Journal of Nutrition & Intermediary Metabolism 8 (2017) 1–7

Contents lists available at ScienceDirect

Journal of Nutrition & Intermediary Metabolism

journal homepage: http://www.jnimonline.com/

Genome- and CD4$^+$ T-cell methylome-wide association study of circulating trimethylamine-N-oxide in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN)

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ARTICLE INFO

Article history:
Received 20 January 2017
Received in revised form
2 March 2017
Accepted 5 March 2017
Available online 8 March 2017

Keywords:
Atherosclerosis
Cardiovascular disease
Genetic
Epigenetic
Methylation
Trimethylamine-N-oxide

ABSTRACT

Background: Trimethylamine-N-oxide (TMAO), an atherogenic metabolite species, has emerged as a possible new risk factor for cardiovascular disease. Animal studies have shown that circulating TMAO levels are regulated by genetic and environmental factors. However, large-scale human studies have failed to replicate the observed genetic associations, and epigenetic factors such as DNA methylation have never been examined in relation to TMAO levels.

Methods and results: We used data from the family-based Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) to investigate the heritable determinants of plasma TMAO in humans. TMAO was not associated with other plasma markers of cardiovascular disease, e.g. lipids or inflammatory cytokines. We first estimated TMAO heritability at 27%, indicating a moderate genetic influence. We used 1000 Genomes imputed data (n = 626) to estimate genome-wide associations with TMAO levels, adjusting for age, sex, family relationships, and study site. The genome-wide study yielded one significant hit at the genome-wide level, located in an intergenic region on chromosome 4. We subsequently quantified epigenome-wide DNA methylation using the Illumina Infinium array on CD4$^+$ T-cells. We tested for association of methylation loci with circulating TMAO (n = 847), adjusting for age, sex, family relationships, and study site as the genome-wide study plus principal components capturing CD4$^+$ T-cell purity. Upon adjusting for multiple testing, none of the epigenetic findings were statistically significant.

Conclusions: Our findings contribute to the growing body of evidence suggesting that neither genetic nor epigenetic factors play a critical role in establishing circulating TMAO levels in humans.

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http://dx.doi.org/10.1016/j.jnim.2017.03.002

List of abbreviations

CpG	5'—cytosine—phosphate—guanine—3′ DNA site
CPMG	Carr-Purcell-Meiboom-Gill (pulse sequence)
CVD	cardiovascular disease
FID	free induction decay (signal)
GOLDN	Genetics of Lipid Lowering Drugs and Diet Network (Study)
MAF	minor allele frequency
NMR	nuclear magnetic resonance
SNP	single nucleotide polymorphism
TMA	trimethylamine
TMAO	trimethylamine-N-oxide

1. Introduction

Trimethylamine-N-oxide (TMAO), a pro-atherogenic metabolite species, has recently emerged as a possible causal risk factor for cardiovascular disease (CVD) [1]. TMAO is synthesized in the liver from trimethylamine (TMA), which in turn is released by the gut flora from TMA-containing dietary phospholipid components such as choline, betaine, lecithin, and L-carnitine. Plasma concentrations of L-carnitine, a nutrient commonly found in red meat and seafood, have been linked to both prevalent and incident CVD in a TMAO-dependent manner [2]. Furthermore, elevated plasma TMAO was associated with increased cardiovascular risk even in low-risk subgroups [3]. Other studies have linked TMAO levels to clinical outcomes in the context of heart failure [4] and chronic kidney disease [5], highlighting its importance in chronic disease pathogenesis.

