Published in final edited form as:

J Proteome Res. 2016 March 4; 15(3): 815–825. doi:10.1021/acs.jproteome.5b00719.

Serum metabolite profiles are altered by erlotinib treatment and the integrin $\alpha 1$ -null genotype, but not by post traumatic osteoarthritis

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Abstract

The risk of developing post traumatic osteoarthritis (PTOA) following joint injury is high. Furthering our understanding of the molecular mechanisms underlying PTOA and/or identifying novel biomarkers for early detection may help improve treatment outcomes. Increased expression of integrin $\alpha 1\beta 1$ and inhibition of epidermal growth factor receptor (EGFR) signaling protect the knee from spontaneous OA, however the impact of the integrin $\alpha 1\beta 1$ /EGFR axis on PTOA is currently unknown.

We sought to determine metabolic changes in serum samples collected from wild type and integrin $\alpha 1$ -null mice that underwent surgery to destabilize the medial meniscus and were treated with the EGFR inhibitor erlotinib. Following 1H nuclear magnetic resonance spectroscopy we generated multivariate statistical models that distinguished between the metabolic profiles of erlotinib-

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Supporting Information

Table S-1. Comparison of statistical measures calculated for the supervised models including and excluding samples from the four female mice with hydrocephalus.

Table S-2. The chemical shifts used for metabolite assignment in ¹H NMR spectra

Table S-3. Concentration (μ M) of quantified compounds detected in mice serum samples

Figure S-1. The 3D PCA score scatter plots for serum samples collected from the treated group

Figure S-2. Pathway analysis for the erlotinib-treated versus vehicle-treated mice based on all significant metabolites

Figure S-3. The 3D PCA score scatter plots for serum samples collected from the untreated mice

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

versus vehicle-treated mice, and the integrin $\alpha 1$ -null versus wild type mouse genotype. Our results show the sex dependent effects of erlotinib treatment and highlight glutamine as a metabolite that counteracts this treatment. Furthermore, we identified a set of metabolites associated with increased reactive oxygen species production, susceptibility to OA and regulation of TRP channels in $\alpha 1$ -null mice. Our study indicates that systemic pharmacological and genetic factors have a greater effect on serum metabolic profiles than site specific factors such as surgery.

Keywords

post traumatic osteoarthritis; destabilization of the medial meniscus; integrin $\alpha 1\beta 1$; erlotinib; metabolomics; ^1H nuclear magnetic resonance spectroscopy; multivariate statistical analysis; mice

1. Introduction

Arthritis affects over 4.6 million Canadians today and by 2036 it is predicted that one in five Canadians will suffer from this debilitating disease¹. Osteoarthritis (OA), a subset of arthritis, involves inflammation of the synovium, degradation of the soft joint tissues (cartilage, menisci) and the growth of osteophytes that together result in joint stiffness, pain and immobility for the patient^{2,3}. Current treatment options for OA (weight loss, exercise, pain medication, surgery to repair articular surfaces or replace joints^{2,3}) address signs and symptoms in the short term. There is presently no treatment available that can stop or reverse the progression of OA²⁻⁴. Thus, deepening our understanding of the molecular mechanisms underlying this disease and/or identifying novel biomarkers early in the disease process that might allow early diagnosis and intervention, are important prerequisites to identifying new treatments that will prevent or slow OA disease progression.

Integrins are heterodimeric pericellular matrix receptors that are capable of influencing the activation of growth factor receptors and transient receptor potential (TRP) ion channels on the cell membrane⁵⁻¹¹. Integrin $\alpha 1\beta 1$ is a major collagen binding receptor expressed by human chondrocytes and responsible for the majority (75%) of chondrocyte adhesion to chondron localized collagen VI^{12,13}. During early spontaneous OA before cartilage degradation begins, chondrocyte expression of integrin $\alpha 1\beta 1$ expands from the growth plate and deep cartilage zone into the superficial zone¹³⁻¹⁵. Interestingly, integrin $\alpha 1$ -null mice develop cartilage degradation, synovial hyperplasia, thickened and more dense subchondral bone and osteophyte growth throughout the knee 3 months earlier than wild type mice^{15,16}. Taken together, these findings suggest that integrin $\alpha 1\beta 1$ offers the knee protection against spontaneous OA when it is upregulated early in the disease process. The influence of integrin $\alpha 1\beta 1$ on post traumatic OA (PTOA) however is unknown.

