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Are metabolites linked to midlife cognition on the causal pathway to Alzheimer's Disease? A Mendelian randomization study

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JL performed analyses and wrote the manuscript, BJ performed analyses, reviewed and edited the manuscript; RG, AW, JX, CLQ and RD reviewed and edited the manuscript; MR and PP, conceptualized the study, reviewed and edited the manuscript.

Abstract

There are currently no disease modifying treatments for Alzheimer's Disease (AD), and an understanding of preclinical causal biomarkers to help target disease pathogenesis in the earliest phases remains sparse. Here, we investigated whether nineteen metabolites previously associated with midlife cognition – a pre-clinical predictor of AD – translate through to later clinical risk, using Mendelian randomization (MR) to tease out AD-specific causal relationships.

Summary statistics from the largest Genome-Wide Association Studies (GWAS) for AD and metabolites were used to perform bi-directional univariable MR. Bayesian model averaging (MR-BMA) was additionally performed to address high correlation between metabolites and to identify metabolite combinations which may be on the AD causal pathway.

Univariable MR indicated four Extra-Large High-Density Lipoproteins (XL.HDL) to be on the causal pathway to AD: Free Cholesterol (XL.HDL.FC: 95% CI=0.78-0.94), Total Lipids (XL.HDL.L: 95% CI=0.80-0.97), Phospholipids (XL.HDL.PL: 95% CI=0.81-0.97), and concentration of XL.HDL particles (95% CI=0.79-0.96); significant at an adjusted $p<0.009$. MR-BMA corroborated XL.HDL.FC to be amongst the top three causal metabolites, additionally to Total Cholesterol in XL.HDL (XL.HDL.C) and Glycoprotein Acetyls (GP). Both XL.HDL.C and GP also demonstrated suggestive univariable evidence of causality ($p<0.05$), and GP successfully replicated within an independent dataset.

This study offers insight into the causal relationship between metabolites previously demonstrating association with mid-life cognition, and AD. It highlights GP in addition to several XL.HDLs – particularly XL.HDL.FC - as causal candidates warranting further investigation. As AD pathology is thought to develop decades prior to symptom onset, progressing these findings could hold special value in informing risk reduction strategies.

Significance Statement

The absence of disease modifying therapeutics for Alzheimer's Disease (AD) continues, and an understanding of early, easily accessible biomarkers to inform treatment strategies remains sparse. To our knowledge, this study is the first to use knowledge of blood metabolites previously associated midlife cognition – a pre-clinical predictor of AD – to systematically investigate causal associations with later AD status. Given that the pathological changes underlying AD are thought to develop years before clinical manifestations of the disease, developing these findings further could hold special utility in informing early treatment intervention.

Main Text

1. Introduction

More than 50 million people worldwide currently live with dementia, and with an aging world population this figure is expected to increase to more than 152 million by 2050 (World Alzheimer Report 2018). The most common dementia type is Alzheimer's Disease (AD), characterised by impaired everyday function, severe cognitive decline - particularly working, episodic, and declarative memory (1) - and a range of neuropsychiatric symptoms (2). It represents a major source of global morbidity and mortality and poses significant human and economic costs (3).

Disappointingly, AD drug development has proven difficult, with a 99.6% failure rate in the decade of 2002 to 2012, and this rate continues at the same low level today (4). Numerous reasons have been proposed as to why such clinical trials have failed, including incomplete understanding of true causal mechanisms and a failure to intervene early enough in the pathological cascade. It is therefore necessary to discover biomarkers that can identify individuals at high risk of developing AD and at the earliest possible stages of pathology onset. Moreover, it is important for these to be potentially modifiable so as to offer targets for preventative or therapeutic strategies.

Metabolomics represents one avenue that may give a deeper insight into AD aetiology. Metabolites are small molecules (<1500 atomic mass units) with a role in metabolism (5). As the products of many biological processes, they sit at the end of the systems biology pathway and therefore represent effective intermediate phenotypes to a given disease due to their proximity to the clinical endpoint (6,7). Due to 1) their non-invasive nature of measurement, 2) the fact that they are potentially modifiable through diet and lifestyle, and 3) the ability of many to cross the blood brain barrier, blood metabolites are both practical and valuable markers of biological processes and disease states in dementia (8).

Markers of lipid metabolism have received particular attention in this context, as the impairment of lipid metabolism has been associated with Alzheimer's disease (5,8–11) and beta-amyloid (Aβ) burden (12,13). Relevant to early intervention, they have also been associated with cognitive performance and brain function during normal ageing (14,15). Recently, using a large British population-based birth cohort, we investigated associations between 233 blood metabolites and both memory and processing speed at 60–64 years of age, as well as changes in these cognitive domains from 60–64 to 69 years old. Associations with several metabolite classes were observed, including fatty acids (FAs), various compositions of high-density lipoproteins (HDLs) and glycoprotein acetyls (GP) (16).

However, it is not yet established whether these metabolites are causally associated with dementia and AD. Using knowledge from these preclinical associations to investigate translatability to later AD risk could hold special utility in informing early treatment intervention, particularly if a causal relationship can be shown. This study therefore aims to expand our observational findings and assess whether nineteen blood metabolites previously associated with late midlife cognition causally associate with later clinical AD status. Both univariable and Bayesian multivariable Mendelian Randomization (MR) approaches are harnessed to interrogate independent as well as group associations, and a range of sensitivity analyses performed to further scrutinize results. Identifying candidate blood metabolites which are detectable pre-clinically and on the causal pathway to later AD diagnosis, will aid in facilitating further research into early intervention strategies and more targeted therapeutics.

