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Authors

Elbadawi-Sidhu, Mona
Baillie, Rebecca A
Zhu, Hongjie
et al.

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Pharmacometabolomic signature links simvastatin therapy and insulin resistance

Mona Elbadawi-Sidhu[1], **Rebecca A. Baillie**[2], **Hongjie Zhu**[3], **Yii-Der Ida Chen**[4], **Mark O. Goodarzi**[5], **Jerome I. Rotter**[4], **Ronald M. Krauss**[6], **Oliver Fiehn**[1,7], and **Rima Kaddurah-Daouk**[8]

[1]West Coast Metabolomics Center, Genome Center, University of California - Davis, Davis, CA, USA

[2]Rosa & Co LLC, 751 Laurel St., Ste. 127, San Carlos, CA 94070, USA

[3]Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC, USA

[4]Institute for Translational Genomics and Population Sciences, Los Angeles Biomedical Research, Institute and Department of Pediatrics, Harbor-UCLA Medical Center, Torrance, CA, USA

[5]Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, USA

[6]Children's Hospital Oakland Research Institute, Oakland, CA, USA

[7]Department of Biochemistry, King Abdulaziz University, Jeddah, Saudi Arabia

[8]Department of Internal Medicine; Department of Psychiatry and Behavioral Sciences, Duke Institute for Brain Sciences, Duke University Medical Center, Durham, NC, USA

Abstract

Introduction—Statins, widely prescribed drugs for treatment of cardiovascular disease, inhibit the biosynthesis of low density lipoprotein cholesterol (LDL-C). Despite providing major benefits, sub populations of patients experience adverse effects, including muscle myopathy and development of type II diabetes mellitus (T2DM) that may result in premature discontinuation of

treatment. There are no reliable biomarkers for predicting clinical side effects in vulnerable individuals. Pharmacometabolomics provides powerful tools for identifying global biochemical changes induced by statin treatment, providing insights about drug mechanism of action, development of side effects and basis of variation of response.

Objective—To determine whether statin-induced changes in intermediary metabolism correlated with statin-induced hyperglycemia and insulin resistance; to identify pre-drug treatment metabolites predictive of post-drug treatment increased diabetic risk.

Methods—Drug-naïve patients were treated with 40 mg/day simvastatin for 6 weeks in the Cholesterol and Pharmacogenetics (CAP) study; metabolomics by gas chromatography-time-of-flight mass-spectrometry (GC–TOF–MS) was performed on plasma pre and post treatment on 148 of the 944 participants.

Results—Six weeks of simvastatin treatment resulted in 6.9% of patients developing hyperglycemia and 25% developing changes consistent with development of pre-diabetes. Altered beta cell function was observed in 53% of patients following simvastatin therapy and insulin resistance was observed in 54% of patients. We identified initial signature of simvastatin-induced insulin resistance, including ethanolamine, hydroxylamine, hydroxycarbamate and isoleucine which, upon further replication and expansion, could be predictive biomarkers of individual susceptibility to simvastatin-induced new onset pre-type II diabetes mellitus. No patients were clinically diagnosed with T2DM.

Conclusion—Within this short 6 weeks study, some patients became hyperglycemic and/or insulin resistant. Diabetic markers were associated with decarboxylated small aminated metabolites as well as a branched chain amino acid directly linked to glucose metabolism and fatty acid biosynthesis. Pharmacometabolomics provides powerful tools for precision medicine by predicting development of drug adverse effects in sub populations of patients. Metabolic profiling prior to start of drug therapy may empower physicians with critical information when prescribing medication and determining prognosis.

1 Introduction

Statins reduce the risk of cardiovascular disease (CVD) by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), thereby reducing circulating LDL-cholesterol (LDL-C) and effectively lowering risk of heart attack, stroke and atherosclerosis (Grundy et al. 2004). In addition to cholesterol reduction, statins provide a broad spectrum of pleiotropic biological effects: they improve endothelial function, provide antioxidant, anti-inflammatory and immunomodulatory effects (Jasinska et al. 2007) and improve asthma (Zeki et al. 2015, 2009). New mechanistic insights are being gained about biochemical events related to the numerous therapeutic benefits of statins, as well as to the side effects.

Results from several studies have recognized new onset T2DM as a potential side effect of statin therapy. For instance, in The Justification for the Use of Statin in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) trial, rosuvastatin increased incident T2DM by 28% in participants with one or more diabetes risk factors and expedited diabetes onset compared to those who developed diabetes while on placebo (Ridker et al. 2012). Additionally, investigators from several other human trials, including HPS, 4S, SEARCH (simvastatin), CORONA, GISSI HF (rosuvastatin), ASCOT-LLA, PROVE-IT TIMI, TNT, IDEAL (atorvastatin), and AFCAPS/TexCAPS (lovastatin) also found an increase in incident diabetes in patients taking statins (Ruscica et al. 2014; Sattar et al. 2010). The USAGE study surveyed over 10,000 patients on various statins and found that 62% discontinued statin therapy due to adverse effects (Cohen et al. 2012). Diabetes risk may be linked to statins' tendency to decrease the production of adiponectin, reduce adipocyte maturation, lower GLUT4 expression in adipocytes and inhibit glucose-induced insulin secretion (Brault et al. 2014). Additionally, several studies have implicated that the risk of the diabetic or the pre-diabetic state (metabolic syndrome) is related to amino acid pathophysiology (Newgard et al. 2009; Tai et al. 2010; Wang et al. 2011; Wurtz et al. 2012).

Adverse and therapeutic response to statins varies greatly and may be due to genetic and/or environmental influences. Variation in response due to genetic polymorphisms (Mangravite et al. 2008), diet (Vaquero et al. 2010), level of immune response (Ridker et al. 2008), environmental conditions, and drug interactions (Bai 2010) have been identified, but do not fully explain response variation.

