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Multiomic approach and Mendelian randomization analysis identify causal associations between blood biomarkers and subcortical brain structure volumes

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ABSTRACT

Alterations in subcortical brain structure volumes have been found to be associated with several neurodegenerative and psychiatric disorders. At the same time, genome-wide association studies (GWAS) have identified numerous common variants associated with brain structure. In this study, we integrate these findings, aiming to identify proteins, metabolites, or microbes that have a putative causal association with subcortical brain structure volumes via a two-sample Mendelian randomization approach. This method uses genetic variants as instrument variables to identify potentially causal associations between an exposure and an outcome. The exposure data that we analyzed comprised genetic associations for 2994 plasma proteins, 237 metabolites, and 103 microbial genera. The outcome data included GWAS data for seven subcortical brain structure volumes including accumbens, amygdala, caudate, hippocampus, pallidum, putamen, and thalamus. Eleven proteins and six metabolites were found to have a significant association with subcortical structure volumes, with nine proteins and five metabolites replicated using independent exposure data. We found causal associations between accumbens volume and plasma protease c1 inhibitor as well as strong association between putamen volume and Agouti signaling protein. Among metabolites, urate had the strongest association with thalamic volume. No significant associations were detected between the microbial genera and subcortical brain structure volumes. We also observed significant enrichment for biological processes such as proteolysis, regulation of the endoplasmic reticulum apoptotic signaling pathway, and negative regulation of DNA binding. Our findings provide insights to the mechanisms through which brain volumes may be affected in the pathogenesis of neurodevelopmental and psychiatric disorders and point to potential treatment targets for disorders that are associated with subcortical brain structure volumes.

1. Introduction

Variations and dysfunctions of subcortical brain structures have been associated with numerous neurological and neuropsychiatric disorders such as Parkinson's disease, different types of dementia, insomnia, schizophrenia, autism spectrum disorder (ASD), depression and post-traumatic stress disorder (PTSD) (Bohnen and Albin, 2011; Nir et al., 2013; Voineskos, 2015; van Rooij et al., 2018; Zhao et al., 2017; Emamian et al., 2021; Wang et al., 2021). These brain structures are involved in various functions such as mood processing, sensory investigations, cognitive control, memory, etc. Changes in these structures in individuals with psychiatric and neurological disorders could explain the phenotypic changes and symptoms observed and could be used as biomarkers to identify individuals at risk for developing the disorders (Voineskos, 2015; Zhao et al., 2017; Emamian et al., 2021). However, it is largely unknown what molecular and biochemical processes may influence disease-related changes and how abnormalities of specific subcortical structures influence different traits and in subcortical brain

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structures. Understanding the relationship between brain volume and structure and neurological disease would help us better determine the underlying pathophysiological pathways. Such analysis could also be important in clinical practice, providing biomarkers that could be useful in disease diagnosis and patient management as well as helping to identify treatment targets for the various disorders associated with abnormalities in subcortical brain structure.

Recent large-scale multicenter studies such as the Enhancing Neuro Imaging Genetics through Meta-Analysis (ENIGMA) and UK Biobank (UKB) have put together neuroimaging and genomic data from tens of thousands of individuals and performed genome-wide association studies. This has led to the identification of genetic variants that are associated with subcortical brain structure volumes (Hibar et al., 2015; Satizabal et al., 2019; Thompson et al., 2020). These studies have been followed by transcriptomic and epigenomic analysis to identify genes and epigenetic markers associated with regional brain volumes (Zhao et al., 2021; Barbu et al., 2022; Jia et al., 2021). However, studies seeking to identify associations between regional brain volumes and other biomarkers such as proteins, metabolites and the microbiome are limited.

Here, we seek to address this gap, exploring the role of the proteome, metabolome, and microbiome in mediating brain structure changes which could lead to neurological disease. Proteins are the final product of gene expression and are an important intermediary phenotype that can provide insight into the cellular processes and functions that influence human biology and disease pathophysiology (Geyer et al., 2016). On the other hand, metabolites are small molecules that are a product and intermediates of cellular metabolism and play a pivotal role in cellular and physiological processes (Nath et al., 2017; Miles and Calder, 2015). The observed levels of such metabolites in biofluids can elucidate these processes. Finally, the human microbiota plays an important role in the fermentation of non-digestible substrates as well as providing protection against foreign pathogens (Gilbert et al., 2018; Valdes et al., 2018). A number of studies have found that changes in the level of different proteins, metabolites and the composition of the gut microbiome are associated with different metabolic, immunological as well as neurological disorders (Yang et al., 2021; Mofrad et al., 2022; Sabatine et al., 2005; Vijay and Valdes, 2022). The importance of the level of different metabolites such as glucose, lactate and pyruvate in the cerebrospinal fluid (CSF) is well known and they are established biomarkers to study inflammation and malignancies in the brain (Zhang and Natowicz, 2013). Numerous studies have been performed to determine metabolic biomarkers of neurological diseases such as Alzheimers Disease and most of the results indicate changes in biochemical pathways related to the energy metabolism, amino acids linked to the glucogenic and ketogenic energy metabolism among others (Quintero Escobar et al., 2021). The gut-brain axis (GBA), which consists of bidirectional communication between the central and the enteric nervous system is heavily influenced by the gut microbiota (Carabotti et al., 2015), establishing the importance of the microbiome in neurological functions and disorders. Experimental studies and systematic analyzes have shown that changes in gut microbiota exert significant effects on CNS and immune cells (change in immune response, altered synapse formation and disrupted maintenance of the CNS), and have been associated to various disorders such as Multiple Sclerosis, Alzheimers, Parkinsons and Autism among others (Park and Kim, 2021).

