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Estimating the long-term health impact of nicotine exposure by dissecting the effects of nicotine versus non-nicotine constituents of tobacco smoke: A multivariable Mendelian randomisation study

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Short title: Health effects of nicotine versus non-nicotine constituents of tobacco smoke

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Abstract

The detrimental health effects of smoking are well-known, but the impact of long-term nicotine use without exposure to the other constituents of tobacco is less clear. Given the increasing long-term use of alternative nicotine delivery systems, such as e-cigarettes, it is increasingly important to understand and separate the effects of long-term nicotine use from the impact of tobacco smoke exposure. Using a multivariable Mendelian randomisation framework, we explored the direct effects of nicotine compared with the non-nicotine constituents of tobacco smoke on health outcomes (lung cancer, chronic obstructive pulmonary disease [COPD], forced expiratory volume in one second [FEV-1], forced vital capacity [FVC], coronary heart disease [CHD], and heart rate [HR]). The results suggest that these smoking-related outcomes are not due to nicotine exposure but are caused by the other components of tobacco smoke; however, there are multiple potential sources of bias, and the results should be triangulated using evidence from a range of methodologies.

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Although the detrimental health effects of smoking are well known [1], it is less clear which individual components of cigarette smoke – such as nicotine – drive these effects. In recent years, it has become increasingly important to fill this knowledge gap, given the increase in the popularity of alternative nicotine delivery systems, in particular e-cigarettes [2]. E-cigarettes are often used by smokers to stop smoking and are generally used for longer periods than conventional cessation aids which also contain nicotine (e.g., nicotine patches) [3, 4]. Although *short-term* use of nicotine without the remaining constituents of tobacco smoke is relatively harmless, the *long-term* harms of nicotine use are poorly understood [5]. Given many smokers choose to use e-cigarettes to reduce their risk of poor health outcomes [2], and that e-cigarettes can be used with or without nicotine, smokers and e-cigarette users need causal evidence regarding the possible health outcomes related to long-term nicotine use to make informed decisions regarding their e-cigarette use.

Randomised controlled trials (RCT) are often employed to explore causal relationships [6], but an RCT would be unethical and impractical in this scenario – non-smokers would need to be unnecessarily exposed to nicotine for decades to understand the (potentially harmful) long-term effects of nicotine use without confounding from exposure to cigarette smoke. Mendelian randomisation (MR) is an alternative method which can be used in scenarios where RCTs are implausible or unethical. MR uses genetic variants associated with the exposure of interest to serve as instrumental variables to estimate the effect of that exposure on a particular outcome [7, 8]. Nonetheless, they require well powered genome-wide association studies (GWAS) to identify such genetic variants. There are currently no available GWAS of e-cigarette use or nicotine exposure without exposure to tobacco.

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Furthermore, there are insufficient data available to conduct a well-powered GWAS. Novel methods are therefore required to explore this research question.

Multivariable MR (MVMR) is an extension of MR that can be used to explore the direct effect of one exposure on an outcome while accounting for the effect of another (potentially correlated) exposure or exposures [9]. The method is robust to pleiotropic effects of the single nucleotide polymorphisms (SNPs) used as instruments through the other exposures included in the model. As large-scale GWAS have identified SNPs that are associated with smoking heaviness [10] (i.e., the number of cigarettes a person smokes per day) and the nicotine metabolite ratio (i.e., how quickly a person metabolises nicotine – see below) [11] among smokers, we can employ this method to explore the effect of nicotine on selected health outcomes. Rather than exploring the total effect of smoking on a health outcome (which includes the effect of nicotine), this method allows us to separate the direct effect of nicotine versus the other constituents of tobacco smoke.

However, direct measurement of nicotine is difficult. Metabolites of nicotine such as cotinine (a direct metabolite) and 3'hydroxycotinine (3HC; a metabolite of cotinine) are often used as objective measures to proxy for nicotine given the short half-life of nicotine itself [12] (Supplementary Figure S1). Cotinine and cotinine plus 3HC are highly specific biomarkers, but both are impacted by metabolism (which can differ between individuals). The nicotine metabolite ratio (NMR), is a measure of how quickly a person metabolises nicotine (3'hydroxycotinine/cotinine) and therefore causally impacts the amount of nicotine in a person's body given a set amount of nicotine exposure [13]. Figure 1 illustrates that a smoker with a higher NMR (Person A) will have less circulating nicotine in their body than a

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85 smoker with a lower NMR (Person B) given the same level of nicotine exposure; however,
86 because smokers with a higher NMR clear nicotine quickly from their system, this often
87 results in them smoking more cigarettes per day (CPD) [14]. In a standard MR model with
88 NMR as an exposure, the result may be ambiguous as it could reflect the effect of higher
89 nicotine exposure resulting from increased smoking heaviness or lower nicotine exposure
90 resulting from faster metabolism. In contrast, when both NMR and CPD are included in an
91 MVMR model (Figure 2), the analysis is analogous to a fixed access experiment in which the
92 amount of smoke exposure / cigarettes smoked is fixed and the effect of nicotine exposure
93 per cigarette smoked can be assessed. Therefore, in an MVMR framework accounting for
94 genetic predisposition to CPD, the results should dissect the direct effect of nicotine levels
95 per cigarette smoked from the direct effect of the other constituents of tobacco smoke,
96 whereby an increase in NMR reflects lower exposure to nicotine (Figure 1).
97
98

