ketone bodies as byproducts. Ketone bodies 3-hydroxybutyrate (BHBA) and acetoacetate were significantly lower following ADT ($p<0.05$ for each).
- Dicarboxylic acids: ω-oxidation is a secondary pathway to fatty acid metabolism. ω-oxidation produces dicarboxylic acids (DCAs) as byproducts. Two DCAs (dodecanedioate and octadecanedioate; $p<0.05$ for each) were significantly lower following ADT.

d. Markers of insulin resistance

Biomarkers of two key processes relevant to insulin resistance (IR) were lower or stable at three months than at baseline (see Table 1d). 2-hydroxybutyrate (2-HB, or AHB)[24] and branch chain keto-acid dehydrogenase (BCKDH) complex products[25] have previously been associated with IR. 2-HB is an organic acid and can be a byproduct of 2-ketobutyrate (α-KB) production. α-KB itself can be produced by amino acid catabolism. 2-HB generally rises during states of disrupted metabolism (e.g. lactic acidosis, ketoacidosis) or in the presence of an elevated NADH/NAD+ ratio.[30] Treatment levels of 2-HB were significantly lower than baseline levels (0.84 fold change, $p=0.03$). BCKAs are a major component of skeletal muscle and tend to rise in plasma during fasting and/or protein catabolism.[31] Many components of branched chain amino acid metabolism did not change significantly, while two were significantly lower on treatment than at baseline (α-hydroxyisovalerate, 2-methylbutyroylcarnitine; $p<0.05$ for each).

Discussion

We carried out unbiased metabolomic analyses on fasting plasma samples from 36 men with prostate cancer at baseline and after three months of ADT. These analyses revealed expected, novel, and unexpected results. Steroid levels fell, consistent with the effects of ADT. We observed novel evidence of increased levels of bile acids and bile acid metabolites. Biomarkers of fatty acid oxidation and insulin resistance were stable to decreased, unexpected results given that ADT has previously been shown to increase fasting insulin. To the best of our knowledge, this is the first study to use metabolomics to characterize the metabolic alterations with ADT.

We found that a number of steroids decreased during ADT. This finding reflects the intended effect of ADT. This expected observation within unbiased metabolomic analyses supports the experimental methodology.

We observed that numerous bile acids and their metabolites were higher during ADT for prostate cancer than at baseline. To our knowledge, this is a novel finding. There are a number of potential implications of the observed rise in bile acids.

Bile acids are well known for their role in solubilizing fats within the digestive tract. Primary bile acids are synthesized by the liver and secreted into the duodenum where they form micelles that aid in the digestion and absorption of dietary fats. Primary bile acids can be modified by intestinal flora to produce secondary and tertiary bile acids. All types are efficiently re-absorbed in the terminal ileum to continue within the enterohepatic circulation. Bile acids also function as hormones that are important in modulating a number of metabolic processes.

The concept of bile acids as regulatory hormones is relatively new. Among other receptors, they bind to both the nuclear farnesoid X receptor (FXR) and to a cell surface membrane G protein-coupled receptor (TGR5) in a variety of target tissues. Bile acid signaling through these receptors appears to play important roles in regulating serum lipids, glycemic control, and energy homeostasis.[32] ADT for prostate cancer is known to cause adverse effects on all of these processes. The possibility of clinically meaningful interaction between ADT, bile acids, and these metabolic derangements has not yet been explored.

Our preliminary observations about changes in bile acids must be validated. The present analyses provide relative changes but do not quantify changes in the bile acid pool or define the relative changes in individual primary, secondary, and tertiary bile acids. Further investigation of the ADT-induced elevations in bile acid levels may yield mechanistic

insights. It may also lead to opportunities for therapeutic intervention as bile acid binding agents have an established role in the management of patients with disordered glucose metabolism.

Orally administered bile acid sequestrants have long been commercially-available as cholesterol-lowering agents that work by preventing reabsorption of bile acids in the enterohepatic circulation, thereby shunting cholesterol production to the replacement what is lost.[33] More recently, therapeutic manipulation of bile acids with sequestrants has been shown to improve whole body insulin sensitivity[34] and to improve markers of glycemic control among diabetic men in the general population.[33, 35] With further investigation of the present observations, these agents may find a rational role in the medical management of men who suffer from treatment-induced metabolic effects such as insulin resistance or hypertriglyceridemia.