Although the levels of these biomarkers in the body (especially metabolites and gut microbiome) are heavily influenced by environmental factors such as diet, medication and lifestyle (Rothschild et al., 2018; Maier and Typas, 2017; Bermingham et al., 2021; Nicholson et al., 2011), twin and family-based studies show that genetics also play an important role and they are highly heritable (Hagenbeek et al., 2020; Goodrich et al., 2016, 2014). With advancements in profiling methods, large-scale studies can measure the levels of thousands of proteins and the various metabolites circulating in the blood and identify genetic variants which influence the level of these biomarkers (Geyer et al., 2016; Shin et al., 2014; Sun et al., 2018). Genome-wide association studies have also been performed to identify genetic variants that are associated with the composition of various bacterial taxa in the gut microbiome (Kurilshikov et al., 2021). With results from these multi-omic studies at hand, there is the opportunity to investigate potential causal associations between such biological markers and subcortical brain structure volumes, using a two-sample Mendelian randomization (MR) approach.

MR analysis is a genetic epidemiological method that can help to determine putative causal associations between an exposure and an outcome using genetic variants as instrument variables (Emdin et al., 2017; Sanderson et al., 2022). The method is conceptually similar to a randomized controlled trial which is based on the idea that the individuals receiving the treatment/drug (the instrument variable) are assigned randomly to the different groups (Hariton and Locascio, 2018). Similarly, in MR studies, the SNPs are randomized by nature, assigned to offspring before birth and are not confounded by any environmental factor - thus satisfying the requirement of a randomized trial (Sanderson et al., 2022; Swanson et al., 2017). This method is very powerful and can use the vast number of publicly available results of GWAS to identify causal associations between different exposures and outcomes. Indeed, studies undertaking this approach have identified causal associations between proteins and disorders such as depression, anorexia, ASD, and many others (Yang et al., 2021; Wingo et al., 2021; Yang et al., 2022a, 2022b). MR studies have also uncovered associations between the gut microbiome and autoimmune and cardiovascular disorders (Xu et al., 2022; Zhang et al., 2022). MR studies for brain structures have also found causal associations between subcortical brain structure and neurological conditions like schizophrenia, anorexia, depression, and other disorders (Wootton et al., 2022; Walton et al., 2019; Shen et al., 2020; Wu et al., 2021). However, so far, no studies have examined associations between the different biomarkers and metrics of subcortical brain structures.

In this study, we sought to better understand the mechanisms and mediators that lead to the observed associations between brain structures and neurological and neuropsychiatric disease. In a systems biology approach, we integrated multi omic data with GWAS for subcortical brain volumes and employed a two-sample MR approach to ask if proteome, metabolome, and microbiome could be causally associated with volume of different subcortical brain structures. The central hypothesis of our study was that specific genetic variants influence subcortical brain volumes by altering levels of different biomarkers from the proteome, metabolome, or microbiome.

2. Methods

2.1. Ethics statement

Only publicly available deidentified summary data was used in this study.

2.2. Study design and datasets

We applied a two-sample MR analysis to determine and identify causal associations between three multi-omic datasets (plasma proteome; metabolome; microbiome) and seven different subcortical brain structure volumes (accumbens, amygdala, caudate, hippocampus, pallidum, putamen, and thalamus) using genetic variants as instrument variables. Fig. 1 shows the overall design of the analysis. The basic principle of MR is that SNPs (genetic instruments), which are significantly associated with modifiable exposure, would be causally associated with the exposure-related outcome. Three important assumptions are required for a valid genetic instrument and MR analysis. First, the instrument must be causally related to the exposure. Second, it must be independent of any confounders; and, finally, it should only be associated with the outcome through the exposure. In our current study, the

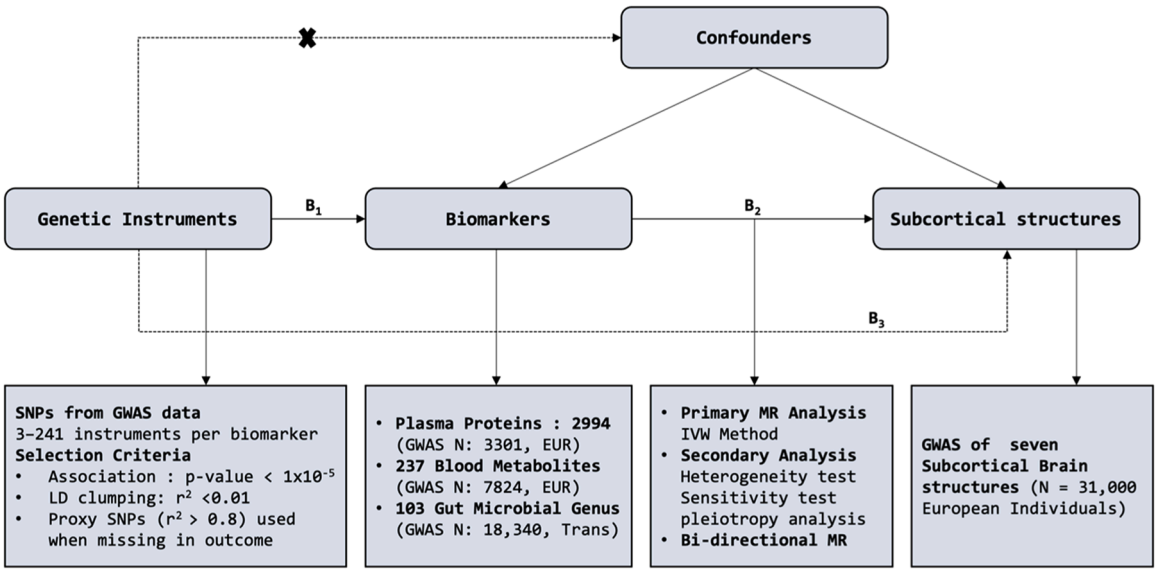

Fig. 1. Study overview and design for MR analysis. SNP information for exposures and outcomes were extracted from GWAS summary statistics for each feature. B2 is the causal association of interest (Effect of Biomarkers on seven different subcortical brain structure volumes), estimated using B2 =B1/B3. B1 and B3 are the direct associations of the genetic variants on the exposure (biomarkers) and outcomes (subcortical structures) obtained from the GWAS studies. We also assume that the SNP instrument selected acts on the outcome only through exposure and not through any confounders. IVW: Inverse Variance Weighted.