One possible mechanism by which integrin $\alpha 1\beta 1$ may offer protection against spontaneous knee OA is through its ability to downregulate epidermal growth factor receptor (EGFR) activation and downstream signaling^{6,7}. In this context, integrin $\alpha 1$ -null renal cells have increased basal levels of EGFR phosphorylation with consequent increased NADPH-mediated superoxide production⁶. Within the context of OA, expression levels of the EGFR ligand TGF α are increased in the synovium, synovial fluid and cartilage of patients with OA^{17,18} and enhanced EGFR activation results in early onset and more severe spontaneous

OA in mice^{19–21}. In contrast to these findings, dampened EGFR signaling using either a genetic or pharmacological approach in a mouse model of PTOA (destabilization of the medial meniscus (DMM)) led to enhanced cartilage damage in male mice²². The role of the integrin $\alpha 1\beta 1$ /EGFR axis in OA is not known.

Erlotinib hydrochloride (Tarceva®) is an EGFR inhibitor approved by the Food and Drug Administration for the treatment of non-small cell lung cancer^{23,24}. The specificity of erlotinib to EGFR is greater than that of the tyrosine kinases v-abl and c-Src and other kinase domains including that of the insulin receptor and insulin like growth factor 1 receptor^{24,25}. In mice, a two-year carcinogenicity study of erlotinib at oral dose of 60 mg/kg/day revealed no carcinogenic or mutagenic effects²⁶. Erlotinib has been used in mice to reduce the severity of collagen-induced arthritis²⁷, however its effects on the development of PTOA have not been previously tested.

Metabolomics is a system level analysis that measures unique metabolic changes in living systems in response to different physiological stimuli or genetic modifications^{28–30}. Metabolomics offers an efficient biochemical assay for clinical diagnosis and biomarker discovery^{31,32}. Metabolic analysis aims to define a *biopattern*, the collection of metabolites in a particular biological sample and it is mainly based on analytical platforms such as nuclear magnetic resonance (NMR) spectroscopy and/or mass spectrometry (MS)^{33,34}. NMR spectroscopy benefits from being very specific, quantitative and highly reproducible while MS is associated with better sensitivity³³. Explanations of the technological basis of both analytical methods, NMR and MS, used for metabolomics studies are available elsewhere^{33,35–37}.

Within the context of OA, metabolomics has been utilized to distinguish between subjects with and without OA, or between different subtypes of OA in both human and animal models^{38–45}. These studies have utilized urine^{39,40}, serum^{41,46}, synovial fluid^{38,42–45} or media from the culture of synovial explants⁴⁷ to compare between groups and have led to the identification of a number of metabolites that may prove valuable as biomarkers for OA. These metabolites include dimethyl sulfone⁴⁶, glutamine⁴⁷, tryptophan⁴¹ and carnitine⁴². Despite these advances, the correlation between the metabolic profiles of different bodily fluids and the development of OA in one or more joints of the body is not fully understood.

The goal of this study therefore, was to compare the metabolite profiles of serum derived from wild type and integrin $\alpha 1$ -null mice following the initiation of PTOA by DMM surgery. Due to the potentially confounding effect of the stress associated with daily oral gavage treatment on metabolite profiles, we chose to analyze the serum obtained from untreated mice or mice treated with the selective EGFR inhibitor erlotinib separately. We hypothesized that erlotinib treatment, genotype, surgery and time post-surgery would result in significantly distinct metabolite profiles of mouse serum, with systemic factors (erlotinib and genotype) having a greater effect than localized factors (surgery and time post-surgery).