Fatty acid oxidation decreased during therapy as evidenced by decreases in markers of lipid β-oxidation (acetyl-carnitines and ketone bodies) and decreases in the byproducts of ω–oxidation, a secondary pathway to fatty acid metabolism. Although this pattern was nearly universal among detected biochemicals, not all decreases were statistically significant. These data suggest that lipolysis and β-oxidation of fatty acids are decreased during the first three months of ADT. This observation was unexpected as it reflects the absence of a catabolic state. Relative resistance to insulin is a previously-observed effect of ADT and causes a catabolic state in the general population. Important future directions include validation of this finding and further investigation of its implications for men receiving ADT for the treatment of prostate cancer. Because the present analysis did not follow men beyond the first three months of treatment, changes in fatty acid oxidation during prolonged ADT remain to be characterized.

We observed significant changes in the opposite of the expected direction for two previously-described markers of insulin resistance. 2-HB fell significantly during the first three months of hormonal therapy and BCKDH complex products were stable to lower at three months. Rise in 2-HB was identified as a biomarker of insulin resistance in the Relationship of Insulin Sensitivity to Cardiovascular Risk (RISK) study. In that cohort of 399 non-diabetic subjects, plasma metabolomic analyses revealed 2-HB to be the biomarker best able to discriminate between insulin sensitive and insulin resistant men (as defined by gold-standard hyperinsulinemic euglycemic clamp testing).[24] Despite the fact fasting insulin has previously been shown to rise during the first three months of ADT[14], 2-HB significantly declined from baseline to three months in the present analysis.

Circulating branched chain keto acids (BCKAs) reflect muscle breakdown and can rise in catabolic states such as insulin resistance. Circulating branched chain amino acid levels have previously been shown to be significantly higher in obese subjects than in lean subjects. Further, branched chain amino acid related metabolites have been shown to correlate positively with HOMA, a validated measure of insulin resistance, even after adjustment for body weight.[25] We observed stability to slight declines in most measured BCKAs.

The clinical significance of these observations about previously-identified biomarkers of insulin resistance is not known but is surprising given that a significant rise in serum insulin has been previously described in this clinical setting.[14] It is possible that the pathophysiology of ADT-induced insulin resistance is different from the pathophysiology of insulin resistance in other clinical settings. It is also likely that biomarkers of early changes in insulin induced by the first three months of ADT are different from biomarkers of insulin resistance that emerge chronically within the general population. For instance, circulating insulin levels are elevated early in the natural history of type 2 diabetes but fall with time.

Further studies are needed to better characterize the mechanism(s) responsible for ADT associated insulin resistance.

The present analyses feature notable limitations. First, metabolomic analyses are limited by the breadth of identifiable compounds within the experimental platform. Many compound peaks were not identified within the Metabolon library (212 unidentified among 504 total, 42%). Of the 88 compounds that changed significantly 32 were unidentified (36%). The identity and biological significance of these compounds is therefore not known. Second, some molecules of clinical relevance to metabolic health were not identified. For example, many products of cholesterol metabolism were identified but testosterone itself was not. Third, this technique carries the potential for chance observations given the large number of molecules screened. Fourth, changes in some biochemicals have been shown to emerge far sooner than 3 months after a drop in testosterone levels (e.g. insulin[36, 37]). ADT-induced metabolic changes can not be fully characterized through the study of just two timepoints. Finally, it is important to note that the metabolomic profile of ADT-induced changes during the first three months of therapy does not speak to the long term effects of treatment. Initiation of a GnRH agonist causes a rapid and drastic change in the host hormonal environment that may later lead to an altogether different metabolomic profile during prolonged treatment.

In addition, it is important to emphasize that the methodology used for these analyses provides only relative quantitation of identified compounds. For example, this analysis detected a statistically significant 1.9 fold rise in the primary bile acid cholate but is not able to accurately specify plasma levels of cholate for the two experimental time points. This limitation is accepted in the context of broad screening but necessitates subsequent quantitative validation of key findings. Key future directions therefore include quantitative analyses of ADT-induced changes in bile acids and in biomarkers of fatty acid metabolism and insulin resistance.

Previously published literature on the metabolic effects of ADT has focused on obesity-related complications such as hyperlipidemia and insulin resistance. In order to search more broadly for clinically important ADT-induced metabolic changes, we carried out unbiased metabolomic analyses on plasma samples from men receiving their first three months of GnRH agonist therapy. These analyses revealed treatment-induced declines in androgens as well as unexpected changes in bile acids and in biomarkers of lipid metabolism and insulin resistance. One key future direction will be to quantitate baseline and on-treatment levels of the compounds identified with this technique. Although metabolic changes clearly occur by three months of treatment, changes in men receiving longer durations of therapy are also clinically relevant and worthy of additional study. Further mechanistic insights are needed in order to better promote the metabolic health of men receiving GnRH agonist therapy for prostate cancer.