genetic instruments for the different exposures were obtained from large-scale GWAS studies for each of the different omic datasets (information on these studies is shown in **Supplementary Table 1**). Overall, we obtained GWAS data on 2994 plasma proteins, 237 blood metabolites and 103 microbial genera (Shin et al., 2014; Sun et al., 2018; Kurilshikov et al., 2021). Our outcome dataset included the GWAS summary statistics for the seven subcortical brain structure volumes (adjusted for intracranial volume) obtained from the ENIGMA consortium (Hibar et al., 2015). All participants in all cohorts in the different GWAS studies gave written informed consent and the sites involved obtained approval from local research ethics committees or Institutional Review Boards.

2.3. Selection of genetic instruments

The first step to performing MR analysis is the selection of instrument variables. We used a threshold of nominal significance ($P < 1 \times 10^{-5}$) to select SNPs from the GWAS summary statistics for each of the exposure variables. Ideally, genome-wide SNPs ($P < 5 \times 10^{-8}$) are used for MR analysis but a relatively relaxed threshold for the genetic instruments has been previously used in MR investigations when there were no or only a few genome wide SNPs available (Yang et al., 2022a, 2022b; Choi et al., 2019; Sanna et al., 2019). To select independent SNPs, we performed LD clumping using PLINK2 with an r^2 threshold of 0.01 within a 500 kb window using the 1000 Genomes European dataset as the reference panel (Auton et al., 2015). The next steps of the analysis were performed using the TwoSampleMR package in R (Hemani et al., 2018). Once the independent SNPs were selected, we harmonized the exposure and outcome datasets to match the effect alleles, obtained the SNP effects and corresponding standard errors, and removed ambiguous SNPs with intermediate allele frequencies. In cases where a SNP was not available in the outcome dataset, a proxy SNPs with high LD with main SNP was used (LD at $r^2 > 0.8$) for the analysis. No overlap was present between the outcome data and the reference LD data used. We then evaluated the instrument strength of each of the exposures by estimating the proportion of variance explained by the SNPs (R^2) and the *F*-statistic for each of the variables (Brion et al., 2013). Typically, an *F* statistic >10 is considered sufficiently informative for MR analysis (Burgess et al., 2013). We extracted a range of seven to 84 SNPs for the proteome data with an average R^2 of 21 % and the minimum *F* statistic was 20.56. The number of SNPs for the metabolites ranged from three to 241 with an average R^2 of 13.1 % and a minimum *F* statistic of 20.52. Finally, for the various microbial genera we extracted 3 to 22, with an average R^2 of 3.2 % and the lowest *F*-statistic of 20.46.The number of instrument variables, R^2 and *F*-statistics for each individual biomarker is shown in Additional file 1.

2.4. Two sample MR analysis and statistical validation

We used the inverse variance weighted (IVW) method of MR analysis to estimate the association between the different exposures and outcomes. The method provides a high-power estimate and assumes that all the genetic instruments used for the analysis are valid. Significant associations of protein, metabolites and microbiomes with the different subcortical brain structures were identified after adjusting for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) threshold of 0.05. We then performed downstream validation using other methods of MR estimation, heterogeneity analysis and pleiotropy analysis for the significant associations. Two methods - the weighted median method and MR-Egger method - were adopted as alternate methods to evaluate the robustness of causality and detect pleiotropy. These methods are useful to validate the results of the MR analysis in case we use SNPs that do not satisfy the assumptions for the analysis. The weighted median method provides a consistent estimate if less than 50 % of the SNPs were invalid instruments (Bowden et al., 2016) and the MR-Egger method was useful when up to 100 % of the SNPs came from invalid instruments (Bowden et al., 2015). Cochran's *Q* test was performed to test for heterogeneity, and pleiotropy was tested by performing an MR-Egger Intercept test and a leave-one out analysis. We used the Mendelian Randomization Pleiotropy RESidual Sum and Outlier (MR-PRESSO) method to test for horizontal pleiotropy and detect any outliers in our analysis (Verbanck et al., 2018). Briefly the method performs a global test for pleiotropy and if significant the outlier SNPs are reported, which can then be removed, and the analysis is repeated without them. The directionality test to validate whether the genetic instruments were acting on the outcome through the exposure was tested using the MR Steiger directionality test, which calculates the variance explained in the exposure and the outcome by the instrumenting SNPs, and tests if the variance in the outcome is less than the exposure (Hemani et al., 2017). We also performed reverse MR analysis

with the subcortical brain structure volume as exposure and the biomarkers as outcomes. This allows us to evaluate if there were any feedback loops between the brain structures and biomarker levels which could lead to false positive results. We used the same thresholds to select the genetic instruments from the GWAS studies of the subcortical structures and used the IVW method to estimate the association.

2.5. Replication analysis

To validate the significant associations identified in our analysis, we obtained independent exposure data for the different biomarkers. For our replication tests we used proteome data from a study of 5368 European individuals (Gudjonsson et al., 2022) and metabolome data from a study of 8871 European individuals (Chen et al., 2023). We then used the same thresholds for instrument selection as described above and performed MR-IVW analysis to test whether the associations are significant in an independent analysis.

2.6. Functional enrichment analysis

Functional enrichment analysis was performed using the gProfiler tool (Raudvere et al., 2019). We tested for enrichment across different gene ontology terms, KEGG and reactome pathway databases, protein complexes and human phenotype ontology databases. A Bonferroni threshold was used to correct for multiple testing for all pathways tested. The pathway and enrichment analysis for metabolites was performed using the MetaboAnalyst platform (Pang et al., 2021).