LDL-C and other biomarkers are insufficient in predicting clinical benefit or side effects; thus, more reliable biomarkers are needed. We previously encompassed the power of metabolomics by using complementary platforms to identify global effects of simvastatin treatment in addition to markers of simvastatin response. Pharmacometabolomics is evolving as a field that compliments and informs pharmacogenomics and serves as a compass for identifying drug-affected biochemical pathways that can be navigated for genetic mutations that may predict drug response and adverse effects (Kaddurah-Daouk and Weinshilboum 2014; Kaddurah-Daouk et al. 2015). Pharmacometabolomics captures the metabolic state of an individual under genetic and environmental influences and permits analysis of the fluidic changes occurring at the biochemical level by sampling pre and post drug treatment (Kaddurah-Daouk et al. 2008; Kaddurah-Daouk and Weinshilboum 2014). Pharmacometabolomics-informed pharmacogenomics as well as pharmacometabolomics-informed pharmacokinetics better elucidates mechanism of drug action, thereby broadening the toolbox for identifying drug effects anddisease pathophysiology while providing powerful tools for precision medicine (Kaddurah-Daouk and Weinshilboum 2014; Kaddurah-Daouk et al. 2015).

Hitherto, we implemented a series of targeted and non-targeted metabolomics platforms to profile samples from the Cholesterol and Pharmacogenetics (CAP) study (Simon et al. 2006), from individuals representing the upper and lower tails of simvastatin response, as well as the full distribution of LDL-C response.

Baseline metabolic profiles of good responders differed from that of poor responders in multiple metabolomic analyses, as did metabolic signatures post drug exposure. Baseline levels of the bacterial-derived bile acids lithocholic acid, taurolithocholic acid and glycolithocholic acid, three bacterial-derived bile acids, as well as coprostanol, a product of enteric bacterial reduction of endogenous cholesterol, predicted simvastatin-induced LDL-C lowering, implicating gut microflora in modulating drug efficacy and reinforcing the interconnection of pharmacometabolomics, pharmacogenomics, the environment and diet on drug response (Kaddurah-Daouk et al. 2011). In the same select groups of CAP study participants, we used targeted lipidomic analysis and found that baseline cholesterol ester and phospholipid metabolites correlated to simvastatin-induced LDL-C reduction, particularly increased arachidonic acid and decreased linoleic acid within multiple lipid classes in all participants, and increased delta-5-desaturase activity in only the good responders. Additionally, we found different signatures that correlate with simvastatin-induced changes in C-reactive protein and in LDL-C lowering, suggesting that distinct metabolic pathways mediate statin effects on these two markers of statin efficacy (Kaddurah-Daouk et al. 2010). Using untargeted gas chromatography time-of-flight mass spectrometry (GC-MS), we determined that simvastatin treatment affected the amino acid degradation pathway (Trupp et al. 2012).

Taken together, our previous findings support metabolomics as an effective tool in identifying markers of simvastatin response (Krauss et al. 2013). These studies led us to hypothesize that pharmacometabolomics can also be used to identify markers of simvastatin adverse effects.

In the present study, we investigate the changes in intermediary metabolism and the risk of developing hyperglycemia and/or insulin resistance as an adverse response to simvastatin.

2 Methods

2.1 Clinical samples obtained from cholesterol and pharmacogenetic study

Plasma samples were analyzed from 148 participants selected from the CAP study cohort, which included a total of 944 Caucasian and African-American men and women treated with simvastatin at 40 mg/day for 6 weeks. Detailed information about the study and the participants of the CAP study has been published by Simon et al. in 2006 while detailed information about the 148 participants included in this investigation is included in the supplementary material presented by Trupp et al. in 2012 (Simon et al. 2006; Trupp et al. 2012). Briefly, participants were 60 years of age with pre-treatment serum cholesterol levels between 160 and 400 mg/dL. The plasma samples used in the present study were collected at baseline and at 6 weeks of therapy after an overnight fast. Clinical measurements of glucose and insulin were taken pre and post treatment and used in the Homeostasis Model Assessment (HOMA) to estimate beta cell function, insulin sensitivity and insulin resistance; c-peptide and cholesterol metabolites were also measured pre and post simvastatin treatment, along with several other clinical tests. Additional information, including medication compliance and diet, have been published (Trupp et al. 2012).

2.2 Metabolomic analysis by GC-TOF mass spectrometry

Metabolomic analysis was performed as previously described (Trupp et al. 2012). Briefly, plasma aliquots were extracted and derivatized for analysis by gas chromatography time-of-flight mass spectrometry (GC-MS) as previously described (Trupp et al. 2012). Peak finding and deconvolution of acquired spectra were processed using the metabolomics BinBase database (Kind et al. 2009; Scholz and Fiehn 2007). BinBase metabolite spectra entries were matched against the Fiehn mass spectral library of 1200 authentic metabolite spectra with retention index and mass spectrum information or against the current NIST commercial library (Kind et al. 2009). Quantitative data were normalized to the sum intensities of all known metabolites and used for statistical investigation.

2.3 Statistical analyses

Right-skewed metabolites with distribution right skewness larger than 2 were log-transformed before further analysis. Metabolites were associated with clinical endpoints using linear regression analysis. First, each metabolite was associated with each clinical endpoint of interest (univariate analysis), while adjusting for clinical covariates that were associated with the clinical endpoint. Covariates for each clinical endpoint were selected using all subset selection based on the Bayesian Information Criterion (BIC). P values were obtained for each metabolite. Storey's q value was calculated to account for multiple testing (Storey 2003). Second, for each clinical endpoint, all subset selection based on BIC was applied to the entire set of metabolites to identify the linear combination of metabolites that was associated with the endpoint (multivariate analysis). During this process, the covariates to be adjusted for were forced staying in the model.