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Key abbreviations

ADT	Androgen deprivation therapy
GnRH	Gonadotropin releasing hormone

MGH	Massachusetts General Hospital
GC/MS	Gas chromatography/mass spectrometry
LC/MS/MS	Liquid chromatography with tandem mass spectrometry
LC+	Liquid chromatography optimized for acid conditions
LC-	Liquid chromatography optimized for basic conditions
RSD	Relative standard deviation
DCAs	Dicarboxylic acids
IR	Insulin resistance
2-HB	2-hydroxybutyrate
BCKDH	Branch chain keto-acid dehydrogenase
α-KB	2-ketobutyrate
FXR	Farnesoid X receptor

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Statement of Translational Relevance

In this study, we present the results of metabolomic analyses that were used to broadly screen for changes in metabolically-relevant biochemicals during the first three months of androgen deprivation therapy (ADT) for prostate cancer. We found that ADT was associated with increased levels of a number of bile acids and decreased levels of previously-identified markers of insulin resistance and lipid beta-oxidation. The translational significance of these findings can be expressed in two primary ways. First, changes in bile acid levels may be among the important metabolic effects of ADT and may with further study become a target for therapeutic intervention. Second, unexpected changes in biomarkers of lipid oxidation and insulin resistance reveal that therapeutic induction of severe hypogonadism using a GnRH agonist causes patterns of disordered metabolism that are distinct from those produced by chronic-onset insulin resistance in the general population.

Table 1

Changes in steroids from baseline to three months

SUB PATHWAY	BIOCHEMICAL NAME	Fold of change, Month 3 vs. Baseline	*p*-value for paired t-test, Month 3 vs. Baseline	q-value for paired t-test, Month 3 vs. Baseline
Sterol/Steroid	**cholesterol**	1.06	0.029	0.120
	dehydroisoandrosterone sulfate (DHEA-S)	0.87	< 0.001	0.002
	epiandrosterone sulfate	0.50	< 0.001	<0.001
	androsterone sulfate	0.50	< 0.001	<0.001
	cortisol	0.84	0.012	0.072
	cortisone	0.89	0.369	0.370
	7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	1.01	0.653	0.478
	4-androsten-3beta,17beta-diol disulfate 1	0.87	< 0.001	0.003
	4-androsten-3beta,17beta-diol disulfate 2	0.70	< 0.001	<0.001
	5alpha-androstan-3beta,17beta-diol disulfate	0.33	< 0.001	<0.001
	pregnen-diol disulfate	0.76	< 0.001	<0.001
	pregn steroid monosulfate	0.76	< 0.001	<0.001
	andro steroid monosulfate 1	0.88	0.029	0.120
	andro steroid monosulfate 2	0.93	0.321	0.356

• All values are normalized in terms of raw area counts.

• “Fold change Month 3 vs. Baseline” denotes <month 3 level> divided by <baseline level> of each biochemical (i.e. fold change of 0.76 denotes a 24% decrease).

• Shading corresponds to degree of change. Thresholds for color intensity are as follows: green denotes ≤ 0.65 fold change, dark green denotes 0.65 - 0.95 fold change, black denotes 0.95 – 1.05 fold change, dark red denotes 1.05 – 1.35 fold change, red denotes ≥ 1.35 fold change.

Table 2

Changes in bile acids from baseline to three months

SUB PATHWAY	BIOCHEMICAL NAME	Fold of change, Month 3 vs. Baseline	*p*-value for paired t-test, Month 3 vs. Baseline	q-value for paired t-test, Month 3 vs. Baseline
Bile acid metabolism	**cholate**	1.94	0.023	0.107
	glycocholate	1.27	0.976	0.553
	taurocholate	2.08	0.435	0.404
	chenodeoxycholate	1.19	0.189	0.310
	taurochenodeoxycholate	1.70	0.698	0.498
	ursodeoxycholate	1.57	0.088	0.222
	hyodeoxycholate	1.33	0.089	0.222
	deoxycholate	1.36	0.398	0.382
	taurodeoxycholate	1.21	0.311	0.356
	glycodeoxycholate	1.19	0.660	0.481
	7-ketodeoxycholate	1.06	0.208	0.324
	glycochenodeoxycholate	1.48	0.136	0.269
	glycolithocholate sulfate*	1.12	0.634	0.468
	taurolithocholate 3-sulfate	1.16	0.982	0.554
	glycocholenate sulfate*	0.96	0.372	0.370
	taurocholenate sulfate*	0.85	0.128	0.266

• All values are normalized in terms of raw area counts.