2. Results

2.1. Metabolite Selection

Metabolite data were obtained from summary statistics of the latest and largest metabolite genome-wide association study (GWAS) which investigated the genetic component of 123 blood metabolites on nearly 25,000 individuals (17) data: http://computationalmedicine.fi/data#NMR_GWAS). Of the 123 metabolites available for analysis, selection was based on our previously published observational study, which investigated associations between blood metabolites and lifetime cognition using data from the MRC National Survey of Health and Development (1946 British birth cohort) (18). Briefly, this study measured the association between three domains of cognition (short-term memory, delayed verbal memory, and processing speed (19), and levels of 233 blood metabolites in 798 participants aged 60-64 (18,20), and then again at age 69 (*N*=633) (18). Twenty metabolites were significantly associated with at least one measure of mid-life cognition in our observational study, and 19 of these were causally investigated within the present study (for further information, see Methods).

2.2. Primary analyses

2.2.1. Bidirectional univariable MR

Using metabolite data from Kettunen et al.(17) together with clinically diagnosed AD data from Kunkle et al. (21) a series of two-sample univariable inverse-variance-weighted (IVW) MR analyses were conducted to investigate the bi-directional causal relationship between each of the selected metabolites and AD. For strong evidence of causality, estimates were required to demonstrate association below an adjusted significance threshold of $p<0.009$ (SI Appendix, Info. S3). By this criterion four metabolites retained strong evidence of an inverse causal association with AD: Free Cholesterol in Very Large HDLs (XL.HDL.FC)(*OR*=0.86, *95% CI*=0.78-0.94, $p=0.001$), Total Lipids in Very Large HDLs (XL.HDL.L)(*OR*=0.88, *95% CI*=0.80-0.97, $p=0.008$), Phospholipids in Very Large HDLs (XL.HDL.PL)(OR=0.89, *95% CI*=0.81-0.97, $p=0.008$), and Concentration of Very Large HDL particles (XL.HDL.P)(*OR*=0.87, *95% CI*=0.79-0.96, $p=0.004$). GP also demonstrated evidence of suggestive causal association, with IVW estimates indicating increased odds of AD given higher GP levels (*OR*=1.20 *95% CI*=1.05-1.38), and both HDL.D and XL.HDL.C demonstrated nominally significant associations in the negative direction (HDL.D: *OR*=0.89, *95% CI*=0.80-0.99, XL.HDL.C: *OR*=0.88, 95% *CI*=0.79-0.99); though p-values did not reach adjusted significance ($p>0.009$)(Dataset S1, Figure 1, and SI Appendix, Fig. S1a-Ss).

For seven large and one small HDL (L.HDLs and S.HDL respectively) (Dataset S2), SNP IVs within the ApoE genomic region were removed prior to analyses due to known violations to core MR assumptions (see Methods). The predicted causal effect for each of the L.HDLs on clinical AD using non-ApoE related IVs were in the negative direction with a similar magnitude of effect across point estimates (*OR* range: 0.89-0.91). 95% confidence intervals remained in the negative direction for all seven L.HDLs (Figure 1, Dataset S1), though only nominal significance was reached ($p<0.05$) (Dataset S1), and not for S.HDL.TG. No other metabolites were found to be genetically predicted by ApoE.

When exposure and outcome were reversed to investigate the potential for reverse causation, there was no evidence of a causal relationship in the opposite direction, from AD to metabolite. Using 24 independent SNP IVs, excluding those within the ApoE genomic region, significance did not exceed $p<0.1$ (Dataset S3, SI Appendix, Fig. S2-S3s).

2.2.2 Bayesian model averaging MR

Metabolites demonstrate notable correlation both phenotypically (22) and genetically (Dataset S4). Consequently, a high degree of instrumental variable overlap is identifiable across metabolites in univariable analyses (Dataset S5). Univariable approaches, whilst useful for identifying individual causal associations, assume exposures to be independent and thus, (1) neglect instances in which "group" relationships may exist, and (2) do not allow for the effect of inter-related exposures to be disentangled by-way of removing non-independent signal. Bayesian model averaging MR (MR-BMA)

offers an alternative approach which allows multiple metabolites to be modelled together. In this way, sub-groups of metabolites which may act together on the causal pathway to AD may be identified and independent metabolites can be appropriately ranked according to their independent causal signal. Thus, this method allows related metabolites to be disentangled to identify which may be driving the true causal signal over others. Like conventional multivariable MR, the inclusion of multiple exposures with overlapping instruments allows for "measured pleiotropy" to be sufficiently handled (22). Unlike conventional multivariable MR (23) however, this method also scales particularly well to high-throughput and highly correlated data (22).

