Plasma Metabolomic Profiling in Rheumatoid Arthritis and the Effect of Methotrexate Therapy

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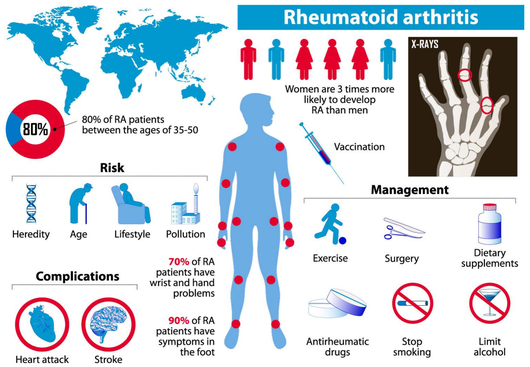
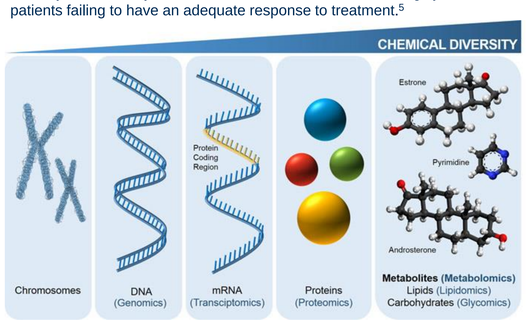
Background



ANRF. "A Brief History of Rheumatoid Arthritis - Arthritis Research: Arthritis National Research Foundation." *Arthritis Research | Arthritis National Research Foundation*, 17 Sept. 2019, curearthritis.org/arthritis-archives-rheumatoid-arthritis/.

Rheumatoid arthritis (RA) is an auto-immune inflammatory disease that impacts joints and a variety of tissues and organs.[1] Disease progression is associated with reduced life expectancy, chronic disability, and handicapping.[2] Roughly 1% of the world's population are afflicted with RA.[3] To date, Methotrexate (MTX) remains the cornerstone disease modifying anti-rheumatic drug (DMARD) due to its efficacy and economic merits.[4] MTX slows the progression of RA while minimizing damage to tissues and joints, however, MTX therapy is often characterized by a highly variable response. In many cases, delayed onset of action is observed, with roughly one third of patients failing to have an adequate response to treatment.[5]



CIT. "Metabolomics Research Introduction, Applications, Sample Types and Handling." *Center for Innovative Technology*, 4 Nov. 2019, www.vanderbilt.edu/cit/introduction-metabolomics-research/.

Metabolomics offers an unbiased method to rapidly measure thousands of endogenous and exogenous low-molecular-weight molecules for identification of clinical biomarkers for early diagnosis, stratification of patient populations, prediction of response to a given treatment, and improvement of our understanding of metabolic changes associated with disease and therapeutic response.[2,6]

Introduction

The variable and unpredictable profile of MTX indicates a need for the identification of clinical biomarkers to guide drug therapy. To date, no reliable biomarkers exist that allow for the stratification of patients that will respond to MTX therapy or will require alternative DMARDs (i.e. biologics).[7] Previous work by our group has used metabolomics as a tool to understand biochemical changes occurring at the cellular level following initiation of MTX therapy to identify metabolic pathways affected by MTX and to identify metabolites and metabolic pathways that represent potential metabolic biomarkers of pharmacological response to MTX.[8]

Plasma samples were collected from a cohort of healthy adult subjects and a cohort of RA patients, prior to and immediately following 16-weeks of MTX therapy. This work utilizes a semi-targeted metabolomics approach to identify changes in the plasma metabolome of patients with RA and determine the effect of MTX therapy on the plasma metabolome in RA patients. The data consists of intermediates of primary metabolism, biogenic amines, and lipids. The metabolomic profiles of the respective cohorts were normalized and evaluated utilizing chemometric and metabolic network enrichment analysis to determine the effects of RA and MTX therapy on the plasma metabolome.

Methods and Materials

Metabolomics Analysis. Raw peak intensity data was obtained and normalized. The normalization ratio was calculated as the ratio of the sum of all peak heights for identified metabolites (mTIC) to the average mTIC for all samples. The observed peak heights for each metabolite were then divided by the normalization ratio, providing the normalized peak height intensity. Duplicate metabolites were combined by mean normalization to ensure equal weighting to a given platform and then averaged. The resulting normalized peak height intensities were then uploaded into MetaboAnalyst 5.0. The data was than submitted to logarithmic transformation and Pareto scaling[9,10], analyzed for fold-change, and visualized via principal components analysis (PCA), variable importance plots (VIP), and volcano plots in order to identify and differentiate those metabolites altered in induction of RA and with MTX therapy.