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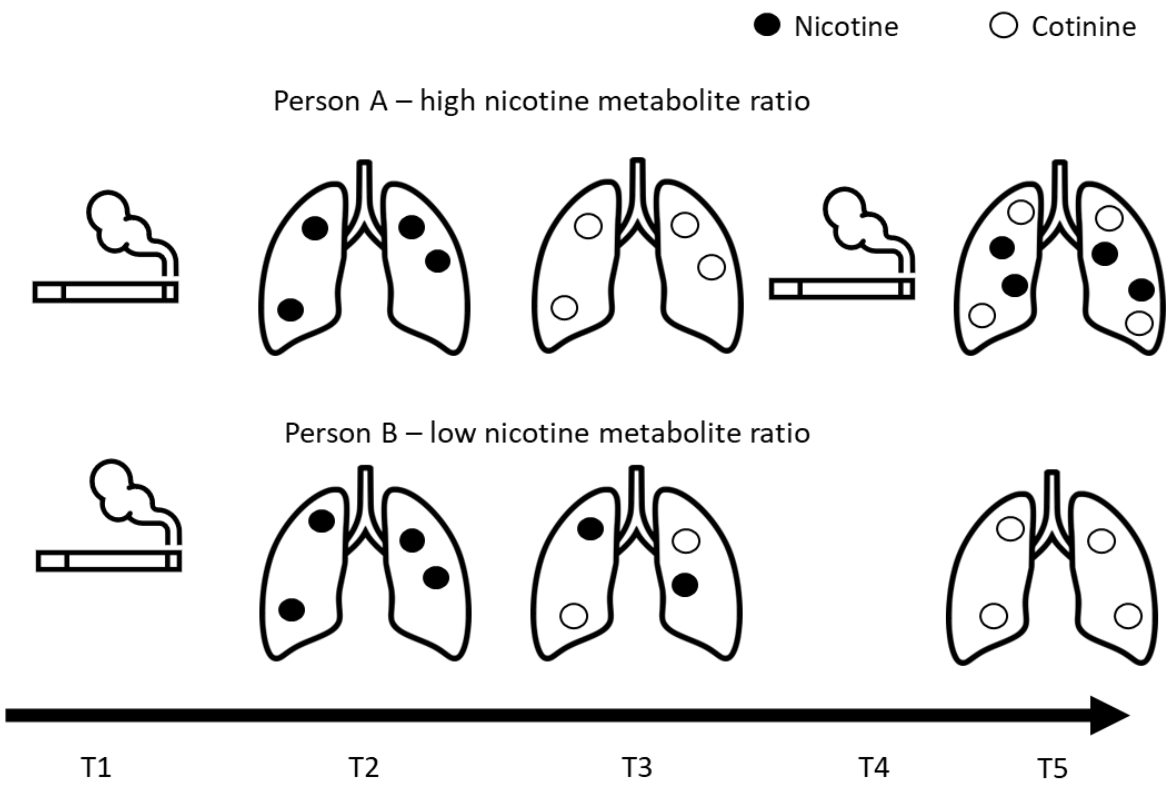
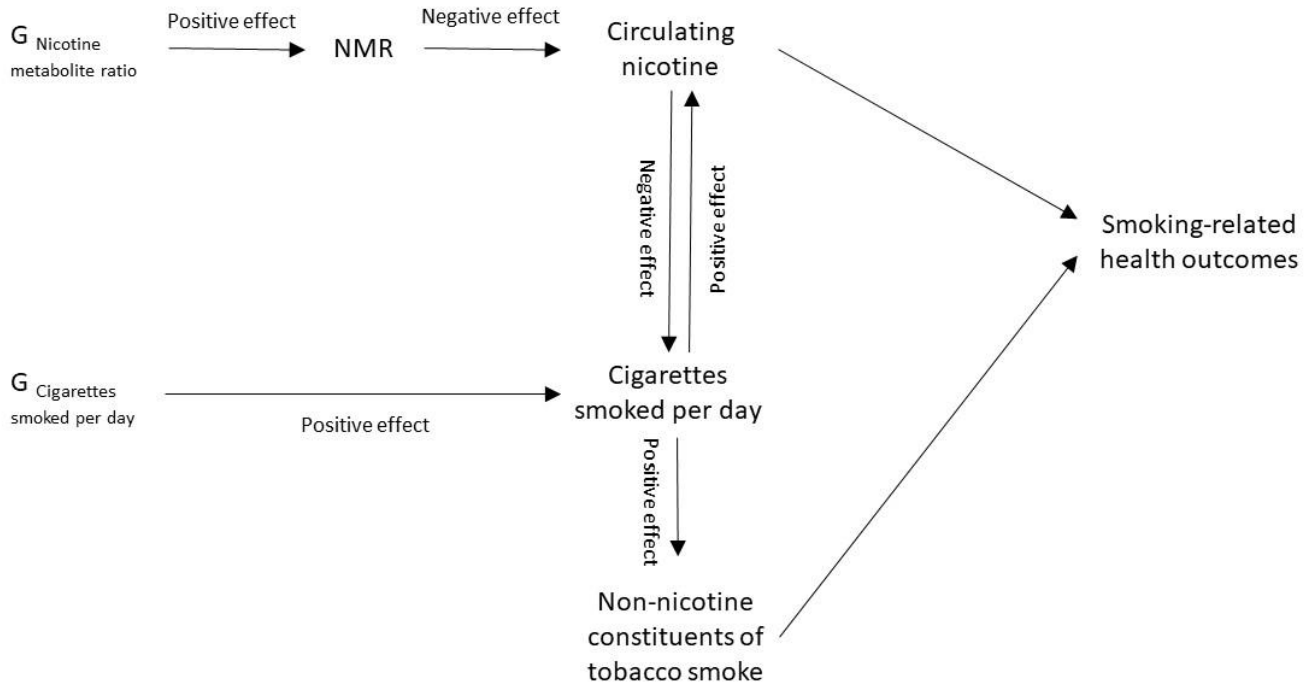
99 Figure 1. Illustration of the impact of the nicotine metabolite ratio on circulating nicotine
100 and smoking heaviness.



101

102 Note: This illustration shows the differences in nicotine exposure between two people who
103 smoke: Person A who has a high nicotine metabolite ratio, and Person B who has a low
104 nicotine metabolite ratio. At timepoint 1 (T1) both Person A and B smoke one cigarette and
105 inhale the same amount of nicotine which can be seen circulating in their body at timepoint
106 2 (T2). Later, at timepoint 3 (T3), Person A will have less circulating nicotine in their body
107 than Person B (despite having inhaled the same level of nicotine) as more of the nicotine
108 has been metabolised into cotinine. However, because smokers with higher nicotine
109 metabolite ratios clear nicotine more quickly from their system, this often results in them
110 smoking more cigarettes per day. So, at timepoint 4 (T4), Person A smokes another cigarette
111 whereas Person B does not. This results in Person A having more circulating nicotine than
112 Person B over the same time period [14].

113 Figure 2. Schematic of the study model



114

115 Note: This schematic shows the proposed causal pathways of the study model. Where the causal directions between variables are known

116 (i.e., evidenced in previous research), the direction of the effect is indicated as 'positive' or 'negative'. NMR = Nicotine Metabolite Ratio. G =

117 genetic variants associated with the named exposure.

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Using this framework, it is possible to explore the effects of nicotine and the effects of the remaining constituents of tobacco smoke on health outcomes known to be impacted by smoking, and which therefore may be impacted by long-term nicotine use, such as cancer and heart and lung function [15-17]. We employed a two-sample MVMR framework to explore the direct effects of nicotine compared with the non-nicotine constituents of tobacco smoke on health outcomes (lung cancer, chronic obstructive pulmonary disease [COPD], forced expiratory volume in one second [FEV-1], forced vital capacity [FVC], coronary heart disease [CHD] and heart rate [HR]). These outcomes include positive and negative control outcomes to aid causal inference; nicotine use is known to increase heart rate, but is not thought to be carcinogenic, so if the results do not provide evidence of an effect of nicotine on heart rate, and / or if they suggest an effect of nicotine on lung cancer, the results may indicate bias from a violation of the assumptions of MR [18-20]. In supplemental analyses, our secondary aim is to compare the results with analyses using alternative proxies for nicotine exposure (i.e., cotinine and cotinine plus 3HC).

Methods

Data Sources

To identify relevant instrumental variables to include in the model, we sourced summary-level genetic data from well-powered, published GWAS. We identified SNPs associated with NMR, cotinine, cotinine plus 3HC and smoking heaviness (measured by CPD) [10, 11, 21]. For the outcomes, we required data to be stratified by smoking status in order to be comparable with the population of the exposure GWAS data. An appropriately stratified GWAS (stratified by smoking status: ever versus never [22]) was only available for one of the outcomes, lung cancer. For the six remaining outcomes, we were not aware of any existing

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142 available GWAS summary-level data that appropriately stratified by smoking status. We
143 therefore used UK Biobank data to conduct GWAS of CHD, COPD, FEV, FVC and HR, stratified
144 by smoking status and restricted to individuals of European ancestry. These analyses were:
145 1) stratified by whether participants had ever smoked, and further stratified into 2) current
146 smoker, or 3) former smoker, with 4) never smoker as the comparator, and were conducted
147 using the MRC Integrative Epidemiology Unit UK Biobank GWAS pipeline (version 2) [23, 24].
148 We also conducted stratified GWAS of body mass index (BMI) using UK Biobank data with
149 the intention of including BMI as an outcome. However, these methods and results are not
150 presented here as we identified a potential interpretation issue: the NMR GWAS was
151 adjusted for BMI, meaning these results would likely be biased [25].
152
153 *Smoking heaviness*
154 Liu and colleagues [10] report summary-level statistics from a GWAS meta-analysis of
155 smoking heaviness (measured by standard deviation change in CPD categories, equivalent to
156 2-3 additional cigarettes per day) among 337,334 smokers of European ancestry in the
157 GWAS and Sequencing Consortium of Alcohol and Nicotine (GSCAN). Smokers were defined
158 as having ever or currently smoked and smoking heaviness (at the time of smoking) was
159 binned into five categories or included as pre-defined bins from the original study (see
160 supplementary material in Liu and colleagues, [10]. Analyses were adjusted for age, age-
161 squared, sex, genetic principal components, and smoking status (current versus former).
162 SNPs associated with CPD explained 4% of variance in CPD. SNPs were reported as
163 independent if they explain additional variance in conditional analyses using a partial
164 correlation-based score statistic [26]. The reported data are available
165 at: https://doi.org/10.13020/3b1n-ff32.

