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Constraint Based Metabolic Modeling Identifies Robust Biomarkers and Therapeutic Target Combinations in Alzheimer Disease

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Abstract

Alzheimer's disease (AD) involves widespread metabolic, mitochondrial, inflammatory, and redox dysfunction. We extended Recon3D with lumped synthesis, degradation, and recycling reactions for AD-associated proteins and inflammatory mediators and integrated brain-region-specific transcriptomic data to reconstruct metabolic models of asymptomatic AD, symptomatic AD, and healthy tissue. Parsimonious multi-objective metabolite-flow variability analysis across five nutrient environments identified medium-robust metabolic alterations, and fuzzy multi-objective optimization prioritized candidate interventions. Model-predicted intracellular production flows and uptake of aromatic and branched-chain amino acids were consistently increased, whereas CoA/itaconate, purine-nucleotide, isocitrate, and glutathione metabolism showed stage- and region-dependent changes. PPA2 upregulation and SUCLG2 downregulation emerged as promising single-enzyme interventions. Two-enzyme combinations (PGM1↓/PPA2↑ and SUCLG2↓/PPA2↑) improved predicted recovery across most disease–medium models. Adding increased uptake of itaconate, omega-3 eicosatetraenoic acid, Mead acid, or LPE20:4 to selected pairs further improved robustness. These findings provide testable hypotheses for metabolic biomarkers and combination interventions in Alzheimer's disease.

Introduction

Alzheimer's disease (AD) is the most common cause of dementia and represents a growing global health burden [1]. Although extracellular amyloid- β (A β) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau are defining neuropathological hallmarks of AD [2], disease progression involves broader disturbances in neuronal function, neuroinflammation, mitochondrial homeostasis, and cellular metabolism [3–11]. Altered glucose and lipid utilization, impaired mitochondrial bioenergetics, amino-acid dysregulation, and redox imbalance can disrupt neuronal and glial homeostasis and contribute to synaptic and neuronal dysfunction [3–7]. Metabolomic studies have likewise identified extensive alterations in energy, lipid, amino-acid, and redox metabolism in the brain and peripheral circulation of individuals with AD [4–7]. These observations support the view that metabolic dysfunction is an integral component of AD pathophysiology rather than simply a downstream consequence of neurodegeneration.

Metabolic dysfunction is closely interconnected with neuroinflammation. Microglia undergo substantial immunometabolic reprogramming as they transition from homeostatic to disease-associated states, involving changes in glucose utilization, lipid metabolism, amino-acid handling, and mitochondrial function [6,8–12]. Persistent or maladaptive microglial activation can in turn promote inflammatory-mediator production, oxidative stress, synaptic dysfunction, and neuronal injury [8–12]. Importantly, metabolic and transcriptional abnormalities may be detectable before overt cognitive impairment. Individuals who remain cognitively intact despite neuropathology

consistent with AD, commonly described as having asymptomatic Alzheimer's disease (AsymAD), therefore provide an informative model for investigating early disease-associated alterations and potential compensatory responses.

Patel et al. profiled post-mortem entorhinal cortex (EC), temporal cortex (TC), frontal cortex (FC), and cerebellum from healthy controls, individuals with probable AsymAD, and patients with symptomatic AD [13]. Their analysis revealed particularly pronounced transcriptional activity in the FC of AsymAD subjects, together with reorganization of energy-related processes, including oxidative phosphorylation and respiratory electron transport. These findings indicate that the transition from asymptomatic neuropathology to symptomatic disease is unlikely to represent a uniform amplification of the same molecular disturbances across the brain. Instead, metabolic reorganization may depend on both disease stage and anatomical region. Consistent with this interpretation, the EC, TC, and FC differ in their vulnerability to AD pathology and in their molecular responses during disease progression. Region-specific metabolic modeling may therefore reveal alterations that would be obscured when the brain is treated as a metabolically homogeneous tissue.

Transcriptomic profiles alone, however, do not directly specify metabolic reaction rates or feasible metabolic states. Cellular metabolism emerges from interactions among enzyme availability, stoichiometric constraints, pathway connectivity, metabolite transport, substrate availability, and cellular objectives. Genome-scale metabolic models (GSMMs) provide a systems-level framework for integrating these factors and translating molecular profiles into feasible metabolic phenotypes.

Constraint-based modeling has previously been applied to AD to integrate transcriptomic information with metabolic networks, predict metabolic biomarkers and candidate targets, characterize altered bile-acid metabolism, and investigate host–microbiome metabolic interactions [14–17]. Recon3D, a comprehensive reconstruction of human metabolism containing compartment-specific metabolites, reactions, genes, and gene–protein–reaction (GPR) associations, provides a suitable reference network for such analyses [18].

Two issues are particularly important when extending constraint-based modeling to AD. First, generic human metabolic reconstructions do not explicitly account for the energetic and biosynthetic demands associated with turnover of AD-related proteins and inflammatory mediators such as A β , tau, interleukin-6 (IL-6), and tumour necrosis factor- α (TNF- α). Incorporating simplified synthesis, degradation, and recycling reactions provides a means of representing these demands within a metabolic-network framework, although such reactions remain computational approximations of complex protein-processing and signaling processes. Second, constraint-based predictions can depend strongly on extracellular nutrient availability, because exchange constraints determine which substrates can enter and leave the network. Predictions obtained under a single medium may therefore represent environment-specific dependencies rather than robust disease-associated metabolic features.

Evaluating models across multiple nutrient environments provides a strategy for distinguishing robust from environment-dependent predictions. Here, DMEM, HAM, human plasma-like medium (HPLM), RPMI, and Virtual Metabolic Human (VMH) medium were applied to each disease-state model and

its corresponding healthy-tissue model. Metabolic alterations that remained strongly and directionally consistent across this predefined panel were prioritized as medium-robust candidate biomarkers. In this context, “medium-robust” denotes consistency across the five tested nutrient environments rather than independence from all possible physiological conditions.