Following the pruning of metabolites with genetic correlations >95% (including the removal of univariably significant XL.HDL.L, XL.HDL.PL, and XL.HDL.P), nine metabolites were jointly analyzed (See Methods, and Dataset S4). Results of single-metabolite causal rankings in accordance with their marginal posterior probability (MIP) are presented in Table 1. As this is a Bayesian method, frequentist p-values are unavailable. Instead inferences can be made on the basis of posterior probabilities and ranking performance. Those ranked with the highest MIP are indicative of being the strongest "true causal" candidates over those of lower rank. Table 1 also confirms corresponding model average causal effect (MACE) estimates, reflecting the average direct effect of each metabolite on AD, independent of contributary signal from any other metabolites included within the model. It is worth noting that the purpose of MR-BMA is to correctly detect (by-way of ranking) true causal risk factors rather than to unbiasedly estimate the magnitude of the direct causal effect, as these will be biased towards the null due to shrinkage applied in variable selection (22). MACE can be used however, to gain insight into the direction of effect and magnitude relative to other metabolites included within the model. GP was estimated as the highest ranked causal metabolite (*MIP*=0.465, *MACE*=0.09), followed by three XL.HDL particles (XL.HDL.C: *MIP*=0.179, *MACE*=-0.02; XL.HDL.FC: *MIP*=0.178, *MACE*=-0.02; XL.HDL.CE *MIP*=0.164, *MACE*=-0.02). When whole models, with variations of metabolite combinations were assessed, these same four metabolites were present within the four highest ranked causal models, with model-based posterior probabilities (*pp*s) of 0.287, 0.113, 0.112, and 0.102 for GP, XL.HDL.C, XL.HDL.FC, and XL.HDL.CE respectively (Table 2).

2.3. Sensitivity analyses

2.3.1 Univariable MR

When causal relationships were re-estimated using MR-Egger and weighted median (conservative methods which are sensitive to pleiotropy and instrument invalidity), directionality of results were in agreement with all nominally significance metabolite exposures ($p<0.05$) from primary analyses. Confidence intervals were, however, wider, resulting in a number of estimates crossing the null (Figure 1). The intercept from MR Egger estimates demonstrated no evidence of horizontal pleiotropy (Dataset S1). Funnel plots also demonstrated symmetrical distribution of SNP effects around the effect estimate for most tests, suggesting balanced pleiotropy, although this was not the case for metabolites with small SNP *N* (SI Appendix, Fig. S4a-S4s). MR-PRESSO – a method for detecting and correcting for outliers within the data – demonstrated attenuated *p*-values for all four metabolites which were strongly associated in primary analyses ($p<0.009$: XL.HDL.FC, XL.HDL.L, XL.HDL.P, XL.HDL.PL). Significance at the 5% level was however, retained and no significant outliers were detected (Dataset S1). Leave-one-out on the other hand, indicated two influential SNPs (rs1532085, rs261291) for most HDL sub-fractions, and one influential SNP was also found for GP (rs77303550) (SI Appendix, Fig. S5a-S5s). Removal of these SNPs resulted in wider confidence intervals, with only XL.HDL.FC retaining significance at $p<0.05$. Leave-one-out analyses when AD was set as the exposure indicated no notable outliers (SI Appendix, Fig S6a-S6s). MR-PRESSO on the other hand, did detect outliers but the corrected p-value upon removal of these remained in agreement with primary tests (Dataset S3). As an additional sensitivity analysis, non-inferable palindromic SNP

instruments were dropped from analyses and MR estimates re-computed. This resulted in almost identical results across IVW, MR-Egger, and weighted median results (Dataset S6).

2.3.2. MR-BMA

Sensitivity analyses consisted of 1) Q-statistic computation to identify heterogeneous/outlier instruments, and 2) Cook's distance (Cd) to identify influential points within the top models identified. Q-statistics indicated no deviant instruments (all Q<10. SI Appendix, Fig. S7a-S7d). The genetic variant with the largest *Cd* was rs1532085, near the *LIPC* gene (SI Appendix, Fig. S8a-S8c and Fig S9a). This had a *Cd*>0.19 in all three XL-HDL models (XL.HDL.C: *Cd*=1.095; XL.HDL.FC: *Cd*=1.25; XL.HDL.CE: *Cd*=1.168). rs2575876 on the *ABCA1* gene (SI Appendix, Fig. S8a-8c and Fig S9b), also demonstrated a high *Cd* in all three XL-HDL models (XL.HDL.C: *Cd* =0.392, XL.HDL.FC: *Cd*=0.247; XL.HDL.CE: *Cd*=0.302), and variant rs247617, near the *CETP* gene (SI Appendix, Fig. S8a-8b and Fig S9c), also had high *Cd* in XL.HDL.C (*Cd*=0.229) and XL.HDL.FC(*Cd*=0.265). Finally, variant rs77303550 on the *TXNL4B* gene (SI Appendix, Fig. S8d and Fig 9d), had a high *Cd* in the GP model (*Cd*=0.518), though was <0.19 in all other models (SI Appendix, Fig. S8a-S8c). A full overview of Q-statistics and *Cds* for the top 4 MR-BMA models are presented in Dataset S7. Removal of influential points reduced MIPs, particularly for HDLs, but did not substantially change results (Dataset S8 and Dataset S9). All MR-BMA results remained consistent when re-ran with non-inferable palindromic SNPs removed (Dataset S10).

2.4. Post-hoc exploratory analyses

2.4.1. LD overlap between influential points and AD

Two core assumptions of MR are 1) the "exchangeability assumption" – that is that the effect of an IV on the outcome does not occur due to confounding – and 2) the "exclusion restriction assumption", which assumes that the association between an IV and outcome occurs only via the exposure of interest (23). Within primary scope (see SI Appendix, Info. S1), any IVs associated with the outcome at genome-wide significance were removed due to potential violations to either of these assumptions. However, violations may also occur if IVs utilised represent the same locus as genes known to significantly associate with the outcome. To explore this further, we visually inspected LD-regions of each influential point and cross checked whether any of these spanned gene-regions previously shown to associate with AD, using information from Kunkle et al. (21). Locus zoom plots are presented within SI Appendix (Fig. S9a-S9d), and confirmation of Kunkle lead SNPs and related genomic regions are presented in Dataset S11. No overlap was observed between any of our influential point regions and genomic regions identified as being associated with AD SNPs. Influential point rs1532085 was however, observed to be located within the LIPC gene, which is located <50kb from ADAM10 – a gene associated with the lead rs593742 SNP from Kunkle et al. (21). To inspect this further, an additional visualisation was produced for rs593742 using data from Kunkle et al. (21) (SI Appendix, Fig. S9e). Whilst rs593742 was found to overlap with the LIPC region, no evidence of LD between the HDL related rs1532085 SNP nor the AD related rs593742 specifically was observed, and there was no evidence of overlap between rs1532085 LD SNPs and ADAM10 – indicating independence of this region (https://www.ncbi.nlm.nih.gov/gene).

