Glutamine/glutamate analysis results

Network analysis of untargeted lipidomics

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05/31/2017
Metabolites of Glutamate Metabolism Are Associated with Incident Type 2 Diabetes

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Background

- Glutamine (Gln) enhances cardiac recovery in mice and human.
- Plasma Glutamate (Glu) levels are \textit{positively} associated with, and Gln levels or glutamine-to-glutamate ratio (Gln:Glu) are \textit{inversely} associated with BMI, blood pressure, and insulin resistance.
- \textbf{Higher} circulating Gln:Glu levels are also associated with a \textit{reduced} type 2 diabetes (T2D) risk.
- However, evidence that relates these metabolites directly and, moreover, relates changes in these metabolites, to T2D risk is limited.

Research questions

• 1) Are baseline levels of these metabolites (Gln, Glu, and their ratio) or their year-1 changes associated with the risk of T2D?
• 2) Do the MedDiet interventions change metabolite levels after 1 year?
• 3) Whether the cardioprotective effects of the Mediterranean diet interventions are modified by these metabolites?
Study design

- Case-cohort design

- 3541 PREDIMED participants Free of T2D at baseline

- A random, non-stratified ~20% of the above participants (N=694, including 53 incident cases)

- All incident T2D cases (N=251)

- 892 participants had plasma levels of metabolites measured
  - 641 non-cases
  - 251 incident T2D cases
Study design

• Case-cohort design

• Metabolomics platform
  – Liquid chromatography tandem mass spectrometry (LC-MS/MS) techniques by Broad Institute
  – Rank-based inverse normal transformations

• Weighted Cox PH models
  – metabolites as both continuous variables and in quartile categories
## Baseline Characteristics

| Table 1. Baseline participant characteristics in the random subcohort and of the cases |
|-----------------|-----------------|----------------|-----------------|
| Subcohort*      | Cases           |
| n               | 694             | 251            |
| Age (years)     | 66.5 (5.7)      | 66.4 (5.7)     |
| Sex (% women)   | 62.8            | 55.0           |
| Intervention group, % |
| MedDiet+EVOO   | 30.7            | 29.9           |
| MedDiet+nuts   | 37.2            | 33.9           |
| Control        | 32.1            | 36.3           |
| Hypertension, % | 90.8            | 96.0           |
| Dyslipidemia, % | 85.0            | 79.7           |
| Smoking, %      |                 |                |
| Never          | 61.0            | 52.6           |
| Former         | 22.6            | 22.3           |
| Current        | 16.4            | 25.1           |
| Waist circumference, cm | 99.5 (10.7)   | 103.4 (10.0) |
| Body mass index, kg/m² | 29.9 (3.6)  | 30.8 (3.3)   |
| Physical activity, METs/d | 238 (238)     | 249 (232)     |
| Education, %   |                 |                |
| Elementary or lower | 75.4          | 76.5           |
| Secondary or higher | 24.6          | 23.5           |
| Total energy intake, kcal/d | 2277 (566)   | 2327 (622)    |
| Score for adherence to Mediterranean diet$^b$ | 8.6 (1.9) | 8.5 (1.8) |
| Fasting Glucose, mg/dl | 99.7 (15.2) | 117.2 (17.6) |

Abbreviation: EVOO, Extra-virgin olive oil; CHD, coronary heart disease; MET, metabolic equivalent. Values are mean (SD) or percentage.$^a$ 37 cases are included in the randomly selected subcohort.

$^b$This score is based on the 14-item dietary screener$^{33}$.
## Risk of incident T2D

