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Integrative Multi-Omics Data Analysis Methods: Vascular Cognitive Impairment Case

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Contents

Abbreviations

AD – Alzheimer's disease;
ALS – amyotrophic lateral sclerosis;
BBB – blood-brain barrier;
CASADIL – cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy;
CBF – cerebral blood flow;
cGMP – cyclic guanosine monophosphate;
CNS – central nervous system;
(c)SVD – (cerebral) small vessel disease;
DAG – diacylglycerols;
DMC – differentially methylated cytosine;
DMR – differentially methylated region;
DNMTS – DNA methyltransferases;
eNOS – endothelial nitric oxide synthase;
EWAS – epigenome-wide association study;
GCN – graph convolution network;
GDI – guanine nucleotide dissociation inhibitor;
GFE – guanine nucleotide exchange factor;
GPA – GTPase-activating protein:
GSEA – gene set enrichment analysis;
GTP – guanosine-5'-triphosphate;
GWAS – genome-wide association study;
I/R – ischemia and reperfusion;
MRI – magnetic resonance imaging;
NO – nitric oxide;
ORA – over-representation analysis;
PC – principal component;
PCA – principal component analysis;
PD – Parkinson's disease;
PE – phosphatidylethanolamines;
PMI – post-mortem interval;
PVS – perivascular spaces;
ROS – reactive oxygen species;
SCFA – short-chain fatty acid;
SD – standard deviation;
sGC – soluble guanylyl cyclase;
SNP – single nucleotide polymorphisms;
SNV – single nucleotide variant;
TMM – trimmed mean of M-values;
VaD – vascular dementia;
VCF – variant call format;
VCI – vascular cognitive impairment;
VEGF – vascular endothelial growth factor;
WMH – white matter hyperintensities.

Introduction

Dementia is a growing global health concern, currently affecting over 55 million people worldwide – a number expected to triple by 2050 (WHO, 2023). Among these cases, approximately 20 % are primarily caused by cerebrovascular pathology, while another 20 % involve vascular contributions to mixed dementia syndromes (Mok et al., 2024). One of the most prevalent yet understudied contributors in this context is vascular cognitive impairment (VCI), which encompasses a broad spectrum of cognitive disorders, ranging from mild cognitive impairment to vascular dementia (Gorelick et al., 2011). VCI is frequently associated with cerebral small vessel disease (cSVD) and often coexists with Alzheimer's disease (AD), amplifying the risk and severity of cognitive decline (Markus & De Leeuw, 2023; Mok et al., 2024). Despite growing recognition of its impact, the mechanisms underlying VCI remain only partially understood. Current evidence suggests that VCI is not driven by a single cause, but rather by a complex interplay of cerebrovascular dysfunction, neurodegenerative processes and environmental influences (Zlokovic et al., 2020). While specific gene mutations have been implicated in rare, inherited forms of cSVD-related VCI, these known variants explain only a fraction of the overall VCI burden (Markus & Schmidt, 2019). Consequently, the broader genetic susceptibility for VCI remains largely unknown, necessitating further research to fully understand its molecular basis across the entire clinical spectrum.

Unravelling the complexity of VCI requires approaches that move beyond studying molecular layers in isolation. Traditional single-omics methods struggle to capture the intricate interactions driving multifactorial diseases. Integrative multi-omics analysis provides a more holistic strategy, enabling the combination of genomic, epigenomic, transcriptomic, and metabolomic data to map the interconnected pathways contributing to disease phenotypes (Hasin et al., 2017). This integrated approach helps bridge the gap between genotype and phenotype, providing a deeper understanding of disease mechanisms and revealing potential novel therapeutic targets. However, while powerful, the optimal methods for integrating such diverse datasets, particularly in the context of complex diseases, are still under development. To address this, our study leverages an integrative multi-omics approach that combines data from four complementary molecular layers: genomics, epigenomics, transcriptomics, and metabolomics.

The goal of this study is, therefore, to explore, evaluate and propose integrative multi-omics data analysis methods that can effectively combine various types of omics data to uncover complex biological relationships underlying VCI.

Objectives:

- Analyse single omics data using widely accepted standard approaches;

- Review existing multi-omics integration methods;
- Apply selected multi-omics methods on VCI dataset;
- Design and propose a comprehensive multi-omics data integration workflow;
- Apply the workflow to investigate the molecular mechanisms underlying VCI.

1. Literature review

1.1. Multi-Omics Data Analysis

In recent years, multi-omics data analysis has emerged as a powerful approach to understanding complex biological systems by integrating diverse layers of molecular information (C. Chen et al., 2023a; Picard et al., 2021; B. Wang et al., 2014; Z. Yang & Michailidis, 2016). Traditional single omics studies often provide a limited perspective, whereas multi-omics approaches enable a more comprehensive view of biological processes by combining several data types. This integrative strategy is particularly valuable in studying complex diseases, where multiple molecular mechanisms interact across different biological levels.

1.1.1. Types of Multi-Omics Data

Genomics is the study of an organism's entire genetic material, encompassing its structure, function, mapping, and evolution. It investigates various genetic variations, including single nucleotide and copy number variants, insertions, deletions, duplications, and inversions. A widely used approach in this field is genome-wide association studies (GWAS), which have identified thousands of genetic variants linked to quantitative traits, single gene complex diseases across diverse populations. In GWAS, large cohorts of individuals are genotyped for millions of genetic markers, and significant differences in minor allele frequencies between cases and controls or correlations with quantitative phenotypes provide evidence of genetic associations. Also, genetic variants identified in GWAS are used to estimate trait heritability, assess genetic correlations between different diseases and traits, and develop polygenic risk scores, which refine individual disease risk predictions (Wray et al., 2007). Next-generation sequencing is commonly used for whole-genome sequencing and whole-exome sequencing, enabling the identification of genetic mutations, single nucleotide polymorphisms (SNPs), and structural variations (Koboldt et al., 2013).

Epigenomics is the study of genome-wide modifications that influence gene expression without altering the DNA sequence. These modifications include DNA methylation, histone modifications, and chromatin accessibility, which regulate gene transcription and cellular identity. Epigenetic regulation plays a crucial role in development, differentiation, and disease pathogenesis, with dysregulation contributing to cancer, metabolic syndromes, cardiovascular diseases, and neurological disorders (Baylin, 2001; Ding et al., 2022; Figueroa-Romero et al., 2012; Hayes et al., 2024; Kim et al., 2010). Additionally, epigenetic modifications are often reversible and influenced by environmental factors, making them promising targets for therapeutic intervention. A widely used approach in epigenomic research is epigenome-wide association studies (EWAS), where

epigenetic modifications linked to complex traits and diseases are identified. For instance, EWAS has linked altered DNA methylation patterns to neurodegenerative diseases, providing insights into disease mechanisms and identifying potential biomarkers for early diagnosis and treatment (Moore et al., 2014; Nabais et al., 2021). High-throughput sequencing technologies have advanced the study of epigenomics, with techniques such as bisulfite sequencing, methylation microarrays, and methylated DNA immunoprecipitation sequencing to characterize DNA methylation patterns. In addition, ATAC-Seq, DNase-Seq, and ChIP-Seq are employed to identify regions of open chromatin and histone modifications. Furthermore, third generation sequencing enables direct detection of DNA methylation without requiring chemical treatments and further facilitate epigenomic data analysis (Hayes et al., 2024; Xiao & Zhou, 2020).

Transcriptomics is the study of RNA produced by the genome and how these transcripts are altered in response to various regulatory processes. It acts as the bridge between genotype and phenotype, providing insight into gene expression and its role in cellular function. The transcriptome offers a direct look at the genes that are actively expressed at a given time, providing a dynamic picture of biological processes (Hayes et al., 2024). Transcriptomics allows studying diverse RNA types, including non-coding RNAs, which are increasingly recognized for their regulatory roles in processes such as chromatin remodelling, transcription, and post-transcriptional regulation (Abdelhamid et al., 2022; Chang et al., 2020; Jia et al., 2022). One of the major goals of transcriptomics is to compare gene expression or alternative splicing profiles across different conditions, such as diseased versus healthy states. Overall, transcriptomics plays a critical role in understanding gene expression at a genome-wide level, providing insights into both the qualitative and quantitative aspects of RNA. Advances in sequencing technologies have led to the identification of novel transcript isoforms and non-coding RNAs, expanding our understanding of gene regulation and the complexities of gene function in health and disease (Hayes et al., 2024).

Proteomics is a large-scale study of proteins, focusing on their structure, function, interactions, and modifications. The proteome is highly dynamic, with proteins being constantly synthesized, modified, and degraded in response to internal and external signals. As proteins rarely act in isolation and normally function in complex networks, studying these protein-protein interactions helps uncover the molecular networks that regulate cellular processes (Kuzmanov & Emili, 2013; Singh, 2024). A cornerstone technology in proteomics is mass spectrometry which is used widely to identify, quantify, and characterize proteins in biological samples (Birhanu, 2023). Proteome studies can provide insights into cellular biology, advancing our understanding of health, disease, and therapeutic interventions. For instance, novel potential therapeutic targets for cardiovascular diseases were identified in a proteomics study, including spondin-1 for atrial

fibrillation and Kunitz-type protease inhibitor 1 for coronary artery disease (Schuermans et al., 2024).

Metabolomics is a comprehensive study of metabolites within a biological system, that provides insights into the biochemical activity within cells. Metabolites, such as simple sugars, amino acids, fatty acids, nucleotides, vitamins, and organic acids, are small molecules that act as intermediates or end-products in metabolic pathways. These metabolites reflect the dynamic state of cellular metabolism and can provide a detailed picture of cellular processes in health and disease. Lipidomics is a specialized branch of metabolomics that focuses on lipids – molecules such as fatty acyls, glycerolipids, phospholipids, sphingolipids, sterols, and other lipid classes. Lipids play a crucial role in maintaining cellular structure, storing energy, and facilitating cellular signaling (Gallart-Ayala et al., 2020). They are involved in various physiological processes, including inflammation, immune responses, and metabolism (Han & Gross, 2022). Both metabolomics and lipidomics studies most commonly use mass spectrometry-based methods or nuclear magnetic resonance spectroscopy to identify and quantify molecules of interest (Moco, 2022). One of key application of metabolomics is in identifying biomarkers for disease progression or drug efficacy in therapeutic interventions (Dalamaga, 2024). For instance, metabolomic profiling has been used to detect biomarkers associated with preeclampsia, aiding in early diagnosis and management of this condition during pregnancy (Cao et al., 2025).

Microbiomics is a large-scale study of microbial communities and their interactions within various environments, including the human body, soil, water, and plant ecosystems. In the human body, the microbiome colonizes the skin, mucosal surfaces, and gut, influencing metabolism, immunity, and disease susceptibility. The gut microbiome alone comprises of approximately 100 trillion bacteria from over 1000 species, with its composition shaped by genetics, diet, environment, and age with microbial imbalance being linked to numerous diseases (Louis et al., 2014; Mars et al., 2020; Org et al., 2015; Sharon et al., 2019). The microbiome can be analysed by amplifying and sequencing specific hypervariable regions of bacterial 16S rRNA genes or sequencing of the entire microbial DNA using shotgun metagenomics sequencing (Hasin et al., 2017). A common approach to study microbiome is through integration of metagenomics and metabolomics, which provides insights into microbial contributions to the biochemical pathways. Although integration of multi-omics data remains challenging, coupling of these two omics have already identified roles of microbial metabolites such as short-chain fatty acids (SCFA) and bile acids in regulation of immune responses, metabolism, and neurological functions in host-associated microbiomes (Muller et al., 2024; Turnbaugh & Gordon, 2008; Yachida et al., 2019).

Single-cell omics, unlike bulk tissue sequencing, allows researchers to analyse individual cells, capturing rare cell types and revealing complex regulatory mechanisms (Dimitriu et al.,

2022). Techniques such as single-cell RNA sequencing (scRNA-seq) characterize gene expression heterogeneity, while single-cell T and B cell receptor sequencing (scTCR-seq, scBCR-seq) delineates immune repertoires. Additionally, single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq) reveals chromatin accessibility and transcription factor activity (X. Wu et al., 2024). Integration with single-cell proteomics using cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) further enhances this analysis by combining protein and transcriptomic data, highlighting both similarities and discrepancies between gene expression and protein abundance (Hao et al., 2021). Spatial transcriptomics combines tissue sectioning with single-cell sequencing, preserving spatial context and compensating for scRNA-seq's limitation in localizing cells within tissues.

While this chapter highlights most commonly used omics disciplines, there are many additional omics layers that can be integrated into multi-omics analyses, such as glycomics, radiomics, and pharmacogenomics. These complementary approaches further enrich our understanding of complex biological systems and disease mechanisms. A summary figure encompassing the omics fields discussed in this chapter can be found in Figure 1.1.

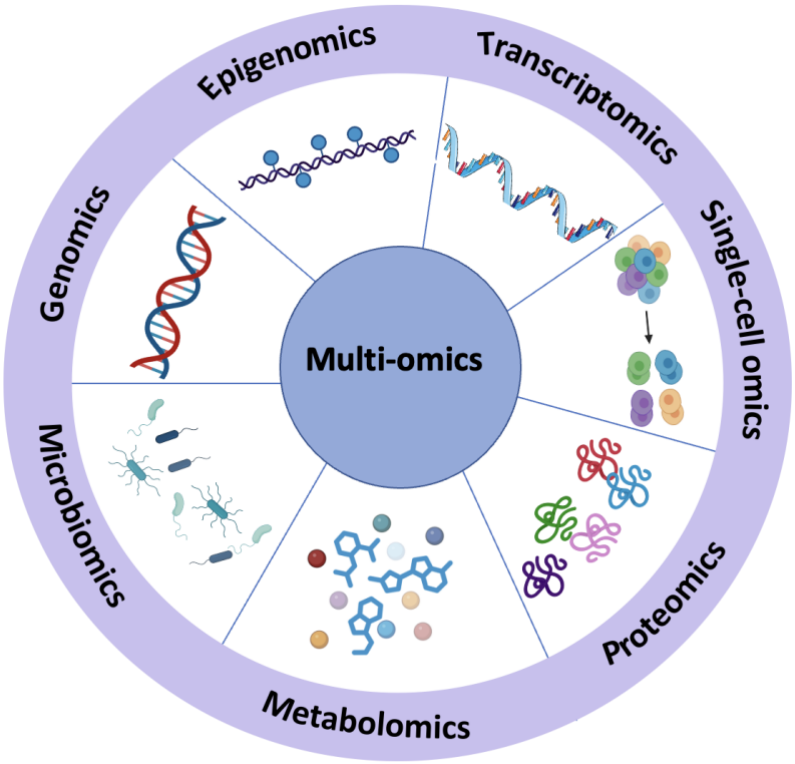

Figure 1.1. Overview of major omics layers integrated in multi-omics analysis

1.1.2. Methods for Multi-Omics Integration

While traditional single omics data provide valuable insights into the molecular underpinnings of diseases, they can be limited in explaining causal relationships and comprehensive

mechanisms of diseases. In contrast, incorporation of multiple omics layers offers a possibility to leverage information from different biological layers and gain a deeper understanding of disease development and progression. As a relatively new field, the development of methodologies for multi-omics is still ongoing with the integration of multi-omics data remaining one of the most significant challenges in multi-omics analysis (C. Chen et al., 2023a). There are two main classes among the methods to integrate the multi-omics data, including supervised and unsupervised integration (Table 1.1). Supervised methods train a model using labelled training data with known outcome variables, such as disease status or exposure to a specific environmental factor. Unsupervised data integration consists of a class of methods that make inferences and find patterns in input data sets without labelled outcome variables (Vahabi & Michailidis, 2022).

Table 1.1. Summary of multi-omics data integration methods

Method	Description	Advantages	Challenges
Supervised integration	Uses labeled training data to build predictive models based on known outcome variables.	Identifies associations with specific phenotypic traits.	Requires large, high-quality labeled datasets.
Unsupervised integration	Identifies patterns in multi-omics data without predefined labels.	Reveals hidden biological structures and relationships.	Interpretation is complex, and validation is challenging.
Network-based	Embeds multi-omics data into biological networks.	Identifies molecular associations and biological modules, provides robust models for describing complex regulatory mechanisms.	Requires sophisticated algorithms and presents challenges in functional interpretation and validation.
Matrix factorization	Decomposes high-dimensional heterogeneous data into meaningful factors.	Captures hidden structures in multi-omics datasets.	Can lose biological interpretability.
Correlation-based	Evaluates correlations between different omics layers.	Simple and computationally efficient.	Weak correlations can limit insights; more advanced association methods are needed.
Machine learning-based	Uses algorithms like SVMs and random forests to identify patterns and predict outcomes.	Effective for biomarker discovery and disease classification.	Prone to overfitting, requires large datasets and high computational resources, may overlook direct omics interactions.
Deep learning-Based	Uses deep neural networks, graphs convolution networks and autoencoders for nonlinear, hierarchical modeling.	Captures complex interactions and aids in feature selection.	Prone to overfitting, requires large datasets and high computational resources.

Network-based approaches are increasingly recognized as powerful tools for integrating multi-omics data. Biological networks, such as protein-protein interaction (PPI), gene co-expression, and signaling pathway networks, offer a valuable framework for understanding the functional relationships between molecules. By embedding multi-omics data into a network context, researchers can uncover molecular associations and identify biological modules or subnetworks associated with specific phenotypes (Vahabi & Michailidis, 2022). One of the most prominent network-based methods is similarity network fusion where individual similarity networks for different omics datasets are constructed and then combined using nonlinear methods. When applied to patient data, this method is effective for identifying disease subtypes by focusing on strong molecular associations across various data types while filtering out weak associations and noise through iterative refinement (B. Wang et al., 2014). Despite the clear advantages, network-based methods are often applied to single omics data types, and further development of sophisticated algorithms that account for interactions across multiple omics layers is still needed (Yugi et al., 2016). In addition, a challenge for downstream functional interpretation and validation of multi-omics findings remains (C. Chen et al., 2023a).

Matrix factorization methods, such as non-negative matrix factorization, are widely used to extract hidden patterns from high-dimensional multi-omics data (Z. Yang & Michailidis, 2016). These techniques decompose large matrices into factors that represent the underlying structure of the data (W. Yang & Sinha, 2017). In the context of multi-omics integration, integrative non-negative matrix factorization methods account for heterogeneous data from multiple omics sources. This method uses a partitioned factorization structure to combine homogeneous and heterogeneous patterns, allowing for better interpretation of multi-omics datasets (W. Yang & Sinha, 2017). Similarly, multi-omics factor analysis is another popular method for integrating multi-omics data. It is an unsupervised technique that uses a probabilistic Bayesian framework to uncover hidden factors across multiple omics layers, facilitating the discovery of shared biological signals and is particularly useful for handling heterogeneous data types, such as continuous gene expression and binary clinical data (Argelaguet et al., 2018).

Correlation-based approaches are among the simplest methods for integrating multi-omics data (C. Chen et al., 2023a). These methods involve evaluating the correlation between data from different omics layers, such as between transcriptomics and proteomics. For example, the correlation between mRNA expression and protein abundance can reveal insights into post-transcriptional regulation (Kumar et al., 2016). One example of a correlation-based integration method is CNAmet, a software tool designed to integrate copy number alteration, DNA methylation, and gene expression data (Louhimo & Hautaniemi, 2011). This method calculates a composite score for each gene by linking expression values to copy number and methylation data. It

helps in identifying genes that are upregulated or downregulated due to genetic alterations, providing valuable insights into disease mechanisms (Louhimo & Hautaniemi, 2011).

Machine learning techniques such as support vector machines, random forests, and neural networks, are commonly applied to integrate different omics data types and predict phenotypic outcomes, such as disease subtypes or patient survival (C. Chen et al., 2023; Cheng et al., 2015; Zou et al., 2017). Several strategies for integrating multi-omics data using machine learning methods have been proposed, including concatenation-based, model-based, and transformation-based approaches:

- **Concatenation-based integration** method involves merging data from various omics layers into a single, large matrix, which is then analyzed using classical machine learning methods like support vector machines or random forests. This strategy is simple and allows for easy comparison of continuous or categorical data across omics types. However, it may face challenges related to mismatched data scales and potential overfitting due to high-dimensionality (Belmonte et al., 2022)(C. Chen et al., 2023; Picard et al., 2021)
- **Model-based integration** method relies on building the separate models for each omics dataset, which are then integrated into a joint predictive model. It is useful for capturing the relationships across different omics types and helps in understanding complex biological phenomena. However, model-based integration may overlook interactions between different omics layers, particularly if these relationships are not explicitly modelled (C. Chen et al., 2023).
- In the **transformation-based integration** method, each omics dataset is converted into an intermediate representation, such as a graph or kernel matrix, which is then integrated with other omics data for further analysis. While this approach is flexible and can handle various data types, it may miss interactions between omics layers, as each omics dataset is transformed independently (C. Chen et al., 2023).