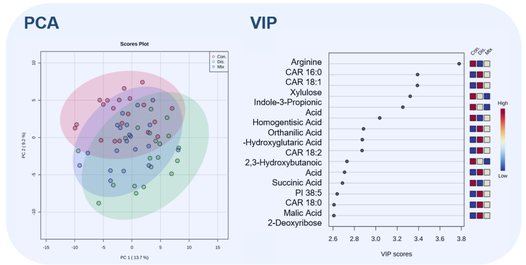
Enrichment Analysis. The fold-change and p-values obtained from MetaboAnalyst were then submitted to chemical and metabolic network analysis. Visualization of biochemical network maps was conducted using MetaMapp. Network maps were generated based upon chemical similarity utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic network database and Tanimoto substructure similarity coefficients and visualized in Cytoscape 3.7.2. Chemical enrichment analysis was performed utilizing the open-source software "Chemical Similarity Enrichment Analysis for Metabolomics" or "ChemRICH".[11] ChemRICH utilizes chemical ontologies and structural similarities to generate non-overlapping sets of identified metabolites. This method does not rely upon the size of a background database or defined biochemical pathways.[12]

Statistical Analysis. Analysis of identified metabolites were evaluated by both univariate and multivariate analysis utilizing MetaboAnalyst 5.0. Metabolites were evaluated for fold-change and statistical significance. A threshold of significance was set at a false discovery rate adjusted *P* value (q value) of less than 0.25 and was used in both metabolomic and chemometric metabolic enrichment analysis. Chemometric analysis was accomplished using ChemRICH, with significant metabolites having a q value of <0.05.

Study Demographics

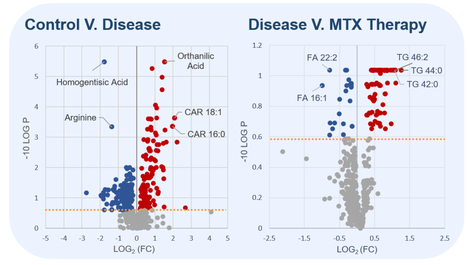
	Control	Patients	p value
N	20	20	—
Female (% Female)	17 (85%)	14 (70 %)	0.77
Age	49 [44, 57]	52 [41, 65]	0.99
Symp. to Diag.	—	11 [5, 10]	—
Diag. to MTX	—	2 [0, 0]	—
SJC	—	5 [1, 7]	—
TJC	—	6 [0, 7]	—
ESR	—	25 [12, 32]	—
Disease Activity	—	39 [19, 56]	—
DAS28	—	4 [3, 5]	—
Baseline	**Non-Responders**	**Responders**	**p value**
N	9	11	—
Female (% Female)	8 (73%)	7 (70%)	0.77
Age	57 [45, 72]	48 [27, 63]	0.3
SJC	6 [3, 7]	10 [6, 14]	0.3
TJC	6 [2, 7]	11 [6, 17]	0.08
ESR	23 [14, 24]	31 [13, 43]	0.4
Disease Activity	50 [38, 52]	48 [34, 63]	0.8
DAS28	5 [4, 5]	4 [4, 5]	0.08
Week 16	**Non-Responders**	**Responders**	**p value**
SJC	4 [1, 5]	0 [0, 1]	0.05
TJC	6 [1, 5]	1 [0, 0]	0.01
Disease Activity	48 [39, 57]	15 [5, 14]	0.0003
ESR	31 [17, 33]	15 [8, 19]	0.1
DAS28	6 [5, 6]	2 [2, 3]	0.001

Results



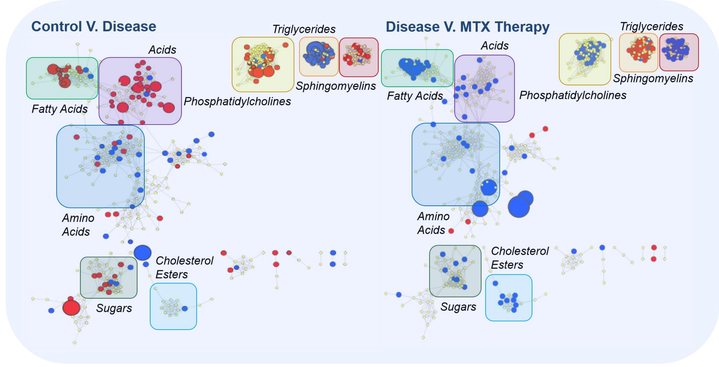
Principal Components Analysis. A total of 647 identified metabolites were analyzed using an unpaired multivariate analysis.