Partial correlation networks were built among metabolites with each clinical endpoint using a sparse partial correlation estimation approach (Peng et al. 2009). A connection between two variables in the network implied conditional dependency between the corresponding variable pairs, conditional on the rest of the variables in the network. The false discovery rate was controlled at 0.05.

Pathway enrichment analysis was performed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) with hypergeometric test used for over representation analysis and relative-betweeness centrality used for pathway topology analysis (Xia et al. 2012, 2009, 2015).

3 Results

Metabolomics results consisted of plasma metabolites identified by GC-MS. Clinical measures consisted of plasma glucose, plasma insulin, HOMA-IR, HOMA-beta and c-peptide/glucose ratio. We focused on the following evaluations: (A) association of baseline metabolite levels versus baseline clinical measures, (B) therapy-induced changes in pre-diabetes risk assessment clinical measures, (C) therapy-induced changes in metabolite levels versus changes in clinical measures, (D) post-treatment metabolite levels versus post-treatment clinical measures (E) baseline metabolite levels versus therapy-induced changes in clinical measures and (F) baseline metabolite levels versus post-treatment clinical measures.

Baseline age and BMI were significantly correlated with baseline fasting plasma glucose levels (p = 0.0012, 0.0110). BMI was also significantly associated with baseline insulin, c-peptide, and HOMA_IR scores (p < 0.0001); thus, these covariates were accounted for in the linear regression analyses. Other covariates, such as sex and race, were not significantly associated with measured clinical scores.

3.1 Baseline glucose, insulin and HOMA correlate with baseline amino acids and fatty acids levels

Baseline clinical measures positively correlated with fatty acids and amino acids (Table 1) both in univariate (p < 0.05) and multivariate analysis. Glucogenic amino acids, including alanine and glutamic acid, were associated with glucose, insulin and HOMA levels; glutamic acid also correlated with c-peptide levels. Fatty acids were also significantly correlated to baseline glucose, insulin and HOMA levels (Table 1). In addition, the ketone body 3-hydroxybutanoic acid negatively correlated with glucose, insulin and HOMA. Aminomalonic acid, a metabolite found in atherosclerotic plaques (Van Buskirk et al. 1984), had significant negative correlation to insulin and HOMA pre-statin treatment. A biomarker for elevated diabetes risk, 2-aminoadipic acid (Wang et al. 2013), was found to positively correlate to baseline HOMA and to glucose/c-peptide ratio, but not to baseline glucose, c-peptide or insulin levels. Azelaic acid was inversely correlated with insulin and HOMA measurements at baseline. Table 1 lists all baseline metabolites that were correlated to baseline clinical measures while Online Resources 1 and 2 detail the p and q values of the univariate and multivariate analyses; pathway enrichment results are listed in Table 2.

3.2 Simvastatin therapy increases risk of T2DM

Despite the short duration of the study, 42% of subjects experienced elevated plasma glucose (>3% increase) post-simvastatin treatment (Fig. 1a). Of these subjects, 6.9% developed fasting hyperglycemia, indicated by fasting plasma glucose greater than 125 mg/dL, and 25.0% became pre-diabetic, as indicated by fasting plasma glucose levels between 100 and 125 mg/dL. Fasting plasma glucose of all subjects was above the lower limit for normal fasting plasma glucose levels of 70 mg/dL; thus, none of the subjects developed hypoglycemia. Insulin increased in 49.7% of subjects following simvastatin treatment; HOMA-IR and HOMA-beta scores increased in 49.0 and 46.0% of subjects, respectively (Fig. 1b–e). Additionally, the glucose/c-peptide ratio, which is used as a measure of insulin secretion, was generally decreased in individuals with increased plasma glucose and increased in individuals with decreased plasma glucose post treatment.

3.3 Change in glucose, isoleucine and ketoleucine correlate to change in T2DM risk assessments following simvastatin therapy

When change in metabolite level pre and post drug treatment was evaluated against change in clinical diabetes measures using a univariate analysis, change in analytical glucose significantly correlated (p < 0.05) to change in clinical glucose, insulin and HOMA-IR prior to FDR correction. Change in isoleucine correlated (p < 0.05) to change in glucose and c-peptide levels while change in ketoleucine correlated to change in insulin. Online Resource 3 details additional simvastatin-induced changes in metabolite levels that correlated to simvastatin-induced changes in clinical measures.

Multivariate analysis revealed several metabolites, primarily amino acids and fatty acids, with change in levels pre and post treatment correlated to change in T2DM risk assessment levels. Alanine correlated to glucose and c-peptide changes; ketoleucine correlated to insulin and HOMA changes. In addition, isoleucine and galactosyl-glycerate (negative correlation) changes correlated to glucose; *N*-methylalanine, glycerol, palmitoleic acid (negative correlation) and glucuronic acid (positive correlation) changes correlated to insulin changes; serine, palmitoleic acid, beta-alanine and an isomer of arachidonic acid (negative correlation) changes correlated to HOMA changes and phenylalanine, isocitric acid, fructose and arabitol (positive correlation) changes correlated to c-peptide changes. Change in isoleucine (negative correlation) pre and post simvastatin treatment correlated to the change in glucose/c-peptide ratio, an indicator of apparent insulin secretion. When all of the above metabolites were used for pathway enrichment analysis, the cyanoamino acid metabolism was the only pathway to be enriched for this analysis (data not shown).