2. Methods

2.1. Animals, surgery and treatment

All methods were approved by the University of Calgary Animal Care Committee. One hundred $\alpha 1$ -null and 100 wild type pure BALB/c mice ⁴⁸, 50 of each sex, were used. Mice were exposed to daily 12-hour light/dark cycles and were provided free access to food and water throughout. At 13 $\pm$ 1 weeks of age, mice were placed under anesthetic (isoflurane), hair was removed from the left leg and skin was sterilised with 0.5% chlorhexidine (Partnar Animal Health Inc., Ilderton, ON). Buprenorphine (Buprenex[®], Recklitt and Coleman Products, Kingston-Upon-Hull, UK) was administered subcutaneously at 0.05mg/kg. Destabilisation of the medial meniscus or sham microsurgery was then performed on the left knee ⁴⁹. After surgery, the joint capsule was closed using 8-0 tapered Vicryl[®] suture, and a single 9mm staple applied to close the skin. Immediately following surgery, mice were weighed and placed in a recovery cage for close observation. The staple was removed one week after surgery. Twenty $\alpha 1$ -null and 20 wild type pure BALB/c mice, with equal numbers of surgery type (DMM/sham) and sex (male/female) were sacrificed at 2, 4, and 8 weeks post-surgery. Forty $\alpha 1$ -null and 40 wild type pure BALB/c mice with the same proportions as above were sacrificed at 12 weeks post-surgery. Mice sacrificed at 12 weeks post-surgery ($\alpha 1$ -null and wild type pure BALB/c) received 50 mg/kg/day erlotinib (Genentech, San Francisco, CA) suspended in 0.5% (w/v) hydroxypropyl methylcellulose (Sigma-Aldrich, St. Louis, MO) (Dow) and 0.1% (v/v) Tween 80 in distilled water or vehicle by oral gavage. Gavage began the day after surgery and continued until the day before sacrifice.

2.2. Euthanasia and Serum Preparation

At their assigned time point post-surgery, mice were euthanized (CO₂) and blood collected via cardiac puncture. Blood was allowed to coagulate on ice for 30mins before undergoing centrifugation to separate serum from red blood cells. The suspended serum was then pipetted into a separate tube and stored at -80°C until further processing.

Serum samples were thawed on ice at room temperature and 150 μ l of each sample was ultrafiltered (3 kDa NanoSep microcentrifuge filters; VWR International, Edmonton, AB, Canada). The volume of each filtrate was then brought to 400 μ l by adding 80 μ l of phosphate buffer (0.5 M NaH₂PO₄, pH 7.0) which contained 2,2-dimethyl-2-silapentane-5-sulfonate (DSS; internal standard), 10 μ l of sodium azide and D₂O. The final concentration of DSS was 0.5 mM for each sample. The samples were stored at 4°C after adding DSS and before NMR spectral acquisition.

2.3. ¹H nuclear magnetic resonance (NMR) spectroscopy and metabolic profiling

One dimensional ¹H NMR spectra were collected on a 600 MHz Bruker Avance NMR spectrometer (Bruker BioSpin Ltd., Canada) using a standard Bruker 1D spectroscopy presaturation pulse sequence (noesypr1d) with optimal water suppression ^{50,51}. The acquisition time for each NMR spectrum was 1 hour and 28 minutes. The spectra were manually corrected (phasing, baseline correction, referencing to the DSS peak at 0.0 ppm) and profiled (metabolite identification and quantification) ⁵¹ in the Chenomx NMR Suite 7.5

software (Chenomx Inc., Edmonton, Alberta, Canada). All ^1H NMR spectra were analyzed in random order to avoid progressive bias. The metabolite peaks that could not be distinguished from noise in the spectra were considered as missing values.

2.4. Data preprocessing and statistical analysis

The metabolite data were pre-processed (median fold change normalization, logarithmic transformation, centering and unit variance scaling ^{52,53}) and then imported into the SIMCA-P+ 12.0.1 software (Umetrics, Sweden) for multivariate statistical analysis ^{53–55}. An unsupervised Principal Component Analysis (PCA) was applied to reveal possible grouping of observations in the dataset, to summarize the major source of variation and to identify outliers. Next, supervised Partial Least Squares (PLS) analysis, Orthogonal PLS (OPLS) analysis and OPLS-Discriminant Analysis (OPLS-DA) were applied to determine the best data discrimination and classification. The OPLS-DA method was carried out to improve model transparency and its interpretability and it was based on metabolites with Variable Influence on Projection (VIP) values larger than 1 ^{53,56}. The supervised models were evaluated by calculating the metrics such as R²Y (the percentage of variation explained by the model), Q² (the predictive ability of the model) and Cross-Validated Analysis Of Variance (CV-ANOVA) p-value during sevenfold CV method ^{53,57,58}. Additionally, based on the sample class prediction during sevenfold CV (Y-predCV) the sensitivity, specificity, accuracy and the area under the receiver operating characteristic (AUROC) were estimated (Metz ROC Software; the University of Chicago, USA). In order to describe potentially important metabolites the OPLS-DA regression coefficients were calculated and only compounds with significant changes in concentration (p-value less than 0.05) were considered. The most perturbed biological pathways were identified using a representative metabolic pathway network constructed in Cytoscape 3.2.1 software with Metscape 3.1.1 application ^{59,60} and KEGG database ⁶¹. Cytoscape is an open source platform widely used for data analysis and visualization of complex networks. The build-in Cytoscape application Metscape allows users to enter experimental data i.e. metabolites and display them in the context of relevant metabolic pathways based on the information stored in the KEGG database. To better visualize the metabolic network all metabolites are represented as nodes while the connecting arrows describe their relationship with reactions within a given metabolic pathway.

