Metabolomics Studies Reveal New Pathways in Cardiovascular Disease with Potential for Diagnostics and Therapeutic Targeting

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Disclosure Information

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http://www.nature.com/nature/journal/v444/n7122/images/4441009a-f1.2.jpg
Phase 1: Discovery-based investigations

Metabolomics screening and structural identification

Phase 2: Clinical validation

Replication and demonstration of clinical utility

Phase 3: Mechanistic studies

Demonstration of causality for a novel pathway
Strategy of metabolomics study design for identifying unbiased small molecule profiles predictive of incident risks for major adverse cardiovascular events

GeneBank (N=10,000)

Learning Cohort
50 cases (3yr MI, CVA, death) vs.
50 age/gender matched ctrls

(ii) Validation Cohort
25 cases (3yr MI, CVA, death) vs.
25 age/gender matched ctrls

HPLC-MS
adjusted -Log(P)>1.3
p for trend < 0.05

58 analytes
43 analytes

HPLC-MS
adjusted -Log(P)>1.3
p for trend < 0.05

29 analytes
25 analytes

40 analytes
24 analytes

18 analytes

(iii) Structural identification of analytes
(iv) Confirm clinical prognostic utility in Independent Prospective Cohort (N>1000)

Plasma analytes with m/z 76, 104, and 118 are associated with CVD, show a dose-response relationship with MACE (3yr MI stroke or death) and are correlated, suggesting participation in a common pathway.

Example data from metabolomics study

Extracted ion LC chromatogram at m/z=76

Mass spectrum of plasma scanned in positive ion mode

Extracted ion LC Chromatogram at m/z=118
Structural identification/validation of plasma analyte at m/z=76 as TMANO (trimethylamine N-oxide)

What is TMANO? - It is proposed to be a gut flora-dependent metabolite of dietary lecithin (phosphatidylcholine, PC)

Dietary egg yolk PC produces increases in analytes with m/z 76, 104, and 118 in both human and mouse plasma.
Dietary phosphatidylcholine enhances levels of the 3 analytes, indicating they are metabolites of PC.

Male mouse, 1.5 mg PC gavage

Female mouse, 1.5 mg PC gavage
Maybe choline is the plasma analyte associated with CVD with m/z 104

Candidate plasma analytes linked to CVD risks with m/z 104

Identity as Choline was confirmed by:
- LC-MS^n
- GC/MS/MS
- NMR
- Isotope tracer studies: d9-choline and d4-choline
Strategy to determine the analyte at m/z=118 by choline deuterated isotopologue feeding study

Choline

\[
\text{Choline} \rightarrow \text{Oral or IP} \rightarrow \text{Methyl transferase} \rightarrow \text{Betaine} \rightarrow \text{Methyl transferase} \rightarrow \text{1- methylcholine} \rightarrow \text{Methyl transferase} \rightarrow \text{2- methylcholine} \rightarrow \text{Methyl transferase} \rightarrow \text{Choline methyl ether}
\]
Isotope challenge studies confirm the identities of TMAO, choline and betaine as the plasma analytes predicting CVD risk.

Choline, betaine and trimethylamine-\(N\)-oxide are plasma analytes associated with CVD.

Identities confirmed by:
- LC-MS\(^n\), \(^1\)H, \(^13\)C, \(^15\)N NMR
- GC/MS/MS, Isotope tracer studies

What is the role of gut flora?