3.4 Post-treatment insulin and HOMA, but not glucose, correlate to post-treatment amino acids and fatty acids levels

Insulin and HOMA plasma levels after simvastatin treatment correlated (p < 0.05) primarily with post-treatment amino acids and fatty acids, several of which were also correlated at baseline (Table 3). Low glycine plasma level was observed in subjects with high insulin or HOMA. In contrast, alanine and glutamic acid post-treatment levels were high in patients with high post-treatment insulin or HOMA levels; however, the relationship did not pass multiple hypothesis testing. These two diabetes risk assessments were also inversely correlated to several fatty acids, including lauric acid, linoleic acid, palmitoleic acid and oleic acid, as well aminomalonate and 3-hydroxybutanoic acid. *N*-methylalanine was inversely related to insulin post-simvastatin treatment. In addition to proline (positive correlation), alanine (positive correlation) and glycine (negative correlation), c-peptide levels post treatment also correlated to lactic acid (positive correlation), succinic acid (negative correlation), lauric acid (negative correlation) and aminomalonate (negative correlation). The non-adjusted p values of these correlations indicate that they may be affected by statin treatment and should be further investigated.

Collectively, post treatment metabolites that correlated to post treatment clinical measures (p < 0.05) were mostly associated with cyanoamino acid metabolism and fatty acid biosynthesis; other enriched pathways are listed in Table 4.

Multivariate regression analysis of post-treatment levels resulted in two metabolites with a significant inverse relationship to glucose post simvastatin treatment: a uridine-like compound (negative correlation) and galactosyl-glycerate (negative correlation) (Online Resource 5). The relationship observed in univariate analysis between insulin and glycine (negative correlation), glutamic acid (positive correlation) and 3-hydroxybutanoic acid (negative correlation) was also observed in the multivariate analysis. Multivariate analysis preserved the relationship observed between HOMA and glycine (negative correlation). Alanine, linoleic acid and octadecanol correlated to HOMA prior to p value adjustment. Despite lack of significant correlations between c-peptide and annotated metabolites during the univariate analysis, multivariate analysis resulted in significant association of glycine

(negative correlation) and alanine (positive correlation) to c-peptide levels post treatment. Tyrosine and azelaic acid post simvastatin treatment were related to post c-peptide levels as well.

3.5 Baseline levels of glucose, branched-chain amino acids and small aminated compounds linked to simvastatin-induced beta cell dysfunction and insulin response

Baseline levels of metabolites were analyzed in relation to changes in clinical measures of diabetes in an effort to identify predictors of a simvastatin-induced pre-diabetic state. Significant correlation of metabolites at baseline to changes in glucose, insulin, c-peptide or HOMA was only observed with non-annotated metabolites, that is, metabolites with current unknown structure. In contrast, several identified metabolites correlated to change in beta cell function and insulin response, as indicated by the glucose to c-peptide ratio (Table 5). Glucose and the branched-chain amino acids isoleucine and valine positively correlated with change in the glucose to c-peptide ratio, indicating that individuals with high baseline levels of these three metabolites had decreased beta cell function after simvastatin treatment. In addition, ethanolamine, a precursor in lipid biosynthesis, and cyclohexylamine, hydroxylamine and hydroxycarbamate also had baseline levels significantly associated ($p <$ 0.05) with change in glucose to c-peptide ratio. Baseline levels of ketoleucine as well as the saturated fatty acid myristic acid and the fatty alcohol hexadecanol correlated with change in the glucose/c-peptide ratio with p value <0.1.

3.6 Pre-drug metabolite levels correlate to post-drug clinical measures of pre-diabetes

Baseline metabolite levels were significantly ($p < 0.05$) correlated with post-treatment clinical measures of pre-diabetes (Table 6). Glutamic acid positively correlated to glucose, insulin and HOMA-IR with $q < 0.2$. Alanine and 3-hydroxybutanoic acid also correlated to these three clinical measures, but the relation to glucose had a high q-value. Baseline levels of several amino acids significantly correlated ($p < 0.05$; $q < 0.2$) to insulin and HOMA-IR, as did aminomalonate, 3-hydroxybutanoic acid, azelaic acid, lactic acid and linoleic acid. Ethanolamine also significantly correlated to insulin. These metabolites were compared to the baseline metabolites that significantly correlated to clinical measures pre-drug treatment to assess which correlations were likely non-drug related. Of the 13 baseline metabolites that were strongly correlated to post-drug treatment clinical measures, two were not correlated to pre-drug treatment clinical measures: *ethanolamine and threonine*. Baseline ethanolamine negatively correlated to post-treatment insulin and threonine positively correlated to insulin and HOMA-IR.

4 Discussion

Statins are widely administered effective drugs for the prevention of cardiovascular disease; however, their use has been linked to increased incidence of T2DM. We have previously published data from the Cholesterol and Pharmacogenetics (CAP) study that detailed the association of metabolites, particularly lipids and amino acids, to simvastatin-induced cholesterol lowering in patients classified as good and poor responders as well as in full range responders (Kaddurah-Daouk et al. 2010; Trupp et al. 2012). We have also published data from the CAP study correlating enteric microbiome metabolites to simvastatin

response, identifying three secondary bacterial-derived bile acids that contributed to prediction of beneficial simvastatin response (Kaddurah-Daouk et al. 2011). In this study, we examined the relationship of metabolites to clinical measures of pre-diabetes. We used a GC-MS metabolomics platform to measure and investigate changes in intermediary metabolism and the associated possibility of developing elevated plasma glucose as an adverse response to simvastatin.

We determined that 6 weeks of daily simvastatin treatment increased plasma glucose and insulin in some subjects, indicating development of insulin resistance. Evaluation of the homeostatic model assessment for insulin resistance (HOMA-IR) and for beta cell function (HOMA-b) indicated that many subjects showed increased insulin resistance (54%) and altered beta cell function (53%) following statin treatment.