Deep learning-based methods, such as deep neural networks and autoencoders, have demonstrated significant promise in multi-omics data integration due to their ability to model nonlinear and hierarchical relationships within the data (Min et al., 2016; Picard et al., 2021). For example, autoencoders are particularly useful for dimensionality reduction and feature selection, making them well-suited for integrating multi-omics datasets (Picard et al., 2021). The general approach involves feature selection and dimensionality reduction firstly for the individual dataset and then on the combined matrix, which is subsequently analyzed using techniques like classification, regression, and clustering (Kang et al., 2022). Graph-based deep learning approaches, particularly graph convolutional networks (GCNs), have become increasingly prominent in multi-

omics data integration (Schulte-Sasse et al., 2021; T. Wang et al., 2021). GCNs are well-suited for analyzing biological networks where samples or molecules exhibit complex interrelationships, as they integrate both feature-level information and topological structure from graphs (Zitnik et al., 2018). In the context of multi-omics, GCNs allow for modeling interactions within and between omics layers by using sample similarity networks or biological interaction graphs. A notable example is MOGONET, a supervised GCN-based framework designed for disease classification using mRNA expression, DNA methylation, and microRNA expression data (T. Wang et al., 2021). MOGONET constructs omics-specific sample similarity graphs using cosine similarity, then trains separate GCNs for each omics layer to generate initial predictions, which are later fused via a cross-omics discovery tensor that captures inter-layer label correlations. This approach outperforms traditional neural networks by leveraging both node features and structural relationships between samples. However, MOGONET primarily focuses on per-omics embeddings and prediction fusion, without directly modeling cross-omics feature interactions; addressing this limitation to better capture the intricate interplay between different molecular layers remains a key challenge and active area of research (T. Wang et al., 2021).

The integration of multi-omics data offers a powerful approach for unravelling the molecular mechanisms that drive complex diseases. As the field progresses, establishing a gold standard workflow for each category of integration method will be essential to enhance reproducibility and facilitate the widespread application of multi-omics approaches in both research and clinical settings.

1.1.3. Application of Multi-Omics in Research

One of the multi-omics applications is its use in subtyping of diseases such as cancers which, historically, were classified based on histopathology features and clinical symptoms (Athieniti & Spyrou, 2023). Using high-throughput omics methods allowed to classify disease stages in higher detail and integration of different omics now shows promise to define disease-specific signatures with even higher precision. For example, multi-omics signatures are now being used to identify heterogeneous cancer subtypes that may respond differently to treatments, allowing to tailor treatments at a single-patient level (Athieniti & Spyrou, 2023). For example, a study utilizing multi-omics analysis of mRNA, lncRNA, miRNA, DNA methylation and somatic mutations classified colorectal cancer into two subtypes with distinct prognoses and tumor microenvironments, facilitating a better understanding colorectal cancer heterogeneity (Ma et al., 2024).

Multi-omics also enables the prediction of drug efficacy and toxicity by identifying molecular signatures that influence how a patient's cells or tissues will respond to a given drug

(Athieniti & Spyrou, 2023; Kreitmaier et al., 2023). While considerable advancements have been made in drug target discovery, no single omics method is sufficient to pinpoint precise molecular targets within complex physiological networks (C. Chen et al., 2023a). In contrast, integrated multi-omics approaches can simultaneously uncover, define, and validate multiple potential targets and mechanisms of candidate natural products (C. Chen et al., 2023a; Santos et al., 2016). For example, a recent study developed a deep learning-based framework to predict cellular responses to known anti-cancer drugs by integrating diverse omics data types, including gene expression profiles, copy number variations, gene mutations, reverse phase protein array expressions, and metabolomics data. The resulting model achieved high predictive accuracy in identifying drug sensitivities across various cancer cell lines (C. Wang et al., 2022).

Multi-omics is used to identify disease-associated molecular patterns that help to uncover co-varying molecules that serve as biomarkers of disease state and progression. Biomarkers are particularly valuable for understanding complex diseases where there is often a disconnect between genotype and phenotype (Athieniti & Spyrou, 2023). For example, a multi-omics study integrating gut microbiota and metabolite profiles linked Crohn's disease to disruptions in sulfur metabolism, leading to the identification of potential biomarkers and therapeutic targets. (Metwaly et al., 2020). Additionally, by combining gene expression data with potential regulatory mechanisms, multi-omics can help to identify key sub-networks that are dysregulated in diseases, offering insights into potential drug targets (Athieniti & Spyrou, 2023). For instance, a recent study integrated genomic, transcriptomic, and epigenomic data to uncover the gene regulatory networks involved in type 2 diabetes. This approach not only provided insights into the molecular mechanisms of the disease but also highlighted potential drug targets for intervention (J. Liu et al., 2022). In summary, multi-omics offers unparalleled opportunities for understanding the complexities of disease at a molecular level with wide applications in diagnostics, personalized treatment plans, and drug development.

1.1.4. Challenges in Multi-Omics Methods

Multi-omics enables a holistic understanding of biological systems by combining diverse layers of omics data, although there are still significant challenges, especially when it comes to data integration (Jamil et al., 2020; Krassowski et al., 2020). One of the major challenges in multi-omics integration is data heterogeneity where data are generated using diverse technologies, including sequencing, mass spectrometry, and microarrays, each with distinct formats, scales, and error profiles. This variation in resolution, targeted versus untargeted approaches, and differing normalization techniques between omics layers complicates data integration (Krassowski et al., 2020). Addressing these discrepancies often involves applying sophisticated batch correction,

scaling, and data harmonization techniques, such as quantile normalization, variance-stabilizing transformations, and feature transformation algorithms (Hasin et al., 2017; Y. Luo et al., 2024).

Another challenge in multi-omics integration is handling missing data, which is common due to technical limitations, low detection thresholds, or incomplete sample collection. Missing values can significantly impact correlation analyses and model predictions. Standard imputation methods such as k-nearest neighbors multiple imputations, or deep learning-based approaches attempt to infer missing values, but these can introduce bias and affect statistical validity. For example, multi-omics analysis often relies on imputing missing values across different omics layers, but this process can distort biological relationships and violate assumptions of independence required for statistical modeling. Therefore, careful consideration of imputation strategies and quality control measures is crucial for ensuring data reliability (Krassowski et al., 2020).

The complexity of multi-omics data analysis methods often poses challenges in result interpretability, making it difficult to extract meaningful biological insights. Machine learning and artificial intelligence have become integral to multi-omics data analysis, enabling pattern recognition and predictive modeling across complex datasets (Picard et al., 2021). However, many of these methods, especially deep learning models, lack transparency in their internal decision-making processes, making it difficult to interpret the underlying biological mechanisms. The complexity of omics data also leads to the "curse of dimensionality", where the number of variables far exceeds the number of samples, increasing the risk of overfitting and reducing model generalizability (Domingos, 2012).

Functional enrichment analysis is commonly used to provide context for multi-omics data, yet it has limitations in uncovering mechanistic interactions across different molecular layers (Canzler & Hackermüller, 2020). Pathway diagrams offer a structured way to interpret multi-omics relationships, but they often lack the flexibility needed to reveal novel regulatory mechanisms (C. Chen et al., 2023a). Network-based approaches, such as those implemented in aPEAR or Cytoscape, allow for a more integrated representation of multi-omics interactions (Kerseviciute & Gordevicius, 2023; Su et al., 2014). However, as datasets increase in size and complexity, these networks can become overly intricate and difficult to interpret (C. Chen et al., 2023a). Developing more interpretable and standardized multi-omics frameworks will be crucial for bridging the gap between computational insights and experimental validation.

Computational complexity is also a limitation in multi-omics integration where extensive processing power, memory, and storage is required for the integration of high-dimensional datasets (Oliveira & Densmore, 2022). High-performance computing clusters with multi-core processors, large RAM capacities, and GPU acceleration are often necessary for handling and processing large datasets efficiently. Furthermore, deep learning and machine learning models, which are

increasingly used for pattern recognition in multi-omics data, demand extensive training and hyperparameter tuning, adding to computational costs. While cloud computing offers a scalable solution, the integration of omics data across platforms remains challenging due to data storage, access, and privacy constraints (Krassowski et al., 2020).

Despite its transformative potential, data integration for multi-omics remains an emerging field with no universally accepted standard for processing, integration, or interpretation. Future developments of robust computational frameworks, improved data harmonization techniques, and transparent analytical approaches will be essential for unlocking the full potential of reproducible multi-omics in biomedical and translational research.

1.2. Vascular Cognitive Impairment

Globally, an estimated 55 million people live with dementia, a number projected to triple by 2050 (World Health Organisation [WHO], 2023). Of these cases, approximately 20 % are predominantly attributable to cerebrovascular pathology, with another 20 % involving vascular contributions to mixed dementia syndromes (Mok et al., 2024). Overall, vascular system-related dementia is estimated to currently affect around 20 million individuals, a figure expected to increase dramatically in the coming decades, particularly in low- and middle-income countries (Mok et al., 2024).

Vascular cognitive impairment (VCI) encompasses a broad spectrum of cerebrovascular pathologies that contribute to cognitive decline. This ranges from subjective cognitive decline to mild cognitive impairment and, ultimately, to dementia (Gorelick et al., 2011). While acute cognitive impairment following a stroke is the most recognizable manifestation of VCI, chronic cerebrovascular conditions, especially cerebral small-vessel disease (cSVD), can lead to a gradual, insidious cognitive decline even in the absence of a clinical stroke (Markus & De Leeuw, 2023). Additionally, cerebrovascular disease frequently co-exists with Alzheimer's disease (AD), amplifying the likelihood of clinical dementia from AD pathology and possibly contributing to the development of AD-related pathologies (Mok et al., 2024). Subcortical ischemic vascular dementia (VaD), predominantly caused by cSVD, has gained increasing global attention (Akinyemi et al., 2019; B. Y. K. Lam et al., 2023). This interest arises from two main reasons: as cSVD is the most common cause of stroke worldwide, and survivors of lacunar strokes, a key feature of cSVD, often live long enough to develop a higher risk of cognitive impairment. In fact, cSVD is likely the leading cause of cognitive impairment and dementia globally (Pantoni, 2010). The clinical presentation of subcortical ischemic VaD often includes motor and executive function slowing, forgetfulness, dysarthria, and a characteristic short-stepped gait resembling Parkinsonism (Kalaria

et al., 2024). These features are believed to result from disruptions in prefrontal cortex-basal ganglia pathways and thalamocortical connections (Kalaria et al., 2024).

VCI is a significant and growing contributor to the global dementia burden, with its prevalence expected to rise, particularly in underserved regions. A deeper understanding of the underlying etiology of VCI is crucial for improving diagnosis, management, and outcomes. Investigating the complex mechanisms driving VCI will provide key insights into its prevention and treatment, ultimately enhancing our ability to address this increasingly prevalent condition.

1.2.1 Mechanisms Underlying VCI

Multiple cerebrovascular etiologies contribute to vascular cognitive impairment, including cSVD, large-artery atherosclerosis, brain hemorrhages, cardioembolism, and other less common stroke causes (Dichgans & Leys, 2017; Rundek et al., 2022). Age, genetics, and lifestyle factors lead to vascular risk factors and subclinical arterial and brain diseases, ultimately causing cerebrovascular dysfunction, which is central to VCI.

The precise mechanisms by which vascular pathologies lead to VCI are not fully understood. Chronic age-related dysregulation of cerebral blood flow (CBF) is the most common underlying mechanism, with other contributing factors including hypoxia, blood-brain barrier (BBB) permeability, endothelial dysfunction, systemic inflammation, and aging-related processes like frailty and cardiovascular aging (Zlokovic et al., 2020). CBF is vital for maintaining brain function, providing oxygen and nutrients, and adapting to fluctuations. In animal models, disruptions in CBF regulation, such as chronic reductions, lead to brain atrophy, white matter damage, and cognitive impairments, including AD (Park et al., 2019). Hypertension significantly impacts CBF regulation, with recent magnetic resonance imaging (MRI) studies showing altered cerebrovascular reactivity in cSVD (Smith & Beaudin, 2018). However, whether CBF dysfunction drives VCI or is a result of aging and neurodegeneration remains unclear (Joutel & Chabriat, 2017; Rundek et al., 2022).

The neurovascular unit, which couples neural activity with CBF, plays a crucial role in VCI. This complex structure comprises endothelial cells, neurons, astrocytes, microglia, and pericytes, and dysfunction within it contributes to various brain diseases, including VCI (Iadecola, 2017). The Circle of Willis, a network of collateral arteries, helps maintain CBF and supports brain function through pial arteries and perivascular spaces (PVS) (B. P. Wood et al., 1975). PVS facilitate waste clearance and energy substrate exchange, and their dysfunction is linked to neurodegenerative diseases and vascular dysregulation (Wardlaw et al., 2020). PVS are commonly observed in SVD and have been associated with cognitive impairment and dementia, although their exact role remains debated. Recent study indicated that the presence of PVS is associated with a higher risk of

cognitive decline (Paradise et al., 2021).

Both small and large cerebral infarcts, often spanning multiple territories, are significant contributors to dementia. Subclinical lesions, such as white matter hyperintensities (WMHs) and microinfarcts, are prevalent in aging populations and are linked to cognitive decline (Au et al., 2006). Subclinical brain lesions, including silent cerebral infarcts, increase dementia risk and are associated with poorer cognitive performance in older individuals (Dong et al., 2015; Vermeer et al., 2003).

Brain hemorrhages, including intracerebral hemorrhages and cerebral microbleeds, also contribute to cognitive decline and dementia. Hypertension and amyloid deposition in cerebral vessels are major factors in these hemorrhages, which disrupt brain networks and impair cognition (Van Veluw et al., 2016). Cerebral microbleeds can be detected using MRI and are believed to affect cognitive function by reducing brain network efficiency. Cerebral amyloid angiopathy, characterized by amyloid-beta deposits in cerebral vessels, is common in AD patients and is associated with cognitive decline, particularly affecting memory, and processing speed. Cerebral amyloid angiopathy is often linked to cortical superficial siderosis, which increases the risk of hemorrhage and cognitive impairment (Biffi & Greenberg, 2011; Boyle et al., 2015).

Cardiovascular diseases, such as heart failure and atrial fibrillation, are also associated with VCI. Heart failure leads to reduced cerebral perfusion, while atrial fibrillation increases thromboembolic risk, both contributing to cognitive decline (Jinawong et al., 2021). Subclinical atherosclerosis, including carotid plaque and increased intima-media thickness, also plays a role in VCI (Kamtchum-Tatuene et al., 2020). Genetic and sporadic arteriopathies affect cerebral vessels and contribute to VCI (Joutel et al., 1996).

Despite the well-established connections between stroke, infarcts, and dementia, the pathophysiology of VCI remains understudied. A deeper understanding of VCI mechanisms is essential for reducing the global burden of cognitive impairment and dementia.

1.2.2 Genetics of VCI

The heterogeneity of VCI presents considerable challenges for elucidating its genetic underpinnings and pathophysiology. Compared to other dementia disorders, such as AD, the genetic basis of VCI remains relatively poorly characterized. To date, most genetic studies of VCI have focused on mechanisms related to stroke and cerebrovascular disease. This focus reflects the observation that monogenic forms of VaD are typically caused by mutations affecting arterial function, particularly in small cerebral arteries. These findings suggest that impaired arterial function may represent a dominant genetic mechanism contributing to VCI (Markus & Schmidt, 2019).

Twin and epidemiological studies estimating the heritability of VaD are sparse. A small twin study suggested that genetic factors play a modest role in VaD, with environmental factors exerting a predominant influence (Bergem, 1997). However, growing evidence from GWAS and epidemiological research indicates significant heritability in the pathological processes underlying VCI. For example, heritability estimates for large artery and small vessel stroke range from 20 % to 40 % (Bevan et al., 2012). Autosomal dominant mutations in genes such as *NOTCH3* (notch receptor 3)*, HTRA1* (HtrA serine peptidase 1), *TREX1* (three prime repair exonuclease 1), *COL4A1* (collagen type IV alpha 1 chain), and *COL4A2* (collagen type IV alpha 1 chain) are linked to hereditary forms of VCI, which often manifest in middle-aged individuals (Markus & Schmidt, 2019). Among these, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CASADIL) is the most frequent hereditary form of VCI, caused by mutations in the *NOTCH3* gene. Clinically, CADASIL is characterized by recurrent strokes, migraine with aura, and psychiatric disturbances. Interestingly, the WMH burden in CADASIL patients shows substantial variability, independent of specific mutations at the *NOTCH3* locus or demographic factors (Dotti et al., 2004; Manso-Calderón & González-Sarmiento, 2016; Rutten et al., 2014; Tikka et al., 2014). A GWAS conducted in CADASIL patients identified multiple genetic variants with small effects influencing WMH volume, providing insights into the mechanisms of WMH formation in both CADASIL and sporadic small-vessel disease (Manso-Calderón & González-Sarmiento, 2016; Opherk et al., 2014)

The complex pathophysiology of VCI involves disruptions within the BBB and neurovascular unit, driven by processes such as cerebral hypoperfusion, hypoxia, inflammation, oxidative stress, and neuronal and glial dysfunction. A reduction in cerebral blood flow triggers a cascade of events, beginning with hypoxia and blood-brain barrier disruption, which are followed by inflammatory and oxidative stress responses. These processes ultimately lead to neuronal and glial malfunction (Manso-Calderón & González-Sarmiento, 2016). Importantly, each of these mechanisms has been associated with specific genetic factors (Figure 1.2.2).

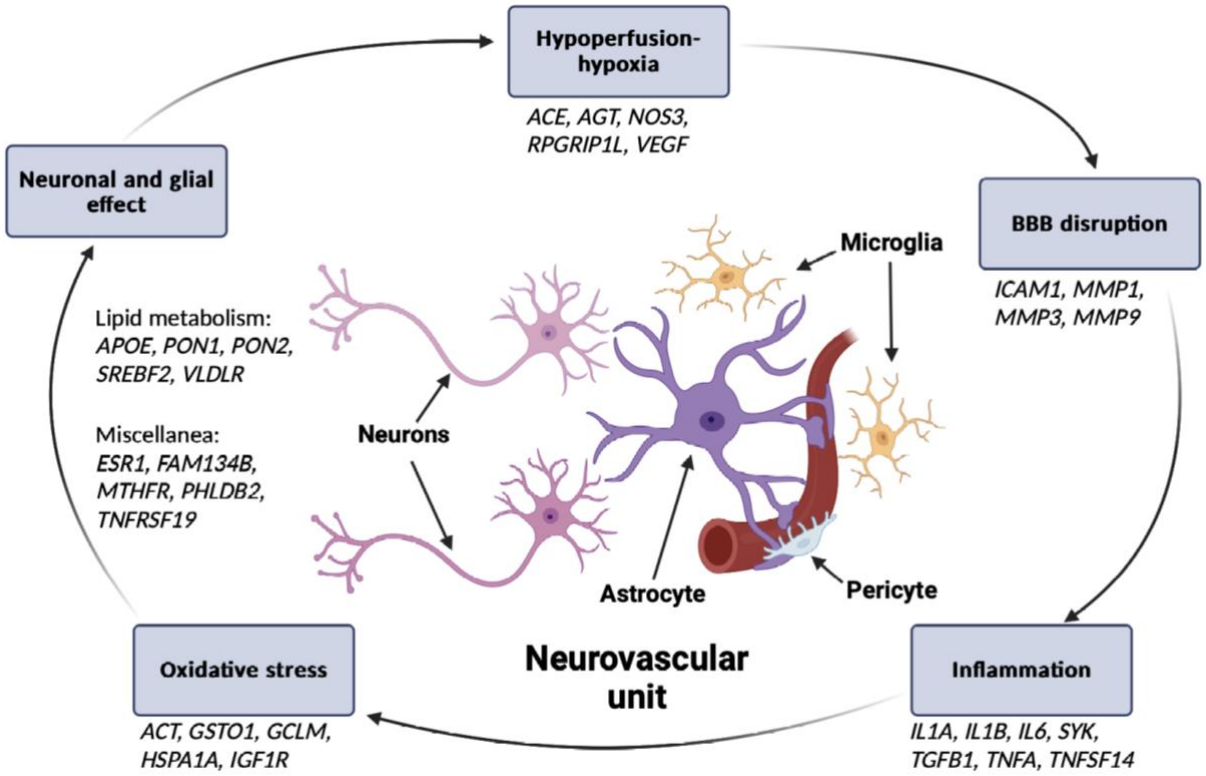

Figure 1.2. Sequence of processes involved in the pathophysiology of sporadic VCI and candidate genes related to each of these possible mechanisms. Figure created with biorender.com and adapted from (Manso-Calderón & González-Sarmiento, 2016).

Genetic factors may play a role in the development or progression of VCI through mechanisms that intersect with AD. The *ApoE* (apoliprotein E) ε4 allele, a well-established genetic risk factor for AD, has also been linked to VaD, potentially contributing to microvascular changes due to its amyloidogenic properties (Baum et al., 2006; L. Yu et al., 2013). Notably, while *ApoE* variants have shown associations with VaD, a definitive connection between *ApoE* and sporadic VCI has yet to be confirmed (Jellinger, 2014; L. Yu et al., 2013). These findings underscore the challenges in interpreting genetic data for VCI, given its heterogeneous pathology and significant overlap with AD (Iadecola, 2013).

Despite advancements in understanding the genetic contributors to VCI, significant gaps remain. The interplay of genetic and non-genetic factors in driving VCI's pathophysiology is not yet fully elucidated. Genetic risk factors have been identified through candidate gene studies and GWAS, but these approaches alone may not uncover the full spectrum of VCI-related pathways. Multi-omics approaches, integrating genomics, transcriptomics, epigenomics, and metabolomics, offer a promising avenue for identifying interconnected biological pathways underlying the VCI phenotype. By leveraging these methods, future research can better unravel the mechanisms contributing to VCI progression, ultimately informing prevention and treatment strategies.

