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A terminal metabolite of niacin promotes vascular inflammation and contributes to cardiovascular disease risk

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Abstract

Despite intensive preventive cardiovascular disease (CVD) efforts, substantial residual CVD risk remains even for individuals receiving all guideline-recommended interventions. Niacin is an essential micronutrient fortified in food staples, but its role in CVD is not well understood. In this study, untargeted metabolomics analysis of fasting plasma from stable cardiac patients in a prospective discovery cohort ($n = 1,162$ total, $n = 422$ females) suggested that niacin metabolism was associated with incident major adverse cardiovascular events (MACE). Serum levels of the terminal metabolites of excess niacin, N1-methyl-2-pyridone-5-carboxamide (2PY) and N1-methyl-4-pyridone-3-carboxamide (4PY), were associated with increased 3-year MACE risk in two validation cohorts (US $n = 2,331$ total, $n = 774$ females; European $n = 832$ total, $n = 249$ females) (adjusted hazard ratio (HR) (95% confidence interval) for 2PY: 1.64 (1.10–2.42) and 2.02 (1.29–3.18), respectively; for 4PY: 1.89 (1.26–2.84) and 1.99 (1.26–3.14), respectively). Phenome-wide association analysis of the genetic variant rs10496731, which was significantly associated with both 2PY and 4PY levels, revealed an association of this

variant with levels of soluble vascular adhesion molecule 1 (sVCAM-1). Further meta-analysis confirmed association of rs10496731 with sVCAM-1 ($n = 106,000$ total, $n = 53,075$ females, $P = 3.6 \times 10^{-18}$). Moreover, sVCAM-1 levels were significantly correlated with both 2PY and 4PY in a validation cohort ($n = 974$ total, $n = 333$ females) (2PY: $\rho = 0.13$, $P = 7.7 \times 10^{-5}$; 4PY: $\rho = 0.18$, $P = 1.1 \times 10^{-8}$). Lastly, treatment with physiological levels of 4PY, but not its structural isomer 2PY, induced expression of VCAM-1 and leukocyte adherence to vascular endothelium in mice. Collectively, these results indicate that the terminal breakdown products of excess niacin, 2PY and 4PY, are both associated with residual CVD risk. They also suggest an inflammation-dependent mechanism underlying the clinical association between 4PY and MACE.

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Data availability

Source data for figures and tables included in this paper were deposited to the *Nature Medicine* website as Source Data. The DNA sequences and sequence maps of AAVs used are available at <https://doi.org/10.5281/zenodo.8357441>. Summary statistics for the meta-analyses for 2PY and 4PY are also available at <https://doi.org/10.5281/zenodo.8357441>. There are restrictions on the availability of some of the clinical data generated in the present study for the US and EU validation cohorts because the informed consent from participants in these studies does not permit sharing data outside each respective institution without authorization. Where permissible, the datasets generated and/or analyzed during the present studies are available from the corresponding author, S.L.H. (hazens@ccf.org), upon reasonable request. Individual-level data used in the present study are available upon application to

the UK Biobank (<https://www.ukbiobank.ac.uk/>). [Source data](#) are provided with this paper.

Code availability

Custom code is available at <https://doi.org/10.5281/zenodo.8357441>.

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Contributions

M.F. participated in the design, performance and analysis of all studies presented and drafted the manuscript, with input from all authors. All authors participated in the preparation of the manuscript. Z.W. and X.L. performed mass spectrometry analysis. J.A. performed chemical synthesis. O.F. and T.C. performed untargeted metabolomics data acquisition. J.R.H., J.A.H. and H.A. performed and assisted in genomic analyses. J.A.D. designed viral genomes. N.S. performed RNA sequencing analysis. A.H., M.W., I.D., M.K., E.S.-T., U.L. and W.H.W.T. provided valuable insights on clinical studies. S.L.H. conceived, supervised and participated in the design of all studies presented.