3. Results

3.1. Surgery and erlotinib treatment

All DMM surgeries were confirmed after sacrifice by observing a medially displaced medial meniscus in microCT images (data not shown). One 8 weeks post-surgery wild type male mouse that underwent sham surgery was identified as having undergone DMM surgery after sacrifice and was reassigned to the equivalent DMM group. One sham wild type female mouse assigned to the 12 weeks post-surgery group and receiving erlotinib was found dead in her cage at 7 weeks post-surgery and was excluded from the study. Four additional female mice assigned to the 12 weeks post-surgery group and receiving erlotinib treatment demonstrated signs of hydrocephalus including an enlarged, domed head, dehydration and depression close to their assigned time point. As recommended by animal care staff, one of

them (wild type DMM) was sacrificed at 10 weeks post-surgery and three others ($\alpha 1$ -null sham) were sacrificed at 11 weeks post-surgery. Their sera were included in the study to maintain equal group sizes and balance in the statistical model. To ensure that the inclusion of the sera from these four female mice with hydrocephalus did not significantly influence the conclusions of our analysis, the supervised models described below were also calculated excluding these samples and the resulting statistical measures compared (see Table S-1).

3.2. Metabolomics

Overall, 64 compounds were detected, assigned and quantified in each serum sample. The percentage of missing values was 2.9% and all missing values were randomly distributed in the dataset. During the multivariate statistical analysis the missing data were interpolated by using a least squares fit giving the missing data no influence on the model. The list of chemical shifts that were used for metabolite assignment and serum concentration of quantified metabolites are presented in the supplementary material (Table S-2 and Table S-3). Due to the potentially confounding effect of the stress associated with daily oral gavage treatment on metabolite profiles, the data set was divided into two groups prior to further analysis - the 12 weeks post-surgery group that had daily gavage treatment, and the 2, 4, and 8 weeks post-surgery groups that did not receive daily gavage (untreated groups).

3.2.1. Statistical modelling for treated mice—The unsupervised PCA model was based on three principal components (PCs) which in total summarized 37% of variation in the dataset (PC1=16.5%, PC2=12.9% and PC3=7.6%). The PCA score scatter plot showed significant grouping between observations associated with mouse sex (Figure 1A) but no other visible trend (Figure S-1). Three samples were detected outside the area of 95% confidence of the Hotelling's T-squared distribution (Figure 1A). These outlying samples were excluded from subsequent analyses as we were unable to find any common pattern of treatment, genotype or surgery type within them which could explain the difference between their metabolic profiles and other samples and, moreover, it is well known that outliers can bias the results of supervised analysis⁵³. Next, the PLS model was constructed implementing all four Y-variables: sex (female/male), treatment (erlotinib/vehicle), genotype ($\alpha 1$ -null/wild type) and surgery (DMM/sham). Two PLS components were calculated via cross-validation demonstrating a very strong association between metabolites and sex (PLS1) and a somewhat weaker association between metabolites and treatment (PLS2) (Figure 1B). The cumulative value of RY2 was 0.32 and Q2 was 0.20. To ensure that sex and treatment were influencing the metabolic alterations detected in treated mice, but genotype and surgery were not, we applied the OPLS method for each Y-variable separately. The OPLS models for sex and treatment showed high validation metrics and significant CV-ANOVA p-values: R2Y=0.88, Q2=0.81, CV-ANOVA p-value=3.1 x 10⁻²⁴ for the Y-variable: sex, and R2Y=0.64, Q2=0.35, CV-ANOVA p-value=3.9 x 10⁻⁶ for the Y-variable: treatment. However, the OPLS models for genotype and surgery were weak and not significant: genotype: the OPLS model could not be fitted; surgery: R2Y=0.52, Q2=0.05, CV-ANOVA p-value=0.55. Consequently, genotype and surgery were not considered as variables in further analyses.