3. Results

3.1. Investigating the causal association between proteome and subcortical brain structures

Using two sample MR analysis, we tested for potentially causal associations between 2994 proteins and seven subcortical brain volumes (Additional file 2). Eleven proteins showed significant causal association with one of the subcortical brain structures as shown in Fig. 2 and **Supplementary Table 2**. Agouti Signaling Protein (ASIP) had the strongest association with putamen volume, with increase in the protein expression resulting in decrease in putamen volume (Beta: 28, *p*-value: 1.2×10^{-8}). Plasma protease C1 inhibitor (SERPING1) and Secretoglobin family 1C member 1 (SCGB1C1) were both found to be causally associated with accumbens volume, with the increase in expression of these proteins being associated with increase in the volume of accumbens (Beta: 6.3–9.7, *p*-value: 3×10^{-5} - 6.9×10^{-7}). Increase in Granzyme A (GZMA) levels was found to be significantly associated with increase in amygdala volume (Beta: 17, p-value: 1.43×10–5). Two proteins had a significant causal association with caudate volume. Increase in Thioredoxin domain containing protein 12 (TXNDC12) levels was associated with increase in caudate volume (Beta: 11.7, *p*-value: 2.3×10^{-6}), whereas Transmembrane protease serine 11D (TMPRSS11D) had a negative association (Beta: -26.8, *p*-value: 7.1×10^{-7}). For the hippocampus, we found four proteins significantly associated and all of them had a negative association with volume of hippocampus. These included Copine-1 (CPNE1), Cardiotrophin-1 (CTF1), Selenoprotein S (VIMP) and Protein CEI (C5orf38) (Beta: -21.2 to -25.9, *p*-value: 4.9×10^{-5} - 9.8×10^{-7}). Finally, we found that increases in Chymotrypsinogen B (CTRB1) were significantly associated with decrease in the volume of thalamus (Beta: -23.9, *p*-value: 1.4×10^{-5}). No proteins were found to be significantly associated with pallidum volume after multiple testing corrections.

Interestingly, we observed that certain proteins such as SERPING1, CTRB1 and ASIP where nominally associated ($p < 0.05$) with other subcortical brain structures as well in similar direction as their primary associations (**Supplementary Fig. 1**).

3.2. Investigating causal association between metabolome and subcortical brain structures

We proceeded to test for potentially causal association between metabolites and subcortical brain structure (Additional file 3). We found six metabolites to be significantly associated with one of the subcortical brain structure volumes (**Supplementary Table 3** and Fig. 3). Among these, two metabolites had a causal association with amygdala volume.

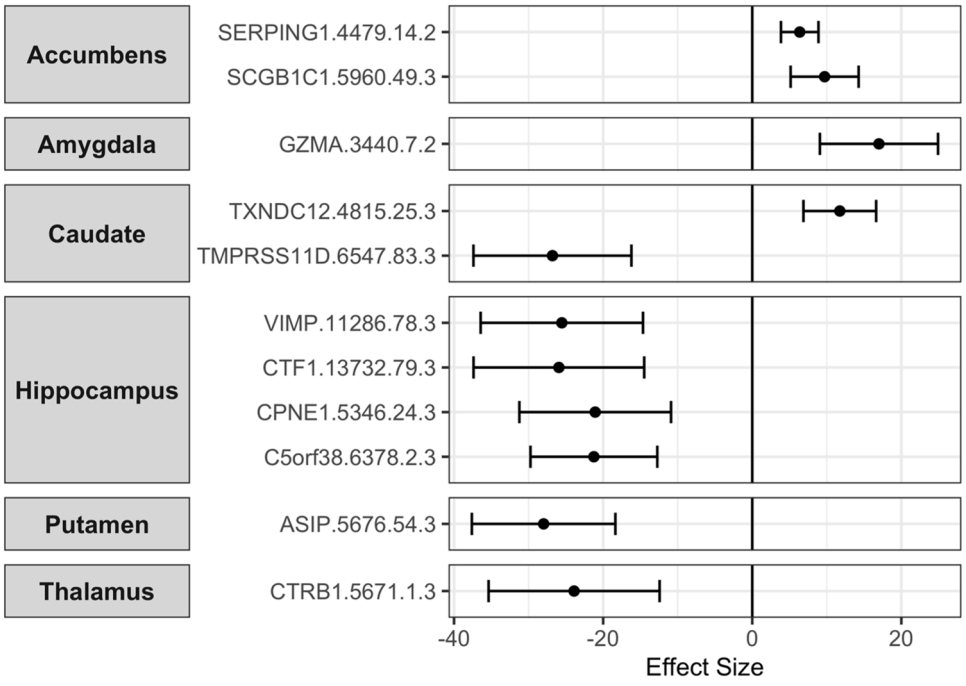

Fig. 2. Significant causal associations between plasma proteins and subcortical brain structure volumes as uncovered via MR analysis. The Proteins were the exposures and the subcortical structures' volume as outcomes. The associations were significant after FDR corrections for multiple testing.