In the present study, Recon3D [18] was extended with lumped reactions representing the synthesis, degradation, and recycling of tau, A β , selected inflammatory mediators, and ubiquitin. Brain-region- and disease-stage-specific transcriptomic profiles were integrated to reconstruct healthy-tissue, AsymAD, and symptomatic AD metabolic models for the EC, TC, and FC. Parsimonious multi-objective metabolite-flow variability analysis (pMOMFA) was then performed across five nutrient environments to identify medium-robust metabolic alterations and candidate biomarkers. In parallel, fuzzy multi-objective optimization was used to prioritize single and combinatorial metabolic perturbations predicted to shift disease-associated metabolic states while preserving essential functions in the corresponding healthy-tissue models. By jointly accounting for disease stage, brain region, and nutrient environment, this framework aims to distinguish conserved metabolic signatures from context-dependent responses and to generate experimentally testable hypotheses for biomarker and therapeutic-intervention studies.

Results

Construction and computational characterization of brain-region-specific metabolic models

The extended Recon3D network comprised 5,872 compartment-specific metabolites, 10,662

reactions, and 2,425 enzyme-encoding genes. Relative to the original Recon3D network [18], the extended reconstruction included 37 additional metabolites, 62 reactions, and 177 genes associated with lumped synthesis, degradation, and recycling reactions for AD-related proteins and inflammatory mediators: tau, A β , IL-6, TNF- α , and ubiquitin (Ub). These additions allow the energetic and biosynthetic demands associated with turnover of these proteins to be represented explicitly within the constraint-based framework.

Brain-region-specific transcriptomic profiles from the EC, TC, and FC of individuals with AsymAD, symptomatic AD, and corresponding healthy controls were integrated with the extended Recon3D network. Tissue-specific GSMMs were reconstructed using the CORDA algorithm [19], generating six disease-state-healthy-tissue (DS-HT) model pairs: AsymAD-HT and AD-HT for each of the three brain regions.

Figure 1 compares the numbers of compartment-specific metabolites, reactions, and enzyme-encoding genes retained in the reconstructed models. Across the six disease-state models, approximately 57–62% of metabolites, 49–54% of reactions, and 73–77% of enzyme-encoding genes in the extended Recon3D network were retained. The corresponding HT models retained approximately 59–69% of metabolites, 50–59% of reactions, and 75–76% of genes. The reconstructed networks thus retained substantial fractions of the reference reconstruction while displaying disease-stage- and brain-region-dependent differences in network composition.

The largest differences in network size occurred in the FC. The AsymAD-FC model contained 3,591 metabolites and 5,720 reactions, compared with 4,026 metabolites and 6,264 reactions in the corresponding HT-FC model. Differences between disease-state and HT models were smaller in the EC and TC, whereas the AD-FC model also diverged substantially from its HT-FC counterpart (Figure 1). These compositional differences are consistent with the prominent transcriptional reorganization previously reported in the FC of individuals with AsymAD [13]. All six DS–HT model pairs were subsequently evaluated under DMEM, HAM, HPLM, RPMI, and VMH nutrient conditions, yielding 30 disease–medium instances for pMOMFVA and therapeutic-target analyses.

Multi-environment simulations identify conserved and context-dependent metabolic alterations

Figures 2 and 3 summarize representative alterations in intracellular metabolite production-flow rates and exchange fluxes across the five nutrient conditions; complete results are provided in Supplementary Data 1. For each disease stage and brain region, the feasible ranges predicted for the DS and corresponding HT models were classified as complete increase (CI), partial increase (PI), inclusive increase (II), unchanged (UC), inclusive decrease (ID), partial decrease (PD), or complete decrease (CD), corresponding to ordinal scores of +3, +2, +1, 0, -1, -2, and -3, respectively. Positive and negative scores indicate higher and lower predicted metabolic activity in the disease-state model, and the score magnitude reflects the degree of separation between the corresponding feasible ranges. A metabolite or exchange flux was considered medium-robust when its mean score across the five media satisfied $|\text{mean}| \geq 2.4$ and the associated statistical criterion (see Methods).

Conserved medium-robust alterations in amino-acid metabolism

The most consistent signature was increased amino-acid production flow and uptake. Lysine, phenylalanine, threonine, tryptophan, and tyrosine showed complete increases, with mean classification scores of +3 across the EC, TC, and FC in both AsymAD and symptomatic AD (Figure 2). The branched-chain amino acids (BCAAs) leucine, isoleucine, and valine were likewise increased across all AsymAD models (mean score +3) and remained strongly increased in symptomatic AD, with mean scores of +2.6 in AD-EC and +3 in AD-TC and AD-FC (Figure 2). Glutamine, histidine, and asparagine also showed consistently positive alterations.

These intracellular production-flow changes were accompanied by increased uptake of many of the same amino acids. Uptake of glutamine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, tyrosine, and valine showed complete increases across nearly all AsymAD and AD comparisons, and phenylalanine uptake was completely increased in most models (mean score +2.4 in AD-FC; Figure 3). The concordance between intracellular production-flow and exchange-flux predictions indicates coordinated remodeling of amino-acid handling and a greater model-predicted dependence on extracellular amino-acid availability.

Not all alterations were uniform across media. For example, in AsymAD-EC the VMH medium gave scores of smaller magnitude than the other four media for coenzyme A, itaconyl-CoA, itaconate, and inosine di- and triphosphates, and ammonium was unchanged under VMH (Figure 2). Mean scores should therefore be read together with the per-medium scores in Supplementary Data 1.

Disease-stage-dependent mitochondrial and immunometabolic alterations

A distinct set of metabolites exhibited pronounced differences between AsymAD and symptomatic AD. Mitochondrial coenzyme A (CoA), itaconyl-CoA, and itaconate showed consistently negative mean classification scores across all three brain regions in AsymAD. In symptomatic AD, these alterations became strongly region dependent, with CoA, itaconyl-CoA, and itaconate shifting to positive mean scores of +2.2 in the FC. Cytosolic and mitochondrial inosine diphosphate (IDP) and inosine triphosphate (ITP) displayed a similar stage- and region-dependent pattern, changing from consistently negative scores in AsymAD to positive mean scores of +1.8 in AD-FC. Together, these findings indicate a transition from broadly reduced mitochondrial CoA/itaconate and purine-associated metabolic activity in AsymAD to more heterogeneous, region-specific alterations in symptomatic AD.