In order to better describe differences in metabolic profiles of erlotinib/vehicle-treated mice without sex as a confounding factor we decided to analyze female and male samples independently using the OPLS-DA method. The OPLS-DA score scatter plot of female samples shows clear separation between erlotinib-treated specimens and controls (Figure 1C) indicating a very strong relationship between the metabolic data and erlotinib treatment. High values of validation parameters calculated for this model $R^2Y=0.73$, $Q^2=0.63$, $AUROC=0.97 \pm 0.02$ and significant CV-ANOVA $p\text{-value}=3.4 \times 10^{-8}$ indicate a very powerful and reliable OPLS-DA model (additional information describing the quantitative evaluation results is provided in Table 1). Interestingly, the discriminatory power of the OPLS-DA model for males was much weaker: $R^2Y=0.42$, $Q^2=0.32$, CV-ANOVA $p\text{-value}=0.001$ than for female samples (Figure 1C, Table 1). The contribution of potentially important metabolites to each of the OPLS-DA models is shown in the regression coefficient plots (Figure 2A and Figure 2B). The concentrations of six metabolites common to females and males were different in erlotinib compared to vehicle treated mice. Increased levels of carnitine, O-acetylcarnitine, dimethyl sulfone, 2-hydroxyisovalerate, butyrate and a decreased trimethylamine N-oxide level were detected in erlotinib-treated mice as compared to controls. The pathway analysis performed on these common metabolites and their concentration changes revealed a number of affected metabolic pathways including fatty acid metabolism, sulfur metabolism, valine, leucine and isoleucine degradation, ketogenesis, butanoate metabolism and trimethylamine metabolism (Figure 2C). Additional pathway analysis was conducted incorporating all significantly disturbed metabolites (those common and unique to the sexes) for erlotinib-treated mice versus controls, separated by sex (Figure S-2). In contrast to female mice, the metabolites unique to males whose levels were altered by erlotinib treatment were involved in an extensive and interconnected metabolic network, indicating a more robust metabolic response in male compared to female mice.

3.2.2. Statistical modelling for untreated mice—During the PCA model construction three PCs were calculated explaining the following percentage of variation in the dataset: $PC1=17.8\%$, $PC2=11.4\%$ and $PC3=9.4\%$ (Figure 3A). The most visible trend revealed by PCA was associated with metabolic differences between female and male samples (Figure 3A, Figure S-3). Additionally, five outliers were identified in the PCA score scatter plot (Figure 3A). As before, we were unable to find any common features such as erlotinib treatment, genotype or surgery type which could justify their unusual behavior, thus they were excluded from all further statistical analyses. The supervised PLS model demonstrated that sex (female/male) and genotype ($\alpha 1$ -null/wild type), but not surgery (DMM/sham) nor time post-surgery (2/4/8 week), were related to metabolic differences in the specimens (Figure 3B). The total account for variance and predictive ability of the PLS model was 0.27 and 0.15 respectively. Next, the OPLS models were created for each Y-variable independently and only two models (Y-variable: sex and Y-variable: genotype) demonstrated a significant discriminative power: $R^2Y=0.90$, $Q^2=0.88$, CV-ANOVA $p\text{-value}=1.0 \times 10^{-36}$ for the Y-variable: sex, and $R^2Y=0.45$, $Q^2=0.13$, CV-ANOVA $p\text{-value}=0.003$ for the Y-variable: genotype. In contrast, the OPLS models for surgery and time post-surgery were not significant ($R^2Y=0.15$, $Q^2=-0.09$, CV-ANOVA $p\text{-value}=1$ for the Y-variable: surgery, and $R^2Y=0.09$, $Q^2=-0.05$, CV-ANOVA $p\text{-value}=1$ for the Y-variable: time post-surgery). Furthermore, supervised models constructed for DMM mice

only, revealed weak and not significant association between metabolite profiles and time post-surgery: $R^2Y=0.27$, $Q^2=0.11$, CV-ANOVA p-value=0.4 for female mice and $R^2Y=0.25$, $Q^2=0.04$, CV-ANOVA p-value=0.9 for male mice. Consequently, we chose not to distinguish between the type of surgery (DMM/sham) or time point post-surgery (2/4/8 week post-surgery) in further statistical analysis.