Numerous publications implicate statin therapy in increasing the risk of new-onset diabetes and worsening glycemic control (Bang and Okin 2014; Macedo et al. 2014; Wang et al. 2014). Statins are associated with 7–25% higher risk of diabetes, with variation of effect based on the particular statin and dose. Although different rankings have been assigned to the statins in terms of their diabetogenic probability, most studies suggest that rosuvastatin, simvastatin and atorvastatin are more likely to cause T2DM than pravastatin, although there is published data that suggest pravastatin is also culpable (Baker et al. 2010; Cho et al. 2015; Koh et al. 2010; Sattar et al. 2010).

The risk of developing T2DM following statin treatment may be higher in some individuals than others, particularly those in a pre-diabetic state (Ridker et al. 2012; Sattar et al. 2010). Those with increased plasma glucose and decreased insulin sensitivity and insulin release may be more likely to fully develop T2DM after taking statins (Cho et al. 2014; Ridker et al. 2012). Several mechanisms are hypothesized for statin effect on diabetes risk, including decreased production of adiponectin, reduced adipocyte maturation, impaired glucose transporter 4 translocation, inhibition of intracellular signaling and diminished beta cell proliferation and insulin secretion due to decreased leptin (Brault et al. 2014).

Clinical diagnosis of T2DM is typically documented after a second test confirms the out-of-normal result. Since assessments in our study were performed once pre-drug treatment and once post-drug treatment, patients within our study were not clinically diagnosed with T2DM. We did, however, link diabetic outcomes to intermediary metabolism at baseline and post-simvastatin treatment. Despite the short duration of the study, several subjects developed hyperglycemia, while many more displayed signs of pre-diabetes and insulin resistance. Insulin and c-peptide concentrations increased in several subjects following simvastatin treatment, as did HOMA-IR and HOMA-beta scores (Fig. 1). These increases predict the potential onset of T2DM, as insulin and c-peptide secretion changes often precede measurable glucose abnormalities. Subjects who had increased plasma glucose in response to statin treatment apparently had decreased insulin secretion post treatment, and vice versa. So in addition to possible changes in insulin resistance, as indicated by changes in HOMA-IR and HOMA-beta, 6 weeks of simvastatin treatment may also alter insulin secretion in susceptible individuals.

4.1 Effect of fasting on energetics is observed at baseline but is disrupted following 6 weeks of simvastatin treatment

Our metabolomics analysis by GC-MS of baseline plasma resulted in many metabolites significantly related to baseline, fasting clinical measures (Table 1). These metabolites are related to intermediary metabolism and most are closely related to the TCA cycle and energy consumption. Many of the identified metabolites would be expected to be elevated during fasting to provide carbon for gluconeogenesis or energy for cells. Additional metabolites that are not directly linked to the TCA cycle also significantly correlated to baseline clinical measures. As expected, fatty acids were low in individuals with high insulin levels, indicating increased lipid biosynthesis in these subjects. Prior to simvastatin treatment, intermediary metabolism appears to be behaving as expected during a fasting state and the impact of gluconeogenesis and the TCA cycle is observed.

The number of metabolites that significantly correlated to clinical measures of diabetes decreased after simvastatin treatment, indicating that the general trends that were associated with a fasting state at baseline were obstructed by individuals' development of pre-diabetes (Table 3).

4.2 Simvastatin therapy alters glycine, alanine and glutamic acid levels

Glycine was repeatedly inversely correlated to clinical measures of diabetes, a possible reflection of the changing metabolic state of subjects post simvastatin administration. Multiple investigators have studied the impact of glycine on glucose and insulin levels in healthy and diabetic individuals. Gannon et al. reported that ingested glycine typically increased insulin secretion or had no effect (2002) and that gelatin, comprised primarily of glycine, significantly increased insulin secretion in untreated diabetics (1988). In our study, a significant decrease in glycine was observed in subjects with increased insulin, HOMA and c-peptide, suggesting simvastatin alters expected outcomes. Post alanine and glutamic acid levels, however, followed expected outcome, with increased levels associated with increased insulin and HOMA post treatment. As major regulators of glucose metabolism, the changes observed in these amino acids suggest that simvastatin treatment alters metabolism towards a more diabetic state.

4.3 Methylated alanine is inversely related to insulin following simvastatin therapy

N-methylalanine, a possible pyruvate precursor, decreased in relation to increased insulin post-treatment; the change in *N*-methylalanine during the course of treatment was also inversely related to the change in insulin. When this same data set was evaluated in regards to amino acid contributions to variation in response to simvastatin, *N*-methylalanine was found to strongly correlate to the branched-chain amino acid valine while negatively correlating with oxalic acid, nicotinic acid, phosphoric acid, asparagine, oxoproline, hexadecanol and octadecanol (Trupp et al. 2012). In 1975, Lin and Wagner purified the enzyme *N*-methylalanine dehydrogenase in pseudomonas MS and characterized its function as responsible for the interconversion of N-methylalanine + H_2O + $NADP^+$ to pyruvate + methylamine + NADPH + H^+ (Lin and Wagner 1975). Despite its use in synthetic and in vitro inhibitory reactions, little is known about the endogenous function of *N*-methylalanine. Pyruvate levels did not significantly change in this study and it is unclear whether the

decrease in *N*-methylalanine is due to decreased synthesis of the metabolite or due to increased consumption. Further research into the biochemical role of *N*-methylalanine is warranted and may help elucidate its link to insulin levels.