Mitochondrial isocitrate was unchanged across all three AsymAD regions but showed complete increases in symptomatic AD, with mean scores of +3 in the EC, TC, and FC. Trans-4-hydroxy-L-proline and 1-pyrroline-3-hydroxy-5-carboxylate were likewise largely unchanged in AsymAD but showed increased production-flow rates in symptomatic AD. Several mitochondrial and immunometabolic alterations therefore differ qualitatively between the asymptomatic and symptomatic disease states rather than simply increasing in magnitude with disease progression.

Brain-region-specific redox and energy-metabolic alterations

Several predicted alterations showed pronounced regional heterogeneity. Cytosolic oxidized glutathione (GSSG) exhibited opposite patterns among brain regions: in AsymAD, mean

classification scores were +3 in the EC, +3 in the TC, and -3 in the FC, and the corresponding symptomatic AD scores were +2.4, +1.2, and -1.4, respectively.

Mitochondrial catecholamine-related metabolites (3-methoxy-4-hydroxyphenylglycolaldehyde and 3-methoxy-4-hydroxymandelate) were also region dependent, with positive scores in AsymAD-EC and AsymAD-TC but negative scores in AsymAD-FC. Similarly, the glycolytic intermediates dihydroxyacetone phosphate and fructose 1,6-bisphosphate varied substantially among the EC, TC, and FC, as did fumarate according to both disease stage and brain region (Figure 2). Collectively, the simulations identified three broad patterns of metabolic alteration: changes that were robust across the five nutrient environments, changes associated with disease stage, and changes that were strongly dependent on brain region.

Combinatorial perturbations improve the robustness of predicted therapeutic responses

Candidate metabolic interventions were evaluated across the six disease-state models and five nutrient environments using disease-state recovery, healthy-tissue preservation, and metabolic-deviation criteria. A higher disease-state recovery score (Eq. 15) represents stronger predicted suppression of tau, A β , IL-6, and TNF- α synthesis while preserving ATP synthesis. The metabolic-deviation score (Eq. 17) additionally evaluates whether the treated disease-state metabolic pattern moves away from its untreated disease-state reference and toward the healthy-tissue state, while the perturbed HT model remains close to its corresponding healthy reference. Both scores range from 0 to 1 (see Methods).

Single-enzyme perturbations

The target-discovery framework identified 59 feasible single-enzyme perturbations, comprising 29 downregulations and 30 upregulations. PPA2 upregulation yielded recovery scores close to 1.0 across all five media in the three AsymAD models and in AD-TC, but its minimum recovery score decreased to approximately 0.4 in AD-EC and 0.2 in AD-FC (Figure 4). SUCLG2 downregulation appears to show lower minimum recovery in AsymAD-EC (about 0.4) and AsymAD-TC (about 0.02) than in the AD models (about 0.8–1.0).

Other single perturbations were less robust across disease stage, brain region, and nutrient environment. For example, PKLR upregulation and PGM1 downregulation yielded substantially lower minimum recovery scores in several disease-specific models (Figure 4). Thus, neither PPA2 upregulation nor SUCLG2 downregulation produced uniformly high recovery across all 30 disease–medium instances.

Two-enzyme combinations improve cross-context robustness

Combining enzyme perturbations substantially improved predicted recovery. PGM1 downregulation combined with PPA2 upregulation produced recovery scores close to 1.0 across all six disease-state models and five nutrient environments (Figure 5). SUCLG2 downregulation combined with PPA2 upregulation also yielded near-maximal recovery in most instances, although the recovery score remained approximately 0.81 for AD-EC under VMH conditions.

In contrast, other combinations retained substantial context dependence. SUCLG2 downregulation

combined with PKLR upregulation yielded a minimum recovery score of 0.02 in AsymAD-FC under VMH, whereas ALDH18A1 downregulation combined with PPA2 upregulation yielded a minimum score of 0.39 in AD-EC. The benefit of combining enzyme perturbations was therefore strongly dependent on the specific target combination rather than being a general consequence of increasing the number of perturbations.

Addition of metabolite-uptake interventions further increases robustness

Adding selected uptake interventions to two-enzyme combinations further improved robustness across brain regions and nutrient conditions. Four combinations were particularly effective: SUCLG2↓/PPA2↑ with increased itaconate uptake; SUCLG2↓/PPA2↑ with increased omega-3 eicosatetraenoic acid (20:4n-3) uptake; SUCLG2↓/PKLR↑ with increased Mead acid (20:3n-9) uptake; and ALDH18A1↓/PPA2↑ with increased LPE20:4 uptake. These combinations produced recovery scores close to 1.0 across nearly all 30 disease–medium instances, while metabolic-deviation scores remained modest (approximately 0.2–0.4 against a theoretical maximum of 1; Figure 6).

Overall, among the prioritized candidates, cross-context robustness generally improved from single-enzyme perturbations to selected two-enzyme combinations and, for several target pairs, to combinations incorporating additional metabolite-uptake interventions. These findings identify candidate metabolic interventions whose predicted responses were less sensitive to variations in disease stage, brain region, and extracellular nutrient composition

Discussion

The present study integrates disease stage, brain region, and nutrient environment within a constraint-based modeling framework to characterize metabolic alterations associated with AD and to prioritize candidate metabolic interventions. Three principal findings emerged. First, several predicted metabolic alterations were conserved across AsymAD and symptomatic AD and remained robust across five nutrient environments. Second, other alterations varied substantially with disease stage or brain region, revealing considerable metabolic heterogeneity. Third, among the prioritized candidate interventions, cross-context robustness generally improved from single-enzyme perturbations to selected two-enzyme combinations and, for several target pairs, to combinations incorporating additional metabolite-uptake interventions. Together, these findings suggest that AD-associated metabolic remodeling comprises both conserved system-level features and context-dependent responses, with potentially distinct implications for biomarker development and therapeutic targeting.

A prominent medium-robust signature involved altered aromatic and branched-chain amino-acid metabolism. Phenylalanine and tyrosine participate in pathways associated with catecholamine metabolism, whereas tryptophan is linked to the kynurenine pathway, whose metabolites exert neuroactive, immunoregulatory, and redox-related effects [20]. BCAAs contribute to nitrogen metabolism, energy homeostasis, and mitochondrial substrate utilization. In the present models, increased intracellular production-flow rates were accompanied by increased uptake of several aromatic and branched-chain amino acids across disease stages and brain regions. These production-

flow rates represent network-level metabolite production and turnover; they should not be interpreted as evidence of de novo biosynthesis, particularly for essential amino acids, or as direct measurements of tissue metabolite concentrations.