1.2.3 Current Approaches to Treatment and Prevention

The overarching goal in managing VCI is twofold: to prevent its onset (primary prevention) and to delay progression from mild impairment to disabling dementia (secondary prevention). Recent epidemiological studies have reported a decline in dementia incidence in several Western countries, with a more pronounced decrease observed in VaD compared to AD. This trend has been attributed to improved control of vascular risk factors, advancements in treatments for cardiovascular and cerebrovascular diseases, better general health, increased educational attainment, reduced burden of cSVD and amyloid pathology, and larger brain volumes compared to earlier cohorts (Satizabal et al., 2016)

Screening for modifiable risk factors is critical in patients with suspected VCI. Factors such as hypertension, atrial fibrillation, hyperlipidemia, impaired glucose tolerance, kidney disease, obstructive sleep apnea, smoking, and heavy alcohol use should be addressed, as they are strongly linked to recurrent stroke risk. Evidence supports that effective management of these vascular risk factors can significantly reduce the risk of recurrent stroke and cardiovascular disease (Dichgans & Zietemann, 2012; O'Donnell et al., 2010). Pharmacological interventions, including antihypertensive agents, glycemic control, and lipid-lowering therapies, present opportunities for both prevention and treatment of VCI. Trials incorporating vascular biomarkers and cognitive outcomes are needed to establish optimal thresholds for vascular risk factors, which may differ from those relevant to other organs like the heart (Berry et al., 2019; Hainsworth et al., 2021).

Currently, cognitive dysfunction in VaD is managed with cholinesterase inhibitors (e.g., donepezil) and NMDA receptor antagonists, which yield modest cognitive improvements, likely due to their effects on coexisting AD pathology (Jellinger, 2014). However, they have not demonstrated significant improvements in global clinical outcomes (Kavirajan & Schneider, 2007). Notably, selective serotonin reuptake inhibitors and dihydropyridine calcium channel blockers have demonstrated short-term cognitive benefits in patients with VaD, while antihypertensive therapy combined with cholinesterase inhibitors has shown promise in preventing post-stroke cognitive decline (Sörös et al., 2013). Other treatments, such as acupuncture, choline alphoscerate, vasodilators, antiplatelet agents and Huperzine A, show potential but require further investigation (Jellinger, 2014; Shi et al., 2014; Xing et al., 2014). Despite these advances, there are no FDA-approved treatments specifically targeting VCI, and ongoing trials continue to explore potential therapeutic candidates (Ritter & Pillai, 2015).

Soluble guanylyl cyclase (sGC), an enzyme activated by nitric oxide (NO) to produce cyclic guanosine monophosphate (cGMP), plays a crucial role in vascular and neuronal health by mediating NO signaling. However, oxidative stress can impair sGC function by oxidizing it into an inactive form, causing heme loss and reducing NO bioavailability through the rapid reaction of NO

with superoxide (O_2^-) and peroxynitrite ($ONOO^-$), which outcompetes the slower enzymatic activation of sGC (Pan et al., 2016). These effects are compounded by endothelial nitric oxide synthase (eNOS) uncoupling, where eNOS produces O_2^- instead of NO, exacerbating oxidative damage and further inhibiting sGC activity (Münzel et al., 2005; Nelissen et al., 2023). While research on sGC modulation in VCI is limited, evidence from cardiovascular studies suggests its potential benefits for cerebrovascular pathology. Targeting sGC may help improve NO-sGC-cGMP signaling in the neurovascular unit, addressing both vascular and neuronal dysfunction (Nelissen et al., 2023).

The complexity of VCI pathophysiology highlights the urgent need for therapies targeting its underlying processes. Multi-omics approaches offer exciting potential for identifying biomarkers and pathways involved in VCI. Advances in artificial intelligence and deep learning can further facilitate the discovery of biomarkers that capture the temporal dynamics of VCI-related processes, improving early diagnosis and monitoring progression. These computational techniques may eventually aid in the development of precision therapies tailored to individual patients.

1.3. Rho family of GTPases

Rho GTPases belong to the Ras-like GTPase superfamily, which includes five main families: Arf/Sar, Rab, Ran, Ras, and Rho (Rojas et al., 2012). These proteins share a conserved three-dimensional structure and possess a G domain that facilitates GTP hydrolysis and GTP/GDP binding (Vetter & Wittinghofer, 2001). Typically, these GTPases act as molecular switches, toggling between an active GTP-bound state and an inactive GDP-bound state. This switching mechanism regulates interactions with downstream effectors, regulating processes such as cell morphology, cytoskeletal dynamics, intracellular transport, autophagy, proliferation, and differentiation (Donovan et al., 2002; H. Huang et al., 2024; Seabra, 1998).

Rho GTPases are divided into two categories: typical or classical and atypical. Classical Rho GTPases, including Rac, Rho, and Cdc42 subfamilies, follow the canonical GDP/GTP exchange cycle. This cycling is regulated by three types of proteins: guanine nucleotide exchange factors (GEFs), which facilitate GDP dissociation and GTP binding; GTPase-activating proteins (GAPs), which enhance intrinsic GTPase activity and promote GTP hydrolysis; and guanine nucleotide dissociation inhibitors (GDIs), which stabilize the GDP-bound state and prevent premature reactivation (Heasman & Ridley, 2008; H. Huang et al., 2024). In contrast, atypical Rho GTPases, such as Rnd3, RhoH, and RhoD, do not follow the classical GDP/GTP cycle. These proteins are constitutively active or exhibit unique regulatory mechanisms, such as rapid GDP/GTP cycling or posttranslational modifications. Atypical Rho GTPases are classified into four subfamilies: RhoU/RhoV, which display enhanced GDP/GTP exchange rates and are transcriptionally regulated

by pathways like WNT and NOTCH; RhoH and Rnd, which lack intrinsic GTPase activity and remain constitutively GTP-bound; and RhoBTB, which are larger proteins with additional domains and are regulated via protein-protein interactions (Aspenström et al., 2007; Dahmene et al., 2020; H. Huang et al., 2024).

Rho GTPases are key regulators of signaling pathways. Upon GTP binding, they undergo conformational changes that enable interactions with specific effectors, triggering downstream biological responses (Vetter & Wittinghofer, 2001). These interactions are highly localized within the cell, with subcellular targeting facilitated by lipid modifications and signals in the C-terminal region (Dahmene et al., 2020; Mitin et al., 2012). Rho GTPases can be regulated by their gene expression and are also extensively modified via posttranslational modifications, which include phosphorylation, lipid addition, ubiquitination, and SUMOylation. These modifications alter Rho GTPases' localization, activity, and stability, ultimately fine-tuning their signaling responses to specific stimuli (Hodge & Ridley, 2016). Additionally, RhoGDIs play a crucial role in controlling Rho GTPase localization and activity. By sequestering GDP-bound Rho GTPases in the cytoplasm, RhoGDIs prevent premature activation while stabilizing the proteins and protecting them from degradation (Dahmene et al., 2020).

While the Rho GTPase subfamily plays a broadly conserved role in regulating cytoskeletal dynamics and cellular functions, their specific contributions vary across different tissues and systems. The following sections will explore their critical functions in the brain and the vascular system, highlighting their importance in both neuronal and vascular biology and complex diseases such as VCI.

1.3.1 Function and Regulation of Rho GTPases in the Brain

Rho GTPases are central regulators of cytoskeletal dynamics, playing critical roles in neuronal development and plasticity. They mediate processes such as neuronal growth cone dynamics, dendritic spine formation, and axonal pathfinding (Stankiewicz & Linseman, 2014). Rho GTPases have dual roles in neuronal survival and death, with Rac activation typically promoting neuronal survival, while Rho activation can lead to neuronal death (Linseman, 2008; L. Luo, 2000). Rac stimulates pro-survival pathways, including p21-activated kinase (PAK signaling) and phosphatidylinositol-3 kinase (PI3K)/Akt pathways, that suppress cellular and mitochondrial apoptosis (Loucks et al., 2006; Stankiewicz et al., 2012). Conversely, Rho signaling, through its downstream effector Rho kinase (ROCK), antagonizes Rac-mediated survival pathways by activating PTEN phosphatase, which inhibits Akt signaling (Lai et al., 2014).

Among the Rac isoforms (Rac1, Rac2, and Rac3), Rac1 is ubiquitously expressed, Rac2 is hematopoietic-specific, and Rac3 is brain-specific, with its expression peaking during

synaptogenesis (Donovan et al., 2002). Rac1 and Rac3 appear to have overlapping roles, as Rac1/3 double knockout mice display hippocampal abnormalities and behavioral deficits (Corbetta et al., 2009). Similarly, RhoA, the most studied Rho isoform, exhibits cell-type-specific functions in nervous system development, with its deletion in neuroepithelial cells leading to embryonic lethality and disruptions in neural progenitor proliferation (Herzog et al., 2011; Katayama et al., 2011).

Downstream of Rho GTPases, effector proteins such as PAKs and ROCKs regulate the actin cytoskeleton and synaptic plasticity. PAKs are activated by Rac/Cdc42 and promote spine morphogenesis, while ROCKs are activated by Rho and regulate synaptic plasticity. Knockout studies in mice have demonstrated that alterations in PAK or ROCK signaling lead to changes in spine density, impaired long-term potentiation and long-term depression (W. Huang et al., 2011; Meng et al., 2005; Y. Wang et al., 2018; H. Zhang et al., 2021). Additionally, RhoA signaling has been implicated in memory formation, with its inhibition impairing conditioned place aversion memory in rats, whereas Rac1 inhibition had no effect (J. Wang et al., 2013). Intriguingly, Rac1 has been linked to memory forgetting, with studies showing that manipulating Rac1 activity in *Drosophila* and mice affects memory decay and reversal learning (Haditsch et al., 2009; Shuai et al., 2010).

The dysregulation of Rho GTPases is implicated in various neurodegenerative diseases. Aberrant Rac and Rho activity have been reported in AD, amyotrophic lateral sclerosis (ALS), and ischemia-related conditions and other neuronal disorders (H. Zhang et al., 2021). In AD, increased Rac1 activity contributes to amyloid-β-induced synaptic dysfunction and oxidative stress and exacerbates the production of reactive oxygen species (ROS), promoting neuroinflammation and synaptic impairments (M. Lee et al., 2002; P. Wang et al., 2009). Dysregulated activity of Rho GTPase leads to neurite retraction and degeneration, further contributing to the neuronal loss and cognitive decline in AD (Huesa et al., 2010). In ALS, mutations in Rac1 regulatory genes and epigenetic regulation of the Rac GEF *ARHGEF16* (Rho Guanine Nucleotide Exchange Factor 16) have been connected to motor neuron degeneration and neuronal apoptosis, respectively signaling (Figueroa-Romero et al., 2012; Hadano et al., 2007; Kanekura et al., 2005). In cerebral ischemia and reperfusion (I/R), dysregulated Rac1 activity has been also linked to neuronal injury and cell death with I/R models showing decreased active Rac1 levels (Gutiérrez-Vargas et al., 2010). Conversely, increase in Rac1 levels was shown to potentially delay cell death (Q.-G. Zhang et al., 2006, 2009). In addition, RhoA upregulation in ischemic conditions leads to actin cytoskeletal changes and neurodegeneration and inhibiting the related Rho/ROCK pathway can protect neurons from ischemic damage (Brabeck et al., 2003; Satoh et al., 2007; Stankiewicz et al., 2012). In summary, dysregulation of Rho GTPases contributes to the pathology of several neurodegenerative

diseases, indicating their important role in regulating neuronal processes and suggesting signaling pathways downstream of Rho GTPases as potential therapeutic targets.

1.3.2 Role of Rho GTPases in the Vascular System

Rho GTPases and their downstream effectors are critical regulators of vascular biology, governing endothelial barrier function, inflammation, leukocyte migration, platelet activation, thrombosis, oxidative stress, and vascular smooth muscle cell behavior (Marinković et al., 2015; Strassheim et al., 2019). In endothelial cells, Rho/Rho-kinase signaling disrupts barrier integrity, promotes ROS production, and induces inflammation. Dysregulated RhoA/ROCK activity destabilizes endothelial cell junctions and enhances vascular permeability, leading to inflammation and leukocyte migration (O. M. Yu & Brown, 2015). Rac1 activation has been shown to regulate cytokine-induced NFκB activity through a redox-dependent pathway, driving inflammation and promoting ROS production (Strassheim et al., 2019). Interestingly, while RhoA and RhoB can either promote or inhibit NFκB activation depending on the context, Rac1 and Cdc42 generally activate NFκB, further emphasizing the complex regulatory roles of these GTPases (Tong & Tergaonkar, 2014). In vascular smooth muscle cells, the RhoA/ROCK pathway promotes contraction, migration, and proliferation, processes critical for maintaining vascular tone and responding to injury (Swärd et al., 2003). RhoA/ROCK signaling enhances smooth muscle contraction through phosphorylation of myosin light chain via inhibition of myosin phosphatase (Brozovich et al., 2016). Elevated Rho-GEFs and myosin phosphatase target subunit 1 have been observed in hypertensive patients, contributing to vascular hypercontractility and increased blood pressure (Strassheim et al., 2019).

Rho GTPases are key mediators of ROS production in the vasculature induced by mechanical stretch, pressure, hypoxia, shear stress, and growth factors (Zhou et al., 2013). ROS production increases endothelial permeability, promotes leukocyte adhesion, and facilitates their extravasation across the endothelial barrier (Boueiz & Hassoun, 2009). These oxidative stress responses are pivotal in the development of vascular diseases, including atherosclerosis, aortic aneurysms, and vascular stenosis (Strassheim et al., 2019). Furthermore, vascular endothelial growth factor (VEGF), histamine, and inflammation-induced permeability depend on Rho GTPase-mediated signaling, with NO playing a critical role. Notably, mice deficient in eNOS or with mutations in the eNOS phosphorylation site S1176 demonstrated reduced vascular permeability and RhoA activation, underscoring the interplay between NO and Rho signaling in maintaining vascular integrity (Di Lorenzo et al., 2013).

Increased ROCK activity has been observed in patients with hypertension, pulmonary hypertension, angina, heart failure, peripheral artery disease, and stroke (Strassheim et al., 2019).

Polymorphisms in genes regulating Rho GTPases, such as *ROCK2*, correlate with a reduced risk of hypertension (Rankinen et al., 2008). Elevated leukocyte ROCK activity has been noted in patients with metabolic syndrome, dyslipidemia, and coronary artery disease, highlighting its potential role as a biomarker for cardiovascular disorders (Shimizu & Liao, 2016). Clinical trials have demonstrated the beneficial effects of ROCK inhibitors like fasudil in improving endothelial function in patients with coronary artery disease (Nohria et al., 2006). Similarly, long-term inhibition of Rac1 using small-molecule inhibitors has shown atheroprotective effects in hypercholesterolemic $ApoE^{-/-}$ mice with reduction of NADPH oxidase activity, decrease in oxidative stress, and improvements in endothelial function (Zimmer et al., 2021).

In conclusion, Rho GTPases play a critical role in both the vascular and central nervous systems by regulating key processes such as cytoskeletal dynamics, endothelial and neuronal function, ROS production, inflammation, and vascular permeability. Targeting Rho GTPase pathways could not only mitigate vascular dysfunction but also address associated cognitive decline. This dual therapeutic potential makes Rho GTPases an exciting focus for advancing our understanding and treatment of VCI and related diseases.

2 Methods

To achieve the objectives of this study, genomics, epigenomics, transcriptomics, and metabolomics datasets were pre-processed and analyzed using state-of-the-art computational methods. Results from individual omics analyses were then integrated to examine multi-omics relationships between datasets, facilitating a deeper understanding of the complex biological mechanisms underlying VCI. A simplified experimental scheme workflow can be found in Figure 2. It is important to note that this research was solely computational; no laboratory procedures were performed by the author of this thesis, and all methods pertain to computational biology.

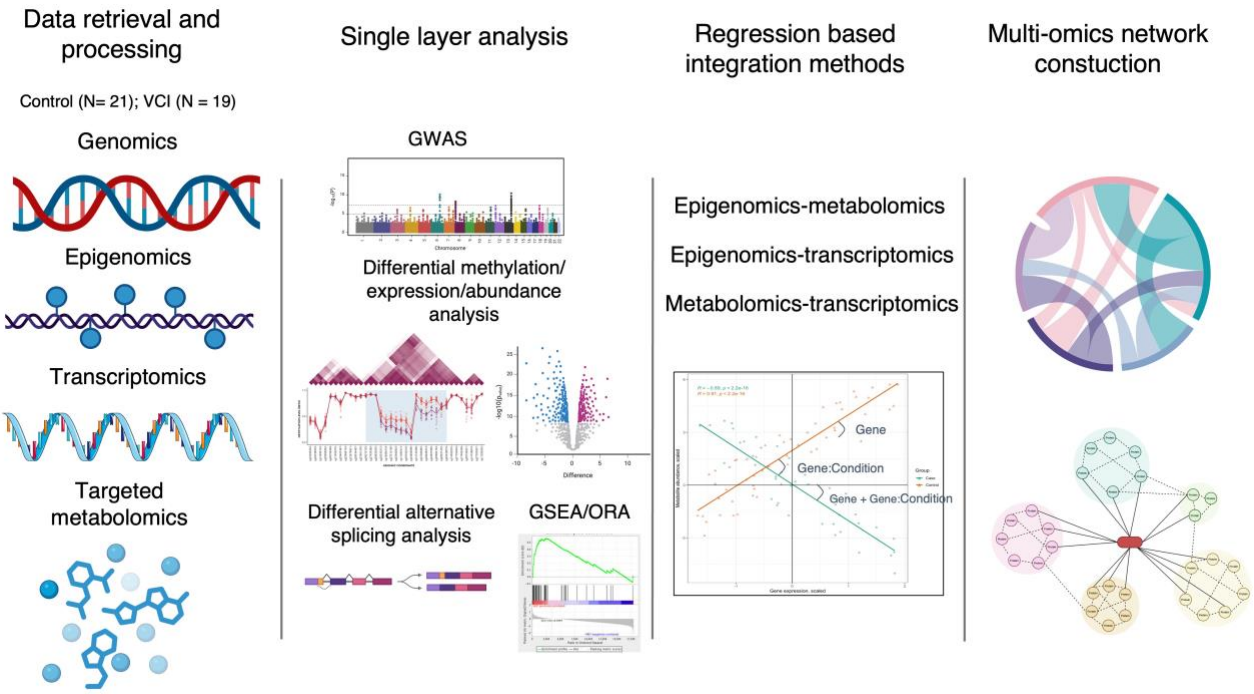

Figure 2. Simplified experimental workflow scheme

2.1 Study Cohort

This study analyzed post-mortem brain tissue samples from the parietal cortex, specifically Brodmann area 7. The study cohort for epigenomics, transcriptomics, and metabolomics data analysis included 40 samples: 21 control samples from individuals with no neuropathological abnormalities, and 19 samples from patients diagnosed with VCI (Appendix Supplementary table 1). Importantly, samples from the same tissue were used across different omics analyses (epigenomics, transcriptomics, and metabolomics), allowing for the integration of multiple omics datasets for each individual sample.

For the genomics analysis, a slightly smaller cohort was available (N = 31), including 10 samples from individuals with VCI and 21 control samples.

2.2 Common Statistical Methods

Several statistical methods were applied consistently across the analysis of different omics datasets (genomics, epigenomics, transcriptomics, and metabolomics) and during the integration phases. These methods are detailed below to avoid repetition within the individual omics analysis sections.

Principal Component Analysis (PCA) for Outlier Detection:

PCA was employed to identify outlier samples based on the distribution of their data in the principal component (PC) space (Pearson, 1901). Typically, PCA was performed on normalized and/or transformed data for each omics dataset. Samples exhibiting values exceeding a specified number of standard deviations (SDs) (e.g., 2 or 3) from the mean along the first three PCs were flagged as potential outliers for further inspection and potential exclusion from downstream analyses, as detailed in the respective omics analysis sections.

Imputation of Missing Values:

The k-Nearest Neighbors (k-NN) algorithm, implemented using the impute.knn function from the impute R package (v1.76.0) (Trevor Hastie, 2017) was used to impute missing values in the epigenomics and metabolomics datasets. This method estimates missing values for a given feature (e.g., methylation probe, metabolite) based on the values of its k nearest neighbors among the samples, where 'k' was set to five.

Linear Regression Analysis:

Linear regression models were implemented using the limma R package (v3.58.1) (Ritchie et al., 2015) to assess the relationship between variables of interest and the outcome (e.g., VCI status). Models were adjusted for relevant covariates, including condition, age, sex assigned at birth, and post-mortem interval (PMI), as specified in the respective omics analysis sections. To account for potential unobserved confounding factors, the RUVSeq R package (v1.36.0) (Risso et al., 2014) was used to estimate and incorporate unwanted variation (RUVg vectors) as additional covariates in the models. Robust aggregation methods with a maximum of 100 iterations were employed for model fitting to provide more stable estimates. Empirical Bayes statistics were subsequently estimated using the eBayes function within limma to improve statistical power.

Pathway Enrichment Analysis:

Two primary approaches were used for pathway enrichment analysis: Over-Representation Analysis (ORA) and Gene Set Enrichment Analysis (GSEA). ORA was performed using the enricher function from the clusterProfiler R package (v4.6.0) (T. Wu et al., 2021). For GWAS and multi-omics network analysis, a gene list comprising genes of interest was tested for enrichment against a background set of all genes identified in the respective omics datasets. The

Bader Lab GMT file (Toronto), containing pathways from various databases, including KEGG, MSigDB, NCI, IOC, NetPath, HumanCYC, Reactome, GO, MSigDB-c3, Panther, PathBank, and WikiPathways, served as the gene set database. GSEA was conducted using the software from gsea-msigdb.org (Mootha et al., 2003; Subramanian et al., 2005). Genes were ranked based on the sign of the fold change multiplied by the negative log-transformed p-value from the differential analysis. Enrichment was assessed for pathways containing 10 to 500 genes, using the classic scoring scheme and meandiv normalization with 1,000 permutations. Enriched pathways were further clustered based on their functional similarities, and their interaction networks were visualized using the aPEAR R package (v1.0.0) (Kerseviciute & Gordevicius, 2023). Clusters were named according to the most influential pathway within each group, identified using the PageRank algorithm (Page et al., 1999), providing a comprehensive view of disrupted biological pathways.