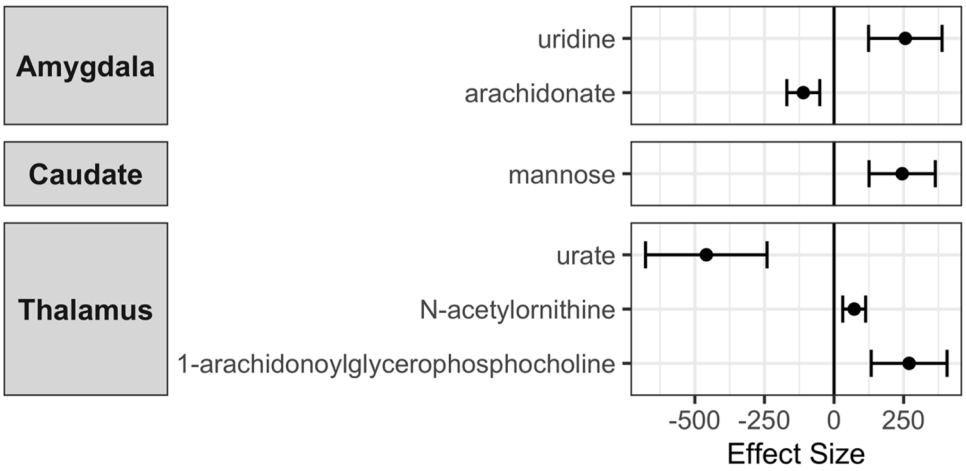

Fig. 3. Significant causal associations between metabolites and subcortical brain structure volumes as uncovered via MR analysis. The metabolites were the exposures and the subcortical structures' volume as outcomes. The associations were significant after FDR corrections for multiple testing.

These included uridine levels which had a positive association (Beta: 255.9, *p*-value: 1.44×10^{-4}) and Arachidonate which had a negative association with amygdala volume (Beta: -110.4, *p*-value: 2.54×10^{-4}). We also found three metabolites significantly associated with thalamus volume which were Urate (Beta: -458.7, *p*-value: 3.7×10^{-5}), 1-arachidonoyl-GPC (Beta: 269.7, *p*-value: 1.1×10^{-4}) and N-acetylornithine (Beta: 72.4, *p*-value: 5.6×10^{-4}). Increase in mannose levels was found to be causally associated with increase in caudate volume (Beta: 244.7, *p*-value: 5.5×10^{-5}). We also observed that Uridine, N-acetylornithine and 1-arachidonoyl-GPC were nominally associated ($p < 0.05$) with other subcortical structures as well (**supplementary Fig. 2**).

3.3. Investigating causal association between microbiome and subcortical brain structures

Here, we pursued MR analysis between 103 microbial genera as exposure and subcortical structure as outcome. Although our analysis did not reveal any significant associations after multiple testing corrections (Additional file 4), 28 associations were found to be nominally significant ($p < 0.05$) **(Supplementary Figs. 3 and 4)** between microbiome and brain volume. The strongest association was observed for Erysipelatoclostridium and Amygdala volume (Beta: 29, *p*-value: 1.1×10^{-3}).

3.4. Heterogeneity, sensitivity and pleiotropy analyzes

To determine the robustness and the validity of our results, we performed downstream statistical analysis to further increase the confidence in the observed associations. For all the significant associations identified in the primary analysis, we repeated the MR analysis using other methods such as the weighted median method and the MR-Egger method. We found that the associations were largely consistent with effects in the same direction and a significant *p*-value for the proteins (**Supplementary Table 4**). The MR-Egger estimate between the metabolites and subcortical brain volumes was found to be non-significant (**Supplementary Table 5**). We then determined if there was any heterogeneity in the genetic instruments used by calculating the Cochran's *Q* statistic and found little to no evidence of heterogeneity (p-value: 0.094–0.99) for all proteins and metabolites (Table 1A and B). Following this, we tested for pleiotropy of SNPs between exposure and outcome

Table 1
Statistical validation of MR results. The table shows the results of heterogeneity and pleiotropy tests performed for all biomarkers that had significant association with subcortical volume. Table 1A shows the results for proteins and 1B shows the results for the metabolites. Q refers to Cochran's Q estimate for the heterogeneity test; DF is the degree of freedom. The Int refers to the MR-Egger intercept for the pleiotropy test and SE is the standard error of the Intercept.
The last 2 columns represent the test-statistic and the p-value of the global test performed using MR-PRESSO.

Biomarker ID (Exposure)	Region (Outcome)	Heterogeneity Test			Pleiotropy test			MR-PRESSO	
		Q	Q_DF	p-val	Int	SE	p-val	Int	p-val
				(1A) Proteins					
SCGB1C1.5960.49.3	Accumbens	7.12	16	0.971	-0.12	0.88	0.893	8.05	0.977
SERPING1.4479.14.2	Accumbens	43.59	35	0.151	0.12	0.63	0.852	47.12	0.182
GZMA.3440.7.2	Amygdala	21.54	26	0.714	-0.45	2.26	0.841	23.35	0.729
TMPRSS11D.6547.83.3	Caudate	58.99	46	0.095	2.85	2.78	0.311	61.2	0.131
TXNDC12.4815.25.3	Caudate	87.65	80	0.261	-2.37	1.63	0.15	90.4	0.269
C5orf38.6378.2.3	Hippocampus	41.42	43	0.540	0.11	1.82	0.952	43.7	0.559
CPNE1.5346.24.3	Hippocampus	28.60	24	0.236	1.62	2.46	0.516	32.3	0.245
CTF1.13732.79.3	Hippocampus	34.77	28	0.177	-1.97	2.60	0.454	36.5	0.214
VIMP.11286.78.3	Hippocampus	24.13	28	0.675	-0.30	2.17	0.891	26.9	0.647
ASIP.5676.54.3	Putamen	55.63	49	0.239	3.79	1.97	0.061	67.18	0.086
CTRB1.5671.1.3	Thalamus	21.99	40	0.991	1.57	2.47	0.528	22.96	0.995
				(1B) Metabolites					
Uridine	Amygdala	11.90	21	0.942	1.1	2.26	0.634	12.98	0.955
Arachidonate	Amygdala	20.25	28	0.855	0.41	1.01	0.686	20.83	0.934
Mannose	Caudate	32.76	30	0.333	4.71	2.81	0.104	35.46	0.329
Urate	Thalamus	23.63	27	0.650	-1.64	3.29	0.623	25.73	0.656
1-arachidonoyl-GPC	Thalamus	25.42	22	0.277	-0.28	2.64	0.915	19.84	0.816
N-acetylornithine	Thalamus	18.74	25	0.809	3.78	2.27	0.109	13.04	0.961