2.4.2. One-sample univariable MR

To further interrogate the validity of findings from MR analyses, baseline individual level data from the Alzheimer's Disease Neuroimaging initiative (ADNI) (24) were utilised to perform a small-scale replication using 2-stage least squares (2SLS) methodology. Here, we obtained NMR metabolite data for those metabolites demonstrating adjusted significance within primary univariable analyses (XL.HDL.FC, XL.HDL.L, XL.HDL.PL, XL.HDL.P) (*N*=878), and for the highest ranked causal metabolite identified by Bayesian model-averaging MR (GP) (*N*=894). An adjusted significance threshold of $p<0.02$ - representing 2.45 independent tests, accounting for correlation structures amongst metabolites (see SI Appendix, Info. S3) - was expected to demonstrate strong evidence of causality. In line with this criterion, GP was the only metabolite to successfully replicate at the adjusted level ($p=0.004$). Directionality was in agreement with primary analyses, with an effect size of

297 greater magnitude, but larger window of uncertainty (*OR*=2.28, *95% CI*=1.3-4.0). No other metabolite
298 reached adjusted significance. However, weighted F statistics for each metabolite ranged from 5.85-
299 8.55, indicating low instrument strength to detect causal estimates (Dataset S12).
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3. Discussion

The absence of disease modifying therapeutics for Alzheimer's Disease (AD) continues, and an understanding of early, easily accessible biomarkers to inform treatment strategies remains sparse. Using knowledge of associations between pre-clinical risk factors and potential biomarkers and assessing how well such markers translate through to later clinical risk could therefore hold special utility in informing early treatment intervention, particularly if a causal relationship can be shown. To our knowledge, this study is the first to use blood metabolites previously associated with midlife cognition to systematically investigate causal associations with later AD status. Using summary data from the largest metabolomics and AD GWASs to date, causality was interrogated using a combination of both bidirectional univariable and Bayesian model-averaging (BMA) Mendelian Randomization (MR), with results further scrutinised using a range of sensitivity and post-hoc measures. Primary analyses indicated an inverse causal relationship between sub-fractions of extra-large HDL molecules – particularly XL.HDL.FC – and AD, indicating a protective effect. Glycoprotein Acetyls (GP) on the other hand, when modelled with consideration of other metabolites, demonstrated evidence of a direct casual effect in the positive direction, indicating that this metabolite may contribute to increased AD risk. GP's risk increasing effect was further supported in an independent small-scale replication using individual level data.

Within the medical literature, higher levels of high-density lipoproteins (HDLs) are commonly referred to as being health promoting, demonstrating vascular protective properties and a consistent association with lowered cardiovascular and stroke risk (25–29). In-line with this health-promoting hypothesis, our primary analyses found evidence for a causally protective effect of XL.HDLs on clinical AD diagnosis. Of these, free cholesterol in extra-large HDLs (XL.HDL.FC) demonstrated particular pertinence, representing the strongest univariable relationship with AD and showing the greatest consistency across both univariable and Bayesian methods. Three addition XL.HDLs (XL.HDL.P, XL.HDL.PL, XL.HDL.L) demonstrated evidence of a protective effect in univariable analyses, significant at $p<0.009$. These were, however, excluded from MR-BMA due to a genetic correlation >95% with other HDLs. This non-independence of genetic signal could indicate that the univariable causal effect of these three metabolites captures signal across the HDL metabolite family as opposed to demonstrating specificity for the individual sub-fractions themselves. The benefit of MR-BMA is that it is able to disentangle these intertwined effects, and indeed, whilst XL.HDL-P,PL and L were removed from BMA models, XL.HDLs remained implicated, with both XL.HDL-FC and -C ranking within the top 3 independent causal metabolites, and effects remaining in the protective direction. Our exploratory post-hoc analyses on the other hand, failed to replicate XL.HDL associations. However, small sample N ($N<900$) and weak instrumental strength (F-statistics <10) imply that this may simply reflect a lack of power in our replication cohort.

Evidence of an protective effect also extended to a number of large HDLs in univariable analyses. Though these did not reach adjusted significance, they demonstrated consistent negative directionality in both primary and sensitivity analyses, and retained significance at the 5% level for inverse variance weighted (IVW) estimates. The protective effect observed for HDLs corroborate our previous observational study which demonstrated positive associations of HDLs and mid-life cognition, indicative of potential neurocognitive protective properties. HDLs have also been implicated more widely in age-related cognitive decline and dementia (30), with evidence from human studies, animal models, and bioengineered arteries of a cerebrovascular protective effect, which commonly show dysfunction in AD (31). Results are also supported by existing AD GWAS, with SNP associations found near genes encoding HDL protein components and biogenesis proteins such as *APOE, ABCA1, APOA1 &2, CLU, LCAT and CETPI* (31). Previous MR studies, including ours (32,33) have failed however, to show a causal link between HDL levels and AD. This is potentially due to insufficiently capturing HDL composition complexity. To our knowledge, this study represents the first to provide deeper granularity through inclusion of specific sub-fractions and sizes of HDL, and to account for the interrelated structure of such sub-fractions through use of Bayesian multivariable methodology.