The concordant increases in production flow and uptake nevertheless suggest coordinated remodeling of amino-acid utilization and identify these metabolites as candidates for experimental validation in brain tissue, cerebrospinal fluid, or peripheral biofluids. This interpretation should remain cautious, because metabolomic studies have reported heterogeneous alterations in amino-acid metabolism in AD [4,6,7]. Recent epidemiological evidence also indicates that circulating branched-chain and aromatic amino acids can show associations with dementia risk that do not necessarily parallel the direction of model-predicted intracellular metabolic activity [21]. The present findings are therefore more appropriately interpreted as alterations in metabolic-network handling and flux capacity than as predictions of uniformly elevated metabolite concentrations.

A second group of alterations differed substantially between AsymAD and symptomatic AD. Mitochondrial CoA, itaconyl-CoA, itaconate, IDP/ITP, isocitrate, and hydroxyproline-related metabolites displayed pronounced disease-stage- and region-dependent patterns. These differences suggest that symptomatic disease may involve qualitative reorganization of metabolic networks rather than a simple amplification of abnormalities already present during AsymAD. This interpretation is consistent with broader evidence implicating impaired metabolic flexibility, mitochondrial bioenergetics, glucose utilization, oxidative stress, and immunometabolic remodeling in AD

[3,8,11,22,23]. However, because the transcriptomic profiles analyzed by Patel et al. [13] were obtained from different post-mortem individuals rather than longitudinally followed subjects, the predicted differences between AsymAD and symptomatic AD should not be interpreted as direct evidence of temporal metabolic progression.

Among these alterations, itaconate is of particular mechanistic interest. Itaconate is an immunometabolite derived from the tricarboxylic-acid-cycle intermediate cis-aconitate [24] and is generated through aconitate decarboxylase 1 (ACOD1), particularly in activated myeloid cells. Itaconate can modulate mitochondrial metabolism, oxidative stress, and inflammatory signaling [25]. These functions are relevant to the extensive immunometabolic reprogramming of microglia implicated in AD [8,11,23,26]. The predicted CoA/itaconate alterations are therefore biologically plausible, particularly in the context of microglial immunometabolism. Nevertheless, neither their predicted stage-dependent direction nor their brain-region-specific distribution has been established experimentally in AD, and the models were built from bulk tissue that cannot resolve microglial contributions. The CoA/itaconate and purine-associated alterations identified here should therefore be regarded as mechanistically plausible, testable hypotheses rather than established features of AD pathophysiology.

Brain-region specificity represents another important dimension of the predicted metabolic phenotype. Oxidized glutathione exhibited opposing alterations in the EC and TC compared with the FC, while catecholamine-related metabolites, glycolytic intermediates, and fumarate also displayed substantial

regional heterogeneity. These results are consistent with the region-dependent transcriptional changes reported in AsymAD and symptomatic AD [13] and with broader evidence that metabolic and oxidative disturbances in AD are heterogeneous rather than spatially uniform [4,7,22]. Region-specific modeling may therefore help distinguish broadly conserved metabolic alterations from responses associated with local vulnerability, compensatory processes, cellular composition, or tissue-specific metabolic organization. Such distinctions are important when interpreting brain metabolomic data, because findings obtained from one anatomical region may not generalize directly to another.

The target-discovery analysis further illustrates the importance of metabolic context. PPA2 upregulation and SUCLG2 downregulation produced the strongest single-enzyme responses, but neither perturbation was uniformly effective across all disease-state models and nutrient environments. PPA2 is a mitochondrial inorganic pyrophosphatase required for pyrophosphate homeostasis [27], and recent experimental evidence links PPA2 to mitochondrial fission, proliferation, and mitophagy [28]. These functions provide a plausible connection between PPA2 and mitochondrial homeostasis, which is extensively disrupted in AD [29,30]. However, the present predictions do not establish PPA2 as an AD therapeutic target, nor do they imply that PPA2 directly regulates A β , tau, IL-6, or TNF- α ; their modeled suppression results from redistribution of energetic and biosynthetic fluxes.

SUCLG2 encodes the GDP-forming β -subunit of succinyl-CoA ligase and participates in succinyl-

CoA metabolism and mitochondrial substrate-level phosphorylation [31,32]; its knockdown has been reported to reduce proliferation in glioblastoma cells [33]. The predicted benefit of SUCLG2 downregulation should be interpreted cautiously, because excessive loss of succinyl-CoA ligase activity could compromise mitochondrial energetic capacity, particularly in metabolically vulnerable neural cells. Similarly, PGM1 interconverts glucose-1-phosphate and glucose-6-phosphate and thereby links glycogen metabolism with central glucose utilization [34,35]; extensive inhibition of PGM1-dependent metabolism could adversely affect cellular energy homeostasis. These considerations support experimental evaluation of graded or partial metabolic modulation rather than assuming that complete inhibition would be therapeutically desirable.

Selected two-enzyme combinations showed greater cross-context robustness than the corresponding single perturbations. In particular, PGM1↓/PPA2↑ and SUCLG2↓/PPA2↑ produced consistently favorable predicted responses across a larger fraction of disease-stage, brain-region, and nutrient contexts. This result suggests that coordinated perturbation of complementary metabolic processes can, within the model, compensate for context-specific limitations of individual targets. It should not, however, be interpreted as evidence that multi-target interventions are intrinsically superior: several other two-enzyme combinations remained ineffective in particular disease–medium conditions. The specific composition of a target combination, rather than simply the number of targets, determines computational robustness.