Intestinal Microbial Organisms Play an Obligatory Role in TMAO Generation from Dietary Egg Yolk PC in Mice

**TMAO is a gut flora dependent metabolite in humans:**

**PC challenge - Oral d9-PC and 2 hard boiled eggs at each visit**

- **6 h post PC challenge**
  - **Plasma**
    - **Pre-antibiotics (visit 1)**
    - **Antibiotics**
      - Suppression of gut flora
    - **Post-antibiotics (visit 2)**
    - **Acquisition of gut flora (visit 3)**
  - **Intensities (a-f)**
    - **Intensity (%)**
    - **Time (min)**
    - TMAO 76 [58]
    - d9-TMAO 85 [66]

- **24 h post PC challenge**
  - **Urine**
    - **Intensities (g-l)**
      - **Intensity (%)**
      - **Time (min)**
      - TMAO 76 [58]
      - d9-TMAO 85 [66]

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Demonstration of causality for a novel pathway
Development of stable isotope dilution LC/MS/MS assays for choline, TMAO and betaine using d9(trimethyl) isotopologues as internal standards

<table>
<thead>
<tr>
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<th>Intraday CV</th>
<th>Interday CV</th>
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<tbody>
<tr>
<td>TMAO (low)</td>
<td>4.7</td>
<td>4.9</td>
</tr>
<tr>
<td>(high)</td>
<td>3.1</td>
<td>4.4</td>
</tr>
<tr>
<td>Betaine (low)</td>
<td>5.2</td>
<td>5.4</td>
</tr>
<tr>
<td>(high)</td>
<td>3.0</td>
<td>6.1</td>
</tr>
<tr>
<td>Choline (low)</td>
<td>4.8</td>
<td>6.9</td>
</tr>
<tr>
<td>(high)</td>
<td>3.4</td>
<td>3.9</td>
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Prospective Cohort: N=1865 Sequential Cardiology Patients
Plasma choline, TMAO and betaine levels predict CVD risks
(N=1865)

Odds ratio (95%CI) adjusted for age, sex, DM, HTN, smoking, LDL, HDL, TG, CRP, eGFR

Plasma levels of the gut flora dependent metabolite TMAO predict incident (3 year) CVD risks

New Independent Cohort: N=4007 Sequential Subjects

Adjusted for age, sex, DM, HTN, smoking, LDL, HDL, TG, CRP, eGFR

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Suppression of gut flora inhibits TMAO formation and dietary choline induced atherosclerosis.

Cholesterol metabolism in cells of the artery wall:

Forward Cholesterol Transport

Reverse Cholesterol Transport (RCT)

Liver

Cholesterol Pool

ABCA1

SR-B1

LDL

MPO

CD36

SR-A

LDL-R

ApoA-I

αHDL

Spherical HDL

LCAT

Nascent “discoidal” HDL

Preβ HDL
TMAO alters cholesterol and sterol metabolism in multiple compartments - net effect - increased atherosclerosis

Carnitine, an abundant nutrient in red meat, is pro-atherogenic too

Carnitine supplementation accelerates atherosclerosis in apoE-/- mice, but not with suppression of intestinal flora (and suppression of TMA/TMAO formation)

Human carnitine tolerance study: There is an obligatory role for gut flora in TMAO production from oral carnitine.

Hypothesis: Dietary patterns alter the composition of the gut microbial community

Omnivores and Vegans/Vegetarians

N=30

Stool Collected

Gut Microbiota Composition

N=23

Blood Collected

TMAO measured by mass spectrometry
TMAO is formed from dietary carnitine in omnivores, but minimally in vegans.

Carnitine challenge: 8oz tenderloin + d3(methyl)-carnitine

Specific microbiota taxa are associated with long-term dietary patterns and plasma TMAO levels

Plasma levels of carnitine in subjects predict cardiovascular risks

Sequential subjects (N=2595) undergoing cardiac evaluation at the Cleveland Clinic Preventive Cardiology Clinic

Plasma levels of carnitine in subjects predict cardiovascular risks

Plasma levels of carnitine in subjects predict cardiovascular risks - only if TMAO is high

Sequential subjects (N=2595) undergoing cardiac evaluation at the Cleveland Clinic Preventive Cardiology Clinic

Metabolomics studies are a powerful tool for discovery of new diagnostic and therapeutic targets.

Gut flora contributes to atherosclerotic heart disease

Bennett B et al (2013) *Cell Metab*
Tang WHW et al (2013) *NEJM*
Acknowledgments

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