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166

167 *Nicotine*

168 Buchwald and colleagues [11] report summary-level statistics from a GWAS meta-analysis of
169 the standard deviation change in NMR among 5,185 current smokers of European descent
170 with cotinine levels ≥10 ng/ml (indicating recent smoking). NMR is a ratio of
171 3'hydroxycotinine/cotinine which indicates how quickly a person metabolises and clears
172 nicotine. Analyses were adjusted for population substructure, age, sex, BMI, alcohol use,
173 and birth year. The SNPs associated with NMR explained ~38% of the variance in NMR. SNPs
174 were reported as independent if they explained additional variance in a step-wise
175 conditional regression using genome-wide complex trait analysis [27]. The reported data are
176 available on request from the authors. Information relating to the nicotine measures used in
177 the supplemental analysis (using cotinine and cotinine plus 3HC data as a proxy for nicotine
178 exposure) can be found in the Supplementary Material (Supplemental Note S1).

179

180 *Lung Cancer*

181 McKay and colleagues [22] report summary-level statistics from a case-control GWAS meta-
182 analysis of overall lung cancer risk (including adenocarcinoma, squamous cell carcinoma,
183 and small cell carcinoma) among 50,046 individuals of European descent using the
184 International Lung Cancer Consortium (ILCCO). Analyses were adjusted for age, gender,
185 country (if applicable) and significant principal components. We received the data via
186 request to the authors and we additionally received the data stratified by smoking status
187 (ever versus never smoking). Definitions of ever and never smoking differed by cohort
188 included in the meta-GWAS. Details of the definitions can be found in McKay and
189 colleagues' [22] supplementary note.

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190

191 *Non-Cancer Smoking-Related Health Outcomes*

192 We conducted GWAS of non-cancer health outcomes using data from UK Biobank, a
193 population-based health research resource consisting of approximately 500,000 people,
194 aged between 38 years and 73 years, who were recruited between the years 2006 and 2010
195 from across the UK [28]. UK Biobank has a particular focus on identifying determinants of
196 human diseases in middle-aged and older individuals who provided a wide range of health
197 information (data available at www.ukbiobank.ac.uk). A full description of the study design,
198 participants, and quality control (QC) methods have been described previously [28, 29]. UK
199 Biobank received ethics approval from the Research Ethics Committee (REC reference for UK
200 Biobank is 11/NW/0382). Written informed consent was obtained from participants prior to
201 their participation in UK Biobank. Approval to use these data was sought from and
202 approved by UK Biobank (project 9142). All studies approved by UK Biobank do not require
203 individual ethical clearance as UK Biobank has approval from the North West Multi-centre
204 Research Ethics Committee as a Research Tissue Bank under which each approved study
205 operates.

206

207 The full data release contains the cohort of successfully genotyped samples (n=488,377).
208 Further information about genotyping and imputation can be found in the Supplementary
209 Material (Supplemental Note S2). Individuals with mismatched sex were excluded and the
210 analyses were restricted to those of 'European' ancestry using an in-house k-cluster means
211 method. The GWAS were conducted using the linear mixed model (LMM) association
212 method as implemented in BOLT-LMM (v2.3) [30], adjusting for genotype array, age and sex
213 [23]. BOLT-LMM association statistics are on the linear scale, so test statistics (betas and

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their corresponding standard errors) relating to binary phenotypes were transformed to log odds ratios and their corresponding 95% confidence intervals (95% CI) on the liability scale using a Taylor transformation expansion series [31]. Each outcome was stratified by self-reported participant smoking status (ever [further stratified by current or former] and never), resulting in four GWAS per outcome. Smoking status was categorised as never, previous, and current smoking in UK Biobank (field ID 20116). From this variable, we derived an 'ever smokers' category which was defined as currently or having previously smoked occasionally, most days or daily (i.e., having smoked more than just once or twice). Current smoking was defined as currently smoking occasionally, most days or daily. Former smoking was defined as not currently smoking but having previously smoked occasionally, most days or daily (i.e., more than just once or twice). Those who had tried smoking once or twice or who had never smoked were categorised as never smokers.

We identified COPD cases as participants who self-reported a doctor's diagnosis of COPD. Lung function (FEV-1 and FVC, in litres) was measured using a Vitalograph spirometer. CHD diagnosis was determined using linked hospital admission data (ICD codes relating to Ischemic Heart Disease). Further information regarding each health outcome (including UK Biobank field IDs) can be found in the Supplementary Material (Supplemental Note S3).

Statistical Analysis

All analyses were conducted using R version 1.4.1.

Selection of genetic variants

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We selected conditionally independent (at the genome-wide significant level, $p < 5 \times 10^{-8}$) genetic variants identified in the GWAS of NMR and CPD for inclusion in the analysis [10, 11]; 55 SNPs associated with smoking heaviness were identified as conditionally independent [10]; 7 SNPs associated with the NMR were identified as conditionally independent.

After removing SNPs which were not available in the outcome GWAS (where no available proxy could be identified), harmonising, and clumping the combined exposure datasets with the outcome dataset, the number of SNPs in each analysis varied. Supplementary Tables S1-S6 detail which SNPs were included and excluded (with reasons) in each analysis, and Supplemental Note S4 gives further details of the proxy search and clumping methods.

We tested instrument strength and validity using the two-sample conditional F-statistic for MVMR and Cochran *Q* statistic [9, 32]. The conditional F-statistic for MVMR indicates instrument strength of each exposure when accounting for the prediction of other exposures in the model (i.e., whether the SNPs jointly predict smoking heaviness after predicting the NMR, and vice versa) [33]. The standard critical values for the F-statistic and Q-statistic can be approximated so F-statistic should be greater than 10 to indicate sufficient instrument strength and *Q* estimates should be less than the number of SNPs included in the model to indicate no excessive heterogeneity [34, 35].

Univariable Mendelian randomisation

For comparison with the MVMR analysis, we considered the total effect of both the NMR and smoking heaviness on each health outcome using MR. We used four complementary

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MR methods (inverse variance weighted [MR-IVW], MR-Egger, weighted median, weighted mode). Details of the univariable MR analysis methods can be found in Supplemental Note S5.

Multivariable Mendelian randomisation

We explored the direct effects of both the NMR and smoking heaviness on health outcomes using MVMR. We used summary data from Buchwald and colleagues' [11], GSCAN [10] and ILCCO [22], and UK Biobank GWAS pipeline results. We repeated these analyses using two complementary methods – MVMR-IVW and MVMR-Egger [9, 36].