• “Fold change Month 3 vs. Baseline” denotes <month 3 level> divided by <baseline level> of each biochemical (i.e. fold change of 0.76 denotes a 24% decrease).

• Shading corresponds to degree of change. Thresholds for color intensity are as follows: green denotes ≤ 0.65 fold change, dark green denotes 0.65 - 0.95 fold change, black denotes 0.95 – 1.05 fold change, dark red denotes 1.05 – 1.35 fold change, red denotes ≥ 1.35 fold change.

Table 3

Changes in markers of fatty acid oxidation from baseline to three months

SUB PATHWAY	BIOCHEMICAL NAME	Fold of change, Month 3 vs. Baseline	*p*-value for paired t-test, Month 3 vs. Baseline	q-value for paired t-test, Month 3 vs. Baseline
Carnitine metabolism	**deoxycarnitine**	0.89	0.023	0.107
	carnitine	1.01	0.799	0.512
	3-dehydrocarnitine*	0.93	0.266	0.356
	acetylcarnitine	0.80	< 0.001	0.011
	hexanoylcarnitine	0.80	0.002	0.026
	octanoylcarnitine	0.72	< 0.001	0.011
	decanoylcarnitine	0.71	< 0.001	0.003
	laurylcarnitine	0.65	0.003	0.029
	palmitoylcarnitine	0.82	0.041	0.144
	stearoylcarnitine	0.77	0.004	0.035
	oleoylcarnitine	0.88	0.049	0.157
Ketone bodies	**3-hydroxybutyrate (BHBA)**	0.36	0.009	0.063
	acetoacetate	0.52	0.002	0.020
Fatty acid, dicarboxylate	2-hydroxyglutarate	0.96	0.728	0.508
	sebacate (decanedioate)	0.85	0.360	0.366
	dodecanedioate	0.80	0.016	0.092
	tetradecanedioate	0.72	0.105	0.237
	hexadecanedioate	0.67	0.053	0.168
	octadecanedioate	0.73	< 0.001	0.001
	3-carboxy-4-methyl-5-propyl-2-furanpropanoate (CMPF)	0.88	0.451	0.408

• All values are normalized in terms of raw area counts.

• “Fold change Month 3 vs. Baseline” denotes <month 3 level> divided by <baseline level> of each biochemical (i.e. fold change of 0.76 denotes a 24% decrease).

• Shading corresponds to degree of change. Thresholds for color intensity are as follows: green denotes ≤ 0.65 fold change, dark green denotes 0.65 - 0.95 fold change, black denotes 0.95 – 1.05 fold change, dark red denotes 1.05 – 1.35 fold change, red denotes ≥ 1.35 fold change.

Table 4

Changes in biochemicals relevant to insulin resistance from baseline to three months

SUB PATHWAY	BIOCHEMICAL NAME	Fold of change, Month 3 vs. Baseline	*p*-value for paired t-test, Month 3 vs. Baseline	q-value for paired t-test, Month 3 vs. Baseline
	2-hydroxybutyrate (2-HB)	0.84	0.030	0.120
Valine, leucine and isoleucine metabolism	3-methyl-2-oxobutyrate	0.96	0.553	0.4393
	3-methyl-2-oxovalerate	0.99	0.548	0.4391
	beta-hydroxyisovalerate	0.94	0.203	0.3186
	isoleucine	0.91	0.066	0.1959
	leucine	0.93	0.118	0.2544
	valine	0.94	0.272	0.3558
	2-hydroxyisobutyrate	1.02	0.856	0.5260
	3-hydroxyisobutyrate	0.94	0.328	0.3558
	4-methyl-2-oxopentanoate	0.97	0.546	0.4386
	alpha-hydroxyisovalerate	0.91	0.016	0.0915
	isobutyrylcarnitine	1.01	0.802	0.5115
	2-methylbutyroylcarnitine	0.88	0.039	0.1414
	isovalerylcarnitine	0.99	0.475	0.4135
	hydroxyisovaleroyl carnitine	0.97	0.797	0.5115
	tiglyl carnitine	0.98	0.839	0.5222
	methylglutaroylcarnitine	0.86	0.072	0.2018

• All values are normalized in terms of raw area counts.

• “Fold change Month 3 vs. Baseline” denotes <month 3 level> divided by <baseline level> of each biochemical (i.e. fold change of 0.76 denotes a 24% decrease).

• Shading corresponds to degree of change. Thresholds for color intensity are as follows: green denotes ≤ 0.65 fold change, dark green denotes 0.65 - 0.95 fold change, black denotes 0.95 – 1.05 fold change, dark red denotes 1.05 – 1.35 fold change, red denotes ≥ 1.35 fold change.