Table 2. Incident diabetes by Baseline Plasma Amino Acid Concentrations in the PREDIMED Trial, 2003–2010: Observed Event Rates and Hazard Ratios (251 cases, 694 participants in sub-cohort)\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Quartile 1</th>
<th>Quartile 2</th>
<th>Quartile 3</th>
<th>Quartile 4</th>
<th>P for trend</th>
<th>P-interaction (^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariable models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.00 (ref)</td>
<td>1.24 (0.83, 1.85)</td>
<td>0.93 (0.61, 1.43)</td>
<td>0.84 (0.54, 1.3)</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.00 (ref)</td>
<td>2.39 (1.43, 3.99)</td>
<td>4.13 (2.54, 6.72)</td>
<td>2.43 (1.44, 4.1)</td>
<td>0.0001</td>
<td>0.66</td>
</tr>
<tr>
<td>Glutamine to glutamate ratio</td>
<td>1.00 (ref)</td>
<td>1.36 (0.93, 2)</td>
<td>0.78 (0.52, 1.19)</td>
<td>0.39 (0.23, 0.64)</td>
<td>0.00002</td>
<td>0.84</td>
</tr>
<tr>
<td><strong>Multivariable models</strong> (^b) (main model)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.00 (ref)</td>
<td>1.14 (0.68, 1.93)</td>
<td>0.8 (0.47, 1.36)</td>
<td>0.64 (0.36, 1.12)</td>
<td>0.04</td>
<td>0.39</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.00 (ref)</td>
<td>2.99 (1.15, 7.74)</td>
<td>3.83 (1.64, 8.94)</td>
<td>2.03 (0.72, 5.73)</td>
<td>0.0002</td>
<td>0.64</td>
</tr>
<tr>
<td>Glutamine to glutamate ratio</td>
<td>1.00 (ref)</td>
<td>1.18 (0.72, 1.93)</td>
<td>0.69 (0.4, 1.2)</td>
<td>0.31 (0.16, 0.57)</td>
<td>0.0001</td>
<td>0.75</td>
</tr>
<tr>
<td><strong>Multivariable models + baseline fasting glucose</strong> (^c) (sensitivity analysis)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Glutamine</td>
<td>1.00 (ref)</td>
<td>0.82 (0.39, 1.71)</td>
<td>0.47 (0.19, 1.18)</td>
<td>0.38 (0.15, 0.96)</td>
<td>0.02</td>
<td>0.92</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.00 (ref)</td>
<td>2.24 (1.22, 4.11)</td>
<td>4.34 (2.42, 7.78)</td>
<td>2.78 (1.43, 5.41)</td>
<td>0.14</td>
<td>0.36</td>
</tr>
<tr>
<td>Glutamine to glutamate ratio</td>
<td>1.00 (ref)</td>
<td>1.17 (0.49, 2.82)</td>
<td>0.55 (0.2, 1.49)</td>
<td>0.34 (0.12, 0.94)</td>
<td>0.03</td>
<td>0.36</td>
</tr>
</tbody>
</table>

\(^a\) Inverse normal transformation was applied to raw values.

\(^b\) Stratified by recruitment center and intervention group (MedDiet+EVOO, MedDiet+nuts, low fat), and adjusted for age (years), sex (male, female), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia and hypertension.

\(^c\) glucose levels were available in 537 participants.

\(^d\) p for interaction with 1 degree of freedom: amino acid level × intervention arm (intervention vs. control)
Correlation with fasting glucose

Table 3 correlation of glucose with amino acids

<table>
<thead>
<tr>
<th></th>
<th>glutamate</th>
<th>glutamine</th>
<th>Glutamine to glutamate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Correlation coefficient</td>
<td>0.10</td>
<td>-0.07</td>
<td>-0.13</td>
</tr>
<tr>
<td>p value</td>
<td>0.02</td>
<td>0.10</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Risk of incident T2D

Table 4. Incident Type 2 diabetes by Changes in Plasma Amino Acid Concentrations in the PREDIMED Trial, 2003–2010: Observed Event Rates and Hazard Ratios (251 cases, 694 participants in sub-cohort) \(^a\)

<table>
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<td><strong>Univariable models</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine change</td>
<td>1.00 (ref)</td>
<td>1.33 (0.80, 2.20)</td>
<td>1.33 (0.80, 2.20)</td>
<td>1.33 (0.80, 2.20)</td>
<td>0.75</td>
</tr>
<tr>
<td>Glutamate change</td>
<td>1.00 (ref)</td>
<td>0.81 (0.52, 1.29)</td>
<td>0.81 (0.52, 1.29)</td>
<td>0.81 (0.52, 1.29)</td>
<td>0.14</td>
</tr>
<tr>
<td>Glutamine to glutamate ratio change</td>
<td>1.00 (ref)</td>
<td>4.88 (2.51, 9.47)</td>
<td>4.88 (2.51, 9.47)</td>
<td>4.88 (2.51, 9.47)</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Multivariable models (^b) (main model)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamine change</td>
<td>1.00 (ref)</td>
<td>1.12 (0.59, 2.13)</td>
<td>1.12 (0.59, 2.13)</td>
<td>1.12 (0.59, 2.13)</td>
<td>0.64</td>
</tr>
<tr>
<td>Glutamate change</td>
<td>1.00 (ref)</td>
<td>1.00 (0.49, 2.04)</td>
<td>1.00 (0.49, 2.04)</td>
<td>1.00 (0.49, 2.04)</td>
<td>0.84</td>
</tr>
<tr>
<td>Glutamine to glutamate ratio change</td>
<td>1.00 (ref)</td>
<td>3.03 (1.37, 6.70)</td>
<td>3.03 (1.37, 6.70)</td>
<td>3.03 (1.37, 6.70)</td>
<td>0.41</td>
</tr>
</tbody>
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\(^a\) Inverse normal transformation was applied to raw values.