using the Egger intercept test and leave one out analysis. We found no evidence of pleiotropy (Egger Intercept *p*-value: 0.06–0.95) and leave one out analysis showed that removing any SNP did not greatly affect the association (Table 1 and additional file 5). Additionally, the MR-PRESSO test showed that there was no horizontal pleiotropy in the genetic instruments (global test $p > 0.05$) used and thus no outliers were present in the analysis (Table 1). One of the assumptions of MR is that the instruments influence the exposure first and then the outcome through the exposure. To evaluate this, we used the MR-Steiger test which calculates the variance explained in the exposure and the outcome by the instrumenting SNPs, and tests if the variance in the outcome is less than the exposure. The test showed that for all the proteins and metabolites that had significant associations with subcortical volume, the variance of the genetic instruments in the exposure is always greater than the outcome - thus validating the assumption of MR (**Supplementary Tables 6 and 7**).

3.5. Reverse Mendelian randomization analysis

We performed the MR analysis with the subcortical brain structure volumes as exposure and the significantly associated biomarkers as outcomes. The results showed that for all proteins except C5orf38, there was no reverse causation observed in our analysis (Table 2A), thus indicating the causal effects of the proteins on the subcortical brain volume were statistically robust and not false positives. No reverse association was found between subcortical brain volume and the six metabolites as well (Table 2B).

3.6. Replication analysis

We validated our significant biomarker – subcortical structure volume associations using independent exposure data (Gudjonsson et al., 2022; Chen et al., 2023) and performed MR-IVW analysis. The results showed that nine proteins (out of the ten tested – one was not available in the dataset) (Table 3A) and five (out of six) metabolites (Table 3B) were associated (FDR p-value < 0.05) with the subcortical brain structure volume, thus providing additional confirmations for our findings.

3.7. Functional enrichment analysis

Analysis of the associated proteins using the g:Profiler platform revealed significant enrichment for various Gene Ontology terms after adjusting for multiple testing (Fig. 4 and **Supplementary Table 8**). These included molecular functions such as endopeptidase activity, peptidase activity and hydrolase activity. We also observed significant enrichment for biological processes such as proteolysis, regulation of the endoplasmic reticulum apoptotic signaling pathway and negative regulation of DNA binding. Most of the proteins were enriched in the extracellular regions of the human system. No significant enrichment was observed for the metabolites across all metabolic pathways.

Table 3
Replication Analysis. The table shows the results of MR analyzes using independent exposure data for the significant (A) proteins and (B) metabolites and subcortical brain structure volume as outcomes. The Adj_P column refers to FDR corrected p-value for the associations. (*) indicates significant after multiple testing correction.

Exposure	Outcome	Beta	SE	P value	Adj_P
		(3A) Proteins			
SERPING1	Accumbens	4.79	0.76	3.27E-10	1.63E-09*
GZMA	Amygdala	11.632	4.58	0.01487	0.01652*
TMPRSS11D	Caudate	-38.07	8.47	6.95E-06	1.39E-05*
TXNDC12	Caudate	11.89	2.38	6.17E-07	2.05E-06*
C5orf38	Hippocampus	-21.94	5.27	3.10E-05	5.17E-05*
CPNE1	Hippocampus	-17.66	3.75	2.54E-06	6.34E-06*
CTF1	Hippocampus	-24.02	6.63	2.91E-04	4.16E-04*
VIMP	Hippocampus	-6.25	10.99	0.56970	0.56970
ASIP	Putamen	-32.17	3.85	6.83E-17	6.83E-16*
CTRB1	Thalamus	10.79	3.83	0.00483	6.04E-03*
		(3B) Metabolites			
Uridine	Amygdala	21.45	8.19	0.01277	0.01533*
Arachidonate	Amygdala	-13.542	4.44	0.004277	0.00641*
Mannose	Caudate	37.32	10.23	2.63E-04	7.9E-04*
1-Arachinoyl-GPC	Thalamus	30.46	7.12	1.90E-05	1.1E-04*
N-Acetylornithine	Thalamus	2.97	6.71	0.65824	0.65824
urate	Thalamus	-44.60	12.69	4.39E-04	8.8E-04*

4. Discussion

Here, pursuing a systems biology, multi-omic approach, we sought to

Table 2
Reverse MR analysis. The table shows the results of MR analysis with the subcortical brain structures as exposure and the biomarkers that were significant in the primary analysis as the outcomes. Table 2A shows the results for proteins and 2B shows the results for the metabolites. N SNPs is the number of genetic instruments used for the analysis.

Exposure	Outcome	N SNPs	Beta	SE	P value
		(2A) Proteins			
Accumbens	SCGB1C1.5960.49.3	7	5.84E-05	0.00143	0.967
Accumbens	SERPING1.4479.14.2	7	0.0024	0.00175	0.159
Amygdala	GZMA.3440.7.2	17	0.0001	0.00048	0.795
Caudate	TMPRSS11D.6547.83.3	24	0.00018	0.00021	0.377
Caudate	TXNDC12.4815.25.3	24	-0.00026	0.00023	0.268
Hippocampus	C5orf38.6378.2.3	18	-0.00066	0.00025	0.008
Hippocampus	CPNE1.5346.24.3	18	-0.00126	0.00119	0.288
Hippocampus	CTF1.13732.79.3	18	-0.00022	0.00025	0.369
Hippocampus	VIMP.11286.78.3	18	-0.00019	0.00025	0.435
Putamen	ASIP.5676.54.3	28	-0.00014	0.00017	0.410
Thalamus	CTRB1.5671.1.3	28	0.00005	0.00015	0.743
		(2B) Metabolites			
Amygdala	Uridine	6	-3E-06	7.28E-05	0.967
Amygdala	Arachidonate	6	7.84E-05	0.0001	0.437
Caudate	Mannose	8	-8.23E-05	4.21E-05	0.051
Thalamus	Urate	12	-7.89E-06	1.69E-05	0.641
Thalamus	1-arachidonoyl-GPC	12	-4.13E-05	3.73E-05	0.267
Thalamus	N-acetylornithine	12	-3.60E-05	5.05E-05	0.475