4.4 Decrease in azelaic acid, a potential treatment for T2DM, observed following simvastatin treatment

Post treatment free fatty acid levels also correlated to post treatment insulin levels, as well as to glucose and HOMA, indicating a shift towards insulin resistance. Interestingly, azelaic acid, a dicarboxylic acid found in wheat, rye and barley that has been shown to reverse diabetic symptoms following a high-fat diet in mice (Muthulakshmi et al. 2015), decreased in relation to increased glucose, insulin, c-peptide and HOMA in all analyses. Although not always with statistical significance, this inverse correlation did meet significance in several comparisons. Muthulakshmi and Saravanan demonstrated that when diabetic mice were fed 80 mg/kg body weight of azelaic acid daily for 5 weeks, plasma glucose and insulin were reduced to near normal levels, suggesting potential therapeutic effect of azelaic acid (Muthulakshmi and Saravanan 2013). The investigators detailed the azelaic acid-induced enhanced activity of hepatic hexokinasae, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase and fructose-1,6-bisphosphatase, thereby favoring glycolysis over gluconeogenesis and lowering plasma glucose levels (Muthulakshmi and Saravanan 2013). Since this endogenous dicarboxylic acid, which is also used to treat inflammation, decreased when subjects in our study developed simvastatin-induced pre-diabetes and apparent full diabetes, it may be inferred that this statin either inhibits azelaic acid production or accelerates its beta-oxidation to acetyl-CoA and malonyl-CoA; alternatively, the reduction of azelaic acid may be due to depletion in response to elevated plasma glucose. In our study, the low endogenous levels of azelaic acid may be rapidly diminished in hyperglycemia.

4.5 Branched-chain amino acids and other metabolites predict T2DM risk following simvastatin therapy

The branched-chain and aromatic amino acids have been reported to predict T2DM and may modulate diabetic onset by impairing insulin secretion and promoting insulin resistance via activation of mTOR, JUN and insulin receptor substrate (IRS) 1 (Wang et al. 2011). We found that baseline valine and tyrosine were significantly correlated to baseline insulin levels and that both, as well as leucine, were correlated with HOMA at baseline; phenylalanine, but not the others, significantly correlated to insulin secretion at baseline. Furthermore, baseline levels of valine and isoleucine correlated to the change in glucose to c-peptide ratio following simvastatin treatment, indicating that their baseline levels do indeed predict diabetes onset. The previously reported biomarker for predicting T2DM, 2-aminoadipic acid, was also identified in our study as correlating with clinical measures of diabetes at baseline, but not post-treatment (Wang et al. 2013).

Several other baseline metabolites were associated with the change in glucose to c-peptide level and monitoring these metabolites prior to simvastatin treatment could aid in predicting the potential of an individual for developing beta cell dysfunction or insulin resistance. The baseline levels of ketoleucine correlated to the change in glucose to c-peptide ratio. Randle et al. documented that ketoleucine, a HMG-CoA and ketone body precursor, stimulates

insulin secretion and is elevated in diabetes (Randle et al. 1987) and our data suggests that this transamination product of leucine may also be a potential biomarker of simvastatin-induced insulin resistance.

Notably, the baseline levels of the saturated fatty acids myristic acid, capric acid, lauric acid and pelargonic acid positively correlated to the change in glucose to c-peptide ratio, whereas the baseline levels of the monounsaturated oleic acid and the polyunsaturated linoleic acid negatively correlated with this ratio. This contrast may illuminate differences in diet and lifestyle of individuals who are more or less susceptible to developing type II diabetes.

As expected, the ketone body 3-hydroxybutanoic acid was inversely correlated to glucose, insulin and HOMA in several of the analyses. Ketone bodies elevate in a healthy fasting state to supply energy in times of low glucose as well as in a type I diabetic state, when lack of insulin production results in high levels of ketone bodies despite high levels of plasma glucose. Ketoacidosis rarely occurs in T2DM and cases are typically limited to select populations, including the elderly, those of Hispanic or African-American descent or those with extensive family history of the disease (Dhatariya et al. 2016; Gabriel and Soni 2014; Jefferies et al. 2015). Although 43 of the 148 subjects in our analysis were African-American, we did not observe an increase in ketone bodies in relation to increased glucose levels.

4.6 Baseline levels of ethanolamine and hydroxylamine linked to simvastatin-induced insulin dysregulation

The strongest correlations between simvastatin-induced change in glucose to c-peptide ratio and baseline metabolites were to ethanolamine and the hydroxylated amino metabolites hydroxylamine and hydroxycarbamate, as well as isoleucine and cyclohexylamine. Hydroxylamine is an intermediate in microbial denitrification and is also an intermediate in the oxidation of arginine to nitric oxide and induces nitric oxide-mediated vasorelaxation (DeMaster et al. 1989). Panagiotidis et al. demonstrated that hydroxylamine inhibited both glucose-induced and L-arginine-induced insulin secretion in freshly prepared mouse islets (Panagiotidis et al. 1995). More recently, Kimura et al. demonstrated that hydroxylamine may reduce hyperglycemia in T2DM by enhancing glucose uptake in C2C12 skeletal muscle cells via insulin receptor substrate 1 activation (Kimura et al. 2014). Thus, the association we observe between baseline levels of hydroxylamine and the change in insulin regulation indicates this intracellular nitric oxide donor may be a potential predictor of patient susceptibility to diabetes onset following simvastatin treatment.

Ethanolamine was the only metabolite to strongly correlate at baseline to both post-treatment and to change in clinical measures of diabetes, indicating that this phospholipid head group may be a potential biomarker of simvastatin-induced insulin resistance and hyperglycemia and may indicate statin-induced alterations in lipid metabolism leading towards a diabetic state. In addition to the membrane lipid phosphatidylethanolamine, ethanolamine is also a component of endogenous lipid signaling molecules, including *N*-palmitoylethanolamine, *N*-acylethanolamine and *N*-arachidonoylethanolamine. These messenger molecules are anti-inflammatory and promote cellular homeostasis (Skaper et al.