Additional metabolite-uptake interventions further improved the predicted robustness of selected

enzyme combinations. The itaconate-containing combination is consistent with the immunometabolic role of itaconate described above [25], whereas omega-3 eicosatetraenoic acid, Mead acid, and LPE20:4 implicate lipid remodeling in the modeled response. LPE20:4 is particularly noteworthy because an integrative human brain-omics analysis identified lysophosphatidylethanolamine-associated alterations across control, AsymAD, and symptomatic AD states [36]. This independent evidence supports the biological relevance of altered LPE metabolism in AD, although it does not validate increased LPE20:4 uptake as a therapeutic intervention. More generally, a beneficial response to increased metabolite uptake in the model should not be equated with dietary supplementation or systemic administration. Uptake perturbations represent modifications of exchange-flux capacity within the reconstructed network; blood–brain barrier transport, extracellular availability, transporter expression, uptake kinetics, dose dependence, and cell-type-specific metabolism are not explicitly represented. The predicted uptake interventions should therefore be viewed as indicators of metabolic dependencies that warrant experimental investigation rather than as nutritional or pharmacological recommendations.

Several limitations should be considered when interpreting these findings. First, the tissue-specific models were reconstructed from bulk post-mortem transcriptomic data and therefore cannot distinguish metabolic states of neurons, astrocytes, oligodendrocytes, microglia, and other cell populations; differences in cellular composition among disease states or brain regions may contribute to the reconstructed network differences. Second, the AsymAD and symptomatic AD groups are

cross-sectional and cannot establish longitudinal transitions during disease progression [13]. Third, transcript abundance was used to guide network reconstruction and parsimonious flux selection but does not directly determine enzyme abundance, catalytic activity, or reaction flux. Fourth, the lumped reactions for A β , tau, IL-6, TNF- α , and ubiquitin approximate energetic and biosynthetic demands but do not reproduce protein modification, trafficking, aggregation, degradation, secretion, or signaling.

The environmental analysis also has limitations. The five nutrient media provide a systematic test of sensitivity to extracellular nutrient constraints, but none fully reproduces the composition, compartmentalization, and dynamic regulation of the human brain extracellular environment. Accordingly, “medium-robust” in the present study should be understood operationally as consistency across the five tested nutrient environments rather than independence from nutrient availability under all physiological conditions. Moreover, pMOMFVA estimates feasible ranges of metabolite production and exchange fluxes rather than intracellular metabolite concentrations. Finally, the target-discovery results represent computational perturbations of reactions and exchange capacities rather than pharmacological interventions. Druggability, dose–response relationships, blood–brain barrier penetration, cell-type selectivity, off-target effects, and toxicity remain to be established experimentally.

These limitations define clear directions for validation. Medium-robust amino-acid signatures should be assessed using independent brain-tissue, cerebrospinal-fluid, and plasma metabolomic datasets,

with particular attention to the distinction between metabolite concentration and metabolic turnover. Stage- and region-dependent predictions involving CoA/itaconate, glutathione, isocitrate, purine-associated metabolism, and glycolytic intermediates would benefit from region-resolved and, where feasible, cell-type-resolved metabolomic measurements. Candidate enzyme and uptake perturbations should be evaluated experimentally in neuronal and glial systems for their effects on ATP production, mitochondrial respiration, redox homeostasis, inflammatory signaling, lipid metabolism, and A β /tau turnover before therapeutic relevance is inferred.

Overall, the findings support a systems-level view in which AD-associated metabolic remodeling depends jointly on disease state, anatomical context, and nutrient availability. Metabolic alterations that remain robust across multiple nutrient environments may provide higher-priority candidates for experimental biomarker validation, whereas stage- and region-specific signatures may offer complementary information on disease heterogeneity. Likewise, the improved performance of selected combinatorial perturbations suggests that coordinated modulation of complementary metabolic processes can provide greater computational robustness than some individual perturbations. The framework therefore offers a systematic approach for distinguishing conserved metabolic features from context-dependent responses and for generating experimentally testable hypotheses for biomarker development and metabolic-intervention studies.

Methods

Figure 7 summarizes the computational framework used for brain-region-specific metabolic model

reconstruction, identification of medium-robust metabolic alterations, and therapeutic-target discovery. Recon3D provided the reference human metabolic network [18], and region- and disease-stage-specific transcriptomic profiles were integrated to generate tissue-specific GSMMs and their associated GPR relationships. The reconstructed models were analyzed under five nutrient environments using pMOMFVA, followed by fuzzy multi-objective optimization for candidate therapeutic-intervention discovery.

Extension of Recon3D with protein synthesis, degradation, and recycling reactions

Recon3D [18] was extended by incorporating lumped reactions representing the synthesis, degradation, and recycling of tau, A β , IL-6, TNF- α , and ubiquitin (Ub). These reactions represent the amino-acid and energetic requirements associated with turnover of AD-related proteins and inflammatory mediators within the constraint-based framework. Similar lumped protein-polymerization formulations have previously been incorporated into human GSMMs [37,38]. Protein-specific synthesis reactions were constructed from the amino-acid composition of the corresponding UniProt sequences. For each protein, synthesis was represented by the generalized lumped reaction



where A_j denotes amino acid j , and S_{A_j} is its stoichiometric coefficient. All coefficients were normalized to the synthesis of 1 g of the corresponding protein. The coefficient of amino acid j ($mmol\ g^{-1}\ protein$) was calculated as

$$S_{A_j} = \frac{10^3 n_{A_j}}{M_{Protein}} \quad (2)$$

where n_{A_j} is the number of residues of amino acid j in the protein sequence and $M_{protein}$ is the molecular mass of the protein ($g \text{ mol}^{-1}$). The total number of residues is

$$N_A = \sum_j n_{A_j} \quad (3)$$

A protein containing N_A residues contains $N_A - 1$ peptide bonds. The energetic requirement for protein polymerization was represented by k_{ATP} , the number of ATP equivalents consumed per peptide bond:

$$S_{ATP} = \frac{10^3 k_{ATP} (N_A - 1)}{M_{Protein}} \quad (4)$$

Protein biosynthesis requires approximately four high-energy phosphate equivalents per peptide bond when amino-acid activation and translation are considered. Following the energetic convention adopted in previous modeling studies [37,38] and the reported range of approximately 4.0–4.3 ATP equivalents per peptide bond [39], k_{ATP} was set to 4.15. Under the simplified convention, each ATP equivalent was represented as hydrolysis of ATP to ADP and inorganic phosphate (P_i):

$$S_{ADP} = S_{P_i} = S_{ATP} \quad (5)$$

The net water coefficient accounts for water consumed during ATP-equivalent hydrolysis and water released during peptide-bond formation:

$$S_{H_2O} = \frac{10^3 (k_{ATP} - 1) (N_A - 1)}{M_{Protein}} \quad (6)$$

With $ATP + H_2O \rightarrow ADP + P_i + H^+$ as the hydrolysis step, the initial proton coefficient is

$$S_{H^+} = S_{ATP} \quad (7)$$

Because the protonation states and charges assigned to ATP, ADP, and phosphate depend on the

metabolite definitions in Recon3D [18], the proton coefficient was adjusted where necessary to maintain elemental and charge balance. It should therefore be interpreted as a reconstruction-specific stoichiometric coefficient rather than a universal proton requirement. The coefficient of the synthesized protein ($mmol\ g^{-1}\ protein$) is

$$S_{Protein} = \frac{1000}{M_{Protein}} \quad (8)$$

such that the complete lumped reaction corresponds to the synthesis of 1 g of protein. Protein-specific degradation reactions were subsequently introduced to represent proteolytic breakdown of tau, A β , IL-6, TNF- α , and Ub, and recycling reactions returned liberated amino acids to the corresponding intracellular amino-acid pools (Supplementary Table 1). Together, these reactions allow the extended Recon3D network to account explicitly for the metabolic and energetic demands of turnover of the selected AD-related proteins and inflammatory mediators.

Reconstruction of brain-region-specific metabolic models

The extended Recon3D network served as the reference for tissue-specific reconstruction. Microarray expression profiles from the EC, FC, and TC of cognitively healthy controls, individuals with probable AsymAD, and patients with symptomatic AD were obtained from Patel et al. [13] (Gene Expression Omnibus accession GSE118553). Gene-expression information was mapped to metabolic reactions through the GPR associations of the extended network. Reaction confidence was then used to reconstruct context-specific networks with the CORDA algorithm [19]. For each brain region, separate models were generated for AsymAD, symptomatic AD, and the corresponding healthy-tissue (HT) condition, giving six DS–HT pairs: AsymAD–HT and AD–HT for the EC, FC, and TC.

Parsimonious multi-objective metabolite-flow variability analysis

Five nutrient environments were considered to evaluate the robustness of predicted metabolic alterations to extracellular nutrient availability: Dulbecco's modified Eagle medium (DMEM), Ham's medium (HAM), human plasma-like medium (HPLM), Roswell Park Memorial Institute medium (RPMI), and Virtual Metabolic Human medium (VMH) (Supplementary Table 1).

pMOMFVA was formulated as a hierarchical optimization framework. At the upper level, the production-flow rate of each metabolite was independently maximized and minimized to determine its feasible range. Each upper-level optimization was constrained by a lower-level metabolic optimization comprising two sequential stages: (1) determination of the metabolic objectives (multi-objective flux balance analysis, MOFBA) and (2) a weighted uniform flux distribution (UFD), in which the total weighted reaction flux was minimized while prescribed fractions of the optimized objectives were retained. The general formulation is given in Eq. (9):

$$\left\{ \begin{array}{ll} \begin{array}{l} \max/\min_{\mathbf{v}_f, \mathbf{v}_b, \zeta_i \in \Omega^o} r_{m_k}, \text{ for each } m_k \in \Omega^m \\ 1^{st} \text{ stage MOFBA problem} \end{array} & \begin{array}{l} 2^{nd} \text{ stage weighted UFD problem} \\ \left\{ \begin{array}{l} \min_{\mathbf{v}_f, \mathbf{v}_b} \sum_{i=1}^n w_i (v_{f,i} + v_{b,i}) \\ \text{subject to} \\ \mathbf{N}(\mathbf{v}_f - \mathbf{v}_b) = \mathbf{0} \\ \mathbf{0} \leq \mathbf{v}_f^{LB} \leq \mathbf{v}_f \leq \mathbf{v}_f^{UB} \\ \mathbf{0} \leq \mathbf{v}_b^{LB} \leq \mathbf{v}_b \leq \mathbf{v}_b^{UB} \\ \text{GPR association} \\ z_i \geq \zeta_i z_i^*, \zeta_i \in [0,1], \\ \text{for each } i \in \Omega^o \end{array} \right. \end{array} \right. \quad (9)$$

In Eq. (9), Ω^m is the set of metabolites evaluated by pMOMFVA, and Ω^o is the set of metabolic objectives. The vectors $\mathbf{v}_f$ and $\mathbf{v}_b$ are the forward and backward reaction fluxes, $\mathbf{N}$ is the $m \times n$

stoichiometric matrix (m compartment-specific metabolites, n reactions), and fluxes were constrained by their lower and upper bounds and by the GPR associations retained in each tissue-specific model. The parameter z_i^* is the optimized value of objective i from the first stage, and ς_i is the minimum fraction of z_i^* that must be preserved during the during weighted flux minimization.

For the AsymAD and symptomatic AD models, the metabolic objectives comprised ATP synthesis together with synthesis of tau, A β , IL-6, TNF- α , and Ub. ATP synthesis alone was the objective for the corresponding HT models. This formulation was intended to represent maintenance of cellular energy metabolism in healthy tissue while explicitly accounting for the energetic and biosynthetic demands associated with disease-related protein and inflammatory-mediator production in the disease-state models.

To incorporate transcriptomic evidence into the parsimonious flux solution, reaction-specific penalties w_i were assigned according to the confidence classification:

$$w_i = \begin{cases} 1/4, i \in \{\text{high confidence reactions}\} \\ 1/2, i \in \{\text{medium confidence reactions}\} \\ 3/4, i \in \{\text{negative confidence reactions}\} \\ 1, i \in \{\text{others or non - gene - expression}\} \end{cases} \quad (10)$$

Smaller penalties reduce the contribution of strongly supported reactions to the UFD objective and thereby preferentially preserve flux through reactions with greater transcriptomic support, whereas reactions with weaker or unavailable expression support receive larger penalties. These weights guide selection among feasible parsimonious flux distributions but should not be interpreted as quantitative estimates of enzyme abundance, catalytic capacity, or reaction rate.