GP – a marker of inflammation – demonstrated a causal association in the positive direction, both in univariable analyses and MR-BMA. As with large HDLs, univariable results remained significant at the $p<0.05$ level only. However, when direct effects were measured using MR-BMA – accounting for interrelation amongst metabolites - GP was estimated to have the largest causal effect of all

metabolites within the model and demonstrated the highest posterior probability of existing within the true causal model. Further, GP was the only metabolite to successfully replicate within a small-scale independent cohort, though instrument power was low (F<10). This risk-increasing relationship aligns with our previous study(16), which observed an association between GP and lower cognitive ability in late midlife; consistent with findings from a large independent cohort (14). Additionally, A1-acid glycoprotein has been shown to be a strong predictor of 10-year mortality (34) as well as all-cause mortality in a recent large meta-analysis of >40K individuals (35). Changes in the level of several glycoproteins have also been observed in the hippocampus and inferior parietal lobe in human AD (36). Some of these glycoproteins interact with neurofibrillary tangles, leading to speculation that changes in their glycosylation may be associated with the pathogenesis of this disease (36).

Interestingly, while our previous observational study found the strongest associations to be between fatty acids and late midlife cognition, the present study found no evidence for causal associations between these and AD. This may in part be due to only a low number of instruments available for fatty acids (five SNPs available for both omega-3 and DHA, and six available for mono-unsaturated fatty acids (MUFA)), resulting in a lack of statistical power to detect a causal relationship between these metabolites and AD. Alternatively, this inconsistency could be attributable to the different outcome phenotypes (cognition verses AD), with fatty acids potentially being associated with non-AD related cognitive decline, but not AD specifically. Finally, observed associations between fatty acids and cognition may simply reflect confounding, highlighting the importance of methods such as MR for disentangling such scenarios. Future research on larger, independent samples will be an important endeavour to better understand the discrepant findings observed here.

Strengths of this study include the use of the largest and most up to date GWASs available for both NMR metabolomics and AD. Being the first of its kind to utilise knowledge from preclinical associations between metabolites and midlife cognition also allows a window of insight into causally relevant metabolites which may hold utility pre-clinically. Moreover, through use of bidirectional MR, relationships were interrogated in both directions as opposed to relying on a-priori (potentially erroneous) assumptions about directionality. Employment of MR-BMA also allowed for correlations between metabolites to be accounted for and for multivariable models of combined metabolites to be proposed. Further, the inclusion of sensitivity analyses across univariable and multivariable models allowed for further interrogation of MR assumptions, ensuring that any notable changes in results could be investigated. This was further extended through the addition of a small-scale post-hoc replication using independent, individual level data.

There remain, however, some limitations. First, power. For several metabolites, less than ten genetic variants were available at genome-wide significance, with two having only five variants available at this level. Whilst steps were taken to ensure individual SNPs did not suffer from weak instrument bias through calculation of per-instrument F-statistics, we cannot exclude the possibility of false negative errors due to insufficient statistical power. Power was also a notable drawback within replication analyses, with a sample *N* of up to 894 in comparison to ~25,000 and ~95,000 for metabolite and AD summary data respectively in a priori analyses. This was reflected in instrument strength, with no metabolite reaching an F-statistic >10. Whilst replication proceeded as an exploratory step, with the view that internal validation when possible, is important to assess consistency of findings, such post-hoc results should be considered with caution until further replications of greater sample size can be considered. Second, due to the absence of available stratified GWA data, the present study was unable to stratify on key variables such as sex – something which our previous observational study indicated may modify many metabolite-cognition associations, and may plausibly too, modify metabolite-AD associations (16).

A third limitation lies with exclusion of ApoE related instrumental variables. This was necessary due to known associations between ApoE and non-AD traits, such as coronary artery disease (37), violating the MR exchangeability assumption. However, as ApoE is directly implicated in the production of lipoproteins and lipid metabolism (38), its removal likely attenuated observed causal associations. This is of particular relevance to large HDLs given that, for those models where ApoE instruments were removed, evidence of a negative causal relationship was observed at the nominal level but failed to reach adjusted significance. It remains plausible – particularly given the opposing direction of ApoE related effect sizes between HDLs and AD, equating to a negative association (see Dataset S13) – that this reflects attenuated power which would otherwise have been recovered with the

addition of ApoE instruments. Finally, whilst several IVW causal associations were observed, sensitivity analyses revealed a number of influential points and wider confidence intervals, resulting in a loss of significance. Influential points may arise for a number of reasons, one of which being due to violations of MR exchangeability and exclusion-restriction assumptions. Whilst instrument validity can never be concluded with certainty, steps were taken to mitigate violations, such as the removal of instruments with known pleiotropy, and exclusion of SNPs demonstrating genome-wide significance with the outcome of interest. Moreover, post-hoc visual analyses indicated no LD between influential points within this study and gene regions associated with lead AD SNPs from the latest GWAS conducted by Kunkle and colleagues (21). Together, these add weight to assumptions of instrument validity. Both MR-Egger and weighted median were introduced as a means for re-estimating causal estimates in the presence of potential pleiotropy. Failure of these to detect a causal effect could therefore indicate violation to MR assumptions. Robust method estimates do however, have greater imprecision than that of IVW estimates. As such, they commonly present with larger windows of uncertainty and lower power to detect causal estimates (39). MR-Egger also provides a test of pleiotropy via its intercept and this indicated no significant pleiotropy across any of our IVW estimates. Moreover, no significant heterogeneity was observed, and consistent directionality for point estimates were maintained across different univariable methodologies. Additionally, MR-BMA – a method able to account for measured pleiotropy – largely corroborated univariable findings, ranking XL.HDLs and GP as the most likely causal metabolites of those included. Taken together, the weight of evidence supports IVW conclusions, with no indication that core model assumptions have been violated. Instead, a loss of significance in sensitivity measures are likely a reflection of higher imprecision and low statistical power.