Multiple Testing Correction:

To control for the increased risk of false positives due to multiple comparisons, p-values from differential analyses and pathway enrichment tests were adjusted using the Benjamini-Hochberg (BH) method (Benjamini & Hochberg, 1995). A q-value (adjusted p-value) threshold of 0.05 was used to determine statistical significance, unless otherwise specified.

Fisher's Exact Test:

Fisher's exact test was applied in specific analyses to evaluate the significance of non-random associations between categorical variables.

2.3 Genomics Data Analysis

Genome-wide sequence data were obtained from the NIMH Data Archive (NDA; https://nda.nih.gov/) under a Data Use Certification (OMB Control Number: 0925-0667; Expiration Date: 06/30/2026). Data were accessed securely using institutional Corewell computers with authorized login credentials. All data were stored in restricted-access folders, with adherence to institutional data management and retention policies. When no longer needed for research or legal obligations, the data will be securely deleted using institution-approved methods. All variant call format (VCF) file manipulations were performed using BCFtools (v.1.21) (Wu et al., 2021). Raw VCF files were merged, retaining only autosomal variants. The filtered VCF was annotated with dbSNP (Sherry et al., 1999) identifiers based on the hg38 reference genome to ensure consistent variant identification. Quality control was performed using PLINK (v.1.9) (Purcell et al., 2007). Genotype missingness was assessed per variant and per individual. Variants with a missingness rate exceeding 0.01 were excluded. Similarly, individuals with a genotype missingness rate above 0.1 were excluded from further analyses. Variants were further filtered based on minor allele frequency and Hardy-Weinberg equilibrium. A minimum minor allele frequency threshold of 0.1 was applied

to retain common variants for association analyses, while variants with a Hardy-Weinberg equilibrium p-value below 0.001 were excluded.

Heterozygosity rates were calculated per individual to identify and flag potential outliers. To control for population stratification, PCA was performed. Before PCA, SNPs were pruned for linkage disequilibrium to reduce correlations among markers, ensuring that PCs captured population structure rather than local linkage effects.

Association testing was conducted in PLINK (v.1.9) (Purcell et al., 2007) using logistic regression for each variant. To control for population structure, the first PC from PCA was included as a covariate in the regression model, along with additional covariates, including sex assigned at birth, age, and PMI, to account for demographic and technical factors potentially influencing genotype-phenotype associations. To evaluate the quality of GWAS results, we calculated the inflation factor (λ), which measures deviation of the observed test statistics from the expected distribution under the null hypothesis.

Pathway enrichment analysis was performed using an ORA approach. SNPs were first annotated using the annotatr (v.1.28.0) R package (Cavalcante & Sartor, 2017), and for SNPs associated with multiple genes, a robust method was developed to assign a single gene name to each SNP based on the most frequent annotation results.

2.4 Epigenomics Data Analysis

Raw data from the Infinium MethylationEPIC v2.0 array were pre-processed using the minfi R package (v1.48.0) (Aryee et al., 2014). Subsequent analyses utilized normalized beta values, pre-processed with the preprocessNoob function from minfi (v1.48.0) R package, applying default parameters. Outlier detection was carried out using PCA as described in the "2.2 Common Statistical Methods" section and two SDs from the mean were used.

For beta value matrix normalization, probes with infinite methylation values or detection p-value lower than 0.01 were marked as missing. Probes with more than a half missing values, as well as those on sex chromosomes, and those containing common SNPs (minor allele frequency > 0.05) were excluded. Remaining missing methylation values were imputed. Variance inflation factor was calculated with the vif function from car (v3.1.0) R package to assess multicollinearity (Daoud, 2017).

Methylation beta values were transformed into M values and differential methylation analysis was conducted. The regression model was adjusted for condition, sentrix ID, age, sex assigned at birth and PMI. One RUVg vector was used. Additionally, differentially methylated region (DMR) analysis was performed, where methylation regions were defined as clusters of probes with similar methylation levels. The normalized beta value matrix was scaled and Euclidean

distances between probe methylation levels were calculated. The distance matrix was then scaled using a sigmoid function and clustered hierarchically, grouping nearby probes with similar methylation levels. After clustering, the beta value matrix was recalculated using cluster methylation values instead of individual probe values. Normalized probe beta values were converted to M values, and the median M value of all probes in each cluster was used. This cluster M-value matrix was then used in linear regression modeling, as previously described in the "2.2 Common Statistical Methods" section.

Pathway enrichment analysis was performed using GSEA method and by annotating each Infinium MethylationEPIC v2.0 array probe with UCSC reference gene names, retaining the CpG with the lowest p-value for each gene.

2.5 Transcriptomics Data Analysis

RNA extraction and sequencing of parietal cortex samples were conducted by colleagues at the Corewell Institute. Initial quality control and pre-processing steps were performed using fastp (v0.23.2) (S. Chen et al., 2018), which included removal of duplicate sequences and adapter trimming with default parameters. Further quality control was conducted with FastQC (v2.13.0) and MultiQC (v1.14.0) software (Ewels et al., 2016). Gene alignment and mapping were performed using STAR (v2.7.9a) (Dobin et al., 2013) with the human reference genome GRCh38 version 44.

Initial filtering by expression levels was conducted using the filterByExpr function from the edgeR (v3.42.4) R package (Robinson et al., 2010) with default settings. Data normalization was then performed with the calcNormFactors function using the Trimmed Mean of M-values (TMM) method (Robinson & Oshlack, 2010). The cpm function was used to convert raw counts into the Counts per Million (CPM) values, and logarithmic transformation was applied for subsequent analysis.

A series of quality control steps were implemented to ensure data integrity prior to downstream analysis. Read mapping quality metrics, including the number of ambiguous, multi-mapping, non-mapped, and mapped reads, were evaluated in relation to the outcome variable. Samples with a percentage of mapped reads below 25 % were designated as outliers. Additionally, samples with mapped read counts exceeding two SDs from the mean were marked as outliers. For clustering, hierarchical clustering was performed using correlation distance to evaluate sample similarity. Low-variability genes were excluded from this analysis to improve clustering accuracy. Samples with correlations lower than two SDs from the overall average correlation were marked as outliers. Data scaling was also applied to standardize the range of independent genes to prevent any single gene from disproportionately influencing the PCs in the PCA. PCs of the scaled, normalized

count matrix were calculated, with any samples exceeding two SDs in the first three PCs marker as outliers.

Normalized gene counts were then used to fit a linear regression model as described previously. Two RUVg vectors were included to account for unobserved sources of variability. Pathway enrichment analysis was performed using GSEA method as described in the "2.2 Common Statistical Methods" section.

Additionally, alternative splicing analysis was performed using the LeafCutter tool (v0.2.9) (Y. I. Li et al., 2018), which uniquely quantifies RNA splicing variation from short-read RNA-seq data. LeafCutter's core approach leverages spliced reads (those spanning an intron) to quantify differential intron usage across samples. Sorted BAM files were indexed with Samtools (v1.12) (Danecek et al., 2021) to enable efficient access and processing of exon-exon junctions. Exon-exon junctions were then extracted from BAM files using Regtools (v.1.0.0) (Cotto et al., 2023) with a minimum anchor length of 8 base pairs on each end, and intron sizes restricted to between 50 and 500,000 base pairs. Intron clusters were identified by setting clustering parameters to allow intron lengths up to 500 kb and requiring a minimum of 20 reads per cluster. Differential intron excision between conditions was then assessed in LeafCutter, using a contrast defined by sample groups, comparing samples from patients with VCI against controls.

2.6 Metabolomics Data Analysis

Targeted metabolomics using mass spectrometry and nuclear magnetic resonance was conducted by colleagues at the Corewell Institute. Abundance measurements were collected for each patient, and mass spectrometry and nuclear magnetic resonance data were combined to provide a comprehensive metabolomics profile.

For quality control, metabolites with zero concentration values were designated as missing. Metabolites with more than 30 % missing values across all samples were excluded from further analysis. A natural logarithmic transformation was applied to the data to reduce skewness. Metabolites with variance below the 5th percentile were considered invariant and removed from the analysis. Samples with more than 80 % missing values were also excluded. Remaining missing values were imputed. Outlier detection was carried out using PCA as described in the "2.2 Common Statistical Methods" section and three standard deviations from the mean were used.

Further normalization was performed both sample-wise and metabolite-wise. Sample-wise normalization was conducted by scaling metabolite intensities within each sample, and then metabolite-wise normalization scaled the intensity values across the dataset. Any metabolites with zero variance post-normalization were removed, and metabolite intensities greater than five (considered erroneous) were marked as missing and subsequently imputed as described previously.

Normalized metabolite concentrations were used to fit a linear regression model as described in the "2.2 Common Statistical Methods" section. Unknown confounders were controlled using three RUVg vectors.

2.7 Regression Based Integration Methods

We performed three types of pairwise multi-omics analyses: epigenomics-metabolomics, transcriptomics-metabolomics, and epigenomics-transcriptomics. These analyses used linear regression models with interaction terms to assess associations between datasets from different omics layers. For epigenomics-metabolomics, we examined associations between all methylation regions from the epigenomics dataset and all metabolites. For epigenomics-transcriptomics, we assessed associations between methylation regions and transcripts from the transcriptomics dataset. Finally, in the transcriptomics-metabolomics analysis, we tested associations between metabolites and transcripts. The primary objective of these analyses was to identify cross-omics associations potentially influenced by VCI.

Prior to analysis, normalized data were pre-processed to ensure quality and improve the robustness of the results. Low-variance features, with variance below 0.01, were also excluded to retain only features with sufficient variability for meaningful association testing.

Processed data was used to fit a linear regression model which was adjusted for condition, the interaction between condition and the feature of interest, the normalized feature values (e.g., methylation or expression), age, sex assigned at birth, PMI and RUVg vectors derived from single omics analyses to correct for unknown sources of variation. For example, the model for epigenomics-metabolomics was structured as follows:

Metabolite abundance ~ 1 + Condition + (CpG × Region methylation) + Region methylation + Age + Sex + PMI + RUVg vectors

For each pairwise analysis, we tested two contrasts to assess the strength and nature of associations:

- **Control-only associations:** Tested whether the association between two features was significant in control samples.
- **Differential associations:** Tested whether the level of association differed between control and VCI samples, indicating a disease-related effect.

Multiple testing correction was performed as described earlier. To interpret the results, we classified associations into four categories:

- **Association gain:** Associations were i) significant only in VCI samples or ii) in both conditions but with a stronger effect size association in VCI, indicated by a greater absolute coefficient value.

- **Association loss:** Associations were i) significant only in VCI samples with or ii) in both conditions but weaker in VCI, indicated by a smaller absolute coefficient value.
- **Association reversal:** Associations were significant in both conditions but showed opposite directions of correlation (positive vs. negative) between control and VCI samples.
- **Unaffected:** Associations were significant only in control samples and unchanged in VCI.

Given the focus on disease-related changes, unaffected associations were excluded from further analyses and significant disease-affected associations (gain of function, loss of function, and function reversal) were visualized using circos plots generated with the circlize R package (v0.4.16) (Gu et al., 2014). These visualizations highlighted cross-omics interactions that were significantly altered in the disease context.

2.8 Multi-omics Network Construction

In earlier steps, we performed pairwise integration analyses between the three omics datasets (transcriptomics-metabolomics, epigenomics-transcriptomics, and epigenomics-metabolomics), identifying significant associations that differed between VCI and control samples. To further investigate the shared characteristics of these altered associations, we analyzed common genes and metabolites across the pairwise comparisons, aiming to identify potential patterns or shared molecular features.

To gain functional insights, we conducted an ORA of genes with altered associations in VCI. The background gene list comprised all genes identified in the transcriptomics and epigenomics datasets, while the list of genes of interest included only those showing significant association changes in VCI samples compared to controls based on the regression-based pairwise integration analyses.

To explore the interactions between the genes identified across all pairwise omics comparisons, we applied the DIAMOnD network diffusion algorithm (Ghiassian et al., 2015), which prioritizes genes based on their connectivity to the initial set. DIAMOnD evaluates the significance of interactions within the network, incorporating genes with strong connections to the initial list. Human brain interactome data were retrieved from the TissueNet v.2 database (Basha et al., 2017), which includes 165,240 tissue-specific protein-protein interactions. Using 200 iterations of the DIAMOnD algorithm, we generated a subnetwork encompassing the initial genes and their network neighborhood, resulting in a gene regulatory network of the top 200 genes. All other DIAMOnD parameters were kept at default settings.

3 Results

To investigate the molecular underpinnings of VCI, we conducted an integrative multi-omics analysis combining genomics, epigenomics, transcriptomics, and metabolomics data. Our goal was to identify pathways consistently disrupted across omics layers that may contribute to VCI pathophysiology.

3.1 Genomic findings suggest Rac GTPase pathway involvement in VCI mechanisms

For the genomics analysis, we analyzed a cohort of 31 samples, including 10 from individuals with VCI and 21 from individuals without a confirmed neurological phenotype. Following quality control, a total of 139 341 sequence and structural variants were analyzed, the but the GWAS identified no variants with significant affects after multiple testing correction. The genomic inflation factor (λ) yielded an elevated value of 1.14, suggesting inflated test statistics, likely due to confounding factors such as population stratification, cryptic relatedness, or genotyping errors, compounded by the study's small sample size (N = 31). Therefore, due to the limited sample size, the genomic analysis was treated as a pilot project aimed at identifying potentially affected pathways and prioritizing candidate genes for further study.

To investigate GWAS results on a pathway-level, we selected variants with GWAS p-value < 0.01, selected unique genes covering these variants and conducted ORA. While no pathways reached statistical significance after multiple testing correction (q-value < 0.05) – likely due to sample size limitations – we identified three pathways with unadjusted p-value below 0.01: the Rac1 GTPase cycle, organophosphate catabolic process, and stimuli-sensing channels.

To explore the genetic association signals in the three pathways highlighted in ORA, we have focused on individual association signals for the single nucleotide variants (SNVs) in genes linked to each of these pathways. For this, we have selected all the variants in the genes linked to the pathways of interest and carried out Fisher's exact test to test if the number of significant associations for the specific set of variants was non-random. This analysis revealed a significant enrichment of associated variants within genes involved in the Rac GTPase cycle (odds ratio (OR) = 4.52, p = 0.0065; Figure 3.1a). No significant enrichment was observed for SNVs in genes linked to organophosphate catabolic process and stimuli-sensing channels, based on the significance threshold set for this pilot study (p-value < 0.01) (Figure 3.1 b, c).

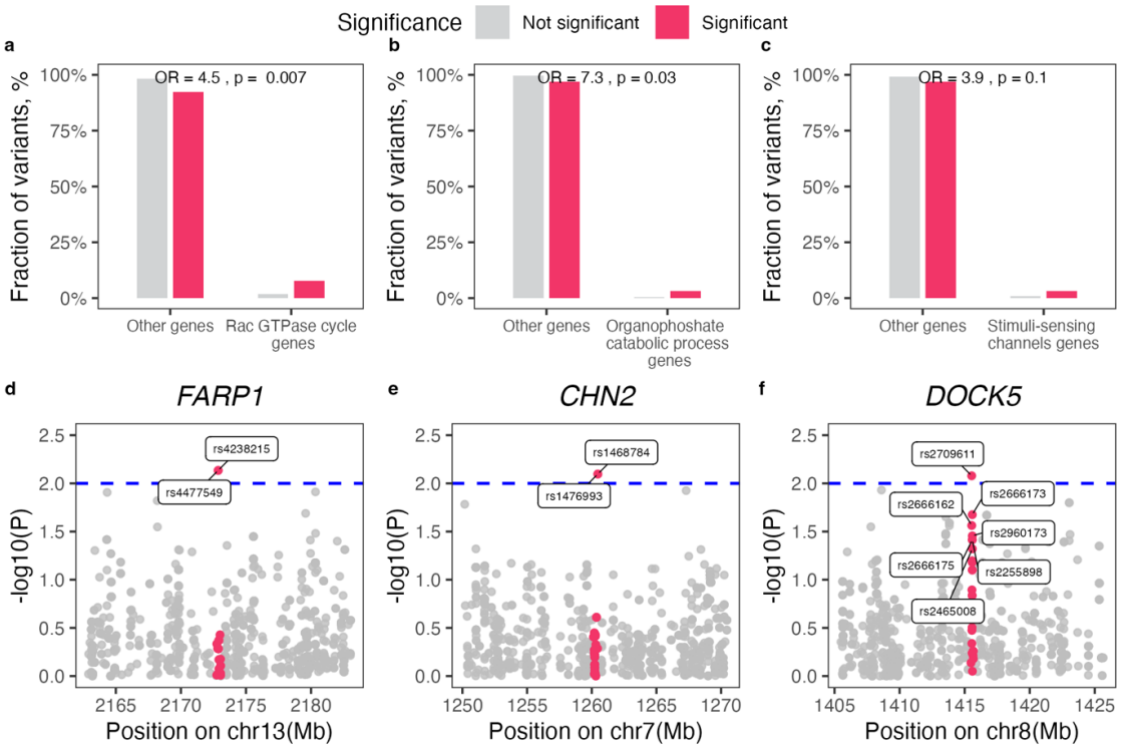

Figure 3.1. Genomics analysis results (a-c). Proportions of variants in Rac GTPase cycle, organophosphate catabolic process, and stimuli-sensing channel pathway genes compared to other genes in statistically significant (pink) and non-significant (grey) groups. Fisher's exact test was used to assess the non-randomness of the associations between significant variants and each pathway. **(d-f)** Locus-level association plots for the selected three Rac GTPase cycle-associated genes: *FARP1*, *CHN2*, and *DOCK5*. Variants with-in the target gene are highlighted in pink and those with p-value < 0.01 are labelled.

Focusing specifically on the Rac GTPase cycle pathway, we further examined individual associations among 1415 variants located in 93 Rac GTPase cycle pathway genes. Five variants were associated with VCI under the suggestive significance threshold (p-value < 0.01). The five variants are in three genes: *FARP1* (FERM, ARH/RhoGEF, and pleckstrin domain-containing protein 1), *CHN2* (Chimerin 2), and *DOCK5* (Dedicator of Cytokinesis 5) (Figure 3.1 d-f, Appendix Supplementary table 2). These findings suggest performing further analysis as dysregulation of the Rac GTPase cycle may contribute to the molecular mechanisms underlying VCI.

3.2 Widespread epigenetic alterations implicate Rac GTPase pathway hypermethylation in VCI

In this part of analysis, DNA methylation profiles were analyzed in 40 subjects, including 19 samples from the superior parietal brain regions (Brodmann area 7) of individuals with VCI and 21 samples from pathologically healthy controls. Quality control analysis using PCs identified two outlier samples. After visual inspection and considering inter-array clustering results, one control sample was excluded to optimize data quality while maximizing overlapping samples (N = 39) for regression-based integration methods (Appendix Supplementary figure 1a).

Following quality control, 740 679 CpG sites were included in the methylation analysis. Differential methylation analysis revealed 3601 differentially methylated cytosines (DMCs) between VCI and controls, with 2953 hypermethylated and 648 hypomethylated CpG sites (Appendix Supplementary table 2). Hypermethylation was significantly over-represented in VCI (OR = 2.42, $p < 2.2 \times 10^{-16}$, Fisher's exact test). In the GWAS analysis (see above) we identified the Rac GTPase pathway as a potentially impacted pathway in VCI compared to controls. Given this finding, we as well prioritized a deeper investigation of Rac GTPase-related genes methylation. To achieve this, we compiled a targeted gene list encompassing all genes associated with the Rac/Rho subfamily GTPase cycle. DMC analysis pinpointed 50 differentially methylated cytosines, 41 of which were hypermethylated, linked to 42 unique genes in the Rac/Rho GTPase pathway.

Further analysis of DMRs, defined as genomic regions of ≥ 5 probes with similar methylation patterns, revealed a stronger predominance of hypermethylation. In VCI condition, DMRs were enriched more than five times for hypermethylation compared to hypomethylation (OR = 5.25, $p < 2.2 \times 10^{-16}$, Fisher's exact test; Figure 3.2a). Of the 463 significant DMRs identified, 12 were associated with the Rac/Rho GTPase pathway (Appendix Supplementary table 2). All genes related to the Rac/Rho GTPase pathway and with significant methylation differences showed hypermethylation, except for *SRGAP1* (SLIT-ROBO Rho GTPase Activating Protein 1), which exhibited significant hypomethylation in VCI compared to controls.