An increased overlap with healthy subjects, albeit incomplete, suggests a normalization of the plasma metabolome following the initiation of MTX therapy.

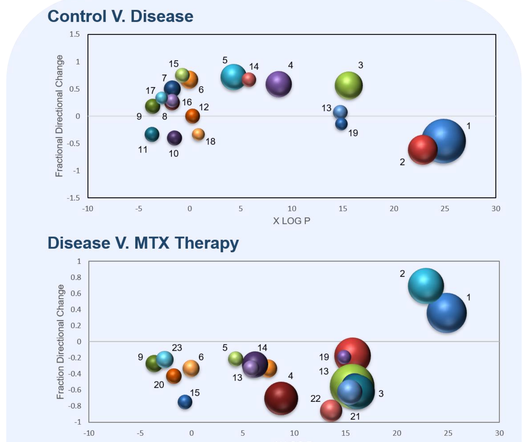


Multivariate analysis revealed 278 metabolites of interest that differentiated healthy subjects and patients with RA, and 108 metabolites of interest (q value < 0.25) altered after MTX therapy.

Red-colored metabolites were found to increase, and blue-colored metabolites were found to decrease (q value < 0.25).



Metabolic Network Maps of induction of RA and MTX therapy. Red denotes metabolites found to increase and blue denotes metabolites found to decrease. Node size is proportional to the fold-change.



1) Unsaturated Triglycerides
2) Saturated Triglycerides
3) Unsaturated Ceramides
4) Unsaturated Fatty Acids
5) Carnitine
6) Hydroxybutyrates
7) Glutarates
8) Sugar Acids
9) Sugar Alcohols
10) Branched Chain Amino Acids
11) Basic Amino Acids
12) Carboxylic Acids
13) Sphingomyelines
14) Lysophosphatidylcholines
15) Succinates
16) Disaccharides
17) Butyrates
18) Indoles
19) Phosphatidylethanolamines
20) Dicarboxylic Acids
21) Plasmogens
22) Phosphatidylinositols
23) Dipeptides

Control V. Disease

Cluster Name	Key Compound	Q Value	Fractional Change	X LOG P
Unsaturated Triglycerides	**TG 59:2**	**1.2E-18**	**-0.460674157**	**24.861596**
Saturated Triglycerides	**TG 54:0**	**0.00000001**	**-0.615384615**	**22.863643**
Unsaturated Ceramides	**Cer d36:1**	**0.00000013**	**0.5625**	**15.5865**
Unsaturated Fatty Acids	**FA 22:2**	**0.00000074**	**0.588235294**	**8.7363333**
Carnitine	**CAR 18:1**	**0.00000074**	**0.714285714**	**4.303**
Hydroxybutyrates	**2-deoxytetronic acid**	**0.0014**	**0.666666667**	**-0.0542**
Glutarates	2-hydroxyglutaric acid	0.0028	0.5	-1.7143333
Sugar Acids	Glyceric Acid	0.0077	0.25	-1.7295
Sugar Alcohols	**maltotriitol**	**0.0089**	**0.181818182**	**-3.6269091**
Branched-Chain AA	Isoleucine	0.012	-0.4	-1.4945
Amino Acids, Basic	Arginine	0.012	-0.333333333	-3.692
Carbocylic Acids	Homogentisic acid	0.012	0	0.2665
Unsat. Sphingomyelins	**SM d43:2**	**0.013**	**0.068965517**	**14.756875**
Saturated LPC	LPC O-16:0	0.015	0.666666667	5.7976667
Succinates	**Succinic Acid**	**0.015**	**0.75**	**-0.72**
Disaccharides	Maltose	0.022	0.285714286	-1.7295
Butyrates	Erythronolactone	0.041	0.333333333	-2.76375
Indoles	indole-3-propionic acid	0.041	-0.333333333	0.8366
Phosphatidylethanolamine	**PE P-40:4**	**0.043**	**-0.142857143**	**14.8576**