Calculation of metabolite production-flow rates

For metabolite m in compartment c , the compartment-specific production-flow rate was calculated as

$$r_{m_c} = \sum_{N_{ij}>0, j=1}^n N_{ij} v_{f,j} - \sum_{N_{ij}<0, j=1}^n N_{ij} v_{b,j}, m_c \in \Omega^c \quad (11)$$

The first term represents production through forward fluxes of reactions in which the metabolite has a positive stoichiometric coefficient, and the second term represents production through reverse fluxes of reactions in which the metabolite is consumed in the forward direction. Consumption fluxes were therefore excluded from the production-flow measure. For metabolites occurring in multiple subcellular compartments, the model-wide production-flow rate was obtained by summing the compartment-specific rates:

$$r_m = \sum_c r_{m_c}, c \in \Omega^c \quad (12)$$

This quantity represents aggregate network production or turnover capacity and should not be interpreted as a measured metabolite concentration or, particularly for essential amino acids, as evidence of de novo biosynthesis.

Priority-based maximizing fuzzy decision formulation

Because the disease-state models contain multiple metabolic objectives, fuzzy set theory was used to transform the multi-objective stage into a priority-based maximizing fuzzy decision (PBMFD) problem, adapted from previously developed fuzzy hierarchical metabolic-optimization frameworks [37,38]. The PBMFD formulation integrates competing metabolic objectives through normalized membership grades while allowing preferential weighting of a designated primary objective. It was coupled with weighted UFD to give the two-stage PBMFD–UFD formulation in Eq. (13):

$$\begin{array}{ll}
1^{st} \text{ stage: PBMFD problem} & 2^{nd} \text{ stage: weighted UFD problem} \\
\left\{ \begin{array}{l} \max_{\mathbf{v}_f, \mathbf{v}_b} (\mu_1 + \min\{\varpi_i \mu_i, i \in \Omega^o\})/2 \\ \text{subject to} \\ \mathbf{N}(\mathbf{v}_f - \mathbf{v}_b) = \mathbf{0} \\ \mathbf{0} \leq \mathbf{v}_f^{LB} \leq \mathbf{v}_f \leq \mathbf{v}_f^{UB} \\ \mathbf{0} \leq \mathbf{v}_b^{LB} \leq \mathbf{v}_b \leq \mathbf{v}_b^{UB} \\ \text{GPR association} \end{array} \right. & \left\{ \begin{array}{l} \min_{\mathbf{v}_f, \mathbf{v}_b} \sum_{i=1}^n w_i (v_{f,i} + v_{b,i}) \\ \text{subject to} \\ \mathbf{N}(\mathbf{v}_f - \mathbf{v}_b) = \mathbf{0} \\ \mathbf{0} \leq \mathbf{v}_f^{LB} \leq \mathbf{v}_f \leq \mathbf{v}_f^{UB} \\ \mathbf{0} \leq \mathbf{v}_b^{LB} \leq \mathbf{v}_b \leq \mathbf{v}_b^{UB} \\ \text{GPR association} \\ \mu_i \geq \zeta_i \mu_i^*; \zeta_i \in [0,1], i \in \Omega^o \end{array} \right. \quad (13)
\end{array}$$

Here, μ_i is the membership grade of objective i , and ϖ_i is its priority weight. These priority weights express the relative importance of the metabolic objectives and are distinct from the reaction-specific penalties w_i in Eq.(10), which are used only in the weighted UFD objective. The parameter μ_i^* is the optimal membership grade obtained in the PBMFD stage, and ζ_i is the minimum fraction of μ_i^* retained during the subsequent UFD minimization. For a maximization objective, the one-sided membership function increases linearly from 0 at the predefined lower bound LB_i to 1 at the upper bound UB_i :

$$\mu_i = \begin{cases} 0, & \text{when } FV_i \leq LB_i; \quad i \in \Omega^o \\ (FV_i - LB_i)/(UB_i - LB_i), & \text{when } LB_i < FV_i < UB_i \\ 1, & \text{when } FV_i \geq UB_i \end{cases} \quad (14)$$

where FV_i is the achieved value of objective i ; larger membership grades indicate greater satisfaction of the objective. The PBMFD–UFD procedure was applied to each disease-state and HT model under each of the five nutrient environments before metabolite-flow variability analysis [37,38].

Analysis of metabolic alterations and identification of medium-robust candidate biomarkers

Figure 7 summarizes the procedure for comparing predicted metabolic activity between disease-state and corresponding HT models. Whereas differential gene-expression analysis identifies transcript-level differences between biological states [40], the present approach evaluates their consequences at

the level of feasible metabolic-network behavior. For each disease stage, brain region, and nutrient environment, pMOMFVA was used to determine feasible ranges of intracellular metabolite production flow and exchange fluxes. Corresponding disease-state and HT ranges were compared according to their direction of change and degree of overlap and classified into seven categories: CI, PI, II, UC, ID, PD, and CD (Figure 7K), with ordinal scores of +3, +2, +1, 0, -1, -2, and -3, respectively. Positive scores indicate higher predicted metabolic activity in the AsymAD or AD model relative to its HT model, and negative scores indicate lower predicted activity. CI and CD correspond to non-overlapping feasible ranges and therefore represent the strongest model-based evidence for increased or decreased activity; the intermediate classes represent increasing degrees of overlap.

For each metabolite or exchange flux, the classification scores obtained under DMEM, HAM, HPLM, RPMI, and VMH were averaged to quantify directional consistency across nutrient environments. A candidate was classified as medium-robust when the absolute mean classification score was ≥ 2.4 and the disease-state-HT comparison satisfied the statistical criterion $p < 0.05$. The 2.4 threshold corresponds, for example, to a score of +3 in four media and 0 in the fifth, and thus tolerates at most one non-responsive medium. Candidate biomarkers were categorized as intracellular production-flow, nutrient-uptake, or metabolite-secretion alterations.

Computational discovery of candidate therapeutic interventions

The therapeutic-target discovery framework is illustrated in Figure 8. Candidate enzyme

perturbations were evaluated in treated disease-state (TD) models and the corresponding perturbed healthy-tissue (PH) models using three complementary criteria: (1) recovery of the TD metabolic state, (2) preservation of PH metabolic function, and (3) treatment-induced metabolic-pattern change relative to the untreated disease-state (DS) and HT reference models. Fuzzy membership functions transformed these heterogeneous objectives into normalized satisfaction grades between 0 and 1, which were integrated into the PBMFD framework, following previously developed fuzzy optimization approaches for metabolic target identification [37,38,41].