The OPLS-DA models were built separately for female and male samples using genotype as an observation class. It can be noticed that the OPLS-DA score scatter plots look very similar for both sample groups (Figure 3C). The calculated validation metrics were: $R^2Y=0.39$, $Q^2=0.26$ and CV-ANOVA p-value= 2.1×10^{-4} for females and $R^2Y=0.4$, $Q^2=0.28$ and CV-ANOVA p-value= 1.1×10^{-4} for males. Also the ROC analysis (Table 1) revealed similar results for both models: AUROC (females)= 0.81 ± 0.06 and AUROC (males)= 0.83 ± 0.05 . We found 16 significant metabolites in female and 10 compounds in male samples that were responsible for the separation between $\alpha 1$ -null and wild type mice (Figure 4A and Figure 4B). Moreover, the concentration of dimethyl sulfone and glutamine was higher whereas the levels of serotonin, 3-hydroxyisovalerate, phenylalanine were lower in both $\alpha 1$ -null female and male groups when compared to wild type mice. These metabolites indicate that the most disturbed pathways in $\alpha 1$ -null compared to wild type mice may include inflammatory mediator regulation of TRP channels, sulfur metabolism and amino acids metabolism (alanine, aspartate and glutamate metabolism; arginine and proline metabolism; tryptophan metabolism; valine, leucine and isoleucine degradation; phenylalanine metabolism; phenylalanine, tyrosine and tryptophan biosynthesis; glycine, serine, alanine and threonine metabolism) (Figure 4C).

4. Discussion

The purpose of this study was to compare the metabolite profiles of serum derived from $\alpha 1$ -null and wild type mice following the initiation of PTOA by DMM surgery. The serum obtained from untreated mice or mice receiving erlotinib treatment were analyzed separately. Our data show a very strong relationship between the metabolic data and erlotinib treatment with a very powerful and reliable OPLS-DA model for females and a weaker model for males. Interestingly, erlotinib treatment resulted in a decrease of multiple signs of PTOA in the female mice in this study including meniscal and fabella bone volume, subchondral bone thickness, and cartilage degradation. In the males however, erlotinib treatment had no effect or a mild worsening effect on OA signs. Together our data suggest that the effects of erlotinib treatment measured in this study were 'on target' and sex dependent. Sex specific effects of erlotinib have been reported both in terms of treatment effectiveness^{62,63} and pharmacokinetics⁶⁴. In terms of pharmacokinetics, a single oral dose of erlotinib results in 25–43% greater exposure in female patients compared to males⁶⁴, however sex differences in the metabolism of erlotinib in mice have also been shown to be dependent on the timescale (single dose vs. multiple doses over weeks) of treatment regimes⁶². Our data provide additional evidence to support the sex dependent response of both tissues and metabolites to erlotinib treatment.

The mechanism by which erlotinib differentially affects males and females is not fully understood. It is known that erlotinib significantly reduces both ligand-dependent and

independent EGFR activation⁶⁵ and that estrogen receptors are involved in ligand-independent activation of EGFR^{66,67}. It is possible therefore that the increased expression of estrogen receptors in female compared with male tissues (e.g. cartilage^{68,69}) may be one mechanism by which erlotinib differentially effects male and female mice. In response to erlotinib treatment, we report changes to the concentrations of six metabolites common to females and males, six unique to females and nine unique to males. Importantly, our pathway analysis demonstrates a more robust influence and interconnectedness of the metabolites unique to males compared to those unique to females (Figure S-2). Such a response may be capable of overcoming and/or neutralizing the erlotinib-related metabolic changes and thus might also contribute to the differential effects of erlotinib in male compared to female mice. In support of this hypothesis, the metabolites unique to males include glutamine whose metabolism has been identified as a potential biomarker for resistance to EGFR tyrosine kinase inhibitors⁷⁰. Together these results suggest that the mechanism by which erlotinib differentially effects males and females may operate at both the receptor and metabolite levels.