Given that the GWAS of smoking heaviness was restricted to ever smokers and the GWAS of the NMR was restricted to current smokers, the analyses were restricted to ever and current smokers as the summary statistics are not applicable to never smokers. However, the ILCCO lung cancer data have only been stratified by ever and never smoking status; therefore, analyses restricted to current smokers were not possible when exploring lung cancer incidence. In supplementary analyses, we additionally stratified the analysis by never smokers to explore potential horizontal pleiotropy – effects observed among never smokers could indicate horizontally pleiotropic effects (i.e., the included SNPs influencing the outcome directly, or via another phenotype, but not through the measured exposure), misreporting of smoking status, or residual population stratification. Horizontally pleiotropic genetic variants are not valid instruments in MR analyses and their inclusion would result in a violation of the exclusion restriction assumption (further details on the assumptions of MR can be found in Supplemental Note S6). Pleiotropy robust methods (e.g., MR-Egger) are robust to horizontal pleiotropy under assumptions of the form that pleiotropy takes. For all

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outcomes except lung cancer (where data are unavailable), we also included supplementary results restricted to former smokers to explore whether any health effects found among current smokers may be recoverable (i.e., not present among former smokers who have recovered following smoking cessation).

Results

Descriptive Statistics

Smoking heaviness data were collected from 337,334 current and former smokers who reported their current or past smoking behaviour respectively (analyses corrected for current versus former status, average bin 3 [SD = 1] equating to 16-25 cigarettes per day) [10]. NMR data were collected from 5,185 current smokers [11]. Case rates and means (with standard deviations) for the outcomes in each of the samples included in the analyses are shown in Table 1.

Table 1. Descriptive statistics for participants included in the genome-wide association studies of lung cancer, coronary heart disease, chronic obstructive pulmonary disease, lung function and heart rate.

	Ever smokers	**Current Smokers**	**Former Smokers**	**Never Smokers**
	International Lung Cancer Consortium			
N	40,187	N/A	N/A	9,859
Lung Cancer (case rate)	57%	N/A	N/A	24%
	UK Biobank			
N	213,341	49,721	163,620	258,056
Coronary Heart Disease (case rate)	11%	11%	11%	6%
Chronic Obstructive Pulmonary Disease (case rate)	3%	6%	2%	<1%

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Forced Expiratory Volume (mean litres [SD])	2.82 (0.78)	2.78 (0.83)	2.83 (0.76)	2.88 (0.78)
Forced Vital Capacity (mean litres [SD])	3.78 (0.97)	3.81 (1.02)	3.78 (0.96)	3.78 (0.99)
Heart Rate (mean beats per minute [SD])	69.05 (11.38)	71.27 (11.58)	68.40 (11.23)	68.93 (11.10)

Note: SD = standard deviation

Instrument strength and heterogeneity

Where the Cochran's Q statistic was greater than the number of SNPs included in the model, it is advised to focus on pleiotropy robust methods as this indicates heterogeneity and potential pleiotropy. MR-Egger and MVMR-Egger give estimates that are robust to directional horizontal pleiotropy under the assumption that this pleiotropy is uncorrelated with the strength of association between the SNP and the exposure [36]. However, using the MR-Egger and MVMR-Egger methods limits the statistical power of the analysis compared to MVMR-IVW. Therefore, although we present the IVW results in the text, we have explicitly stated where the sensitivity analyses differ substantially (i.e., where the results could lead to different conclusions) and in these cases, additionally compare the Egger results (and weighted mode and weighted median results) in the MR analyses.

The F-statistics indicate that the SNPs included in these analyses are strong instruments for assessing the direct effects of smoking heaviness while accounting for the effect of the NMR (Fs = 33.96 and 34.17) and for assessing the direct effects of the NMR while accounting for smoking heaviness (Fs = 30.17 and 49.08).

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Binary outcomes

The total and direct effects of the NMR and smoking heaviness on lung cancer, CHD, COPD, and are shown in Figure 3 (ever smokers). These results are also included in Supplementary Tables S7 (lung cancer), S8 (CHD), and S9 (COPD) along with the F-statistics, Q-statistics and Egger intercept and the results among current, never and formers smokers. We have focussed on the results among ever smokers rather than current smokers here as receiving a diagnosis of lung cancer, CHD or COPD may increase the likelihood of someone quitting smoking, so the results are more relevant and interpretable among ever smokers. The results among current smokers can be found in Supplemental Note S7 and Supplementary Figure S2. Additionally, we have included the median and mode weighted MR sensitivity analyses in Supplementary Table S10. Results are presented as odds ratios per standard deviation (SD) increase in the exposure phenotype (i.e., per SD increase in the NMR or cigarettes per day).

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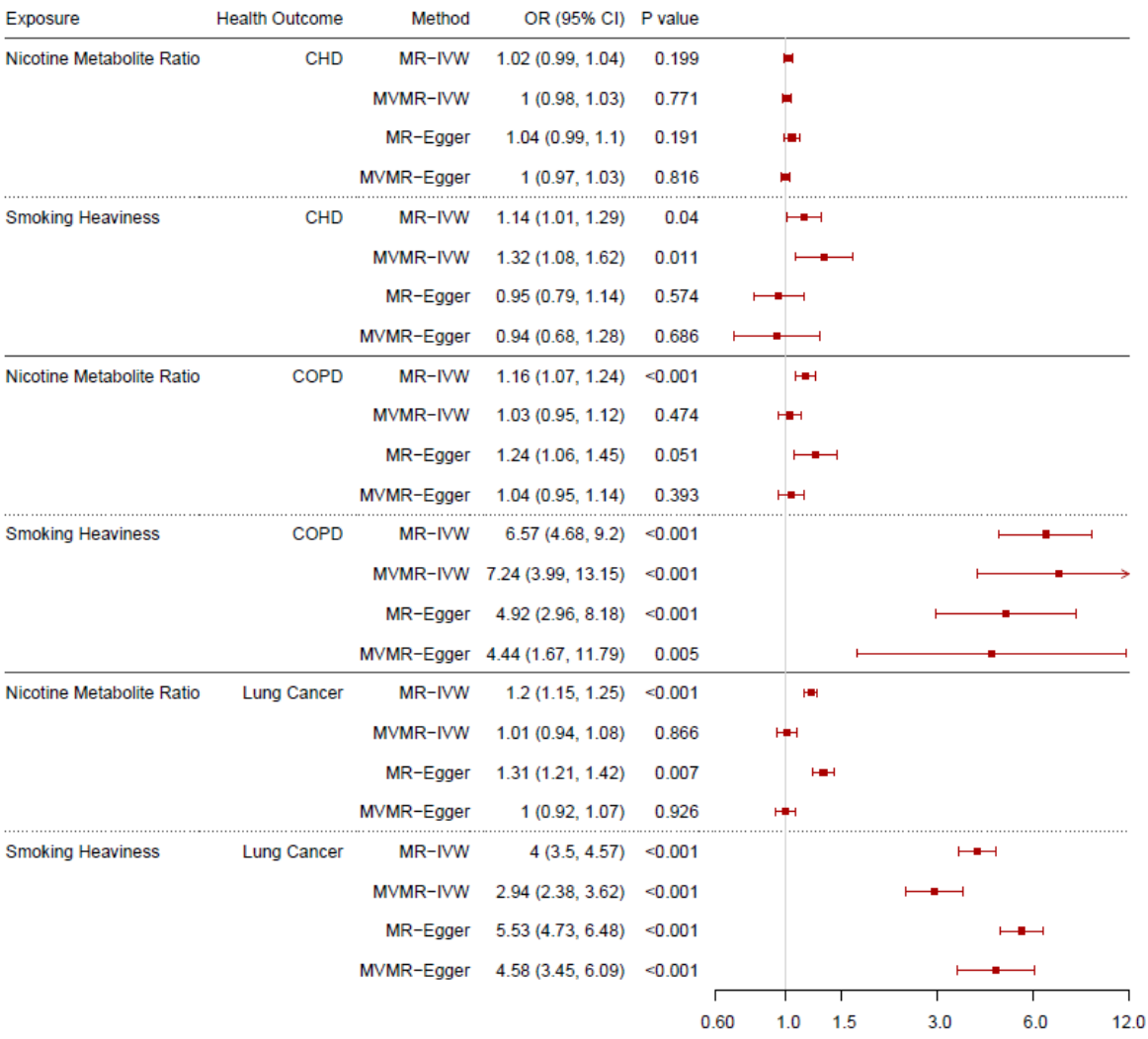