2015). Thus, alterations in plasma ethanolamine levels may indicate a disruption in protective mechanisms.

4.7 Pharmacometabolomics identifies decarboxylation and oxidation mechanism in simvastatin-induced hyperglycemia and insulin resistance

Decarboxylation was found to be a recurring theme in metabolites that were significantly associated with clinical measures of T2DM. Ethanolamine, the metabolite we identified as most likely to predict simvastatin-induced diabetic risk, is biosynthesized via decarboxylation of serine. Hydroxylamine is a decarboxylation product of *N*-hydroxycarbamate, both of which were found to have pre-drug plasma levels correlated to post-drug adverse effects. Azelaic acid is a dicarboxylic acid known to have T2DM therapeutic properties and was inversely correlated to diabetes risk in our study; the reduction of azelaic acid may possibly be due to simvastatin-induced decarboxylation. This may be due to drug-induced oxidative stress, as decarboxylation of aliphatic dibasic acids is known to be an oxidation mechanism.

Cyclohexylamine is a toxic metabolite formed by desulfonation of cyclamate by intestinal bacteria and enhances oxidation of proteins. The statistical significance of cyclohexylamine in pre drug treated plasma to post drug treated glucose and insulin levels is surprising, since the artificial sweetener cyclamate is banned in the United States. This interesting finding needs further investigation and may lead to novel discoveries involving the gut microbiome and oxidative stress mechanism in statin induced adverse effects.

4.8 Conclusions

Collectively, the correlations observed between metabolites and clinical measures of diabetes demonstrate that oral administration of 40 mg/day of simvastatin for 6 weeks increases the risk of developing elevated plasma glucose particularly in susceptible individuals. Patients with mild insulin resistance are more likely to develop full insulin resistance after simvastatin treatment, which may drive their metabolism towards a more gluconeogenic state. Metabolic profiling prior to simvastatin treatment may assist in identifying individuals who are at higher risk of adverse side effects. The metabolites identified in our study should be further investigated and their potential as biomarkers of simvastatin-induced hyperglycemia and insulin resistance validated. Pharmacometabolomics provides powerful tools for precision medicine by predicting development of drug adverse effects in sub populations of patients. Metabolic profiling prior to start of drug therapy may empower physicians with critical information when prescribing medication and determining prognosis.

Supplementary Material

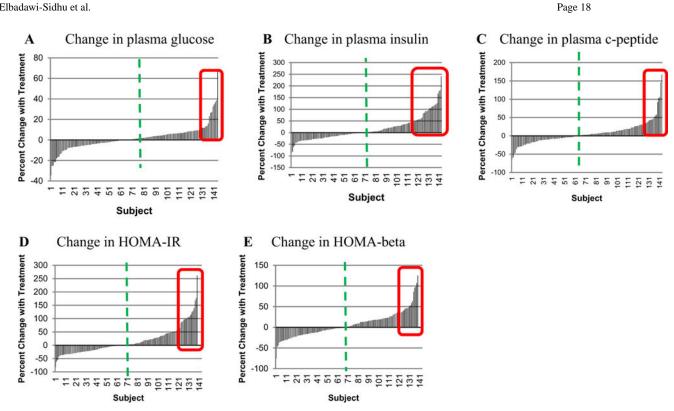Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Changes in clinical measures after simvastatin treatment. Changes in clinically measured, **a** glucose, **b** insulin, **c** c-peptide, **d** HOMA-IR and **e** HOMA-beta are represented with negative values indicative of decrease after treatment and positive values indicative of increase after treatment. Patients with the greatest elevation in clinical measures are highlighted within the *red boxes*. The *dashed vertical line* represents the mean

Table 1

Baseline metabolites significantly (p < 0.05) associated with baseline clinical measures

	Glucose	Insulin	HOMA	C-peptide
a. Univariate analysis				
Glucose	Positive	Positive	Positive	Positive
Lactic acid	Positive	Positive	Positive	Positive
Glutamic acid	Positive	Positive	Positive	Positive
Glycine	NS	Negative	Negative	Negative
Asparagine	NS	Negative	Negative	Negative
Aminomalonate	NS	Negative	Negative	Negative
Alanine	Positive	Positive	Positive	NS
3-Hydroxybutanoic acid	Negative	Negative	Negative	NS
Glycerol	Negative	Negative	Negative	NS
Linoleic acid	Negative	Negative	Negative	NS
Azelaic acid	Negative	Negative	Negative	NS
2-Aminoadipic acid	NS	NS	Positive	Positive
Nicotinic acid	Negative	NS	Negative	NS
Valine	NS	Positive	Positive	NS
Leucine	Positive	NS	Positive	NS
Aspartic acid	NS	Positive	Positive	NS
Hydroxylamine	NS	Negative	Negative	NS
5-Oxoproline	NS	Negative	Negative	NS
Palmitoleic acid	NS	Negative	Negative	NS
Tyrosine	NS	Positive	Positive	NS
Oleic acid	NS	Negative	Negative	NS
Fructose	NS	NS	NS	Positive
Malate	NS	NS	NS	Positive
Proline	NS	NS	Positive	NS
Uric acid	Positive	NS	NS	NS
Sucrose	Negative	NS	NS	NS
b. Multivariate analysis				
Glucose	Positive	NS	Positive	Positive
3-Hydroxybutanoic acid	Negative	Negative	Negative	NS
Glutamic acid	Positive	NS	Positive	NS
Glycine	NS	Negative	Negative	NS
Asparagine	NS	NS	NS	Negative
2-Aminoadipic acid	NS	NS	NS	Positive