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Ethics declarations

Competing interests

S.L.H. and Z.W. report being named as co-inventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. S.L.H. and Z.W. also report having received royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heart Lab, a fully owned subsidiary of Quest Diagnostics, and Procter & Gamble. S.L.H. is a paid consultant for Zehna Therapeutics and Procter & Gamble and has received research funds from Zehna Therapeutics, Procter & Gamble, Pfizer and Roche Diagnostics. W.H.W.T. is a consultant for Sequana Medical A.V., Cardiol Therapeutics, Genomics plc, Zehna Therapeutics and Renovacor and has received honoraria from Springer Nature for authorship/editorship and from the American Board of Internal Medicine for exam writing committee service. All other authors declare no competing interests.

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Extended data

Extended Data Fig. 1 Network module-based pathway analysis identifies MACE-associated pathways containing MACE-associated metabolites.

(**center scatterplot**) MACE-associated metabolite enrichment scores versus enrichment P values for metabolic pathways (each point represents a pathway) defined in the Human MFN genome-scale metabolic model, as released with Metaboanalyst v4.0 ([Supplemental Methods](#)). Previously, Human MFN was constructed as a network by defining all human metabolites listed in KEGG (release 81.0), SMPDB v2.0, HMDB v4.0, ChEBI (release 131), and Biocyc (release 17.0) as points (nodes) and all chemical reactions (enzymatic or otherwise) as connections (edges) between metabolites ([Supplemental Methods](#)). Thus, any two molecules (nodes) participating in the same enzymatic reaction are connected with a line (edge). Metabolites (nodes) are separated into pathways (modules) to maximize the ratio of within-group to between-group connections. Pathway enrichment scores (ratio of observed to expected MACE-associated metabolites) and enrichment P values were determined with mummichog (v1.0.10), as implemented in Metaboanalyst v4.0 ([Supplemental Methods](#)), which includes adjustment for multiple testing. In the center scatterplot, each point represents the enrichment score (ratio of observed to expected MACE-associated metabolites) and the P value for the pathway (module). Each pathway (module) with enrichment P value < 0.05 and at least one component metabolite (node) with prospective 3-year MACE hazard ratio (highest vs lowest quartile) P < 0.005 is

highlighted in red. P values for all metabolites and pathways are shown in Supplemental Table 5. (**outer modules**) Shown are nine pathways (network modules) that were enriched with MACE-associated metabolites (enrichment P values < 0.05) and contained at least one component metabolite associated with prospective 3-year MACE (hazard ratio P < 0.005, two-sided Wald test assuming a univariate Cox model with no adjustment for multiple testing). Spectral features detected during untargeted metabolomics analysis were assigned to any molecule with a predicted mass-to-charge ratio within measurement error of the observed m/z , producing multiple assignments per feature ([Supplemental Methods](#)). Names of each network module-based pathway are shown, and within each pathway, lines (edges) indicate a shared metabolic conversion between the two connected metabolites (nodes), and metabolites (nodes) are colored according to the magnitude of the prospective 3-year MACE hazard ratio. Note that the “Vitamin B3 Metabolism” (niacin/NAD metabolism) pathway module was enriched with MACE-associated metabolites (enrichment P = 0.048) and the top unidentified metabolite (by incident MACE risk hazard ratio), with $m/z = 153.0656$ Da was assigned to both 2PY and 4PY within the “Vitamin B3 Metabolism” pathway module.

[Source data](#)

Extended Data Fig. 2 Overview of vitamin B3 metabolism, as defined in the Human MetaFishNet (MFN) model.