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There were no statistically significant differences in mean first-year changes of metabolites between intervention and control groups, after adjustment for age (years), sex (male, female), body mass index (kg/m2), smoking (never, current, former), leisure-time physical activity (metabolic equivalent tasks in minutes/day), dyslipidemia, hypertension, and baseline level of respective amino acid.
Conclusion

• Our results suggest that participants with higher Glu levels may have a higher risk, and those with a higher Gln:Glu might have a lower risk of T2D.

• The intervention diets may not change these metabolite levels after 1 year.
Untargeted Lipids Signed Network Analysis in PREDIMED preliminary results

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Weighted correlation network analysis

- Weighted correlation network analysis (WGCNA) is a widely used data mining method based on *pairwise correlations between variables*.

- WGCNA can be used for
  - finding clusters (modules) of highly correlated metabolites,
  - summarizing such clusters using the module eigengene or an intramodular hub gene/metabolite,
  - relating modules to one another and to external clinical traits.

https://en.wikipedia.org/wiki/Weighted_correlation_network_analysis
https://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/
### Data outline

<table>
<thead>
<tr>
<th>CVD project</th>
<th>T2D project</th>
</tr>
</thead>
<tbody>
<tr>
<td>• 6359 metabolites (201 named lipids and 6158 unnamed lipids)</td>
<td>• 3374 metabolites (301 named lipids and 3073 unnamed lipids)</td>
</tr>
<tr>
<td>• Samples were from 984 participants at baseline</td>
<td>• Samples were from 1017 participants at baseline</td>
</tr>
<tr>
<td>• Metabolite levels were natural log-transformed before any further analysis</td>
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</tr>
</tbody>
</table>
Data pre-management

Using WGCNA package for R:
https://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/

Detected and deleted metabolite outliers due to >50% missing samples or zero variance, and 5633 metabolites were left for further analysis in CVD project and 3327 metabolites in T2D project.

Sample clustering to detect sample outliers

X-axis: each sample (individual); Y-axis: measure of similarity between samples based on metabolites; I did not exclude any sample based on this figure—984 samples for further analysis.
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Sample clustering to detect sample outliers

X-axis: each sample (individual); Y-axis: measure of similarity between samples based on metabolites;
I did not exclude any sample based on this figure—>1017 samples for further analysis
Incorporating metabolite and trait data-CVD project

The upper plot: X-axis: each sample (individual); Y-axis (Height): measure of similarity between samples based on metabolites;

The lower heat-map: each row represents a trait, each column represents a sample (individual); white means a low value, red a high value, grey a missing value. E.g., most individuals had hypertension at baseline, and thus the hypertension row is almost red.
Incorporating metabolite and trait data-T2D project

The upper plot: X-axis: each sample (individual); Y-axis (Height): measure of similarity between samples based on metabolites;

The lower heat-map: each row represents a trait, each column represents a sample (individual); white means a low value, red a high value, grey a missing value. E.g., most individuals had hypertension at baseline, and thus the hypertension row is almost red.
Signed Network construction (choosing the soft-thresholding power)

I choose the **power 9**, the lowest power for which the scale-free topology fit index reaches 0.80 (the default value is 0.90) with a relatively decent mean connectivity.

Analysis of network topology for various soft-thresholding powers. The left panel shows the scale-free fit index (y-axis) as a function of the soft-thresholding power (x-axis). The right panel displays the mean connectivity (degree, y-axis) as a function of the soft-thresholding power (x-axis).
Signed Network construction-CVD project
(I choose the minimum module size=100)
Signed Network construction-T2D project
(I choose the minimum module size=100)
I use diabetes as the only outcome in the following analysis; I select the most significant module: GREEN for the further analysis.
I use diabetes as the only outcome in the following analysis; I select the most significant modules: Brown and Black for the further analysis.
Select metabolites within module-CVD project

Measuring the contribution of each metabolite to the module: the correlation of the module eigenvalue and the individual metabolites within this module. 226 metabolites were included for visualization.