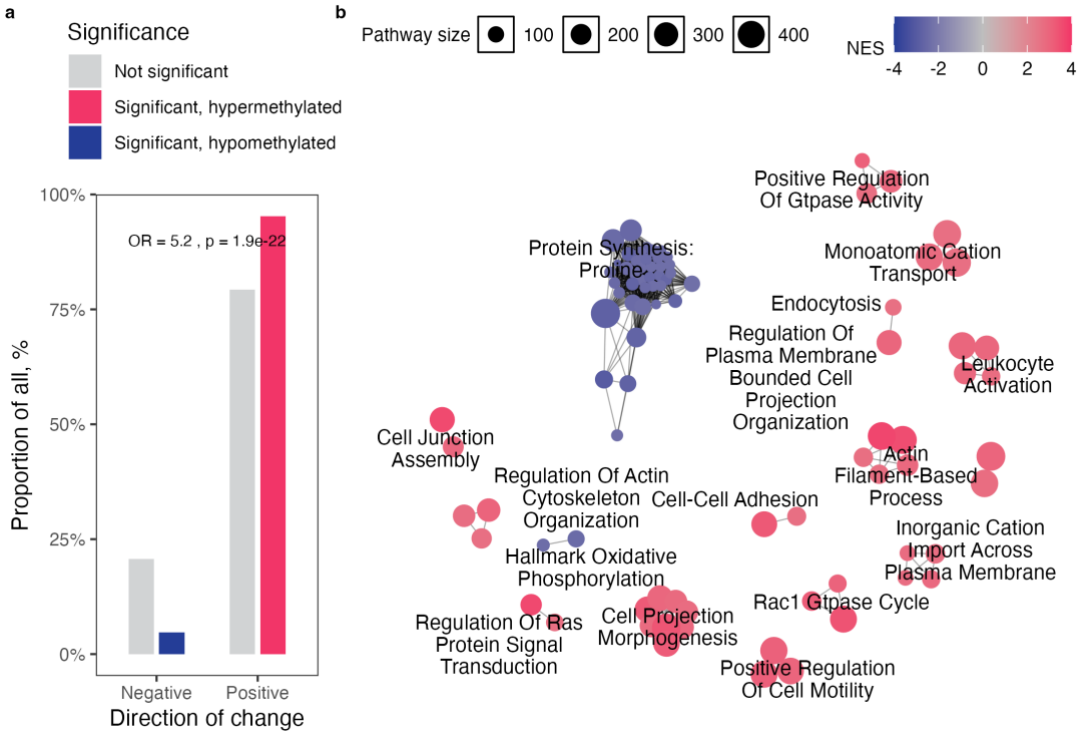

Figure 3.2. Epigenomics analysis results. (a) Proportion of hypermethylated and hypomethylated CpGs in statistically significant (pink for hypermethylation and blue for hypomethylation) and non-significant (grey) groups. Fisher's exact test indicates whether there is a tendency towards hyper-/hypo- methylation in VCI. **(b)** Network and clustering of significantly

affected pathways identified through GSEA. Each cluster is labelled with its most prominent pathway, determined using the PageRank algorithm. Node color represents the normalized enrichment score (with positive scores indicating a tendency for genes in the pathway to be hypermethylated), while node size corresponds to the number of genes identified within each pathway.

Pathway enrichment analysis based on DMR data revealed 180 and 45 pathways significantly enriched in hypermethylated and hypomethylated genes, respectively, in VCI compared to controls ($q < 0.05$; Appendix Supplementary table 3). Among the most affected clusters, pathways related to "Actin-filament-based process" and "Regulation of actin cytoskeleton organization" – regulated by Ras-related GTP-binding proteins (Rho, Ras, Cdc42) (Tapon, 1997) – were highly enriched in hypermethylated genes. Notably, hypermethylated pathways associated with the Rac/Rho GTPase cycle, such as "Rac1 GTPase Cycle", "Positive Regulation of GTPase Activity", and "Regulation of Ras Protein Signal Transduction", were significantly enriched (Figure 3.2b).

GSEA based on DMC data further supported these findings, revealing 1887 significantly enriched pathways (Appendix Supplementary table 3). Among the 1830 pathways enriched with hypermethylated genes, several were related to the disrupted Rac/Rho GTPase cycle (N = 25), neuroinflammation (N = 40) and cytoskeleton reorganization (N = 18), consistent with previous analyses. Numerous significantly enriched pathways were associated with oxidative stress, such as "Nitric Oxide Stimulates Guanylate Cyclase", "Regulation of Response to Oxidative Stress", and "Response to Hypoxia". Together, these results highlighted the widespread epigenetic dysregulation of the Rac/Rho GTPase pathway in VCI, with cascading effects on oxidative stress, hypoxia, and neuroinflammation.

3.3 Transcriptomics reveals dysregulated lipid metabolism, GTPase activity and oxidative stress in VCI

We further investigated the VCI cohort of 21 patients with and 19 controls by looking at gene expression levels in the brain tissues. Although PCA has identified three outlier samples, they were retained after visual inspection to ensure consistency with other omics datasets (Appendix Supplementary figure 1b). Variance inflation analysis revealed multicollinearity among age, sex assigned at birth, and post-mortem interval variables. Despite this, we preserved all variables in the differential expression models due to their biological relevance and to maintain consistency with the epigenomics data analysis approach. Following QC and pre-processing, 19 613 transcripts were reliably quantified across all samples.

Differential expression analysis revealed that 670 transcripts were significantly differentially expressed between VCI and control samples (Appendix Supplementary table 2). Gene

downregulation was significantly over-represented in VCI (OR = 1.55, $p < 3.36 \times 10^{-8}$, Fisher's exact test), with 403 genes significantly downregulated. Significant downregulation of gene expression is consistent with the observed trend of hypermethylation in the epigenome analysis and its established link to reduced gene expression. Eight out of 403 downregulated transcripts were linked to to the Rac/Rho GTPase cycle pathway, supporting the idea that the Rac/Rho GTPase cycle may contribute to the molecular mechanisms underlying VCI, notably, only three transcripts in this pathway were upregulated (Appendix Supplementary table 2).

In addition to differential expression analysis, we performed differential alternative splicing analysis, which identified local splicing variations within genes rather than overall gene activity. This analysis revealed 177 transcripts with significant differential events between VCI and control samples (Appendix Supplementary table 2). Five of these transcripts were associated with Rac/Rho GTPase cycle-associated genes – *ABCD3* (ATP Binding Cassette Subfamily D Member 3), *SRRM1* (Serine And Arginine Repetitive Matrix 1), *NSFL1C* (NSFL1 Cofactor), *CYFIP2* (Cytoplasmic FMR1 Interacting Protein 2), and *SPTAN1* (Spectrin Alpha, Non-Erythrocytic 1) (Figure 3.3a). These splicing variations in Rac/Rho GTPase cycle and other genes highlight an additional layer of regulatory complexity in VCI.

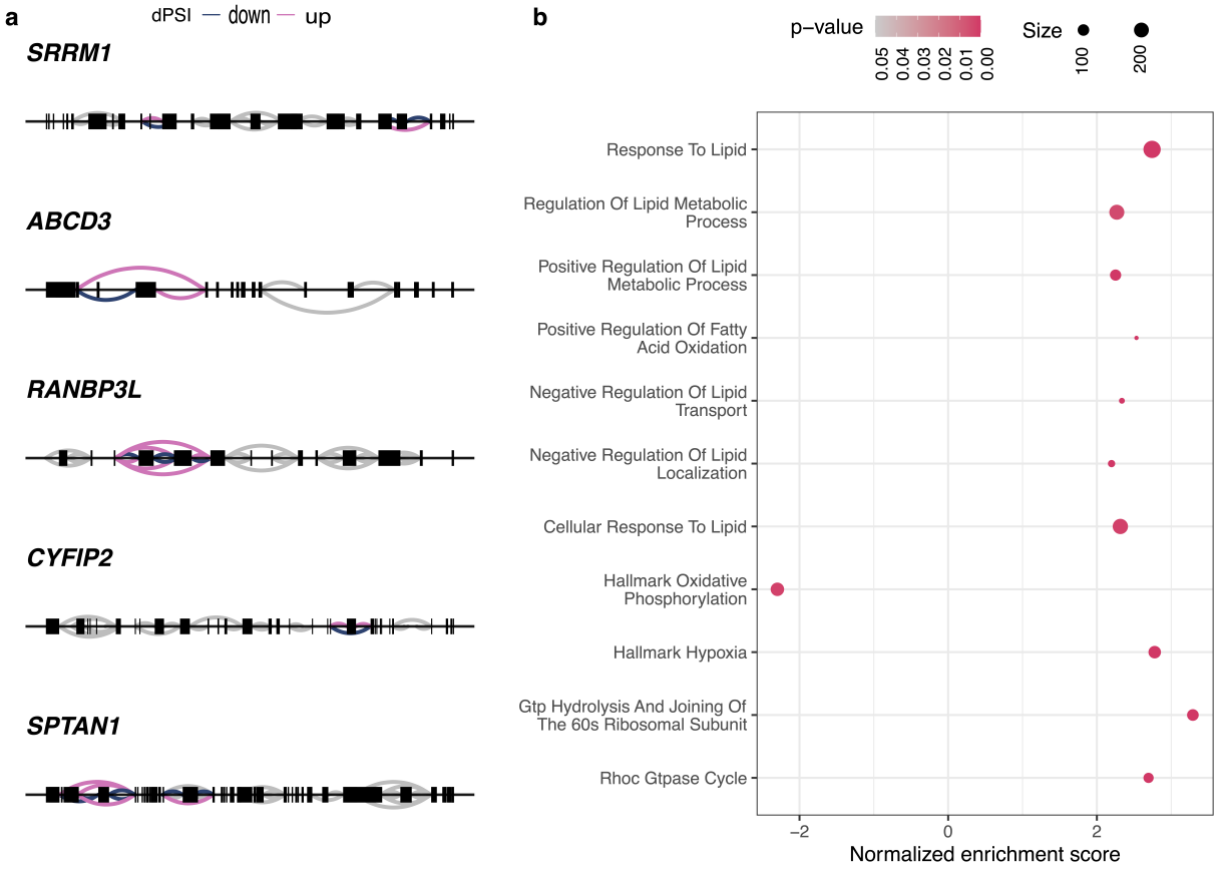

Figure 3.3. Transcriptomics analysis results. (a) All alternative splicing clusters found in Rac/Rho GTPase pathway associated genes. Significantly differentially spliced are colored in blue and pink, representing negative and positive dPSI (delta percent spliced-in) values.

Negative dPSI indicates or reduced inclusion of a specific splice site, while positive dPSI indicates increased alternative splicing in VCI. **(b)** Normalized enrichment scores of significantly enriched pathways. Node color represents the p-value, with more saturated color indicating higher statistical significance, and node size corresponds to the number of genes identified within each pathway.

GSEA of the differential expression analysis results identified 152 statistically significantly upregulated and 222 downregulated pathways ($q < 0.05$) when comparing VCI to control samples (Appendix Supplementary table 3). Among the significantly affected pathways were those related to lipid metabolism, hypoxia activation, and the RhoC GTPase cycle. (Figure 3.3b). These findings align closely with results from the epigenomics analysis, reinforcing the hypothesis that Rac/Rho GTPase pathways, along with oxidative stress mechanisms, are potential key players in VCI.

3.4 Epigenetic alterations in Rac GTPase pathway genes and metabolite associations in VCI

Metabolomics analysis was performed on 40 individuals, testing 533 metabolites. Three metabolites showed significant changes: PC.O-40:1, Betaine, and Cer.d18:1/20:0-OH, all of which were downregulated (Appendix Supplementary figure 2).

For the integrated analysis in this study, we have matched 39 samples for the analysis, excluding one sample that was identified as an outlier during epigenome analysis through PCA and inter-array clustering. To uncover disease-specific alterations in methylation-metabolite interactions in VCI, we conducted an all-versus-all analysis (examining all metabolites and methylation regions that passed quality control) to investigate the associations between these regions identified from the epigenomics dataset and metabolites.

This regression-based pairwise integration analysis identified 5210 significant methylation region-metabolite associations with altered effects in VCI, from an initial pool of 1 850 043 associations examined in the all-versus-all analysis (Appendix Supplementary table 4). These changes were categorized as association gains (new significant associations in VCI), association losses (associations present in controls but absent in VCI), and association reversals (where the direction of the association, positive or negative, was reversed between controls and VCI).

To further investigate the genes where methylation changes showed statistically significant associations with metabolite abundance in VCI, we performed an ORA. This analysis identified 50 significantly enriched pathways, with main clusters of these pathways associated with the Rac1 GTPase cycle and negative regulation of phosphate metabolic processes (Figure 3.4). Notably, one fifth of the enriched pathways were related to the Rac/Rho GTPase cycle, further suggesting that epigenetic alterations in Rac/Rho GTPase pathway genes contribute metabolic dysregulation in VCI.

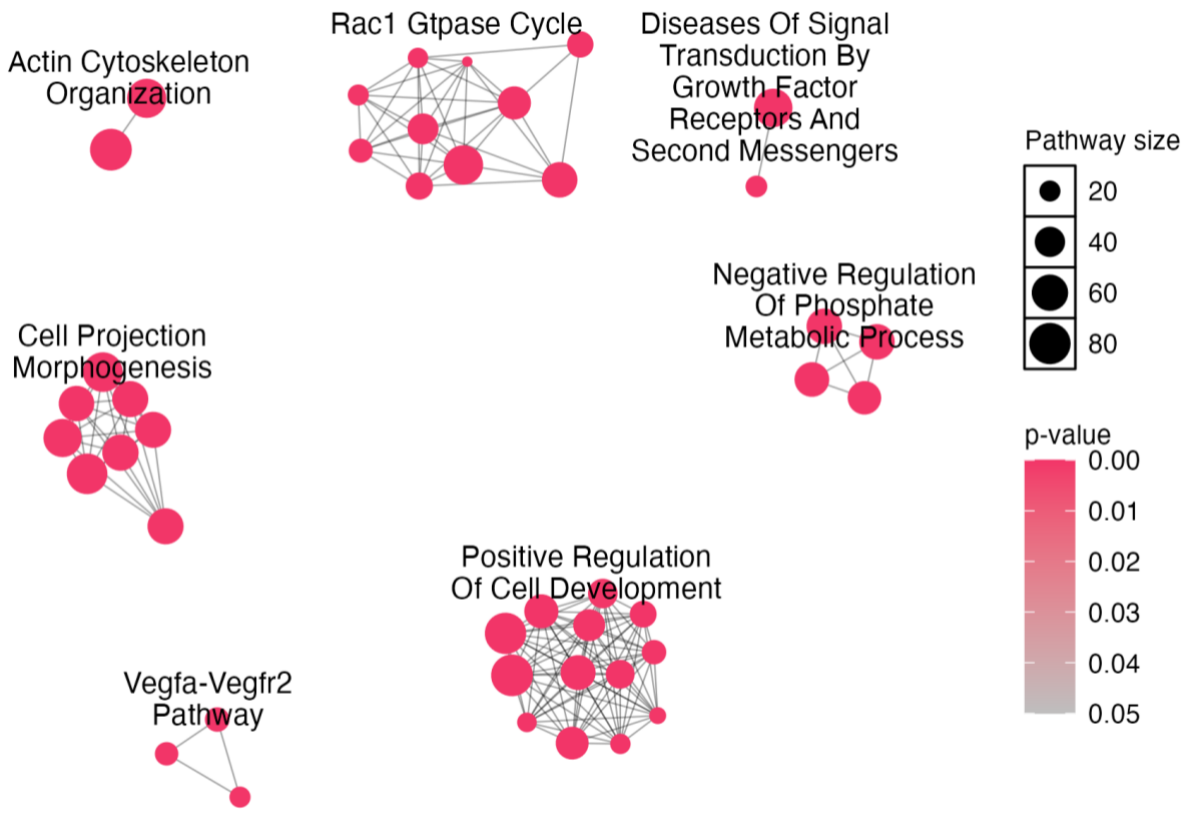

Figure 3.4. Network and clustering of significantly affected pathways identified through over-representation analysis of genes showing significant association changes with metabolites in VCI samples. Each cluster is labelled with its most prominent pathway, determined using the PageRank algorithm. Node color represents the p-value, with darker shades indicating higher significance, and node size corresponds to the number of genes identified within each pathway.

To delve deeper into the Rac/Rho GTPase pathway and its relationship with metabolites, we filtered the results to focus exclusively on associations involving Rac/Rho GTPase-related genes. This analysis revealed 178 significant methylation-metabolite association changes in VCI. Of these, the majority were association gains (N = 85), followed by 67 association losses, and 26 association reversals, where the regulatory effect between gene methylation and metabolite abundance shifted directionally in VCI.

These findings emphasize the pivotal role of epigenetic modifications in the Rac/Rho GTPase pathway and their contribution to metabolic dysregulation in VCI. The predominance of altered associations, particularly gains, underscores the complexity of epigenetic-metabolic interactions in the disease and highlights potential mechanistic links to Rac GTPase-mediated processes.

3.5 Multi-omics data analysis reveals Rac GTPase-driven dysregulation of diacylglycerol and phosphatidylethanolamine metabolism in VCI

In earlier steps, we performed pairwise comparisons across the three omics datasets (transcriptomics-metabolomics, epigenomics-transcriptomics, and epigenomics-metabolomics) to identify significant associations that differ in VCI compared to controls. To explore shared characteristics of these altered associations, we focused on identifying common genes across the pairwise comparisons, aiming to uncover potential patterns. ORA of genes with altered associations in VCI revealed significant enrichment in pathways related to the regulation of GTPase activity and the Rac1 GTPase cycle. These findings further corroborate the disruption of the Rac/Rho GTPase pathway observed across other omics layers, reinforcing its central role in VCI pathophysiology (Figure 3.5a, Appendix Supplementary table 5).

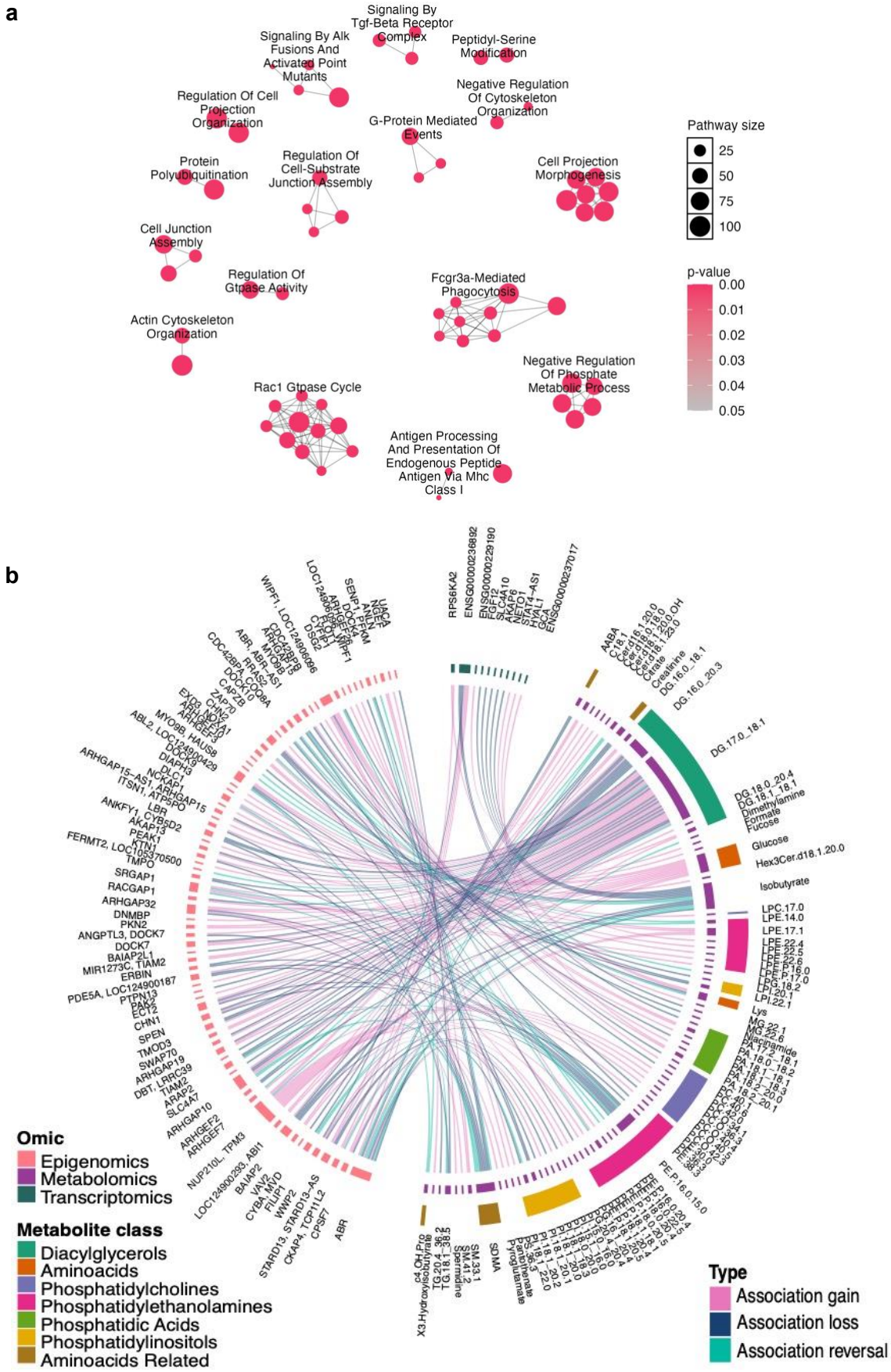

Figure 3.5. Multi-omics analysis results. (a) Network and clustering of significantly affected pathways identified in the over-representation analysis of genes exhibiting significant changes in associations across different omics in VCI samples compared to controls. Each cluster is labelled with its most prominent pathway, determined using the PageRank

algorithm. Node color represents the p-value, with darker shades indicating higher significance, and node size corresponds to the number of genes identified within each pathway. **(b)** The circos plot illustrates the multi-omics correlations between transcripts, metabolites, and methylation profiles of Rac GTPase-associated genes. Edge colors represent the different omics layers. The strips connecting metabolites and genes indicate changes in associations between VCI and control samples. The most abundant metabolite classes are highlighted to enhance the visualization of their contributions to the dysregulation of the Rac GTPase pathway in VCI.