(a) Network module for vitamin B3 (niacin/NAD) metabolism. Previously, Human MFN was constructed as a network by defining all human metabolites listed in KEGG (release 81.0) SMPDB v2.0, HMDB v4.0, ChEBI (release 131) and Biocyc (release 17.0) as points (nodes) and all chemical reactions (enzymatic or otherwise) as connections (edges) between metabolites ([Supplemental Methods](#)). Thus, any two molecules (nodes) participating in the same enzymatic reaction are connected with a line (edge). Metabolites (nodes) are separated into pathways (modules) to maximize the ratio of within-group to between-group connections. Pathway enrichment P values (MACE cases vs controls) were determined with mummichog (v1.0.10), as implemented in Metaboanalyst v4.0 ([Supplemental Methods](#)), which includes adjustment for multiple

testing. The vitamin B3 network module was enriched with MACE-associated metabolites (enrichment $P = 0.048$), and a mass spectral feature ($m/z = 153.0656$ Da) assigned to metabolites (nodes) 2PY and 4PY was associated with prospective 3-year MACE risk (highest vs lowest quartile, unadjusted hazard ratio (HR) [95% confidence interval (CI)] = 2.77[1.59–4.86], $P = 0.005$ [two-sided Wald test, no adjusted for multiple testing]). Further CVD risk analysis of this mass spectral feature is shown in Fig. [2a-b](#). Metabolite (node) colors indicate the MACE hazard ratio (highest vs lowest quartile), while grey indicates the metabolite was not detected. Edges indicate a shared metabolic conversion between the two connected metabolites. Cofactors are not labeled. P values for all metabolites and pathways are shown in Supplemental Table [5](#). **(b)** Pathway representation of vitamin B3 metabolism using the molecules and reactions defined in the Human MFN model. The conversion of iminoaspartate to quinolinate was inferred in Human MFN, and so no enzyme is listed. NAD, nicotinamide adenine dinucleotide; ACMS, aminocarboxymuconate semialdehyde; NAMN, nicotinic acid mononucleotide; SAM Sadenosyl methionine; SAH, S-adenosyl homocysteine.

[Extended Data Fig. 3 Serum metabolite \(\$m/z = 153.0656\$ Da\) produces CID spectrum similar to a mixture of synthetic 2PY and 4PY.](#)

Untargeted metabolomics revealed a serum analyte ($m/z = 153.0656$ Da) associated with prospective residual MACE risk. Network module-based pathway analysis suggested this serum analyte ($m/z = 153.0656$ Da) was a mixture of the structural isomers 2PY and 4PY, which have the same elemental composition ([Supplemental Methods](#)). As the collision-induced dissociation (CID) mass spectrum of the MACE-associated serum analyte ($m/z = 153.0656$ Da) included characteristic daughter ions of both 2PY and 4PY, we explored whether a mixture of synthetic 2PY and 4PY could reproduce the CID mass spectrum of the serum analyte before conducting further structural studies and developing a method of independently quantitating 2PY and 4PY. Chemical synthesis of 2PY and 4PY is described in [Supplemental Methods](#), and $^1\text{H-NMR}$ spectra of synthetic material are shown in Supplemental Figs. [1](#) and [2](#). **(a)** Comparison of the high-resolution collision-induced dissociation (CID) mass spectra in positive ion mode of the unknown MACE-associated serum analyte with $m/z = 153.0656$ Da and calculated elemental formula