As the pathological changes underpinning AD are thought to develop at least a decade prior to the onset of symptoms, it is important to identify modifiable targets for intervention at an early stage, before AD pathology has caused major irreversible damage. This study represents the first to utilise knowledge of pre-clinical associations between metabolites and mid-life cognition to investigate causal associations between early candidate biomarkers and later AD risk. Findings highlight GP as a particularly promising risk-increasing metabolite, and XL.HDLs – particularly XL.HDL.FC – warrant further follow-up as protective candidates on the AD causal pathway. Progressing these findings could hold special value in informing future risk reduction strategies.

4. Methods

A flow diagram summarising the methodology is detailed in Figure 2. A Document containing further details on motivation and scope in line with MR reporting guidelines outlined by Burgess et al. (39) is provided in SI Appendix, Info. S1.

4.1. Data sources

Summary statistics from the latest and largest metabolite GWAS were used for all MR analyses (17) (data: http://computationalmedicine.fi/data#NMR_GWAS). This GWAS investigated the genetic component of 123 blood metabolites on nearly 25,000 individuals using NMR spectroscopy. This platform provides a detailed characterisation of metabolite measures and ratios representing a broad molecular signature of systemic metabolism. Multiple metabolic pathways were covered, including: lipoprotein lipids and lipid sub-classes, FAs and FA compositions, and amino acids and glycolysis precursors. Specific details are described elsewhere (40–42).

Of the twenty metabolites previously associated with cognition, all had at least one single nucleotide polymorphism (SNP) association at genome wide significance (GWS)($p<5*10^{-8}$). However, as only two GWS SNPs were available for Pyruvate, this metabolite was removed due to power concerns, leaving nineteen metabolites for MR. To avoid weak instrument bias, a computed F-statistic of at least 10 was also required for all SNP instruments.

For AD, summary statistics from the latest GWAS of clinically diagnosed late-onset AD (LOAD) by Kunkle and colleagues were utilised (21). This study consisted of three stages; 1) a discovery phase of 63,926 samples, 2) a replication phase of 18,845 samples, and 3) a post replication phase of 11,666 samples. For MR with AD as an outcome, stage 1 summary data were utilised, and for MR with AD as an exposure, stage 1&2 data were employed.

4.2. Mendelian Randomisation

4.2.1. Univariable analyses investigating metabolites as causal risk factors for AD

4.2.1.1. SNP Selection

All data extraction, pre-processing, and analyses were performed within R.3.6.1. using the MRBase package(v.0.4.25) (43). SNP instruments selected for each metabolite were those available within the metabolomic quantitative trait loci (mQTL) catalogue within MRBase. All mQTLs available within this catalogue were pre-curated using the data from Kettunen et al. (17), and only independent instruments made available for selection. For each metabolite, summary statistics consisting of effect sizes, standard errors and p-values for all GWS SNPs were extracted from each of the GWAS datasets (17). SNPs associated with AD at GWS were excluded due to potential violation of the MR exchangeability assumption (39), which assumes SNP instruments are not associated with confounding risk factors. Any SNPs within the ApoE genomic region (chromosome 19, base-pairs 4500000-4580000) were also excluded for this reason, as ApoE is an established risk factor for traits additional to AD, such as coronary artery disease (37). This resulted in SNP exclusions from large HDL subclasses only (Dataset S2, Dataset S13). Data were harmonised between AD and metabolite datasets, and SNPs with MAF<0.01 were excluded. All GWAS were assumed to be coded on the forward strand, thus no palindromic SNPs were excluded from analyses. However, Additional sensitivity analyses were performed excluding non-inferable palindromic SNPs (MAF>0.40), with metabolite MAFs used to infer AD allele frequencies, due to MAF non-availability within the AD dataset.

4.2.1.2. Primary analyses

Total causal estimates were computed using inverse variance weighted (IVW) two-sample MR, setting each metabolite as the exposure in turn and AD as the outcome. Briefly, IVW-MR uses a univariable model to regress SNP-instrument associations with an outcome on SNP-instrument associations with an exposure, weighted by the inverse of the variance in SNP-outcome associations (44). To reflect MR's 'exclusion restriction assumption', which states that SNP instrument(s) must only be associated with the outcome via the exposure (44), the IVW intercept is constrained to zero. Results are presented in OR per 1-SD unit to enable a comparison of the magnitude of effect across all exposures.