To further investigate the shared characteristics of multi-omics associations in VCI, we examined common genes across the pairwise comparisons and constructed a comprehensive gene list. This list comprised all genes with significant changes in associations across different omics layers in VCI samples compared to controls. Using the DIAMOnD algorithm, we expanded this gene set by identifying its network neighborhood. The DIAMOnD algorithm evaluates the significance of connections within a gene network to identify genes closely linked to the initial set. After 200 iterations, we generated a gene regulatory subnetwork containing the top 200 genes (Appendix Supplementary table 5). Notably, 40 out of these 200 genes were associated with the Rac/Rho GTPase cycle, demonstrating significant enrichment (OR = 6.84, $p < 2.2 \times 10^{-16}$, Fisher's exact test, Supplementary table 5). These results emphasize the pivotal role of the Rac/Rho GTPase pathway in VCI and its widespread influence across molecular interactions.

To delve deeper into the intricate relationship between Rac/Rho GTPase cycle gene methylation and other omics layers, we constructed a multi-omics network. This analysis integrated multiple associations: 178 methylation region-metabolite associations identified as significantly altered in VCI, 12 metabolite-transcript associations where the metabolite or transcript was also significantly linked to Rac/Rho GTPase cycle gene methylation, and five methylation-transcript associations that exhibited significant changes in VCI and were also significantly associated with metabolites. Altogether, this yielded 195 associations spanning different multi-omics layers, visualized in the circos plot shown in Figure 3.5b.

Next, we investigated the metabolite classes most disrupted by the combined effects of Rac/Rho GTPase cycle gene methylation and transcriptomic alterations. Diacylglycerols (DAGs) and phosphatidylethanolamines (PEs) emerged as the two most prominently affected classes (Table 3.5). Both showed a predominance of association gains with other omics layers, although association losses and reversals were also observed.

Table 3.5. Metabolite classes affected by Rac/Rho GTPase gene methylation and transcriptomic alterations in VCI

Metabolite class	Association gain	Association loss	Association reversal
Diacylglycerols	29	22	2
Phosphatidylethanolamines	14	11	7
Aminoacids	12	1	1

Table 3.5. Metabolite classes affected by Rac/Rho GTPase gene methylation and transcriptomic alterations in VCI

Metabolite class	Association gain	Association loss	Association reversal
Phosphatidic Acids	6	2	0
Phosphatidylcholines	6	3	0
Ceramides	3	0	0
Phosphatidylinositols	3	9	1
Sphingomyelins	3	0	0
Acylcarnitines	2	0	0
Phosphatidylglycerols	2	0	1
Biogenic Amines	1	0	0
Dihydroceramides	1	0	0
Glycosylceramides	1	0	0
Monoacylglycerols	1	1	0
Phosphatidylserines	1	0	0
Aminoacids Related	0	11	6
Triacylglycerols	0	3	1

To further explore the role of Rac/Rho GTPase gene methylation in VCI, we focused on its associations with specific metabolites, particularly isobutyrate. As short-chain fatty acids (SCFAs), such as isobutyrate, are gut microbiota-derived metabolites known for their roles in neuroinflammation and potential impact on cognitive function (Sarkar et al., 2016). We identified nine methylation regions in Rac/Rho GTPase genes that exhibited significant associations changes with isobutyrate, all of which showed either a loss or a reversal of effect in VCI samples compared to controls. Specifically, five of these associations were losses, while the remaining four exhibited reversed effects. This consistent pattern of disruption suggests a potential link between Rac/Rho GTPase gene methylation and isobutyrate metabolism in VCI, implicating SCFA metabolism, which plays a crucial role in regulating oxidative stress and inflammatory processes in the brain. In addition to the epigenomics-metabolomics associations, our transcriptomics-metabolomics analysis revealed five significant associations involving isobutyrate, all of which were losses. Interestingly, the genes that showed significant association changes with isobutyrate also exhibited altered associations with DAGs and PEs. Collectively, these findings highlight a potential complex interplay between Rac/Rho GTPase gene regulation, isobutyrate metabolism, DAGs and PEs metabolism, and vascular impairment, offering new insights into the novel pathways that may underlie VCI pathophysiology.

4 Discussion

We employed a bottom-up, integrative multi-omics strategy to uncover molecular mechanisms underlying vascular contributions to VCI. This data-driven approach integrated genomic, epigenomic, transcriptomic, and metabolomic profiling to identify convergent molecular signatures. Rac/Rho GTPase signaling emerged as a key molecular axis potentially driving VCI pathophysiology, supported by genetic associations, widespread epigenetic silencing, and transcriptomic downregulation in pathway-related genes. Broader disturbances in oxidative stress and lipid metabolism were also observed, suggesting system-level disruption. Integration of methylation and metabolomics data further linked epigenetic changes in Rac/Rho GTPase pathway genes to altered metabolic states. This pathway-focused, exploratory approach offers novel insights into the neurovascular pathology of VCI that might be missed by hypothesis-driven models.

4.1 Genomic insights into the molecular mechanisms of VCI

Our findings provide preliminary insights into the potential role of genetic factors in VCI, specifically implicating the Rac1 GTPase cycle pathway. Despite the limitations of our study, including a small sample size and other potential confounders contributing to the observed inflation in our analyses, our results highlighted candidate genes that warrant further investigation. This suggests that GWAS with smaller sample sizes can serve as pilot studies for hypothesis generation and the identification of candidate genes or pathways. Our analysis revealed an enrichment of genetic variants in the Rac1 GTPase cycle pathway, with five variants reaching suggestive significance (p-value < 0.01) in the genes *FARP1*, *CHN2*, and *DOCK5*. Given the known roles of these genes in neurodevelopment, synapse formation, and vascular integrity, their potential contribution to VCI is biologically plausible. *FARP1* has been implicated in synapse formation and intron retention within this gene has been associated with AD in mouse models (H. Li et al., 2021). Furthermore, single-cell RNA sequencing studies have demonstrated over-expression of *FARP1* transcripts in microglia of AD patients, and its role in WMH volume has also been noted (Brase et al., 2023; Lopez et al., 2015). The involvement of *FARP1* in dendritic filopodial dynamics further supports its relevance in neuronal connectivity and cognitive function (Cheadle & Biederer, 2012). *CHN2*, encoding beta2-chimaerin, is positively correlated with Na^+/K^+-ATPase α1 expression, suggesting a role in maintaining neuronal function and ion homeostasis (Jiao et al., 2022). As a Rac-GTPase-activating protein, beta2-chimaerin inhibits Rac1 activity, thereby suppressing growth factor-induced proliferation and migration of vascular smooth muscle cells (Maeda et al., 2006). This positions *CHN2* as a key modulator of vascular remodeling and atherogenesis. A 3,958 bp deletion at the *DOCK5* locus, associated with Lewy body dementia, was also found to correlate

with motor function (Vialle et al., 2025). However, the specific neural function of *DOCK5* remains largely unexplored. Genetic studies, nonetheless, suggest a potential link between *DOCK5* and Parkinson's disease (PD) (Pankratz et al., 2011). *DOCK5* contributes to vascular inflammation primarily by modulating neutrophil-driven processes, especially neutrophil extracellular traps formation, which is increasingly recognized as a pathogenic mechanism in inflammatory vascular disorders. Through its role in Rac activation, *DOCK5* also indirectly supports the production of ROS and chemotactic responses that amplify local inflammation in blood vessels (Watanabe et al., 2014). Given the established functions of these genes in brain and vascular health, it is plausible that some of the identified genetic variants influence brain structure and contribute to VCI pathogenesis.

Our study suggests that the Rac1 GTPase cycle, organophosphate catabolic process, and stimuli-sensing channels may be enriched in the VCI population. Rac1 pathway, in particular, has been implicated in BBB disruption under simulated microgravity, suggesting a potential role in neurovascular dysfunction (Yan et al., 2021). Furthermore, Rac1 GTPase has been shown to mediate I/R injury-induced NADPH oxidase activation, ROS generation, and oxidative stress in the hippocampus, contributing to neuronal degeneration and cognitive impairment following cerebral ischemia (Raz et al., 2010). Notably, Rac1 alterations have been detected in the fronto-cortical brain lysate and plasma of AD patients, with the severity of alteration correlating with cognitive impairment, highlighting its potential as a biomarker for neurodegenerative diseases (Borin et al., 2018). Although our Fisher's exact test did not reveal significant enrichment of variants in genes associated with the organophosphate catabolic process, this finding remains relevant given the increasing discussion about the potential link between organophosphate pesticide exposure and the risk of dementia and AD (Y. Chen et al., 2024; Lin et al., 2015).

However, several limitations must be considered when interpreting these findings. The elevated genomic inflation factor suggests potential confounding effects such as population stratification or cryptic relatedness. While we applied principal component analysis to mitigate these issues, our small sample size likely exacerbated statistical inflation. Future studies should utilize larger, more diverse cohorts to validate these findings and minimize potential biases introduced by population structure. Additionally, as none of the identified pathways remained statistically significant after multiple testing correction, our results should be regarded as exploratory rather than definitive.

4.2 Epigenomic insights into the molecular mechanisms of VCI

Our epigenomic analysis provides compelling evidence of widespread DNA methylation alterations in individuals with VCI, particularly implicating the Rac/Rho GTPase pathway. These

findings complement our genomic analysis, reinforcing the central role of this pathway in the pathophysiology of VCI. Despite the relatively small sample size, the consistent hypermethylation signature observed across both DMCs and DMRs strongly suggests a coordinated epigenetic shift that may impact key molecular processes involved in vascular and neurodegenerative pathology.

During data pre-processing, PCA identified two outlier samples. After visual inspection and considering clustering patterns, only one sample from the control group was excluded due to its extremely elevated PC1 and PC2 values. This conservative approach ensured the removal of a likely technical artifact while maintaining the integrity and balance of the dataset for downstream analyses (N = 39).

The predominance of hypermethylation in our results, affecting 2953 CpG sites and 441 regions, could also be reflected in other researchers' studies on conditions related to vascular health and dementia. DNA hypermethylation is strongly associated with the development of vascular aging-related disorders (Xu et al., 2021). Another compelling study of ischemic stroke, one of the major risk factors for VCI, suggests that 25 % – 30 % of ischemic stroke survivors develop immediate or delayed VCI (Kalaria et al., 2016). The global level of DNA methylation tends to be greater in animal models of ischemic stroke and is associated with higher activity of DNA methyltransferases (DNMTs) in the brain (Endres et al., 2000). In fact, pharmacological inhibition of DNMTs was shown to decrease DNA methylation, reduce infarct size, and alleviate cerebral ischemic damage in the middle cerebral artery occlusion rat model (Dock et al., 2015). In the field, there has been discussion about the potential of methylation of cytosines as a biomarker for various neurodegenerative and cerebrovascular disorders. Researchers from the Euroespes Biomedical Research Center confirm that global DNA methylation decreases in blood samples from patients diagnosed with AD, Parkinson's disease (PD), and VaD (Martínez-Iglesias et al., 2020). Interestingly, our results revealed the opposite effects of global methylation, and these discrepancies could be due to differences in sample sources. For instance, a study conducted on frontal cortex samples from patients with AD showed increased global methylation levels (Rao et al., 2012). It is also worth noting that VCI is a complex disease, and the results do not always reflect those observed solely in neurodegenerative disorders.

As genomic data analysis results shed light on the association of the Rac/Rho GTPase pathway and its genes with the disease, we focused on the methylation of these genes and observed the same trend as for global epigenetic regulation – these genes exhibited hypermethylation. Similar to our observations, *ARHGEF16* was hypermethylated and downregulated in post-mortem sporadic ALS spinal cord samples when compared to samples from neurologically normal controls (Figueroa-Romero et al., 2012). Different studies have implicated Rac1 in global cerebral ischemia,

with Rac1 inhibition resulting in decreased activation of the pro-apoptotic signaling kinase JNK in the hippocampal region, leading to neuronal protection (Raz et al., 2010).

Notably, the epigenetic repression of genes within the Rac1 GTPase cycle pathway (e.g., "Regulation of Ras Protein Signal Transduction", "Positive Regulation of GTPase Activity") could impair intracellular signaling and cellular motility, potentially contributing to vascular instability and impaired neurovascular coupling. This hypermethylation could lead to transcriptional silencing of key regulators, disrupting cellular processes essential for vascular health. These findings align with prior studies linking Rac1 activity to endothelial permeability, oxidative stress regulation, and cytoskeletal integrity (Tapon, 1997). Interestingly, the literature shows that ROS produced by the Nox1/Rac1 complex play a detrimental role in neurodegeneration in an animal model of PD, while Rac1 inhibition significantly protects the nigrostriatal dopaminergic neurons (Choi et al., 2012).

A particularly important aspect of our findings is the convergence of epigenetic alterations with oxidative stress-related pathways. The enrichment of pathways such as "Response to Hypoxia", "Nitric Oxide Stimulates Guanylate Cyclase", and "Regulation of Response to Oxidative Stress" suggests that methylation changes may compromise cellular antioxidant responses, thereby exacerbating hypoxia-induced neuronal injury and suppressing the corresponding responses. These observations resonate with previous studies implicating Rac1 GTPase in NADPH oxidase regulation and ROS production during ischemic insult, where its dysregulation led to cognitive impairment and hippocampal neuron degeneration. However, their results showed that inhibition of Rac GTPase activation markedly decreased oxidative neuronal damage to hippocampal CA1 region lipids, proteins, and DNA following ischemia/reperfusion injury (Raz et al., 2010). Based on our results, the genes in the pathways of Rac/Rho GTPase regulation and response to oxidative stress were predominantly hypermethylated, suggesting the downregulation of these processes leading to altered reactivity to potential outcomes of ROS. These findings suggest that Rac/Rho GTPase cycle dysregulation may disrupt downstream processes critical for managing ROS, potentially leading to hypoxia and neuroinflammation. Additionally, this finding could support the observed global hypermethylation trend, as Thienpont et al. showed that oxygen deprivation (hypoxia) promotes DNA hypermethylation by interfering with the activity of ten-eleven translocation methylcytosine dioxygenases (TETs) – enzymes that are involved in DNA demethylation (Thienpont et al., 2016). The widespread hypermethylation suggests that this could even impair immune surveillance.

In summary, our epigenomic findings point to significant hypermethylation and possible suppression of Rac/Rho GTPase-related pathways in VCI, with potential downstream effects on oxidative stress, hypoxia response, and neuroinflammatory signaling. These results not only validate the pathway's relevance observed in genomic analyses but also emphasize its potential as a central regulator in VCI pathogenesis.

4.3 Transcriptomic insights into the molecular mechanisms of VCI

Our linear modeling analysis of the transcriptomics dataset identified 668 differentially expressed transcripts in VCI samples, with a predominance of downregulated genes. This overall suppression of gene expression mirrors the hypermethylation pattern observed in our DNA methylation dataset and reinforces the established association between promoter hypermethylation and transcriptional silencing (Jones, 2012). Similarly, the vast majority differentially expressed transcripts associated with Rac/Rho GTPase cycle were downregulated in VCI. This, together with our observations from the epigenomic analysis, suggests that epigenetic silencing and reduced expression of the genes in this pathway may contribute to impaired signal transduction, disrupted cytoskeletal organization, and altered cellular stress responses. As all these processes are essential for neurovascular stability, our findings reinforce the central role of the Rac/Rho pathway in VCI pathophysiology.

GSEA revealed extensive transcriptional pathway changes, including strong overlap with pathways identified in the methylation data. Among these were hypoxia-related signaling, and notably, the Rhoc GTPase cycle. This alignment of the two omics suggests that Rac/Rho signaling suppression in VCI is not a random observation but reflects a biologically meaningful mechanism. The enrichment of hypoxia pathways in transcriptomic data also aligns with hyper- and hypomethylated pathways identified in our epigenomic analysis. This overlap supports a mechanistic cascade in which oxygen deprivation reduces Rac1 activity, leading to increased RhoA-mediated actomyosin contractility and ultimately to endothelial barrier dysfunction (Wojciak-Stothard et al., 2005). In parallel, Rac1 has also been shown to be essential for HIF-1α induction under hypoxia, suggesting dynamic regulation of this pathway in stress adaptation (Hirota & Semenza, 2001). These findings support the hypothesis that the Rac/Rho GTPase cycle and hypoxia-response pathways could potentially act in concert to drive vascular and cognitive impairment in VCI.

In addition to hypoxia related and Rac/Rho cycle disruptions, GSEA identified significant enrichment of pathways related to lipid metabolism. This is especially relevant because VCI often damages white matter, a brain region abundant in lipids (Qin et al., 2021a)The enriched pathways included "Cellular Response to Lipid", "Positive Regulation of Lipid Metabolic Process", "Regulation of Lipid Metabolic Process", and "Response to Lipid". This observed dysregulation in our transcriptomic analysis reinforces the growing evidence linking disrupted lipid metabolism in blood to VCI pathogenesis (Y. Liu et al., 2020), underscoring the vulnerability of the brain's lipid-rich components.

Our analysis also revealed 177 transcripts with significant alternative splicing changes between VCI and control samples. This observation of significantly altered splicing in 3.4 % of transcripts aligns with the growing recognition that widespread disruptions in alternative splicing are a common feature of neurodegenerative diseases, where studies have reported significant changes involving hundreds of exons (Tollervey et al., 2011). Overall, the importance of alternative splicing in brain tissue is well recognized; the brain exhibits the highest diversity of alternatively spliced transcripts across human tissues, contributing to its complexity (Mills et al., 2013). Aberrant splicing and mutations in RNA-binding proteins have been implicated in several neurodegenerative disorders, including ALS (Dredge et al., 2001), frontotemporal lobar dementia (Arai et al., 2006), PD (Trabzuni et al., 2012), and AD (Rockenstein et al., 1995). In AD, for example, alternative splicing of *APP, TAU*, and *ApoE4* may directly influence disease pathology and have become targets for therapies such as antisense oligonucleotides ('Alternative Splicing in Alzheimer's Disease', 2015). Together, these findings underscore that alternative splicing disruptions are a driving force in many neurodegenerative disease – and our results suggest that similar mechanisms may extend to cerebrovascular conditions such as VCI.

Among the genes with significant splicing alteration, five were related to the Rac/Rho GTPase cycle (*ABCD3, SRRM1, NSFL1C, CYFIP2,* and *SPTAN1*) displayed significant splicing alterations in VCI. Hypoxia-induced alternative splicing of *SPTAN1* has previously been shown to mediate angiogenesis and cytoskeletal changes in endothelial cells (Weigand et al., 2012), and pathogenic mutations in *SPTAN1* have been linked to cognitive impairment (Luongo-Zink et al., 2022). Moreover, splicing regulators such as QUAKING have been shown to modulate microexon inclusion in Rho GTPase pathway genes, influencing microglial homeostasis and immune responses (J. Lee et al., 2020). The broader biological significance of splicing dysregulation is also underscored by studies showing that hypoxia impacts splicing machinery. In liver cells, hypoxic conditions affected over 3000 splicing events across 2000 genes (Jakubauskienė & Kanopka, 2021; Kanopka, 2017). Similar hypoxic microenvironments are believed to play a role in neurodegenerative diseases by promoting Aβ aggregation, tau phosphorylation, BBB dysfunction, and calcium dysregulation (Snyder et al., 2017). Moreover, alternative splicing is implicated in the pathogenesis of ischemic stroke, particularly in astrocytes and microglia, supporting its broader relevance in neurovascular conditions like VCI (Jin et al., 2024).

To sum up, our transcriptomic analysis reveals significant dysregulation in gene expression and alternative splicing, an provides further evidence for the involvement of the Rac/Rho GTPase signaling pathway. These alterations align closely with the epigenomic changes observed in our analysis, suggesting and important or even the central role of this pathway in the pathophysiology of VCI. Notably, the results underscore the interplay between oxidative stress and lipid metabolism

in the disease process. Oxidative stress-related pathways, including those governing hypoxia and Rac/Rho GTPase signaling, appear to drive key cellular responses, such as endothelial dysfunction, that contribute to vascular damage and cognitive decline in VCI. Additionally, disruptions in lipid metabolism, a critical aspect of brain and vascular function, may further exacerbate neurovascular impairment.

4.4 Methylation-metabolite interactions uncover cytoskeletal and vascular pathway disruption in VCI

To build on the insights from the single-omics analysis, we have carried out pairwise integration analysis for all the different omics datasets. One of such integrations involved epigenomic and metabolomic datasets where we aimed to investigate how DNA methylation changes may contribute to metabolic dysregulation in VCI. To perform this analysis, we applied a regression-based pairwise integration framework that incorporated interaction terms between disease status (VCI vs. control) and DNA methylation levels. This modeling strategy enabled us to move beyond simple correlation analyses by directly quantifying how methylation–metabolite associations are modified in the disease context. In other words, we could determine not only whether a methylation region was associated with metabolite abundance, but whether that relationship was significantly altered in VCI – a crucial step toward capturing disease-specific molecular rewiring. Our approach aligns with methods like IntLIM, which use interaction terms in linear models to identify condition-specific gene–metabolite relationships (Siddiqui et al., 2018). Additionally, our model allowed for the inclusion of covariates such as age, sex assigned at birth, and PMI, ensuring control over non-disease-related variability. This integrative analysis identified 5210 significant methylation–metabolite associations that were differentially regulated in VCI versus controls. These findings suggest that VCI is not only marked by isolated molecular disruptions but involves systemic reprogramming of the regulatory interface between the epigenome and metabolome.