In addition to the significant effects of erlotinib treatment on mouse serum metabolites, we also show that the $\alpha 1$ -null genotype influenced the metabolic profile but in a sex independent manner. We show five altered metabolites common to males and females, and all are influenced (increased or decreased) in the same manner independent of sex. Dimethyl sulfone levels for example are larger in serum from $\alpha 1$ -null compared to wild type mice. Dimethyl sulfone is the major oxidation product of dimethyl sulfoxide (DMSO) which is well known to act as a scavenger of free radical species⁷¹. Moreover, dimethyl sulfone and DMSO have strong analgesic and anti-inflammatory properties which may inhibit the degenerative changes occurring with OA⁷²⁻⁷⁴. Glutamine levels were also higher in $\alpha 1$ -null compared to wild type serum. Since glutamine is a precursor of glutathione, an important antioxidant molecule, its elevated level in blood can help maintain glutathione concentrations in order to avoid oxidative stress damage^{75,76}. Additionally, it has been shown that glutamine protects articular chondrocytes from heat stress and reactive oxygen species (ROS)-induced apoptosis⁷⁷ and thus, high levels of glutamine may protect cartilage from the degeneration associated with OA. We have shown that the phenotype of $\alpha 1$ -null mice involves increased production of ROS⁶ and increased susceptibility to OA^{15,16}, thus systemic metabolic changes resulting in the reduction of either oxidative stress and/or the development of OA in these mice as described above may be expected as a systemic response attempting to counteract the $\alpha 1$ -null phenotype.

The decreased concentration of serotonin in $\alpha 1$ -null compared to wild type mice indicates its expanded utilization and significant disruption in tryptophan metabolism. Importantly, compounds specific to tryptophan metabolism have been highlighted as being related to OA progression and as potential biomarkers for this disease^{41,47}. It is interesting to note that serotonin is also involved in inflammatory mediator regulation of TRP channels. There is increasing evidence in support of interplay between integrins and members of the TRP channel family including TRPV4 and TRPC5⁸⁻¹¹. Most pertinent to the present study, we have shown impaired functioning of TRPV4 in integrin $\alpha 1$ -null compared to wild type chondrocytes as measured by intracellular calcium responses *ex vivo*⁹. The identification of

dysregulation in pathways associated with inflammatory mediator regulation of TRP channels in $\alpha 1$ -null mice provides further evidence of integrin-TRP channel interplay.

Perhaps our most surprising finding is that there was no clear association between PTOA and metabolite profiles, despite there being significant alterations in both soft (cartilage degeneration and synovitis) and calcified (increased bone volume and density of menisci and fabella, and thickening of subchondral bone) knee tissues post DMM surgery. This finding underlines the challenges in applying the metabolic profile of systemic fluids (serum, urine) rather than synovial fluid to diagnose a disease such as PTOA that is often localized to one specific joint in the body, despite the obvious advantages of this less invasive approach. The correlation coefficient between metabolite profiles of plasma and synovial fluid in OA patients is low (0.23)^{38,42}, and our study further highlights the potential confounding effects of pharmaceutical and/or genetic factors in influencing metabolite profiles of systemic fluids.

In conclusion, we have shown that erlotinib treatment and the $\alpha 1$ -null genotype significantly influence the metabolite profile of mouse serum, however DMM surgery does not. The metabolic profile of $\alpha 1$ -null mice appears to both contribute to (serotonin) and counteract (dimethyl sulfone and glutamine) known characteristics of the $\alpha 1$ -null phenotype including heightened ROS production⁶, increased susceptibility to OA^{15,16} and disrupted TRP channel function⁹. Furthermore, our results add evidence in support of the sex dependent effects of erlotinib treatment and highlight glutamine as a potential biomarker for resistance to EGFR tyrosine kinase inhibitors. It will be important to account for pharmacological and genetic factors in metabolite profiling in future studies, especially when systemic fluids such as serum or urine are used to diagnose joint specific diseases such as PTOA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Carin Pihl and Dawn Martin for expert assistance with animal care, genotyping and DMM surgery, Hakan Kadir and Charlie Shin for gavage assistance, Erica Floreani, Lisa Milo and Dilene Mugenzi for serum collection and processing, and Genentech, Inc. and Astellas Pharma, Inc. for provision of erlotinib hydrochloride.