4.2.1.3. Sensitivity analyses

Two robust methods – MR-egger and weighted median – were utilised to re-estimate casual associations with IVW assumptions relaxed. Briefly, MR-egger re-estimates IVW causal estimates whilst removing the intercept constraint. Large deviations from 0 are taken as evidence of violation to MR's exclusion restriction and exchangeability assumptions (45); and large discrepancies between egger and IVW estimates are indicative of pleiotropy. Weighted median provided an alternative estimate which remains valid provided 50% of instruments are valid (46). Briefly, causal estimates for each instrument are ordered and weighted by their association strength. The final estimate is then taken as the 50th weighted percentile of the ordered estimate. Influential points were investigated using leave-one-out analyses, and Cochran's Q was calculated to test for heterogeneity amongst instruments (Q-p<0.05 indicating significant heterogeneity). MR Pleiotropy RESidual Sum and Outlier (MR-PRESSO) test was further utilised to identify and correct for potential bias in estimates due to pleiotropy (47). Briefly, this test consists of up to three parts, with 1) the "global test" providing an estimate for the degree of horizontal pleiotropy (significant pleiotropy indicated by p<0.05), 2) the "outlier corrected causal estimate" providing a corrected estimate for any significant pleiotropy detected, and 3) the "distortion test" providing an estimate for the degree to which the original and corrected estimates differ (p<0.05 indicating a significant difference following corrections for pleiotropy). Tests 2 and 3 are implemented only in cases where p<0.05 for global test estimates.

4.2.2. Univariable analyses investigating AD as a causal risk factor for metabolite levels

To explore causality in the opposite direction, AD was set as the exposure with each metabolite in turn set as the outcome. The same analysis pipeline followed as above, testing the association of GWS SNPs from Stages 1&2 of Kunkle et al. (21). Following clumping (using an R^2 threshold of 0.001), and the removal of ApoE SNPs or those with MAF<0.01, 24 SNPs were utilised as instrumental variables in causal analyses (Dataset S14).

4.2.3. Bayesian Model Averaging

4.2.3.1. Data preparation

MR-BMA adopts a multivariable framework, whereby multiple exposures can be included within the model, provided a) they are each robustly associated with a least one SNP-instrument used within the model, and b) they do not induce multi-collinearity (22). As with univariable models, criterion a) was met through inclusion of only GWS instruments which also had a computed F-statistic of >=10. To meet criterion b), pairwise genetic correlations (rg) across metabolites were computed using linkage-disequilibrium score regression (LDSC) (48). In preparation for this, all GWAS summary statistics underwent a process of data munging. During this, if data were reported with a mean chi^2 statistic <1.02, that dataset was dropped from LDSC analyses (Dataset S15) due to non-suitability as advised by the software authors (48). Any metabolites with rg>0.95 were assumed non-independent and pruned according to the stepwise criteria outlined in SI Appendix (Info. S2). This resulted in nine metabolites being taken forward to MR-BMA (Dataset S4).

4.2.3.2. Primary analysis

Following LDSC pruning, pre-curated, independent mQTLs made available within the MRBase database were extracted for each of the metabolites for use as instruments. Following removal of ApoE SNPs and removal of a SNP for which a suitable proxy (R^2>0.8) could not be obtained, 21 instruments remained. As with univariable analyses, all SNPs were assumed to be on the positive strand and sensitivity analyses were performed excluding palindromic SNPs.

Full details of the MR-BMA methodology can be found elsewhere (22). Briefly, with consideration of all exposures specified, MR-BMA iterates over many potentially "true" causal models, with variations of exposure sub-groups included within each of these (with exposure inclusion determined by binary parameter - γ). For each exposure, an MIP was computed, representing the pp of metabolite x appearing within the true causal model given z iterations. Metabolites ranked highest and with a MIP >0.1 were interpreted as being the strongest "true causal" candidates of all those provided within the model. A model averaged causal effect (MACE) was also estimated, representing the estimated direct (independent) effect of metabolite x on outcome y, averaged across each pp. It is worth noting that MACE will be biased towards the null due to shrinkage applied in variable selection (22). This metric can, however, be used to gain insight into the direction of effect and magnitude relative to other

metabolites included within the model. Finally, computed models were ranked by their posterior probabilities to provide best model-fit estimates for metabolite combinations and their combined association with AD. As with MIP, the highest ranked metabolite combinations, with pp>0.1, were interpreted as showing the strongest evidence as the true causal models for metabolite combinations. For all BMA analyses, we set *z* to 10,000, the prior probability to 0.1, and prior variance (σ2) to 0.25.

4.2.3.3. Sensitivity analyses

Q-statistics quantified potential instrument outliers, and Cook's distance (*Cd*) was used to identify influential points in the top four MR-BMA models (with *pp*>0.1). Diagnostic plots were generated to investigate the predicted versus observed associations for each of the top 4 models. Any SNPs with Q-statistic >10 or *Cd*>0.19 (4/total SNP *N),* were flagged and MR-BMA repeated with the SNP(s) omitted. Metabolite-AD associations remaining after the removal of potential outliers were considered to be more reliably associated with AD.

4.3. Post-hoc exploratory analyses

4.3.1. LD overlap between influential points and AD

Any IV which demonstrates evidence of overlap with genomic regions associated with an outcome in MR analyses risks violating core MR assumptions and, in turn, call into question IV validity. Steps were taken within primary analyses to avoid such scenarios, such as excluding any IVs associated with AD at genome-wide significance. However, influential points signpost unusually large associations which, whilst could be due to particularly strong and biologically relevant associations with the exposure, may also reflect spurious factors such as shared LD with an outcome-specific genomic region. To further explore the validity of influential points, we therefore visually inspected regions of LD, and cross-checked these with genes closest to top AD-related SNPs, as reported within the latest AD GWAS by Kunkle et al. (21). Briefly, summary statistics for each metabolite showing evidence of an influential point was uploaded to the publicly available visualization tool, "Locus Zoom" (http://locuszoom.org/). LD regions were specified using the influential SNP as the reference, together with a flanking region of 400kb. Genomic regions located below any SNP in LD with the reference point, at R^2>0.2 were cross-checked against Kunkle related genomic regions.