$C_7H_8O_2N_2$ (top), with synthetic 2PY (middle) and 4PY (bottom). 2PY and 4PY both have the elemental formula $C_7H_8O_2N_2$, and their observed m/z values are within measurement error (5 ppm) of 153.0656 Da. A CID parent-to-daughter transition characteristic, but not unique to 2PY ($m/z = 110.0605$ Da) is shown in red while a CID transition characteristic to 4PY ($m/z = 136.0395$ Da) is shown in blue. The spectrum of the serum analyte (top) contained parent-to-daughter transitions characteristic of 2PY and 4PY. **(b)** Parent-to-daughter transitions that are predicted to be unique to 2PY ($m/z = 126.0555$ Da) and 4PY ($m/z = 95.0133$ Da). These unique daughter fragments are detected at very low intensities as shown in Source Data. **(c)** We hypothesized the serum analyte with $m/z = 153.0656$ Da was a mixture of 2PY and 4PY. Without the capability to chromatographically separate 2PY and 4PY and compare to pure authentic chemical standards, we estimated the molar ratio of 2PY and 4PY in human serum in order to compare the presumed mixture to an equivalent mixture of 2PY and 4PY chemical standards. The molar ratio of 2PY:4PY in solution can be calibrated with the ratio of unique predicted parent-to-daughter transition intensities. The calibration curve prepared using 2PY and 4PY chemical standards in deionized water is shown. This molar ratio calibration curve was only used for exploratory structural studies and does not estimate absolute concentrations of 2PY and 4PY. Later, different calibration curves were used for independent quantitation of 2PY and 4PY using chromatographic separation. **(d)** Comparison of CID spectra of serum analyte with $m/z = 153.0656$ Da (top) with a solution of 2PY and 4PY in deionized water at the estimated molar ratio.

[Source data](#)

[Extended Data Fig. 4 Illustration of methods development to selectively monitor and quantify serum levels of 2PY and 4PY.](#)

Shown is the baseline chromatographic resolution of 2PY and 4PY in serum, coupled with the selective MRM transitions used to quantify 2PY and 4PY. Briefly, isotope labeled synthetic 2PY and 4PY (d3-2PY and d3-4PY) were added to serum samples, protein precipitated with methanol, and then following injection on silica column, the indicated isotopologues of 2PY and 4PY were resolved and monitored by the indicated MRM

transitions. The selectivity of the MRM transitions for either 2PY (red) or 4PY (blue) are indicated, with change in font size used to indicate selectivity for either 2PY or 4PY (or their corresponding d3-isotopologue internal standards). Chemical synthesis of 2PY and 4PY is described in [Supplemental Methods](#), and ¹H-NMR of synthetic material are shown in Supplemental Figs. [1](#) and [2](#). Note that the three distinct MRM transitions selected for monitoring natural abundance 2PY, 4PY, and their heavy isotope-labeled are also shown. The selected MRM transitions are also shown in Fig. [2d](#). Note how the silica column-based method baseline resolves 2PY and 4PY within the MACE-associated serum analyte with $m/z = 153.0656$ Da.

[Source data](#)

[Extended Data Fig. 5 Verifying the MACE-associated serum analyte with \$m/z = 153.0656\$ Da is comprised of 2PY and 4PY.](#)

The MACE-associated serum analyte with $m/z = 153.0656$ Da was resolved into two chromatographically separable structural isomers, 2PY and 4PY, using the new HPLC method. The two analytes have retention times of 2.4 min (metabolite 1; 2PY) and 3.4 min (metabolite 2; 4PY). **(a)** High resolution CID spectrum of serum metabolite 1 with retention time 2.4 min compared to synthetic 2PY (retention time 2.4 min). Peaks are labeled with their measured m/z in Da. **(b)** Sizes of predicted fragments of 2PY. Predicted fragments are labeled with their predicted m/z in Da. **(c)** CID spectrum of metabolite 2 with retention time 3.4 min compared to synthetic 4PY (retention time 3.4 min). Peaks are labeled with their measured m/z in Da. **(d)** Sizes of predicted fragments of 4PY. Predicted fragments are labeled with their predicted m/z in Da. Chemical synthesis of 2PY and 4PY is described in [Supplemental Methods](#), and ¹H-NMR spectra of synthetic material are shown in Supplemental Figs. [1](#) and [2](#). Differences in m/z among predicted fragments and those observed for synthetic and serum 2PY and 4PY were within expected experimental error and are shown in Supplemental Table [6](#).

[Source data](#)

Extended Data Fig. 6 Comparison of 2PY and 4PY levels in US and European Validation Cohorts.