Disease V. MTX

Cluster Name	Key Compound	Q Value	Fractional Change	X LOG P
Unsaturated Sphingomyelins	**SM d42:2**	**1.2E-18**	**-0.551724138**	**24.8616**
Unsaturated Triglycerides	**TG 42:3**	**3.1E-15**	**0.359550562**	**15.68639**
Unsat. Phosphatidylcholines	PO O-38:2	9.8E-13	-0.174603175	15.65769
Saturated Triglycerides	**TG 44:0**	**1.9E-12**	**0.692307692**	**14.8576**
Unsaturated Ceramides	**Cer d34:1**	**1.1E-11**	**-0.625**	**22.86364**
Unsaturated Fatty Acids	**FA 22:2**	**7.4E-11**	**-0.705882353**	**7.46525**
Unsat. LPC	LPE 22:6	0.000001	-0.285714286	15.8855
Cholesterol Esters	CE 18:2	0.000001	-0.555555556	8.736333
Plasmalogens	PE P-38:6	0.000002	-0.625	-3.62691
Phosphatidylinositols	PI 36:2	0.000043	-0.857142857	6.2037
Saturated Fatty Acids	Heptadecanoic Acid	0.0014	-0.333333333	16.15725
Dipeptides	Phenylacetylglutamine	0.0019	-0.222222222	-1.7295
Sugar Alcohols	**Threitol**	**0.0023**	**-0.272727273**	**15.42157**
Hydroxybutyrates	**3-Hydroxybutyric Acid**	**0.0027**	**-0.333333333**	**13.61457**
Saturated Sphingomyelins	SM d40:0	0.0053	-0.333333333	4.303
Dicarboxylic Acids	Malic Acid	0.0079	-0.428571429	5.797667
Carnitine	**CAR 14:2**	**0.0089**	**-0.214285714**	**-2.62333**
Succinates	**Succinic Acid**	**0.016**	**-0.75**	**-0.0542**
Phosphatidylethanolamines	**PE 38:5**	**0.018**	**-0.19047619**	**-0.64175**

Chemometric Network Maps. Metabolites associated with RA mapped to 57 nonoverlapping chemical classes, 19 of which were found to be statistically significant (q value < 0.05). Metabolomic data associated with MTX therapy mapped to 56 nonoverlapping chemical classes, 19 of which were found to be statistically significant (q < 0.05). Each group of chemicals was assessed based on lipophilicity, fold-change (FC), and q value and plotted above; node size is directly proportional to the negative logarithm of the q value for each class. Bolded metabolites were observed in both "Control V. Disease" and "Disease V. MTX".

Discussion

Dyslipidemia is a common feature among a variety of rheumatic diseases, and lipids have been shown to play an important role in adaptive immunity and inflammation.[13]
- Decreases in both saturated and unsaturated triglycerides occur upon induction of RA
- A correction is observed following MTX therapy, with increases in saturated and unsaturated triglycerides

Dysregulation of fatty acid metabolism has been observed in RA
- Increases in both saturated and unsaturated fatty acids were observed in RA
- Initiation of MTX therapy resulted in significant decreases in saturated and unsaturated fatty acids and a return towards healthy control levels

High levels of LPC in plasma have been shown to be a reliable measure of inflammation. [14]
- Levels of lysophosphatidylcholines (LPC) were found to be higher in patients with RA and has been previously observed.
- Following MTX therapy decreases in LPC were observed, correcting towards the healthy control.

Carnitines are metabolites necessary in lipid metabolism and are responsible for the transport of long-chain fatty acids[15]
- Carnitine levels were shown to increase in association with RA, which has also been previously reported
- Initiation of MTX therapy resulted in a reduction of carnitine levels.

The normalization of fatty acid metabolism following initiation of MTX, towards levels observed in the healthy control population, indicates a correction in the dysregulation of glucose metabolism associated with pharmacological response to MTX therapy.

α-ketoglutarate has been shown to accumulate under hypoxic conditions, which may play a role in chronic inflammation[16]
- Levels of α-ketoglutarate were observed to increase in RA

Arginine is involved in two metabolic pathways critical to RA disease pathogenesis, nitric oxide synthase uncoupling and citrullination[17]
- Reduced levels of arginine observed in RA may play a role in the production of reactive oxygen species (ROS), a hallmark trait of inflamed tissues

Future Directions

Future work will focus on the identification of metabolites levels that correlate with DAS28, stratification of patients according to DAS28 score to identify responders and non-responders to MTX therapy, and identification of clinical biomarkers associated with response to MTX therapy.

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Conflict of Interest

The authors declare no conflict of interest.

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