4.3.2. One-sample univariable MR

Baseline NMR metabolite and AD case-control data from the Alzheimer's Disease Neuroimaging Initiative (ADNI) were obtained to allow for a small scale, exploratory replication of significant associations observed within primary analyses. Full details regarding ADNI can be found elsewhere (24). Briefly, ADNI is a longitudinal initiative, beginning in 2003 and following participants through multiple study phases; collecting multi-omic, cognitive, and phenotyping information relevant to AD risk. At baseline, metabolite information across 241 metabolite sub-fractions were available for almost 1,700 individuals. Metabolites demonstrating evidence of a causal association with AD within primary analyses were extracted from the wider dataset of ADNI metabolites. Genotype information were also extracted for all individuals at baseline (Distinct sample *N*=1,674). This underwent full quality control (QC) and was subsequently imputed (QC and imputation details can be found within SI Appendix, Fig. S10 and Dataset S16). Samples retained following QC were then merged with available metabolite data, extracting only genetic instruments utilized within primary univariable analyses and excluding samples for which metabolite information were missing (missing GP=1, missing HDLs=17). Following data cleaning and merging, metabolite, genetic, and diagnostic information was available for up to 894 individuals (515 AD cases, 379 controls). Metabolite data was standardized to a mean of 0 and standard deviation of 1, and data square-root transformed to achieve normality.

For each metabolite separately, one-sample univariable MR was performed using two-stage least squares (2SLS). Briefly, instrumental variables were first flipped such that each represented the risk-increasing allele for the metabolite exposure of interest. Each metabolite was then regressed on all of its represented IVs, weighted by the relative strength of the genetic instrument. Predicted values from stage one were then regressed on the case/control outcome to obtain a final causal estimate. To avoid estimates being biased by selection or reverse causation (due to calculating with single-person data), stage one estimates were restricted to controls only (49). Overall IV strength for each metabolite was assessed through computation of a weighted F-statistic (IVs combined and weighted by their per-IV instrumental strength). As with primary analyses, an F-statistic<10 was considered

evidence of weak instrument bias – indicating low statistical power.

4.3.3. Association analyses for top causal metabolites

Subsequently to performing our one-sample Mendelian randomization using the ADNI cohort, an additional exploratory observational analysis was performed using ADNI data for each of the metabolites identified as causal candidates within primary analyses. This was to assess whether evidence of an observational relationship between metabolites of interest and AD status could be found within the ADNI cohort. As the scope of this study was to interrogate causal relationships, we refrain from discussing the details of these observational analyses here. However, further information can be within our supplementary material (SI Appendix, Info. S4).

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Availability of data and materials

Metabolite data used within primary analyses is publicly available within the MRBase catalogue (https://www.mrbase.org/). AD GWAS data used within primary analyses is publicly available for download at https://www.niagads.org/datasets/ng00075. Metabolite and genomic data used within post-hoc analyses can be found in the ADNI database (http://adni.loni.usc.edu).

Additional information

A proportion of data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.ucla.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.ucla.edu/research/active-investigators/. Data used in preparation for a proportion of this article were also generated by the Alzheimer's Disease Metabolomics Consortium (ADMC). As such, the investigators within the ADMC provided data but did not participate in analysis or writing of this report. A complete listing of ADMC investigators can be found at: https://sites.duke.edu/adnimetab/team/]

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Figures and Tables

Figure 1. Association of metabolites associated with AD at *p*<0.05 in primary univariable analyses.

Standardized odds ratio (μ=0, *SD*=1) and 95% confidence interval error bars for inverse variance weighted, MR Egger, and Weighted median estimates (*N*=12). Orange bars represent estimates from primary univariable analyses. Grey bars represent conservative estimates from MR-Egger and weighted median sensitivity analyses. Sensitivity estimates appear in grey to indicate lower precision of these estimates relative to primary analyses, resulting in larger windows of uncertainty. HDL=High Density Lipoproteins, XL.HDL= Very Large High Density Lipoproteins, L.HDL=Large High Density Lipoproteins, FC=Free Cholesterol, P=Concentration of Particles, PL=Phospholipids, L=Total Lipids, C=Total Cholesterol, D=Mean Diameter, GP=Glycoprotein Acetyls.

Figure 2. Study design.

Flow chart describing sequence of analytical steps in-line with core study scope.

Table 1. Metabolites ranked by their marginal inclusion probability (MIP) and model average causal effect (MACE) in MR-BMA analyses.

Metabolite	MIP	MACE
GP	0.465	0.088
XL-HDL-C	0.179	-0.022
XL-HDL-FC	0.178	-0.022
XL-HDL-CE	0.164	-0.017
S-HDL-TG	0.107	-0.015
L-HDL-C	0.098	-0.007
L-HDL-CE	0.096	-0.007
DHA	0.044	-0.003
PUFA	0.024	0.001

Table 2. Top 9 causal models based on whole-model posterior probabilities estimated within MR-BMA analyses.

Exposure Combinations	Posterior Probability
GP	0.287
XL-HDL-C	0.113
XL-HDL-FC	0.112
XL-HDL-CE	0.102
L-HDL-C	0.050
L-HDL-CE	0.049
Gp,XL-HDL-C	0.020
XL-HDL-CE,Gp	0.019
Gp,S-HDL-TG	0.019