To interpret these associations in a biological context, we conducted an ORA on genes linked to methylation regions involved in altered metabolite associations. This yielded 50 significantly enriched pathways, notably clustering around the Rac/Rho GTPase cycle and the negative regulation of phosphate metabolism. The latter is particularly relevant, as endothelial dysfunction – a critical early event in atherogenesis – is influenced by eNOS, which is downregulated under sustained high-phosphate conditions (Ellam & Chico, 2012). Moreover, increased phosphate toxicity has been shown to trigger mitochondrial oxidative stress and activate cellular stress responses (Fasano et al., 2019; Rroji et al., 2022).

Interestingly, 20 % of the enriched pathways were related to Rac/Rho GTPase signaling, reinforcing our previous findings that epigenetic dysregulation of this pathway may be a central mechanism in VCI. Beyond cytoskeletal remodeling, pathway analysis also highlighted the VEGFA–VEGFR2 signaling axis, a key regulator of angiogenesis, vascular permeability, and endothelial homeostasis (Abhinand et al., 2016). This pathway is well-established in maintaining neurovascular integrity and is responsive to both hypoxic stress and epigenetic modulation. Its involvement in altered methylation–metabolite associations underscore the possibility that epigenetic dysregulation in VCI compromises vascular repair and endothelial signaling, contributing to white matter vulnerability and metabolic imbalance. The convergence of Rac/Rho GTPase and VEGFA–VEGFR2 pathways reflects a broader collapse in cytoskeletal coordination, vascular remodeling, and cellular stress responses. The interplay between these pathways is well-supported: Rho GTPases modulate VEGFA signaling in both cancer and endothelial cells (El Baba et al., 2020), and knockdown of RhoA and RhoC in astrocytoma cells significantly reduces VEGF expression (El Atat et al., 2019). Interestingly, hypoxic conditions were also shown to downregulate PI3K, RhoA, and RhoC activity, leading to decreased VEGF expression (Nicolas et al., 2019). Furthermore, the VEGFA–VEGFR2 axis itself regulates RhoA, Rac1, and Cdc42 activity (Vader et al., 2011). While Rac1 has been implicated in VEGFA-induced vascular permeability, cell migration and even ROS production (Eriksson et al., 2003; Monaghan-Benson & Burridge, 2009). Notably, VEGF is also upregulated in the AD brain, likely in response to hypoxia, vasoconstriction, and amyloid-β pathology (Harris et al., 2017).

Taken together, our results indicate that Rac/Rho GTPase signaling, VEGFA–VEGFR2 signaling, and associated hypoxia-responsive pathways likely function as interconnected regulatory hubs that are epigenetically and metabolically disrupted in VCI. Altered methylation in genes involved in these networks appears to distort their metabolic crosstalk, leading possible to endothelial dysfunction, impaired vascular remodeling, and redox imbalance. This dysregulation might be increased by hypoxia-induced shifts in phosphatase activity and VEGF signaling. Altogether, these insights support a model in which VCI is driven by integrated disruptions across epigenetic, vascular, and metabolic layers, with multi-omics integration offering a powerful approach to unravel such complex disease mechanisms.

4.5 Multi-Omics data analysis reveals the relationship between Rac/Rho GTPase signaling and lipid dysregulation in VCI

One of the most consistent findings was the disruption of the Rac/Rho GTPase signaling pathway across all omics layers. These small GTPases are well-known regulators of cytoskeletal dynamics, endothelial barrier integrity, and vascular tone – all of which are altered in VCI (Rundek

et al., 2022; Tzima, 2006). The repeated identification of Rac/Rho GTPase-related genes and pathways across all pairwise comparisons strongly suggest that this pathway is not a secondary response but rather a core driver of disease-associated molecular remodeling. ORA of regression-based multi-omics associations further revealed consistent enrichment of small GTPase signaling clusters. Using a network-based approach with the DIAMOnD algorithm, we also showed that the Rac/Rho GTPase pathway functions as a central hub, interacting with numerous components across molecular domains, reinforcing its pivotal role in VCI pathophysiology.

Our multi-omics network analysis of Rac/Rho GTPase cycle related genes also identified pronounced alterations in lipid metabolism, particularly involving DAGs and PEs, which were significantly associated with changes in the methylation and expression of Rac/Rho GTPase cycle genes. Interestingly, both lipid classes displayed a predominance of gained associations with these genes in VCI, suggesting a potential rewiring of regulatory networks that link cytoskeletal gene expression to lipid homeostasis. DAG dysregulation is a well-established hallmark of neurodegenerative diseases, especially those characterized by protein aggregation and synaptic dysfunction (P. L. Wood et al., 2018). Elevated DAG levels have been observed in the plasma of AD patients (L Wood et al., 2014), the frontal cortex of AD subjects (P. L. Wood, Barnette, et al., 2015), and the temporal cortex of individuals with mixed dementia comprising AD and subcortical ischemic VaD (S. M. Lam et al., 2014). These studies suggested that DAG accumulation may occur early in the progression of cognitive decline. Although our study did not observe significant differences in DAG abundance between VCI and control samples, the altered associations between DAGs and Rac/Rho GTPase gene regulation may indicate an upstream regulatory influence that could modulate DAG levels and function indirectly. Interestingly, previous work of other researchers has demonstrated that DAG signaling can stabilize α1-chimaerin, a Rac-GTPase activating protein, thereby modulating Rac1 activity in neurons independent of PKC pathways (Marland et al., 2011). These findings provide a mechanistic link between our observed changes in DAG associations and the downstream regulation of Rac1, a pathway we identify as consistently dysregulated across multi-omics layers in VCI. Notably, the reduction of PE in the brain has also been reported in AD, independent of changes in other major phospholipids like phosphatidylserines, phosphatidylinositols, or phosphatidylcholines (P. L. Wood, Medicherla, et al., 2015). This selective depletion of PE could contribute to increased DAG levels, as PE degradation – potentially through phospholipase A2 (PLA2) activity – may lead to elevated DAG production (Qin et al., 2021b). These findings suggest a potential feedback mechanism whereby Rac/Rho GTPase cycle dysregulation alters PE abundance, which in turn contributes to DAG accumulation, creating a self-reinforcing metabolic loop. Further supporting this, a study in myotube cultures showed that PE concentrations were significantly reduced under hypoxic conditions, with reductions ranging from

20 % - 70 % in a dose-dependent manner (Vacek et al., 2022). In vivo studies have also demonstrated that DAG accumulation, possibly resulting from phosphatidylcholine PC breakdown, occurs following ischemic or hypoxic exposure (Gysembergh et al., 2000). These results align with the hypothesis that hypoxia-induced oxidative stress may intersect with Rac/Rho GTPase signaling, contributing to disruptions in lipid metabolism and vascular integrity.

A particularly intriguing finding from our study was the disruption of isobutyrate associations with Rac/Rho GTPase cycle genes in VCI. We identified nine methylation regions within Rac/Rho GTPase genes that exhibited significant changes in their associations with isobutyrate in VCI compared to controls. Isobutyrate, a short-chain fatty acid (SCFA), is primarily produced through the microbial fermentation of dietary fiber in the gut and subsequently enters systemic circulation (Le Poul et al., 2003). Although the precise physiological functions of isobutyrate remain underexplored, emerging evidence suggests that SCFAs contribute to BBB integrity, a crucial factor in maintaining CNS homeostasis and brain health (Silva et al., 2020). Notably, previous studies have reported decreased fecal isobutyrate levels in individuals with AD (L. Wu et al., 2021), and plasma isobutyrate concentrations have been shown to decline progressively with increasing cognitive decline (Conde et al., 2024). SCFAs are also known to exhibit anti-inflammatory properties, with isobutyrate and other SCFA-mediated signaling through GPR43 shown to inhibit ROS production and reduce oxidative stress (W. Huang et al., 2017). In our analysis, genes with altered associations to isobutyrate also displayed disrupted interactions with DAGs and PEs. This triad of dysregulation suggests a complex molecular interplay between SCFA metabolism, lipid signaling, and cytoskeletal gene regulation.

Our findings raise new questions about the role of sGC signaling in VCI, particularly given its position downstream of NO and its established role in regulating vascular tone and endothelial homeostasis. Impaired NO–sGC–cGMP signaling has been linked to cognitive decline and cerebrovascular insufficiency, and in VCI, reduced sGC activity may exacerbate vascular dysfunction and hinder neurovascular coupling (Nelissen et al., 2023). Our analysis hints that this dysfunction could be potentially connected to epigenetically altered Rac/Rho GTPase signaling. Importantly, another study demonstrated that platelet-derived growth factor (PDGF) regulates sGC expression and cGMP production in vascular smooth muscle cells, and that PDGF reduces sGC expression via PIK3 (Phosphoinositide 3-kinase) and Rac1 activation, leading to altered Notch ligand signaling (Hildebrand et al., 2022). This is especially relevant in the context of VCI, where we observe epigenetic and metabolic disruption in the Rac/Rho GTPase pathway.

Additionally, circulating PDGF-BB levels have been associated with increased WMH volume, a hallmark of VCI (Kapoor & Nation, 2022). PDGF-D has also been reported to be transiently induced in brain endothelial cells after ischemic stroke, further suggesting its role in

vascular remodeling and stress response (Kapoor & Nation, 2022). Taken together, these findings suggest that sGC dysfunction in VCI may result not only from upstream NOS/NO disturbances but also from PDGF-mediated Rac1 activation, which impacts both vascular signaling and endothelial gene expression. Our study hints at a model in which epigenetic dysregulation of Rac1-related signaling and altered sGC function contribute to a feedback loop, potentially exacerbating vascular dysfunction, white matter damage, and cognitive decline in VCI.

Moreover, a recent study demonstrated that repetitive hypoxic conditioning induces intergenerational epigenetic resilience to VCI, adds compelling support to the idea that epigenetic mechanisms play a central role in cerebrovascular health and cognitive resilience (Belmonte et al., 2022). This aligns closely with our findings, where we observed extensive rewiring of DNA methylation–metabolite associations, particularly involving genes related to Rac/Rho GTPase signaling, endothelial regulation, and lipid metabolism. These epigenetic alterations may not simply be bystanders but could represent adaptive or maladaptive molecular responses to vascular stress. Taken together, these insights reinforce the notion that epigenetic regulation is a crucial and dynamic component of VCI pathophysiology, potentially mediating long-term changes in vascular integrity, metabolic balance, and cognitive outcomes.

Our integrated multi-omics approach reveals a complex interplay of molecular events in VCI, with the Rac/Rho GTPase signaling pathway emerging as a central hub of dysregulation. Our findings across different omics highlight the altered gene expression in VCI, including epigenetic silencing, manifested as hypermethylation-linked gene downregulation, and changes in alternative splicing. Hypoxia, an important factor in VCI, could contribute to the observed epigenetic and transcriptomic differences through hypoxia-induced inhibition of TET enzymes and hypoxia-activated alternative splicing events affecting cytoskeletal regulation (Thienpont et al., 2016; Weigand et al., 2012). Whereas DAG dysregulation has been reported previously in neurodegenerative diseases (P. L. Wood et al., 2018; P. L. Wood, Medicherla, et al., 2015), an exact mechanism of action for these lipids in such contexts is not yet fully understood. Here, we provide novel evidence derived from the multi-omics integration, showing significantly altered regulatory associations between lipids, specifically DAGs and PEs, and Rac/Rho GTPase cycle genes. Our observations not only suggest a potential upstream role for the Rac/Rho GTPase cycle genes in lipid homeostasis within the VCI pathology, but also bridges knowledge from previous studies and provides evidence for a potential novel feedback mechanism. Specifically, we propose a hypothesis where dysregulation within the Rac/Rho GTPase cycle perturbs PE levels which then contributes to the accumulation of DAGs (Qin et al., 2021a), creating a self-perpetuating cycle of metabolic disruption. Adding another layer to this complexity is our novel finding of disrupted associations between Rac/Rho GTPase cycle genes and isobutyrate, a SCFA with emerging roles in BBB

integrity and neuroinflammation (Silva et al., 2020). Altogether, differential methylation of Rac/Rho GTPase genes along with their altered associations with DAGs, PEs and isobutyrate points towards a potentially critical, yet previously unappreciated, link between cytoskeletal signaling, lipid metabolism, and gut-derived neuroactive metabolites in VCI. The interplay between these processes could, at least some extent, explain ROS production in VCI, with SCFA such as isobutyrate known to inhibit ROS (W. Huang et al., 2017). Moreover, our findings align with established links between Rac1 activation and increased ROS generation (M. Lee et al., 2002; P. Wang et al., 2009), and further suggest that epigenetically altered Rac/Rho GTPase signaling may disrupt the neuroprotective NO–sGC–cGMP axis (Nelissen et al., 2023) potentially through ROS-mediated sGC inactivation (Pan et al., 2016). Collectively, these alterations contribute to downstream consequences such as oxidative stress, lipid dysregulation, and BBB compromise, ultimately impairing white matter integrity and cognitive function.

The interconnectedness of these events, culminating in a cascade of vascular damage and cognitive decline, is visually summarized in Figure 4.5, which depicts the proposed potential mechanism centered on Rac/Rho GTPase signaling and its interactions with hypoxia, oxidative stress, lipid dysregulation, and sGC/cGMP signaling.

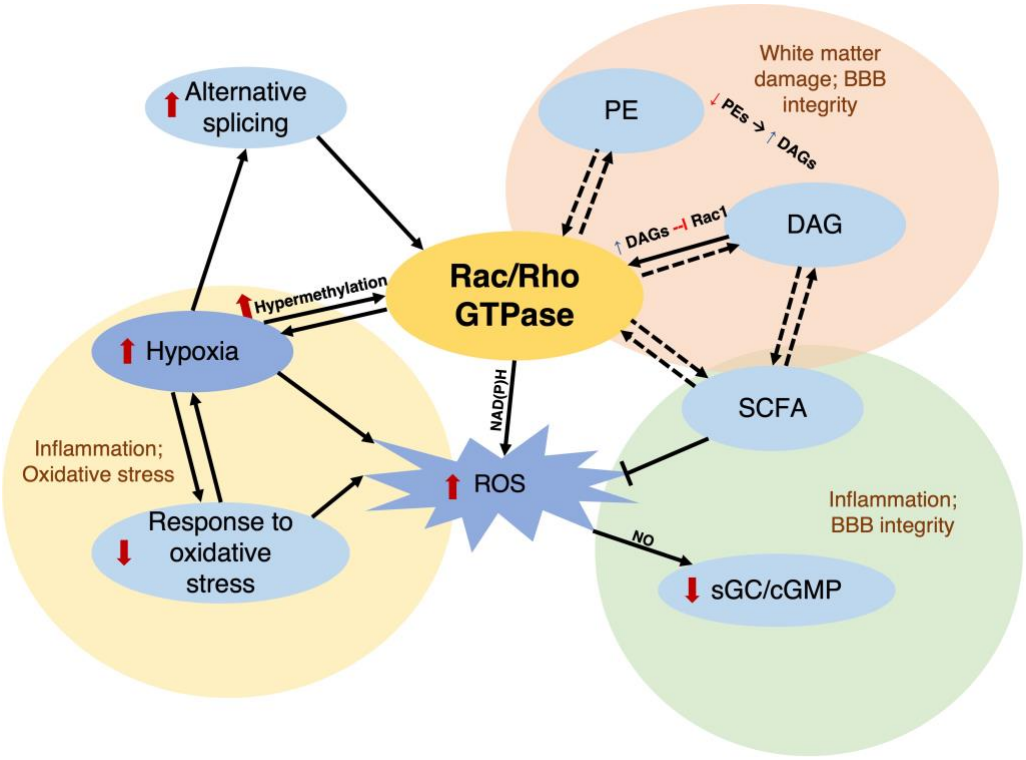

Figure 4.5. Proposed potential mechanism of VCI pathophysiology centered on Rac/Rho GTPase signaling. The central yellow oval highlights the Rac/Rho GTPase signaling pathway, demonstrating its interconnectedness with various molecular alterations across different biological layers. Red upward arrows indicate upregulation or increased activity, while downward arrows indicate downregulation or decreased levels. Solid arrows represent direct effects or associations identified in other studies, while dashed arrows

indicate relationships from our findings. The shaded areas in orange, yellow and green colors represent broader cellular and tissue-level consequences associated with VCI.

To conclude, this study presents a comprehensive, multi-layered approach to investigating VCI by integrating epigenomic, transcriptomic, and metabolomic data. By combining these distinct molecular layers, we were able to uncover critical disease-specific associations that would likely remain undetected in single omics studies. Our findings highlight the power of integrative multi-omics approaches in identifying key regulatory pathways and offer a more holistic understanding of the molecular disruptions that contribute to VCI pathogenesis. The consistent identification of Rac/Rho GTPase signaling and its metabolic interactions with DAGs, PEs, and isobutyrate across molecular layers suggests that components of this pathway – such as altered gene methylation or specific lipid and SCFA profiles – could serve as early biomarkers for VCI detection. For instance, blood-based measurements of DAG or isobutyrate levels (L. Wood et al., 2014; Conde et al., 2024), in combination with methylation signatures from circulating cells, may provide a non-invasive diagnostic panel for early-stage VCI. Moreover, given the role of SCFAs in modulating oxidative stress (W. Huang et al., 2017) and BBB integrity (Silva et al., 2020), dietary interventions targeting the gut microbiota – such as increased intake of fermentable fibers or specific probiotic strains – may enhance isobutyrate production and confer vascular resilience. Pharmacological modulation of Rac/Rho GTPase signaling or restoring sGC–cGMP signaling (Nelissen et al., 2023) also present promising therapeutic avenues, especially given that Rac1, a component of this pathway, is already being explored as a therapeutic target in other diseases like cancer (Bailly et al., 2022). Ultimately, targeting this interconnected network may offer a multifaceted approach to both VCI prevention and treatment.

4.6 Limitations and recommendations for future research

While our integrative multi-omics approach has provided valuable insights into the molecular mechanisms underlying VCI, several important limitations must be considered.

First, the cross-sectional design of the study inherently limits our ability to draw conclusions about causality. The associations observed between molecular features and disease status may reflect downstream consequences rather than causal drivers of the disease. To establish a clearer understanding of the temporal sequence of molecular alterations, longitudinal studies are essential. Tracking dynamic changes in DNA methylation and metabolic pathways over time would enable a more precise understanding of the progression and onset of vascular cognitive impairment.

Second, this study relies on post-mortem brain tissue, which offers invaluable insight into disease-related changes in specific brain regions. However, it is important to acknowledge that post-mortem samples are subject to several confounding factors, such as post-mortem interval, tissue

degradation, and variability in disease stage at the time of death. Although we accounted for several potential confounders (e.g., age, sex, and post-mortem interval), the possibility of residual confounding remains. To mitigate these limitations, future studies could benefit from prospective studies using biofluid samples such as cerebrospinal fluid or blood, which would allow for more direct access to dynamic molecular processes in living individuals.

Third, the sample size presented a significant limitation, particularly for integrating high-dimensional multi-omics data. A modest sample size inherently reduces statistical power, making it challenging to detect subtle yet biologically relevant interactions. This limitation becomes particularly acute when addressing potential outliers or ensuring consistent sample inclusion across different omics layers, as removing even a few samples can disproportionately affect analytical power and necessitate a more conservative approach than might be employed in larger studies. Indeed, these constraints influenced specific analytical decisions within our study. For instance, although PCA identified some potential outliers, all samples for transcriptomics and metabolomics datasets were retained after visual inspection to maintain dataset integrity and ensure consistent cross-omics comparisons – a harmonization step critical for integrative analyses but potentially preserving variance that might be filtered in a larger dataset. Similarly, despite observed multicollinearity among age, sex assigned at birth, and post-mortem interval, all variables were retained in the differential expression model due to their biological relevance and to align with the modeling strategy used in the epigenomic analysis. Consequently, larger, well-powered cohorts are essential to validate and generalize these findings, as well as to explore potential population-specific variations across diverse demographic groups.

Another limitation lies in the regional focus of our study. Although we concentrated on Brodmann area 7, focusing on a single brain region limits the ability to draw conclusions about the broader neurovascular network in VCI. The involvement of other regions, such as the frontal cortex or hippocampus, in VCI pathology warrants further investigation. Expanding the analysis to multiple brain regions in post-mortem studies would offer a more comprehensive understanding of the disease and its systemic effects.

Additionally, the Infinium MethylationEPIC v2.0 array used for DNA methylation analysis, while comprehensive, relies on a predefined set of probes and may not capture all methylation sites or rare epigenetic patterns. This limitation could overlook crucial regulatory regions in the genome, potentially affecting the comprehensiveness of the methylation data and its associations with disease pathology. Future studies could benefit from whole genome methylation sequencing to increase coverage and capture a broader range of methylation events.

Finally, while our analysis highlighted several critical molecular pathways, including Rac/Rho GTPase signaling and lipid metabolism, functional validation is still lacking.

Experimental follow-up using cell culture based or animal models is necessary to confirm the causative roles of these pathways in VCI pathophysiology. This would help establish direct links between the observed molecular alterations and the cognitive deficits associated with VCI.