C24:1 SM
Select metabolites within BLACK module-T2D project

Measuring the contribution of each metabolite to the module: the correlation of the module eigenvalue and the individual metabolites within this module
Select metabolites within BROWN module-T2D project

Measuring the contribution of each metabolite to the module: the correlation of the module eigenvalue and the individual metabolites within this module.
### Implications

#### Prevalent T2D
- C34:2 PC
- C36:2 PC
- C34:3 PC plasmalogen
- C34:1 PC plasmalogen-A
- C36:3 PC plasmalogen
- C36:2 PC plasmalogen
- C36:1 PC plasmalogen
- C40:7 PC plasmalogen
- C38:2 PE
- C24:0 Ceramide (d18:1)
- C14:0 SM
- C16:1 SM
- C16:0 SM
- C18:2 SM
- C18:1 SM
- C18:0 SM
- C20:0 SM
- C22:1 SM
- C22:0 SM
- C24:1 SM
- C24:0 SM
- C16:0 CE
- C18:2 CE
- C18:1 CE
- C54:10 TAG

#### Incident T2D

##### • Black module
- C16.1.LPC
- C16.0.LPC
- C18.2.LPC
- C18.1.LPC
- C18.0.LPC
- C20.4.LPC
- C20.3.LPC
- C22.6.LPC
- C16.0.LPE
- C18.2.LPE
- C18.1.LPE
- C18.0.LPE
- C20.0.LPE
- C22.0.LPE

##### • Brown module
- C14.0.LPC
- C30.0.PC
- C32.2.PC
- C32.1.PC
- C34.4.PC
- C34.1.PC
- C36.1.PC
- C34.0.PE
- C36.3.PE
- C36.2.PE
- C36.1.DAG
- C36.0.DAG
- C34.1.DAG
- C36.0.DAG
- C49.3.TAG
- C49.2.TAG
- C50.4.TAG
- C50.3.TAG
- C50.2.TAG
- C50.1.TAG
- C51.3.TAG
- C51.2.TAG
- C51.1.TAG
- C52.5.TAG
- C52.4.TAG
- C52.3.TAG
- C52.2.TAG
- C52.1.TAG
- C52.0.TAG
- C53.3.TAG
- C53.2.TAG
- C53.1.TAG
- C54.3.TAG
- C54.2.TAG
- C54.1.TAG
- C55.3.TAG
- C55.2.TAG
- C55.1.TAG
- C56.4.TAG
- C56.3.TAG
- C56.2.TAG

#### Incident T2D

##### • Black module
- C16.1.LPC
- C16.0.LPC
- C18.2.LPC
- C18.1.LPC
- C18.0.LPC
- C20.4.LPC
- C20.3.LPC
- C22.6.LPC
- C16.0.LPE
- C18.2.LPE
- C18.1.LPE
- C18.0.LPE
- C20.0.LPE
- C22.0.LPE

##### • Brown module
- C14.0.LPC
- C30.0.PC
- C32.2.PC
- C32.1.PC
- C34.4.PC
- C34.1.PC
- C36.1.PC
- C34.0.PE
- C36.3.PE
- C36.2.PE
- C36.1.DAG
- C36.0.DAG
- C34.1.DAG
- C36.0.DAG
- C49.3.TAG
- C49.2.TAG
- C50.4.TAG
- C50.3.TAG
- C50.2.TAG
- C50.1.TAG
- C51.3.TAG
- C51.2.TAG
- C51.1.TAG
- C52.5.TAG
- C52.4.TAG
- C52.3.TAG
- C52.2.TAG
- C52.1.TAG
- C52.0.TAG
- C53.3.TAG
- C53.2.TAG
- C53.1.TAG
- C54.3.TAG
- C54.2.TAG
- C54.1.TAG
- C55.3.TAG
- C55.2.TAG
- C55.1.TAG
- C56.4.TAG
- C56.3.TAG
- C56.2.TAG

- C34.2.DAG..M.NH4.
- C34.1.DAG..M.NH4.
- C49.3.TAG..M.NH4.
- C50.3.TAG..M.NH4.
- C50.2.TAG..M.NH4.
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- C52.4.TAG..M.NH4.
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- C52.2.TAG..M.NH4.
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- C53.2.TAG..M.NH4.
- C54.3.TAG..M.NH4.
- C54.2.TAG..M.NH4.
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- C55.3.TAG..M.NH4.
- C55.2.TAG..M.NH4.
- C56.4.TAG..M.NH4.
- C56.3.TAG..M.NH4.