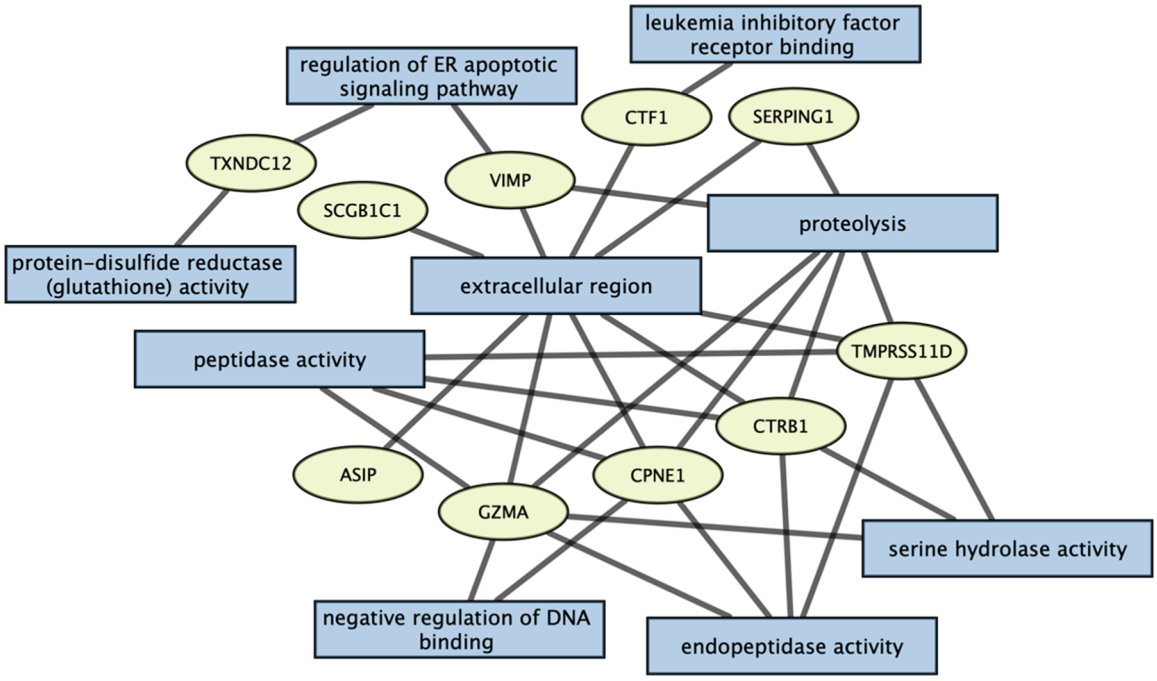

Fig. 4. Enrichment analysis of proteins using the g:Profiler tool. The rectangles correspond to the various enriched Gene Ontology terms and the proteins associated with each term are shown in ellipses.

provide insights into the mechanisms and mediators that underlie known associations of brain structures and neuropsychiatric disease. To do this, we performed a two-sample MR analysis to identify potentially causal associations between the genetically predicted levels of different biomarkers (plasma proteome, blood metabolome and gut microbiome) and the volumes of seven subcortical brain structures. Analyzing available summary statistics from large-scale GWAS, we identified eleven proteins and six metabolites to have a significant causal association with at least one subcortical structure after correcting for multiple testing. Replication analysis using large independent exposure datasets revealed significant associations for nine proteins and five metabolites. Heterogeneity and pleiotropy analysis showed low to no deviation from null thus validating our associations as truly significant. Bi-directional MR analysis for the significant associations showed no reverse causation for any proteins or metabolites except one (C5orf38, which is an unknown protein). Finally, enrichment analysis of the associated proteins showed significant enrichment for proteolytic processes including endopeptidase, peptidase, and hydrolase activities. No significant causal associations were observed between different bacterial genera in the gut microbiome and subcortical brain structures.

The molecular functions and the roles of the different proteins identified in this analysis as causally associated with subcortical brain volumes point to various pathways and mechanisms that could also help explain the relationship between subcortical structures and neuropsychiatric disorders. For example, SERPING1, which is a Plasma Protease inhibitor is a glycosylated protein involved in the regulation of the complement cascade and has been previously found to be associated with influencing frontal cortical thickness (Stelzer et al., 2016; Allswede et al., 2018). The complement system itself has been implicated in depression, schizophrenia, and other neurodegenerative disorders as well (Yi et al., 2019; Druart and Le Magueresse, 2019). The nucleus accumbens has been an important brain region for regulating behaviors related to schizophrenia, depression and addiction (Xu et al., 2020; Forns-Nadal et al., 2017) and our results indicate that this regulation could be driven by levels of SERPING1, which is causally associated with accumbens volume. Similar relationships can also be observed for many of our identified proteins. GZMA, which is a serine protease involved in pyroptosis (Lieberman, 2010), is also found to have a lower expression in patients with major depressive disorder (MDD) compared to healthy controls (Sun et al., 2022). Patients with MDD also tend to have decreased amygdala volume (Hamilton et al., 2008) which, based on our results, could be driven by GZMA. Another interesting example is that of TXNDC12, which is a member of the thioredoxin (Trx) superfamily. The Trx system is an antioxidant system that is important in maintaining sulfhydryl homeostasis protecting against oxidative stress (Arodin et al., 2014). Studies have pointed to the role of Trx-mediated oxidative stress in Parkinson's disease-associated dopaminergic neuron degeneration, thus indicating that this protein might be an important regulator of the dopamine reward system (Garcia-Garcia et al., 2012; Liu et al., Feb. 2021). The caudate which is part of the striatum and connected to the substantia nigra is heavily involved in the reward system where the dopaminergic neurons are produced (Driscoll et al., 2022). Changes in caudate volume have been found to be associated with disorders such as anorexia and Parkinsons disease (Gupta et al., 2022; Pitcher et al., 2012). The results of our analysis suggest that TXNDC12 could be a potential mediator of these associations and could thus be used as a potential target for diagnosis and treatment.