Conclusions

- Single omics analyses independently identified significant molecular disruptions in VCI, highlighting differential gene expression and methylation patterns in pathways related to Rac/Rho GTPase cycle and hypoxia, among other pathways.
- Our integrative multi-omics analysis revealed novel associations and highlighted the Rac/Rho GTPase cycle as the single most prominent pathway in our cohort. While this pathway was implicated in individual omics analyses, its potentially central role might have been overlooked amid the numerous changes in other molecular pathways. This underscores the critical value of comprehensive integration of omics datasets for identifying robust and consistent disease-related pathways.
- The integration of epigenomic and transcriptomic data with metabolomics unveiled significant metabolic dysregulation in VCI, highlighting disturbances within DAG and PE lipid classes. Notably, these metabolic shifts were linked to epigenetic and transcriptomic alterations in genes belonging to the Rac/Rho GTPase pathway, suggesting a mechanistic connection.
- The proposed multi-omics integration workflow is generalizable and can be readily applied to other complex disease datasets, offering a robust framework for uncovering molecular mechanisms through cross-layer data integration.

Author's Personal Contribution

The research presented in this thesis was conducted as a collaborative effort, with significant contributions made by me throughout the process. I contributed to the development of the data analysis workflow, performed the analysis, and proposed the hypothesis that the Rac/Rho GTPase cycle may play a role in the pathogenesis of VCI. The workflow for the bioinformatics analysis was proposed by me and further refined through continuous discussions with my supervisor, Juozas Gordevičius, academic consultant Vilija Lomeikaitė, and colleague Miglė Gabrielaitė. I was responsible for performing all integrative bioinformatics analyses described in the thesis, with the exception of GWAS, which was carried out by my colleague and academic consultant Vilija Lomeikaitė. Although much of the single omics analysis was conducted using established pipelines, these tools were collaboratively developed within the VUGENE bioinformatics team. The data used in this research were provided by the Dr. Stewart F. Graham lab, which also secured the funding that supported the project. I wrote the entire thesis independently. The text was subsequently reviewed, corrected, and occasionally rephrased by my academic consultant and supervisor. For minor grammar refinement, I also used OpenAI-based tools. In parallel with thesis preparation, I co-authored a manuscript alongside my colleagues. The initial drafts of the methods and results sections were written by me, while the discussion section was developed collaboratively with Sangeetha Vishweswaraiah. The final version of the manuscript was refined and edited by all co-authors. All figures presented in the thesis were created by me using *BioRender.com* or generated via the R programming language.

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VILNIAUS UNIVERSITETAS

GYVYBĖS MOKSLŲ CENTRAS

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Metodai integruotai daugiaomikinių duomenų analizei: kraujagyslinio kognityvinio sutrikimo atvejis

SANTRAUKA

Kraujagyslinis kognityvinis sutrikimas yra viena iš pagrindinių ir sparčiai augančių demencijos priežasčių, tačiau jo molekuliniai mechanizmai tebėra mažai ištirti. Taikant daugiaomikinių duomenų integraciją, šiame tyrime mes siekėme išsamiau apibūdinti molekulinius pokyčius pomirtiniuose kraujagyslinio kognityvinio sutrikimo pacientų smegenų mėginiuose. Mūsų analizė, apjungianti genomikos, epigenomikos, transkriptomikos ir metabolomikos duomenis, parodė nuoseklią Rac/Rho GTPazės signalizacijos kelio disreguliaciją visose analizuotose omikose. Genomikos analizė parodė reikšmingą genų variantų praturtinimą genuose, koduojančiuose šio kelio komponentus. Epigenominė analizė nustatė plačiai paplitusį hipermetilinimą, ypač paveikiantį genus, susijusius su su Rac/Rho GTPazės signalizacijos keliu. Transkriptomikos analizė parodė pakitusią šio kelio genų raišką ir alternatyvius splaisingo variantus, taip pat su hipoksija susijusius pokyčius. Kartu šie rezultatai parodė, kad Rac/Rho GTPazės signalizacijos kelias gali būti vienas pagrindinių kraujagyslinio kognityvinio sutrikimo patofiziologijos veiksnių. Be to, mūsų integratyvi epigenomikos, transkriptomikos ir metabolomikos duomenų analizė atskleidė reikšmingus metilinimo regionų, genų raiškos ir metabolitų asociacijų pokyčius. Šiame tyrime nustatėme pakitusias sąveikas tarp Rac/Rho GTPazės kelio genų raiškos ir metilinimo būklės bei specifinių lipidų klasių, įskaitant diacilglicerolius ir fosfatidiletanolaminus, bei izobutyratą. Tai leidžia manyti, kad epigenetinė Rac/Rho GTPazės signalizacijos disreguliacija prisideda prie medžiagų apykaitos sutrikimų, ypač lipidų apykaitos keliuose, kurie yra būtini normaliai neurovaskulinei funkcijai. Apibendrinant, šis tyrimas pabrėžia daugiaomikos integracijos vertę atskleidžiant sudėtingus molekulinius ryšius kompleksinėse ligose, tokiose kaip kraujagyslinis kognityvinis sutrikimas. Nuoseklus Rac/Rho GTPazės kelio disreguliacijos nustatymas įvairiuose omikos lygiuose, jo sąsajos su oksidaciniu stresu ir pakitusia lipidų apykaita išryškina šio kelio svarbą kraujagyslinio kognityvinio sutrikimo patogenezei bei jo potencialą tapti terapiniu taikiniu. Geresnis šių mechanizmų supratimas gali paskatinti naujų gydymo strategijų, skirtų kraujagyslių pažeidimui ir kognityviniam nuosmukiui sergant kraujagysliniu kognityviniu sutrikimu, kūrimą.

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Master's thesis

Integrative Multi-Omics Data Analysis Methods: Vascular Cognitive Impairment Case

ABSTRACT

Vascular cognitive impairment (VCI) represents a significant and growing contributor to dementia, yet its underlying molecular mechanisms remain incompletely understood. To address this gap, we employed an integrative multi-omics approach to characterize molecular alterations in post-mortem brain tissue, providing a comprehensive view of VCI-associated changes through the integration of genomics, epigenomics, transcriptomics, and metabolomics data. Our analysis revealed a consistent pattern of dysregulation in the Rac/Rho GTPase signaling pathway across multiple omics layers. Genomic analysis showed an enrichment of specific genetic variants within genes of this pathway. Epigenomic profiling demonstrated widespread hypermethylation, particularly affecting Rac/Rho GTPase-related genes. Transcriptomic analysis further revealed altered gene expression and splicing within this pathway, alongside changes in signaling related to hypoxia. These findings suggest that Rac/Rho GTPase signaling could be one of the key contributors to VCI pathophysiology. Furthermore, integrative analysis of epigenomics, genomics and metabolomics data highlighted significant alterations in the associations between methylation regions, transcripts, and metabolite levels. Notably, we observed altered interactions between Rac/Rho GTPase-related genes expression and methylation and specific lipid classes, including diacylglycerols (DAGs) and phosphatidylethanolamines (PEs), as well as isobutyrate. This suggests that epigenetic dysregulation of Rac/Rho GTPase signaling contributes to metabolic disturbances, particularly in lipid pathways, which are critical for neurovascular function. In conclusion, this study demonstrates the power of multi-omics integration to uncover complex molecular relationships in VCI. The consistent identification of Rac/Rho GTPase pathway disruption across omics layers, alongside its interplay with oxidative stress and altered lipid metabolism, highlights this pathway as a potential therapeutic target. A deeper understanding of these mechanisms may pave the way for the development of novel interventions aimed at mitigating vascular damage and cognitive decline in VCI.

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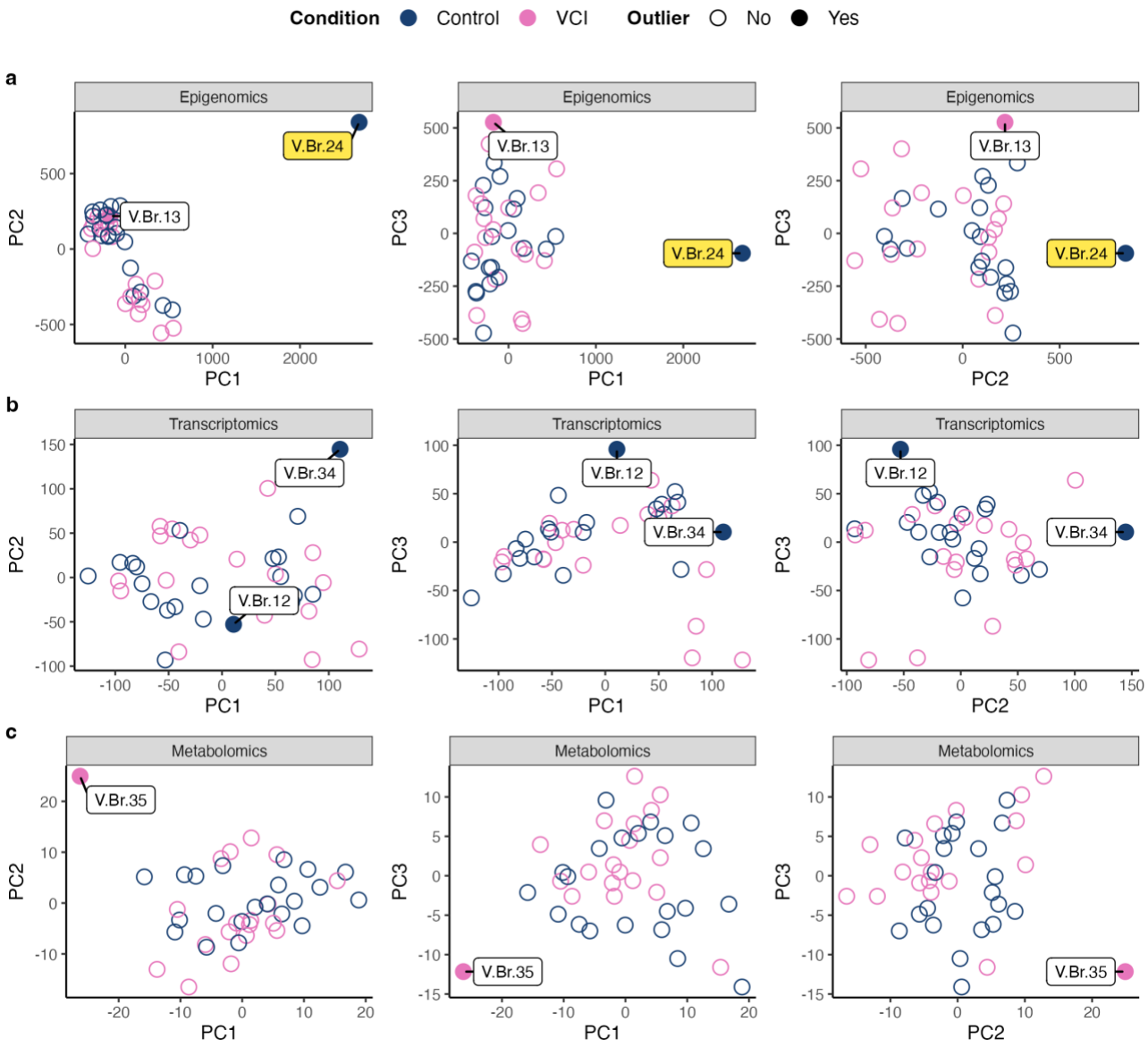
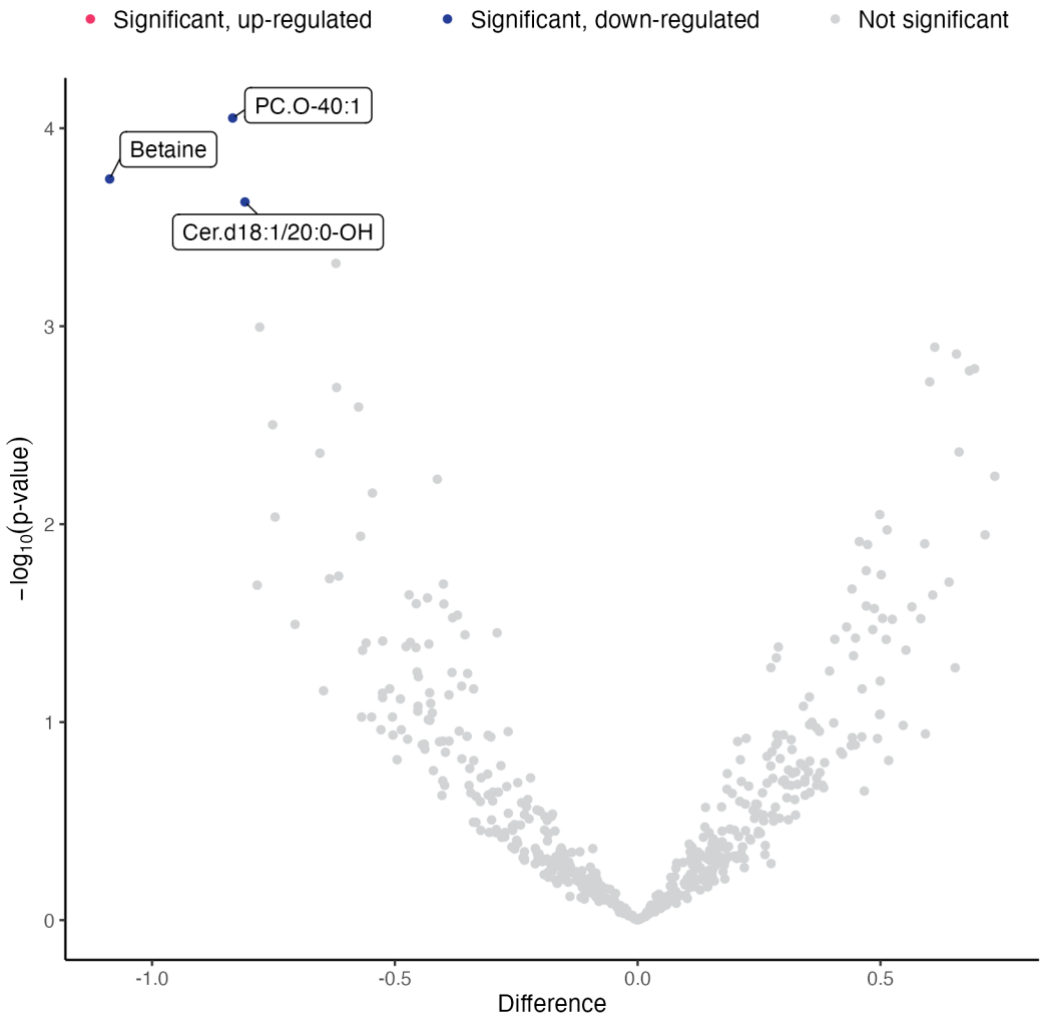
Appendix



Supplementary figure 1. PCA using the first three PCs. Samples exceeding with two SDs from the mean are marked as outliers. Colors represent the different conditions, while a yellow label indicates that samples were removed from further analysis. **a)** corresponds to epigenomics, **b)** to transcriptomics, and **c)** to metabolomics.



Supplementary figure 2. Volcano plot from metabolomics analysis

Supplementary table 1. Demographic information of the samples, detailing key characteristics such as age, sex assigned at birth, and clinical status

Sample_Name	Age	Sex	Race	PMI	Condition	Removed
V.Br.1	65	Female	White	2,43	VCI	No
V.Br.10	91	Female	Black.or.African.American	4,08	VCI	No
V.Br.11	81	Male	White	4,67	VCI	No
V.Br.12	66	Male	White	8,4	Control	No
V.Br.13	80	Male	Black.or.African.American	7,08	VCI	No
V.Br.14	60	Male	Asian	3,25	VCI	No
V.Br.15	90	Male	Black.or.African.American	4,08	VCI	No
V.Br.16	81	Female	Not.reported	8	VCI	No
V.Br.17	108	Female	Black.or.African.American	4	Control	No
V.Br.18	91	Female	White	3,08	VCI	No
V.Br.19	63	Male	Not.reported	5,58	VCI	No
V.Br.2	62	Male	Black.or.African.American	10,1	Control	No
V.Br.20	64	Male	Black.or.African.American	3,22	VCI	No
V.Br.21	102	Female	White	7,05	Control	No
V.Br.22	79	Female	Not.reported	7,67	Control	No
V.Br.23	82	Male	White	11,38	Control	No
V.Br.24	69	Male	Asian	7,42	Control	Yes
V.Br.25	79	Male	White	5,92	Control	No
V.Br.26	84	Female	Black.or.African.American	9	Control	No
V.Br.27	89	Female	White	4,83	Control	No
V.Br.28	95	Female	White	4,5	VCI	No
V.Br.29	64	Female	White	5,42	Control	No
V.Br.3	86	Male	Black.or.African.American	7,55	Control	No
V.Br.30	79	Female	White	10,08	VCI	No
V.Br.31	88	Female	Not.reported	3,25	VCI	No
V.Br.32	94	Female	Black.or.African.American	2,08	Control	No
V.Br.33	84	Female	Black.or.African.American	3,67	VCI	No
V.Br.34	97	Female	White	2,33	Control	No
V.Br.35	86	Female	White	2,25	VCI	No
V.Br.36	89	Female	Black.or.African.American	3,25	VCI	No
V.Br.37	85	Female	White	5	Control	No
V.Br.38	98	Female	White	1,83	VCI	No
V.Br.39	72	Male	White	2,42	VCI	No
V.Br.4	94	Female	Not.reported	7,82	Control	No
V.Br.40	92	Female	White	5,58	Control	No
V.Br.5	90	Female	White	4,17	Control	No
V.Br.6	98	Female	White	1,42	Control	No
V.Br.7	92	Male	Black.or.African.American	3,17	Control	No
V.Br.8	96	Male	Black.or.African.American	6,5	Control	No
V.Br.9	85	Male	Black.or.African.American	2,75	VCI	No

Supplementary table 2. Differential analysis results from all the omics. Due to the size of the tables, only the first 5 rows are presented. The full tables are uploaded to Google Drive and can be accessed using this link.
(https://docs.google.com/spreadsheets/d/1TiIpIyojTfgaS8WzD2xvaqMhrcm0nHDT/edit?usp=share_link&ouid=100376210791955047186&rtpof=true&sd=true)

SNP	Chr	Start	BP	A1	OR	STAT	P	SLP	Symbol
rs2862927	chr10	100024772	100024772	A	0,28	-0,98	0,33	-1,12	
rs622829	chr10	100322393	100322393	C	0,43	-0,70	0,49	-0,72	PKD2L1
rs2657506	chr10	10043201	10043201	G	1,33	0,31	0,76	0,28	
rs1540956	chr10	10045068	10045068	G	0,53	-0,57	0,57	-0,57	
rs2863073	chr10	100879404	100879404	A	2,52	0,89	0,37	0,98	

Supplementary table 3. Pathway enrichment analysis results from all the omics. Due to the size of the tables, only the first 5 rows are presented. The full tables are uploaded to Google Drive and can be accessed using this link.
(https://docs.google.com/spreadsheets/d/17P0xyaOoa6JyWSpoI1-a3wyoos3uM5wN/edit?gid=1437147864#gid=1437147864)

Description	GeneRatio	BgRatio	pvalue	qvalue	geneID	Count
RAC1 GTPASE CYCLE	3/15	91/5468	0,00	0,20	FARP1/CHN2/DOCK5	3
ORGANOPHOSPHATE CATABOLIC PROCESS	2/15	47/5468	0,01	0,20	PLA2G4C/SUCLG2	2
STIMULI-SENSING CHANNELS	2/15	49/5468	0,01	0,20	ASPH/TRPM3	2
RHO GTPASE CYCLE	3/15	177/5468	0,01	0,20	FARP1/CHN2/DOCK5	3
GLYCEROPHOSPHOLIPID BIOSYNTHETIC PROCESS	2/15	68/5468	0,01	0,20	DGKH/PLA2G4C	2

Supplementary table 4. Results from regression-based integration methods analysis. Due to the size of the tables, only the first 5 rows are presented. The full tables are uploaded to Google Drive and can be accessed using this link.
(https://docs.google.com/spreadsheets/d/1_hSXy5cohH5aUkxnnBUnPfCVtisxvimf/edit?usp=share_link&ouid=100376210791955047186&rtpof=true&sd=true)

Gene	Metabolite	Estimate methylation in control	P-value methylation in control	Q-value methylation in control	Estimate change	P-value change	Q-value change	Estimate methylation in VCI
UGT1A10	C2	20,95	0,00	0,04	-18,11	0,00	0,16	2,85
UGT1A10	MG.20.1	-17,89	0,00	0,19	28,77	0,00	0,05	10,88
AGAP1	Acetone	-17,01	0,00	0,04	19,23	0,00	0,07	2,22
AGAP1	PI.18.1_22.0	-6,59	0,04	0,44	16,19	0,00	0,05	9,60
AGAP1	SM.33.1	2,88	0,37	0,84	-16,45	0,00	0,05	-13,57

Supplementary table 5. Results from multi-omics data network construction analysis. Due to the size of the tables, only the first 5 rows are presented. The full tables are uploaded to Google Drive and can be accessed using this link.
(https://docs.google.com/spreadsheets/d/1Fao1Ybvacys_rm_oPMfJvfv4FJm1oJSz/edit?gid=783216626#gid=783216626)

Description	GeneRatio	BgRatio	pvalue	qvalue	Count
RAC1 GTPASE CYCLE	55/2324	173/15840	8,2E-09	6,2E-05	55
RHO GTPASE CYCLE	101/2324	424/15840	3,0E-07	1,1E-03	101
RHOA GTPASE CYCLE	43/2324	142/15840	1,5E-06	2,9E-03	43
REGULATION OF SMALL GTPASE MEDIATED SIGNAL TRANSDUCTION	61/2324	229/15840	1,5E-06	2,9E-03	61
CELL MORPHOGENESIS	102/2324	448/15840	2,4E-06	3,6E-03	102