Figure 3. Forest plot displaying the effect of nicotine metabolite ratio and smoking heaviness on coronary heart disease (CHD), chronic obstructive pulmonary disease (COPD) and lung cancer incidence: univariable Mendelian randomisation (MR) and multivariable Mendelian randomisation (MVMR) results among ever smokers.

Lung Cancer

The MR-IVW results indicate strong evidence to suggest that increased NMR and increased smoking heaviness both increase the risk of developing lung cancer among ever smokers (odds ratio [OR] = 1.20, 95% confidence interval [CI] 1.15 to 1.25; OR = 4.00, 95% CI 3.50 to 4.57 respectively). However, the MVMR-IVW results do not provide clear evidence to suggest an effect of NMR on lung cancer risk (OR = 1.01, 95% CI 0.94 to 1.08), and indicate

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that only increased smoking heaviness increased lung cancer risk (OR = 2.94, 95% CI 2.38 to 3.62). Although there is considerable evidence of heterogeneity and potential pleiotropy in the smoking heaviness analyses among ever smokers, these results are supported by the MR-Egger results. Furthermore, there is no clear evidence of pleiotropy or bias due to population stratification indicated by the results among never smokers.

Coronary Heart Disease

The MR-IVW results provide no clear evidence that NMR affects CHD risk (OR = 1.02, 95% CI 0.99 to 1.04). This is supported by the MR-Egger results, but the weighted median and weighted mode results suggested there may be a weak effect (OR = 1.03, 95% CI 1.01 to 1.05, OR = 1.03, 95% CI 1.01 to 1.05 respectively). The results provide some evidence that increased smoking heaviness increases CHD risk (OR = 1.14, 95% CI 1.01 to 1.29) among ever smokers. The weighted median and weighted mode results support this finding (OR = 1.14, 95% CI 1.02 to 1.29, OR = 1.14, 95% CI 1.02 to 1.27 respectively); however, the MR-Egger analysis does not support this (OR = 0.95, 95% CI 0.79 to 1.14), and there is some evidence of heterogeneity indicated by the Q-statistics and the Egger intercept indicated weak directional pleiotropy which is supported by evidence of a protective effect among never smokers. The MVMR-IVW results provide no clear evidence of an effect of NMR on CHD risk (OR = 1.00, 95% CI 0.98 to 1.03) but some evidence that increased smoking heaviness increases CHD risk (OR = 1.32, 95% CI 1.08 to 1.62). As with the univariable results, the MVMR-Egger analyses do not support this (OR = 0.94, 95% CI 0.68 to 1.28) and there was evidence of heterogeneity and potential directional pleiotropy and some evidence of horizontal pleiotropy or bias due to population stratification in the analysis among never smokers.

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372

373 *Chronic Obstructive Pulmonary Disease*

374 The MR-IVW results indicate that increased NMR and smoking heaviness increase the risk of
375 developing COPD among ever smokers (OR = 1.16, 95% CI 1.07 to 1.24, OR = 6.57, 95% CI
376 4.68 to 9.20 respectively). However, the MVMR-IVW results indicate no clear effect of NMR
377 on COPD risk (OR = 1.03, 95% CI 0.95 to 1.12) but suggest that increased smoking heaviness
378 increases COPD risk among ever smokers (OR = 7.24, 95% CI 3.99 to 13.15). These results
379 are supported by the MR sensitivity analyses and MVMR-Egger results and there is no clear
380 evidence of heterogeneity or directional pleiotropy or horizontal pleiotropy or bias due to
381 population stratification among never smokers (where precise null effects were observed).

382

383 **Continuous outcomes**

384 The total and direct effects of the NMR and smoking heaviness on lung function (FEV-1 and
385 FVC) and heart rate are shown in Figures 4 (ever smokers) and 5 (current smokers). These
386 results are also displayed in Supplementary Tables S11 (FEV-1), S12 (FVC), and S13 (heart
387 rate) along with the F-statistics, Q-statistics and Egger intercept and the results among ever,
388 current, never and formers smokers. Additionally, we have included the median and mode
389 weighted MR sensitivity analyses in Supplementary Table 10. Results are presented as betas
390 per standard deviation (SD) increase in the exposure phenotype (i.e., per SD increase in the
391 NMR or cigarettes per day). As per MR-STROBE guidelines [37], we have reported the results
392 in text on an interpretable scale (i.e., difference in outcome in relevant units e.g., mL).
393 However, to aid comparability across outcomes, we present the results per standard
394 deviation change in the forest plots.