Some of the proteins we identified had an established role in brain development (Park et al., 2012; Peng et al., 2017; Solovyev, 2015). For example, the proteins we found causally associated with hippocampus volume (in both discovery and replication) were Copine-1 and Cardiotrophin-1. Copine 1 is a calcium dependent phospholipid binding protein and plays a role in neuronal progenitor cell differentiation and induces neurite outgrowth (Park et al., 2012). Similar to Copine-1, Cardiotrophin-1 is also involved in the differentiation of neuronal stem cells via a protein kinase dependent signaling pathway (Peng et al., 2017).

Apart from these proteins, we also identified six metabolites that were causally associated with subcortical brain volume. Previous studies have shown that these metabolites have an important role in the functioning of the central nervous system and are also associated with different neurological disorders involved in various functions such as antioxidation and neuro-inflammatory responses. Antioxidants act directly to scavenge oxidizing radicals and regenerate oxidized

biomolecules in organisms to protect the brain from oxidative stress (Lee et al., 2020). Uric acid and uridine which we found associated with subcortical structure volume are considered key antioxidants in humans (Becker, 1993). Interestingly high level of uric acid has been associated with increased risk of disorders such as ASD and ADHD (Page and Coleman, 2000; Sutin et al., 2014). Both of these disorders are also associated with reduced thalamic volume (Tamura et al., 2010; Xia et al., 2012). The association could be potentially explained by higher levels of uric acid as seen in our results. Additionally, both uric acid and uridine are implicated in the development of Lesch-Nyhan syndrome which is a congenital disorder that affects brain structure and behavior of the affected individuals (Jinnah, 2009). Other metabolites such as mannose and arachidonate which were identified in our study have also been found to be associated with disorders like anxiety and depression in mouse model systems (Xu et al., 2021; Larrieu and Layé, 2018; Yu et al., 2021).

We also observed that certain proteins and metabolites such as SERPING1, ASIP, CTRB1 and 1-arachidonoyl-GPC that were significantly associated with a specific subcortical structure were also nominally associated ($p < 0.05$) with other structures as well. This could indicate that these biomarkers are important in functioning of different subcortical brain structures and additional analysis with larger sample sizes could lead to stronger and increased number of associations.

No significant associations were obtained between microbiome and the subcortical brain structures, but several nominal associations were observed. This could be due the larger impact of environment on microbiome levels and potentially a larger study that captures greater level significant genomic variations associated with microbiome is needed. We should also note that the microbiome GWAS study used is based on a trans-ancestry sample while the brain volume GWAS studies are only European and hence there could be a loss of power in the MR analysis.

There are certain limitations of this study. First, there were very few or no genome-wide significant SNPs to be used as instrument variables for many biomarkers in the MR analysis. To address this, we used a more exploratory threshold of 1e-05 for selecting genetic instruments, like previously done in multiple previous studies (Zhang et al., 2022; Wootton et al., 2022; Choi et al., 2019; Sanna et al., 2019). We evaluated the strength for these genetic instruments using different statistical methods and found that they were valid for MR analysis. Second, the proteins and metabolites were quantified in the plasma for the GWAS analysis, which is a natural choice for biomarker-focused applications considering its convenience; however, we do not know whether these biomarkers would have had similar levels in specific brain regions, because of the existence of the blood-brain-barrier. To address this, we checked for the expression and presence of the different proteins and metabolites in the CNS. We found that most of them are highly expressed in different parts of the brain (Uhlén et al., 2015) and play an important role in its development and function (**Supplementary Table 9**). We would also like to point out that, we performed an MR study and identified several statistically causal risk factors associated with the subcortical brain volume, but these findings need further biological validation using experimental verification in cells and model systems. Based on statistical analysis, our study points to the most reliable targets for downstream investment, analysis and experimental validation and provides novel insights into the physiology of brain structures.

In conclusion, we identified several proteins and metabolites that are causally associated with the volume of subcortical brain structures. Our study highlighted the role of proteolytic and anti-oxidative components in the development and functioning of the brain. The biomarkers we identified could mediate the relationship between subcortical structures and different neurological and neuropsychiatric disorders. The results of these analysis highlight the importance of plasma proteins and metabolites as potential biomarkers and could help in early detection of neurological disorders and even subcortical changes. Future analysis could examine other characteristics of the brain such as neuronal activity, gray matter volume, and white matter connectivity which could further improve our understanding of the functioning of the central nervous system and its association to disease. The results of this study not only provide novel insight for understanding subcortical brain structure, but also help in uncovering potential diagnostic markers and drug targets for the many disorders that are associated with changes in brain structures.

Statement of contribution

PJ, MY, CR, and PP designed the study, and performed primary analysis. All authors provided data, materials, and methods. All authors contributed to interpreting results, writing, reviewing, and editing the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of Interest.

Data availability

The GWAS summary statistics used for the analysis were downloaded from publicly available sources. The subcortical brain volume GWAS were downloaded from the ENIGMA consortium (https://enigma.ini.usc.edu/). The proteome GWAS were downloaded from the SomaLogic Plasma Protein GWAS study (http://www.phpc.cam.ac.uk/ceu/proteins/). The metabolites GWAS were obtained from Metabolites GWAS server (http://metabolomics.helmholtz-muenchen.de/gwas/) and the microbiome GWAS results were downloaded from https://mibiogen.gcc.rug.nl/.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2023.120466.

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