Animal studies have shown that circulating TMAO levels are regulated by genetic and environmental factors [6]. Specifically, a genome-wide association study conducted in mice identified robust associations between sequence variation and TMAO levels; however, these findings were not replicated in a large-scale human sample [7] and the heritability of TMAO in humans remains to be established. In addition to DNA sequence variants, methylation loci may play a role in TMAO homeostasis because epigenetic processes integrate both genetic and environmental inputs such as diet. For example, betaine—one of the dietary substrates for TMAO production—can serve an alternate methyl source for converting homocysteine to methionine [8], increasing DNA methylation and altering gene expression. Consistent with that hypothesis, a recent human study reported inverse associations between plasma TMAO and methylation capacity, reflected in altered concentrations of *S*-adenosylhomocysteine and *S*-adenosylmethionine [9]. Despite the biological plausibility of epigenetic associations with TMAO in humans, such links have not yet been investigated on a genome-wide level. Although TMAO concentrations in urine are an order of magnitude higher than in plasma and easier to measure, using plasma concentrations of TMAO reduces variation due to acute dietary intake, resulting in more reliable measurements [10]. Using family data from the metabolically healthy population of the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN), we present the first heritability estimates of circulating TMAO as well as the first human epigenome-wide study of DNA methylation in relation to this promising biomarker.

2. Methods

2.1. Study population

The GOLDN study [11] recruited families of European descent with at least two siblings at two centers of the NHLBI Family Heart Study (Minneapolis and Salt Lake City). The primary aim of the study was to characterize genetic and epigenetic predictors of variability in lipid response to two interventions, namely a high-fat meal and a 3-week fenofibrate challenge. Both DNA and plasma TMAO for the current study were quantified on pre-intervention (baseline) samples to exclude potential effects of the diet and drug interventions. All participants provided written informed consent. Institutional Review Boards at University of Minnesota, University of Utah, and Tufts University/New England Medical Center approved the study protocol. GOLDN screened ~1350 individuals and excluded those with age <18 years; fasting triglycerides ≥1500 mg/dL; recent history of myocardial infarction, coronary bypass surgery, or coronary angioplasty; self-report of a positive history of liver, kidney, pancreas, or gall bladder disease, or a history of nutrient malabsorption; current use of insulin; abnormal liver or kidney function; in women of childbearing potential, pregnancy, breastfeeding, not using an acceptable form of contraception, yielding a net sample of 1048 individuals that consented to the use of their DNA in research.

2.2. TMAO measurements

We measured TMAO levels by proton nuclear magnetic resonance (NMR) spectroscopy using a Vantera® NMR Clinical Analyzer at LipoScience (now LabCorp, Raleigh, NC). Briefly, plasma was diluted with citrate/phosphate buffer (3:1 v/v) to lower the pH to 5.3 in order to move the TMAO signal away from the overlapped signal from betaine. The diluted specimen was placed in a barcoded sample vial, from which 200 μL was then automatically injected with preheating to 47 °C into the flowcell of a 400 MHz superconducting magnet. Spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence by signal averaging 48 transients with a total acquisition time of 5.5 min per sample. Free induction decay (FID) signals were multiplied by an exponential window function with a 0.1 Hz line broadening, Fourier transformed, and automatically phased and baseline corrected. The TMAO methyl signal at ca. 3.30 ppm was quantified using a proprietary non-negative linear least squares analysis that models the line shape as a mix of Gaussian and Lorentzian peak shapes. The derived TMAO signal amplitudes were then transformed into μmol/L concentrations using a conversion factor determined from analysis of dialyzed plasma samples spiked with known amounts of TMAO. NMR-derived TMAO concentrations are highly correlated ($r^2 = 0.98$) with those measured using the liquid chromatography/mass spectrometry assay developed at the Cleveland Clinic [2].

2.3. Epigenetic phenotyping

We measured DNA methylation in GOLDN on the epigenome-wide scale using the Illumina Infinium HumanMethylation450 Beadchip (Illumina, San Diego, CA) as previously described [12,13]. Briefly, to reduce the effect of cell type, we restricted the measurements to $CD4^+$ T-cells that were isolated from peripheral blood frozen buffy coat samples. We isolated DNA using commercially available DNeasy kits (Qiagen, Venlo, Netherlands). We quantified