(a - b) Serum levels of 2PY and 4PY were highly correlated in both the US and European Validation Cohorts. P values for Pearson correlations determined from t distributions with $n-2$ degrees of freedom ($P = 1.2 \times 10^{-1757}$ for the US validation cohort and $P = 2.1 \times 10^{-986}$ for the European validation cohort). P values for Spearman correlations determined with two-sided asymptotic t tests, and exact P values $< 2 \times 10^{-16}$ could not be determined. (c) Spearman correlations among 2PY, 4PY, and risk factors for MACE. P values determined with two-sided asymptotic t tests and adjusted for multiple testing using the false discovery rate method. Baseline clinical cohort characteristics are shown in Supplemental Table 2. ** $p < 0.0001$, * $p < 0.05$.

Extended Data Fig. 7 Sensitivity analysis of 2PY and 4PY association with MACE in the merged cohort.

US and European Validation Cohorts were merged ($n = 3,163$), and Hazard Ratio (quartile 4 (Q4) versus quartile 1 (Q1); open circle) for 4PY (panel a) and 2PY (panel b) association with MACE (3 yr) risks for the indicated subgroups are shown. Baseline clinical cohort characteristics are shown in Supplemental Table 2. Symbols represent hazard ratios and error bars represent 95% confidence intervals. Ref, reference group. Interaction P values were determined with two-sided Wald tests and adjusted for multiple testing using the method of Benjamani and Hochberg.

Source data

Extended Data Fig. 8 Transcriptomic analysis of human endothelial cells exposed to 2PY or 4PY.

(a) Human endothelial cells were cultured with 2PY, 4PY, or vehicle control, and RNA was harvested after 4 hours. *VCAM1* mRNA levels determined by RNA sequencing were elevated in 4PY-treated cells. The bar plot with error bars shows the mean plus or minus one standard error. P values were determined with two-sided Kruskal-Wallis (KW) and Wilcoxon tests. (b) Volcano plot shows differentially expressed genes for vehicle vs 4PY. (c) Differentially expressed genes for 2PY vs 4PY. Genes whose mRNA expression is

impacted by 4PY compared to either control are shown in red. Fold change P values were determined using the log ratio test as implemented in edgeR and adjusted using the false discovery rate method. **(d)** Gene sets (Gene Ontology terms) whose member genes are enriched with the differentially expressed genes between vehicle and 4PY are shown. Dot size indicates the number of differentially expressed genes in the set. P values determined with two sided Fisher Exact tests and adjusted using the false discovery rate method. Only tip terms are shown, that is enriched GO terms with enriched child terms are not shown. All enriched GO terms are included in Source Data. **(e)** Genes associated with the cellular response to tumor necrosis factor (TNF) (shown in red in **(d)**) tend to have higher expression in 4PY-treated cells than vehicle- and 2PY-treated cells. Expression in transcripts per million transformed to Z-scores per gene.

[Source data](#)

Extended Data Table 1 Meta-analysis results for lead variants associated with circulating 2PY, 4PY and sVCAM-1 levels

Extended Data Table 2 2PY, 4PY and sVCAM-1 levels across associated genome-wide significant variant (rs10496731) genotypes in the US validation cohort

Supplementary information

[Supplementary Information](#)

Supplementary Tables 1–8 and Supplementary Figs. 1–6.

[Reporting Summary](#)

Source data

[Source Data Fig. 1](#)

Statistical source data.

Source Data Fig. 2

Statistical source data.

Source Data Fig. 3

Statistical source data.

Source Data Fig. 4

Statistical source data.

Source Data Fig. 5

Statistical source data.

Source Data Fig. 5g

Uncropped intravital microscopy images of auricular venules of mice treated with 2PY, 4PY or vehicle control.

Source Data Extended Data Fig. 1

Statistical source data.

Source Data Extended Data Fig. 3

Statistical source data.

Source Data Extended Data Fig. 4

Statistical source data.

Source Data Extended Data Fig. 5

Statistical source data.

Source Data Extended Data Fig 7

Statistical source data.

Source Data Extended Data Fig 8

Statistical source data.

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