395

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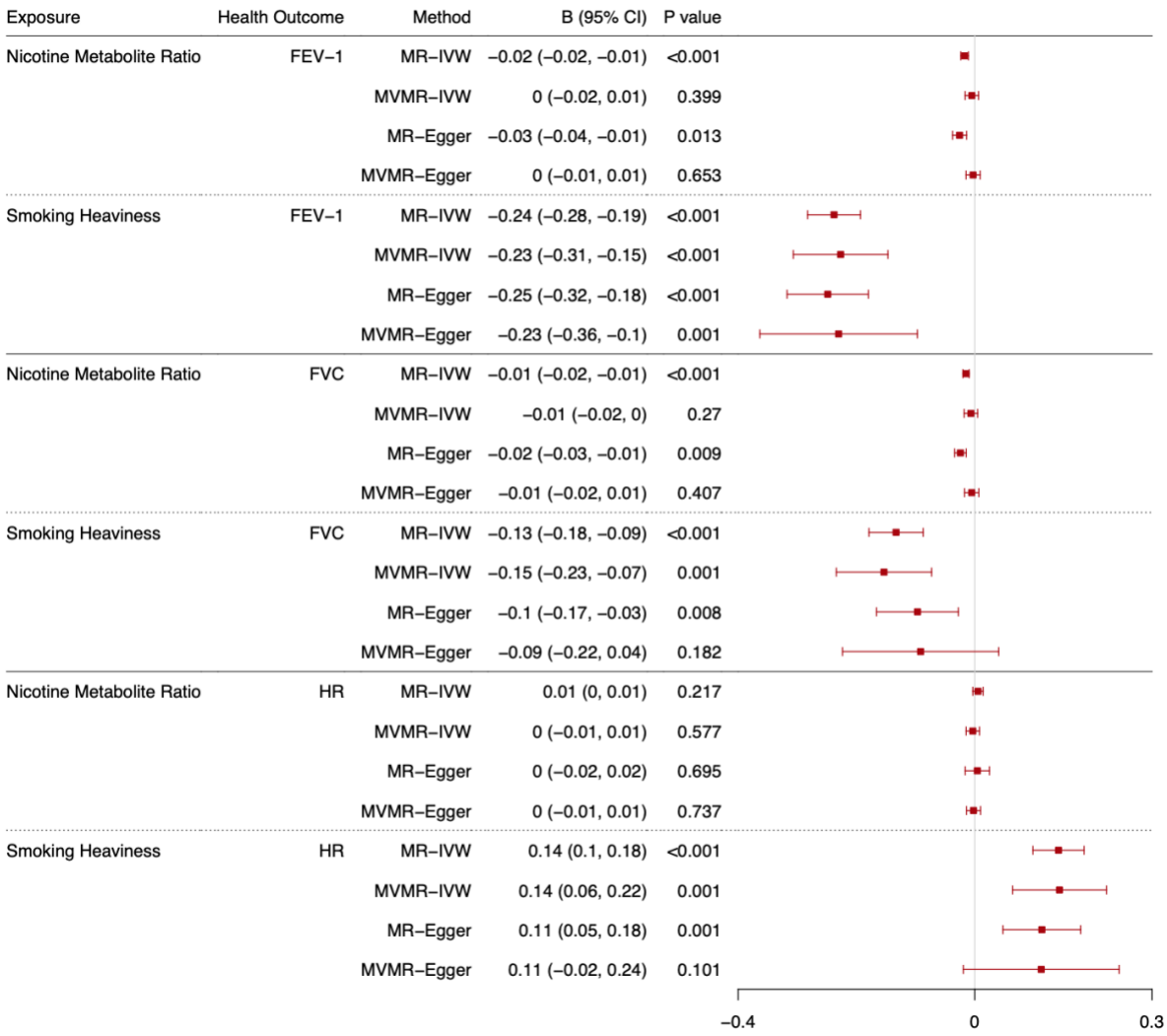

Figure 4. Forest plot displaying the effect of nicotine metabolite ratio and smoking heaviness on standard deviations of lung function (FEV-1 and FVC), and heart rate (HR): univariable Mendelian randomisation (MR) and multivariable Mendelian randomisation (MVMR) results among ever smokers.

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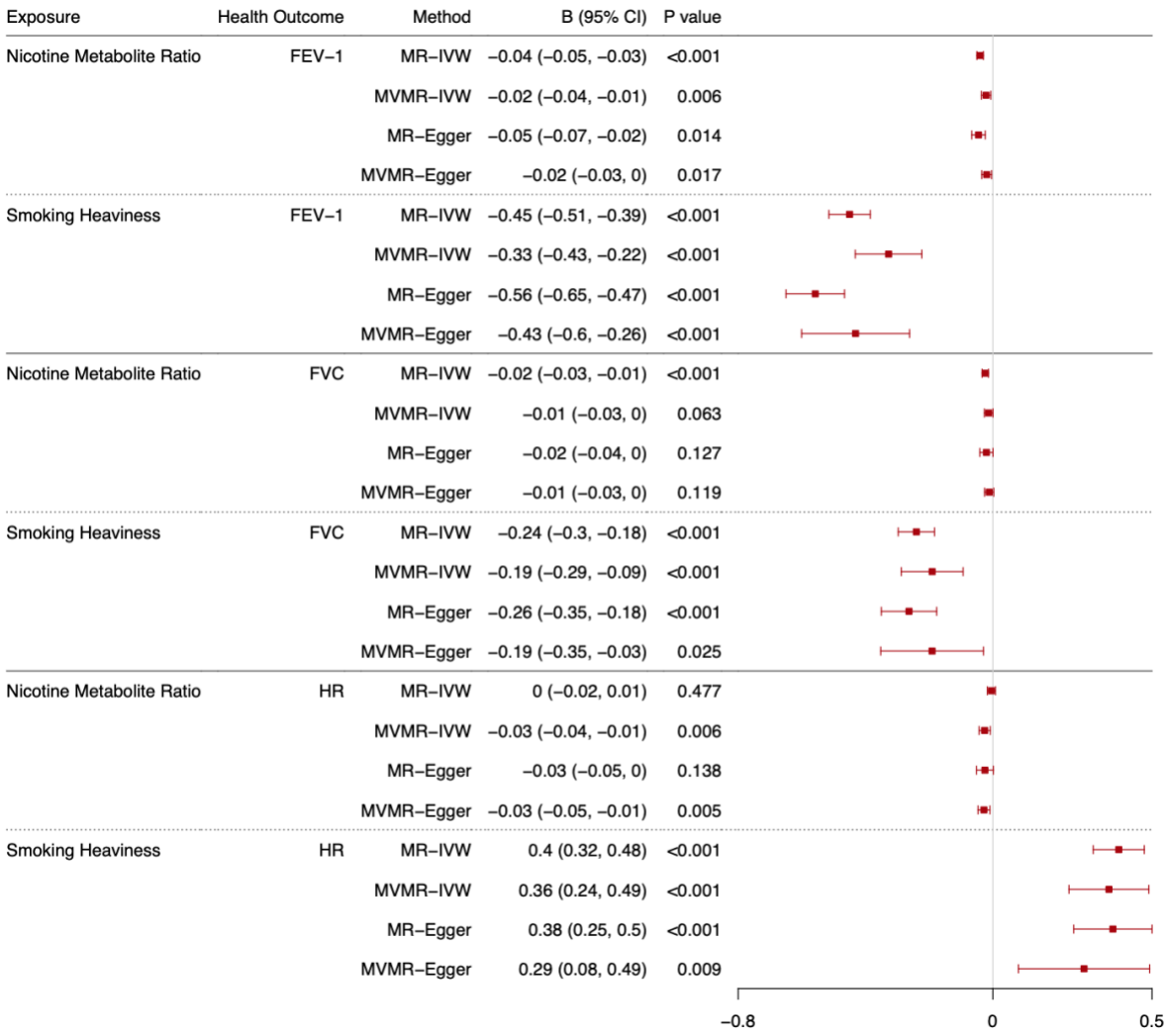
404



405

406 Figure 5. Forest plot displaying the effect of nicotine metabolite ratio and smoking
407 heaviness on standard deviations of lung function (FEV-1 and FVC), and heart rate (HR):
408 univariable Mendelian randomisation (MR) and multivariable Mendelian randomisation
409 (MVMR) results among current smokers.

410

411 *Forced Expiratory Volume in 1 Second*

412 The results relating to FEV-1 are presented in the text as changes in millilitres (mL) of

413 expiration per standard deviation increase in the exposure. The MR-IVW results indicate

414 that increased NMR and smoking heaviness both decrease FEV-1 among ever smokers

415 (Figure 4, b = -13.35, 95% CI -18.38 to -8.33, b = -185.49, 95% CI -220.39 to -150.60

416 respectively). However, there is no clear evidence of an effect of NMR on FEV-1 in the

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MVMR-IVW analysis (b = 3.82, 95% CI -12.63 to 4.98), whereas there is evidence to suggest increased smoking heaviness decreased FEV-1 among ever smokers (b = -176.78, 95% CI -239.15 to -114.41). These results are supported by the MR sensitivity analyses and MVMR-Egger results and there is no evidence of directional pleiotropy in the analyses, but there is evidence of heterogeneity in all analyses except for the NMR MR analyses. However, there is evidence of horizontal pleiotropy or bias due to population stratification in the NMR MR analyses among never smokers.