Metabolite levels pre-statin treatment correlated with clinical measurements pre-statin treatment using (a) univariate regression analysis and (b) multiple regression analysis. Positive/negative indicates direction of correlation between metabolite and clinical measure

NS not significant

Table 2

Pathway enrichment analysis: baseline metabolites related to baseline clinical measures

Pathway	#Hits/total compounds	P value	FDR
Nitrogen metabolism	4/39	0.000153	0.004939
Aminoacyl-tRNA biosynthesis	5/75	0.00016	0.004939
Cyanoamino acid metabolism	3/16	0.000185	0.004939
Thiamine metabolism	2/24	0.013228	0.21165
Alanine, aspartate and glutamate metabolism	2/24	0.013228	0.21165
Valine, leucine and isoleucine biosynthesis	2/27	0.016601	0.22135
Glycolysis or Gluconeogenesis	2/31	0.021608	0.24695
Propanoate metabolism	2/35	0.027166	0.26518
Glutathione metabolism	2/38	0.031675	0.26518
Valine, leucine and isoleucine degradation	2/40	0.034837	0.26518
Galactose metabolism	2/41	0.036463	0.26518
Synthesis and degradation of ketone bodies	1/6	0.044084	0.29389
Fatty acid biosynthesis	2/49	0.050486	0.31069

Baseline metabolites that correlated to baseline clinical measures with $p < 0.05$ and $q < 0.2$ were used for pathway enrichment analysis. Pathways with FDR<0.3 are listed below

Table 3

Post-treatment metabolites significantly (p < 0.05) associated with post-treatment clinical measures

Univariate analysis	Glucose	Insulin	HOMA	C-peptide
Glucose	Positive	Positive	Positive	NS
Glutamic acid	Positive	Positive	Positive	NS
Maltose	Positive	NS	NS	NS
Lauric acid	Negative	Negative	Negative	Negative
Myristic acid	Negative	Negative	NS	NS
Linoleic acid	NS	Negative	Negative	NS
Alanine	NS	Positive	Positive	Positive
Glycine	NS	Negative	Negative	Negative
Palmitoleic acid	NS	Negative	Negative	NS
Isocitric acid	NS	NS	Positive	Positive
3-Hydroxybutanoic acid	NS	Negative	Negative	NS
Aminomalonate	NS	Negative	Negative	Negative
Oleic acid	NS	Negative	Negative	NS
Azelaic acid	NS	NS	Negative	NS
N-Methylalanine	NS	Negative	NS	NS
Proline	NS	NS	NS	Positive
Succinic acid	NS	NS	NS	Negative
Lactic acid	NS	NS	NS	Positive

Metabolite levels post-treatment correlated with clinical measurements post-statin treatment using (a) univariate regression analysis and (b) multiple regression analysis. Positive/Negative indicates direction of correlation between metabolite and clinical measure

NS not significant

Table 4

Pathway enrichment analysis: post-treatment metabolites related to post-treatment clinical measures

Pathway	#Hits/total compounds	Raw p value	FDR
Fatty acid biosynthesis	4/49	0.000231	0.0185
Cyanoamino acid metabolism	2/16	0.00471	0.1884
Citrate cycle (TCA cycle)	2/20	0.007343	0.1958
Glycolysis or Gluconeogenesis	2/31	0.017221	0.3444
Propanoate metabolism	2/35	0.021697	0.3472

Metabolites and clinical measures of pre-diabetes were measured after 6 weeks of simvastatin treatment. Correlations with $p < 0.05$ were used for pathway enrichment analysis. Pathways with FDR < 0.3 are listed below

Table 5

Baseline metabolites related to change in glucose:c-peptide ratio

Metabolite	Correlation	P value	Q value
Ethanolamine	Positive	0.0042	0.14
Cyclohexylamine	Positive	0.014	0.15
Hydroxylamine	Positive	0.026	0.18
Isoleucine	Positive	0.032	0.19
Hydroxycarbamate	Positive	0.042	0.22
Glucose	Positive	0.049	0.23
Valine	Positive	0.059	0.24
1-Hexadecanol	Positive	0.067	0.24
Ketoleucine	Positive	0.069	0.24
Myristic acid	Positive	0.12	0.32

The direction of correlation as well as p and q values are listed for metabolites measured pre-drug treatment that were associated with change in the glucose:c-peptide ratio. The ratio is indicative of pancreatic beta cell function and insulin response

Table 6

Pre-treatment metabolites significantly (p < 0.05) associated with post-treatment clinical measures

Univariate analysis	Glucose	Insulin	HOMA	C-peptide
3-Hydroxybutanoic acid	Negative	Negative	Negative	NS
Alanine	Positive	Positive	Positive	NS
Aminomalonate	NS	Negative	Negative	NS
Asparagine	NS	Negative	Negative	Negative
Aspartic acid	NS	Positive	Positive	NS
Azelaic acid	NS	Negative	Negative	NS
Ethanolamine	NS	Negative	NS	Negative
Glutamic acid	Positive	Positive	Positive	NS
Glycine	NS	Negative	Negative	Negative
Lactic acid	NS	Positive	Positive	Positive
Linoleic acid	NS	Negative	Negative	NS
Threonine	NS	Positive	Positive	NS
Uridine	NS	NS	NS	Negative
Valine	NS	Positive	Positive	NS

Metabolite levels pre-treatment correlated with clinical measurements post-statin treatment using (a) univariate regression analysis and (b) multiple regression analysis. Positive/Negative indicates direction of correlation between metabolite and clinical measure

NS not significant