Funding Sources

This work was supported by CIHR MOP 136800 (ALC), CIHR Studentship (SYS), Alberta OA Team Studentship (SYS), NSERC CREATE Incentive Award (SYS), University of Calgary Faculty of Kinesiology (SYS), the Veterans Affairs Merit Reviews 1101BX002025-01 (AP) and the National Institutes of Health grants DK095761 (AP). BM was supported by the Alberta Sepsis Network and the Alberta Osteoarthritis Team. HJV holds the Armstrong Chair in Molecular Cancer Epidemiology Research.

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Figure 1.

The score scatter plots for serum samples collected from the treated group. A) 3D PCA score scatter plot, samples are colored according to sex: purple – female, blue – male; B) PLS score scatter plots, samples are colored according to the Y-variables: sex (purple – female, blue – male), treatment (erlotinib – yellow, vehicle – black); C) OPLS-DA score scatter plots for female and male mice, erlotinib – yellow, vehicle – black. The sphere (A) and long dashed lines (B and C) show 95% confidence interval of the Hotelling's T-squared distribution.

Figure 2.

Potentially important metabolites and pathway analysis for the erlotinib-treated mice versus vehicle treated mice. The OPLS-DA regression coefficient plots for female (A) and male mice (B). Positive coefficients (the upper parts of the diagrams) indicate larger metabolite concentrations in erlotinib-treated mice while negative values (the lower part of diagrams) present lower metabolite concentrations in erlotinib- compared to vehicle-treated mice. Only significant metabolites are shown ($p < 0.05$). Metabolites in-common between females and males for each model are marked in red. C) Pathway analysis based on the significant

metabolites for the erlotinib-treated mice common for females and males. Hexagons – metabolites (significant metabolites are marked in red; size of red hexagons illustrate higher or lower metabolite concentration as compared to vehicle treated mice), pink diamonds – reactions, green squares – enzymes, blue circles – genes.

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Figure 3.

The score scatter plots for serum samples collected from the untreated mice. A) 3D PCA score scatter plots, samples are colored according to sex: purple – female, blue – male; B) PLS score scatter plots, samples are colored according to the Y-variables: sex (purple – female, blue – male), genotype ($\alpha 1$ -null – orange, wild type – green); C) OPLS-DA score scatter plots for female and male mice, $\alpha 1$ -null – orange, wild type – green. The sphere (A) and long dashed lines (B and C) show 95% confidence interval of the Hotelling's T-squared distribution.

A)

B)

Figure 4.

Potentially important metabolites and pathway analysis for the untreated $\alpha 1$ -null mice versus untreated wild type mice. The OPLS-DA regression coefficient plots for female (A) and male mice (B). Positive coefficients (the upper parts of the diagrams) indicate larger metabolite concentrations in $\alpha 1$ -null mice while negative values (the lower part of diagrams) present lower metabolite concentrations in $\alpha 1$ -null mice as compared to wild type mice. Only significant metabolites are shown ($p < 0.05$). Metabolites in-common between females and males for each model are marked in red. C) Pathway analysis based on the significant

metabolites for the untreated $\alpha 1$ -null mice common for females and males. Hexagons – metabolites (significant metabolites are marked in red; size of red hexagons illustrate higher or lower metabolite concentration as compared to wild type mice), pink diamonds – reactions, green squares – enzymes, blue circles – genes.

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Table 1

Comparison of the statistical measures for the supervised OPLS-DA models

Metabolomics data		R2Y	Q2	CV-ANOVA p-value	Sensitivity : Specificity	PPV : NPV	ACC	AUROC
Treated mice (erlotinib versus vehicle)	Females	0.73	0.63	3.4 x 10 ⁻⁸	0.90 : 0.95	0.94 : 0.90	0.92	0.97 ± 0.02
	Males	0.42	0.32	0.001	0.68 : 0.80	0.77 : 0.73	0.74	0.82 ± 0.07
Untreated mice (α1-null versus wild type)	Females	0.39	0.26	2.1 x 10 ⁻⁴	0.62 : 0.77	0.72 : 0.68	0.70	0.81 ± 0.06
	Males	0.40	0.28	1.1 x 10 ⁻⁴	0.87 : 0.66	0.72 : 0.83	0.72	0.83 ± 0.05

R2Y - variation explained by the model; Q2 – predictive ability of the model, CV-ANOVA - cross-validated analysis of variance
PPV – positive predictive value; NPV – negative predictive value, ACC – accuracy,
AUROC – area under the receiver operating characteristic.