Among current smokers (Figure 5), the MR-IVW results indicate that increased NMR and smoking heaviness decreased FEV-1 (b = -33.33, 95% CI -41.76 to -24.90, b = -374.28, 95% CI -428.21 to -320.35 respectively). However, the MVMR-IVW results indicate a protective effect of nicotine exposure (i.e., lower nicotine exposure, due to higher NMR, lowers lung function) when smoking heaviness is accounted for among current smokers (NMR b = -17.77, 95% CI -29.92 to -5.62). The MVMR-IVW analyses support the MR-IVW results for smoking heaviness (b = -272.36, 95% CI -359.31 to -185.42). These results are supported by the MR sensitivity analyses and MVMR-Egger analyses. There is some evidence of heterogeneity in all analyses except the NMR MR analyses, directional pleiotropy in the MR analysis of smoking heaviness, and horizontal pleiotropy or bias due to population stratification in the NMR MR analyses among never smokers.

Forced Vital Capacity

The results relating to FVC are presented in the text as changes in mL of capacity per standard deviation increase in the exposure. The MR-IVW results indicate that increased NMR and smoking heaviness decreased FVC among ever smokers (Figure 4, b = -14.34, 95%

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CI -19.42 to -9.26, b = -129.63, 95% CI -174.27 to -85.00 respectively). However, there is no clear evidence of an effect of NMR on FVC in the MVMR-IVW analysis (b = -6.32, 95% CI -17.41 to 4.77), whereas there is evidence to suggest increased smoking heaviness decreased FVC among ever smokers, after accounting for NMR (b = -149.55, 95% CI -228.07 to -71.03). These results are supported by the MR sensitivity analyses and MVMR-Egger results, although there is less clear evidence of an effect in the MVMR-Egger results for smoking heaviness than in the MR-Egger results. There is some evidence of heterogeneity in all analyses except the NMR MR analyses, but no clear evidence of directional pleiotropy. There is weak evidence to suggest potential horizontal pleiotropy / bias due to population stratification in the NMR MR analyses and the smoking heaviness MR-IVW analysis among never smokers.

Among current smokers (Figure 5), the MR-IVW results suggest that increased NMR and smoking heaviness decreased FVC (b = -24.50, 95% CI -34.61 to -14.39, b = -246.13, 95% CI -304.36 to -187.89 respectively). MVMR-IVW results support the evidence for an effect of smoking cessation on FVC but indicate weak evidence of a protective effect of nicotine exposure when smoking heaviness is accounted for (NMR b = -13.50, 95% CI -27.39 to 0.39). The MR median and mode weighted analyses support these findings, and the MR-Egger and MVMR-Egger results are in the same direction but provide weaker support for the NMR results. There is evidence in the MVMR-IVW analysis to suggest that increased smoking heaviness decreases FVC (b -195.31, 95% CI -294.72 to -95.90), which is supported by the MVMR-Egger results. There is some evidence of heterogeneity in all analyses except the NMR MR analyses, but no clear evidence of directional pleiotropy. There is weak evidence

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464 to suggest potential horizontal pleiotropy or bias due to population stratification in the NMR
465 MR analyses and the smoking heaviness MR-IVW analysis among never smokers.
466
467 *Heart Rate*
468 The results relating to heart rate are presented in the text as changes in beats per minute
469 per standard deviation increase in the exposure. Among ever smokers (Figure 4), the MR-
470 IVW results suggest that increased NMR does not affect heart rate (b = 0.06, 95% CI -0.04 to
471 0.16), but increased smoking heaviness does increase heart rate (b = 1.61, 95% CI 1.12 to
472 2.10). The MVMR-IVW results support the results for both NMR and smoking heaviness (b =
473 -0.04, 95% CI -0.16 to 0.09, b = 1.63, 95% CI 0.73 to 2.54 respectively). The MR sensitivity
474 analyses and MVMR-Egger results also support these findings. There is some evidence of
475 heterogeneity in all analyses, but no clear evidence of directional pleiotropy. There is weak
476 evidence to suggest potential horizontal pleiotropy or bias due to population stratification in
477 the MR-IVW NMR, MR-IVW and MR-Egger smoking heaviness analyses among never
478 smokers.
479
480 Among current smokers (Figure 5), the MR-IVW results indicate no clear effect of NMR on
481 heart rate (b = -0.05, 95% CI -0.20 to 0.09) but indicate that increased smoking heaviness
482 increases heart rate (b = 4.58, 95% CI 3.65 to 5.51). However, the MVMR-IVW results
483 suggest that decreased NMR when accounting for smoking heaviness (i.e., increased
484 nicotine exposure per cigarette) increases heart rate among current smokers (b = -0.30, 95%
485 CI -0.50 to -0.10) and increased smoking heaviness accounting for nicotine exposure
486 increases heart rate (b = 4.22, 95% CI 2.77 to 5.68). The MR sensitivity and MVMR-Egger
487 results support the MR-IVW and MVMR-IVW findings. There is some evidence of

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488 heterogeneity in all analyses except the MR NMR analyses, but no clear evidence of
489 directional pleiotropy. There is weak evidence to suggest potential horizontal pleiotropy /
490 bias due to population stratification in the MR-IVW NMR, MR-IVW and MR-Egger smoking
491 heaviness analyses among never smokers.
492
493 **Interpreting the NMR MVMR results**
494 In the MVMR results above, we interpret an increase in the direct effect of NMR as a
495 reduction in nicotine exposure per cigarette smoked. Supplementary Table S14 illustrates
496 how the estimated direct effects of NMR found can be flipped to indicate the effect of
497 increased nicotine exposure per cigarette smoked on the outcome.
498
499 **Sensitivity and supplementary analysis**
500 The results of the sensitivity analyses whereby cotinine and cotinine plus 3HC were used as
501 alternative proxies for nicotine exposure can be found in Supplementary Tables S7-S13. The
502 results are generally in line with the main results with some exceptions. Increased cotinine
503 and cotinine plus 3HC exposure appears to increase risk of lung cancer (to a lesser extent
504 than non-nicotine constituents of tobacco smoke). There is also less evidence to suggest a
505 protective effect of nicotine on COPD among current smokers in the analyses alternatively
506 including cotinine and cotinine plus 3HC and there is less evidence of an effect on FEV-1 and
507 heart rate among current smokers in the analyses including cotinine plus 3HC.
508
509 The results among former smokers can be found in Supplementary Tables S7-S13. The
510 findings suggest that there are likely lasting detrimental effects of smoking, but they are
511 unlikely to be attributable to nicotine exposure.

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Discussion

Overall, we found little evidence to suggest a major detrimental effect of nicotine exposure on health when using an MVMR model to distinguish the direct effects of nicotine from the direct effects of non-nicotine components of tobacco smoke (using NMR and CPD as proxies). Our model suggests that, among current smokers, increased nicotine exposure per cigarette smoked increases heart rate but may have protective effects on lung function and COPD. Our findings in relation to smoking heaviness were generally as expected except for CHD, for which there was limited evidence of an effect. In line with previous research [15-17], increased smoking heaviness detrimentally impacted risk of lung cancer, COPD, and CHD, as well as lung function and heart rate. As expected, and in line with evidence from a range of human and animal studies [19], our analysis suggests that exposure to nicotine without the remaining constituents of tobacco smoke increases heart rate (the positive control).

In contrast to previous evidence, in which increased smoking heaviness was found to increase risk of CHD, we found limited evidence of an effect of smoking heaviness on CHD among ever smokers. Previous MR studies have also shown minimal evidence of this effect using UK Biobank data [38], which could indicate that the outcome GWAS sample may not be representative of the general population. Indeed, UK Biobank participants are more likely to describe themselves as female, have fewer health issues, live in less socioeconomically deprived areas, and be more highly educated, than the general population and thus the results may suffer from “healthy volunteer” selection bias [39]. Given that CHD may disproportionately affect men [40] and people with generally poorer health, this may

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explain the limited findings. Additionally, we may have had limited power to detect this effect given the range of causes of CHD and relatively few cases in UK Biobank.

Another unexpected finding was the apparent protective effects of nicotine on lung health (COPD, FEV-1 and FVC) among current smokers. However, this finding may be due to those with a lower NMR (and greater circulating nicotine per cigarette) needing to inhale less smoke and other non-nicotine constituents per cigarette. Measuring smoking heaviness by the number of cigarettes smoked per day will not completely capture the variation in smoking intensity, so there could be a small effect of intensity which is captured in the instrumental variable for NMR because increased NMR may lead to increased inhalation per cigarette. Given the magnitude of effects found for NMR are much smaller than for smoking heaviness, the residual variance in smoking intensity impacting the NMR estimates appears to be limited. Furthermore, if there is a true protective effect it may not be clinically important given the magnitude of the effects found. For example, patients only perceive differences in FEV-1 with changes of 112 mL [41] whereas the MVMR-IVW analyses indicated an increase of only 17.77 mL per SD increase in NMR.

In the supplementary analyses, we also saw apparent effects of cotinine and cotinine plus 3HC on lung cancer risk in the MVMR models. Given that nicotine is not thought to be carcinogenic, these findings could indicate pleiotropic pathways are involved for these additional exposures (e.g., via metabolism). This highlights that NMR may be a more appropriate proxy for nicotine exposure in this context; NMR is a measure of nicotine metabolism, therefore by including it in an MVMR model which is robust to pleiotropy via each exposure in the model, we account for pleiotropic pathways via metabolism.

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559

560 Although this is the first study to use MVMR to explore the impact of long-term nicotine
561 inhalation in humans and it may provide an early indication of the health impact of inhaling
562 nicotine via e-cigarettes, the study is not without limitations. In addition to potential issues
563 with "healthy volunteer" selection bias, the study is limited by the potential presence of
564 horizontal pleiotropy in some analyses (as indicated by effects seen in the analyses
565 restricted to non-smokers and the Egger analyses), which violates an assumption of the
566 method. The effect estimates among never smokers cannot be meaningfully interpreted as
567 we know that never smokers (or at least those who accurately self-report never smoking) do
568 not smoke any cigarettes per day despite being predisposed to heavier smoking, but
569 evidence of an effect in never smokers (along with a high Cochran's Q statistic) is indicative
570 of horizontal pleiotropy. Despite this, no effects were observed in the relationship between
571 NMR and the health outcomes in the MVMR analyses restricted to never smokers.
572 Additionally, while the MR-Egger test of directional pleiotropy indicated directional
573 pleiotropic effects in the relationship between smoking heaviness and health outcomes,
574 there was no clear evidence of directional horizontal pleiotropic effects in the relationship
575 between NMR and health outcomes among ever and current smokers. Therefore, these
576 sensitivity analyses suggest our interpretation of the direct effects of nicotine on the
577 selected health outcomes should not be impacted by directional horizontal pleiotropy or
578 population stratification.

579

580 Further limitations of the study relate to the adjustment and inclusion criteria of the GWAS
581 used for analysis. The first issue pertains to differences between the exposure GWAS
582 inclusion criteria: the GWAS of NMR was restricted to current smokers whereas the GWAS

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583 of smoking heaviness was restricted to ever regular smokers. However, smoking heaviness
584 was assessed as current or past smoking heaviness (i.e., the measure is representative of
585 the behaviour of current smokers despite being a retrospective measure, therefore we are
586 assuming that the two populations are similar). If this assumption in incorrect, we may have
587 introduced bias into our findings. Second, in contrast to the GWAS of smoking behaviour
588 and the health outcomes, the NMR GWAS was adjusted for BMI. Adjusting for BMI in one
589 exposure variable meant that we could not clearly assess the effect of nicotine on BMI,
590 however it also may impact our interpretation of results where the outcomes are associated
591 with BMI (i.e., where BMI is a plausible co-variate of the exposure and outcome
592 phenotypes) [25]. As lung and heart health could be impacted by BMI [42, 43], some of the
593 effect sizes observed may be biased towards the null. Although there are approaches
594 available to correct for this bias [44], it is not feasible to implement this correction with the
595 limited number of SNPs included in this analysis. Therefore, we must interpret these
596 findings with caution. Third, we did not use data from an updated CPD GWAS with a larger
597 sample size and more diverse sample that was released in 2022 [45] after analysis was
598 complete for this study. The study team agreed that it would be detrimental to use the new
599 data release restricted to European ancestry. The number of SNPs found to be associated
600 with smoking heaviness was three times greater in the 2022 data release compared to the
601 2019 data release, which would cause a problematic imbalance/mismatch between the
602 number of SNPs used to instrument smoking heaviness versus NMR, and potentially lead to
603 weak instrument bias [33]. Furthermore, we were unable to use data from the multi-
604 ancestry GWAS of smoking heaviness [45] as the NMR GWAS did not conduct similar
605 analyses and was restricted to those of European descent and an assumption of MVMR is
606 that all data included in an MVMR model are from the same underlying population [9].

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Future work could focus on the inclusion of multiple ancestries if appropriate NMR GWAS become available to explore whether the findings generalise to non-European ancestries.

This work highlights the limited impact that long-term nicotine exposure via inhalation (e.g., via smoking or potentially vaping) is likely to have on heart and lung health. Given that it will not be possible to explore the long-term effects of vaping for some years yet, and the impact of long-term nicotine use is usually confounded by smoking, this is an important addition to our knowledge with respect to the harms of vaping. However, there are many more potential health outcomes (e.g., the effects on cancer progression, mental health, sleep, perinatal outcomes etc.) which should be explored in future research. Additionally, the effect of vaping nicotine could be impacted by nicotine absorption potentially occurring by a different route to smoking (e.g., via oral mucosa absorption rather than lungs), or by the difference in vehicle-delivery (i.e., via liquid droplets of propylene glycol and glycerol rather than particulates) [46]. Furthermore, these findings cannot inform our understanding of non-nicotine e-cigarette vapour constituent exposure; further research is therefore needed to understand the health impact of long-term exposure to propylene glycol, vegetable glycerine and common e-liquid flavourings. Nevertheless, the findings support the safe long-term use of traditional pharmaceutical nicotine replacement therapies such as nicotine patches.

Conclusions

In conclusion, the present findings indicate that long-term nicotine use (via inhalation) when accounting for exposure to non-nicotine constituents of tobacco smoke may increase heart rate but does not increase risk of COPD or CHD and does not appear to adversely impact

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631 lung function. We found that, aside from effects on heart rate (which were expected given
632 our knowledge of effects of short-term nicotine use), there was no evidence to suggest that
633 long-term nicotine exposure is responsible for the detrimental effects of smoking on the
634 outcomes that were included in this analysis. Although further research is necessary to
635 explore other health outcomes and triangulate these findings, our results support existing
636 evidence which suggests nicotine use is not a major risk factor in the development of
637 smoking-related disease.
638

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