Evaluation of disease-state recovery and healthy-tissue preservation

The response of the TD model to each candidate intervention was quantified by the disease-state recovery score η_{CV}^{TD} , which combines the membership grades for suppression of tau, A β , IL-6, and TNF- α synthesis with the membership grade for preservation of ATP synthesis:

$$\eta_{CV}^{TD} = [(\sum_{i=1}^4 \mu_{P_{n_i}}^{TD} + \mu_{ATP}^{TD})/5 + \min\{\mu_{P_{n_i}}^{TD}, i = 1, \dots, 4\}]/2 \quad (15)$$

Here, $\mu_{P_{n_i}}^{TD}$ is the membership grade for suppression of synthesis of protein or inflammatory mediator $i \in \{\text{tau}, \text{A}\beta, \text{IL-6}, \text{TNF-}\alpha\}$, and μ_{ATP}^{TD} is the membership grade for preservation of ATP synthesis.

The mean component represents overall performance across the five objectives, whereas the minimum protein-suppression term prevents an intervention from receiving a high score if any one of the four disease-associated objectives is poorly satisfied. Larger η_{CV}^{TD} values indicate a more favorable model-predicted response. For each minimization objective, the membership grade was calculated from its predefined bounds and constrained to [0, 1]:

$$\mu_{Pn_i}^{TD} = \frac{UB_{Pn_i} - FV_{Pn_i}}{UB_{Pn_i} - LB_{Pn_i}}, i \in \{\text{tau}, A\beta, IL - 6, TNF - \alpha\} \quad (16)$$

A value of 1 represents maximal satisfaction of the suppression objective and 0 represents failure to achieve the prescribed reduction. FV_{Pn_i} is the achieved production rate, and LB_{Pn_i} and UB_{Pn_i} are the predefined lower and upper bounds. The corresponding PH model was evaluated using the healthy-tissue viability-preservation score η_{CV}^{PH} , with ATP synthesis as the principal energetic objective; larger values indicate greater preservation of healthy-tissue metabolic function after application of the candidate intervention.

Metabolic-pattern comparison

Global metabolic responses were evaluated by comparing the metabolic patterns of the TD and PH models with those of the untreated DS and HT reference models. Reaction fluxes and metabolite production-flow rates were calculated using the parsimonious multi-objective formulation described above, with the objective-retention parameters set to 1.0 so that optimized objective levels were fully retained during weighted flux minimization. A desirable intervention was defined as one in which the TD pattern became more similar to the HT template and more dissimilar from the untreated DS template, whereas the PH pattern remained similar to the HT template and dissimilar from the DS template. These properties were quantified using fuzzy similarity and dissimilarity membership functions and combined into the metabolic-deviation score:

$$\eta_{MD}^{TP} = [(\mu_{MD}^{TD,HT} + \mu_{MD}^{TD,DS} + \mu_{MD}^{PH,HT} + \mu_{MD}^{PH,DS})/4 + \min\{\mu_{MD}^{TD,HT}, \mu_{MD}^{TD,DS}, \mu_{MD}^{PH,HT}, \mu_{MD}^{PH,DS}\}]/2 \quad (17)$$

Larger η_{MD}^{TP} values represent a more favorable combination of disease-state redirection and healthy-tissue preservation.

Fuzzy similarity and dissimilarity functions

The similarity membership function is a two-sided function centered on the standard reference value ST , and the dissimilarity function is its complement:

$$\mu_{sim} = \max \left\{ \min \left\{ \frac{FV-LB}{ST-LB}, \frac{UB-FV}{UB-ST}, 1 \right\}, 0 \right\} \quad (18)$$

$$\mu_{dissim} = \min \left\{ \max \left\{ \frac{ST-FV}{ST-LB}, \frac{FV-ST}{UB-ST}, 0 \right\}, 1 \right\} \quad (19)$$

Here FV is the calculated reaction flux or metabolite production-flow rate, and LB , UB , and ST are the lower bound, upper bound, and standard reference value, respectively. When experimental or clinical reference values were unavailable, these parameters were estimated from the corresponding DS and HT metabolic templates. The two-sided functions quantify the extent to which an individual metabolic variable approaches or departs from its specified reference state. The fuzzy minimization, maximization, similarity, and dissimilarity objectives were integrated into the PBMFD target-discovery framework shown in Figure 8 [37,38,41].

Optimization of candidate interventions

The resulting hierarchical target-discovery problem was solved using the nested hybrid differential evolution (NHDE) algorithm [42,43]. Candidate perturbations were iteratively generated and evaluated through the corresponding lower-level metabolic optimization problems for the TD and PH models. Iteration continued until the predefined termination criteria were satisfied, and candidate solutions were ranked according to the PBMFD fitness function. Detailed NHDE procedures and parameter settings are provided in Supplementary Note 1.

Data availability

The gene expression dataset from human brain tissues associated with Alzheimer's disease used in this study is available in the Gene Expression Omnibus (GEO) under accession number GSE118553.

The Recon3D metabolic model is available at Virtual Metabolic Human (<http://vmh.life>).

Code availability

The computational code used to identify metabolic alterations and therapeutic interventions, together with the reconstructed tissue-specific genome-scale metabolic models, is available in the CBM-AD-ToolBox GitHub repository (<https://github.com/chmfsw/CBM-AD-ToolBox>).

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Author contributions

F.-S.W. conceived and designed the study, performed the computations, and contributed to drafting the manuscript. P.-Y.L. reconstructed the model. R.-F.S.H. was primarily responsible for securing funding, coordinated the study, and contributed to drafting the manuscript. All authors approved the final manuscript for publication and agree to be accountable for all aspects of the work.

Competing interests

The authors declare no competing interests.

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Supplementary information

Supplementary Data 1: Metabolic alterations for intracellular metabolite production-flow rates and exchange reactions.

Supplementary Note 1: Mathematical formulation of the therapeutic target discovery framework and computational procedures for its solution using the nested hybrid differential evolution algorithm.

Supplementary Table 1: Additional reactions integrated into Recon3D, and composition of the five nutritional media and components common to all media.

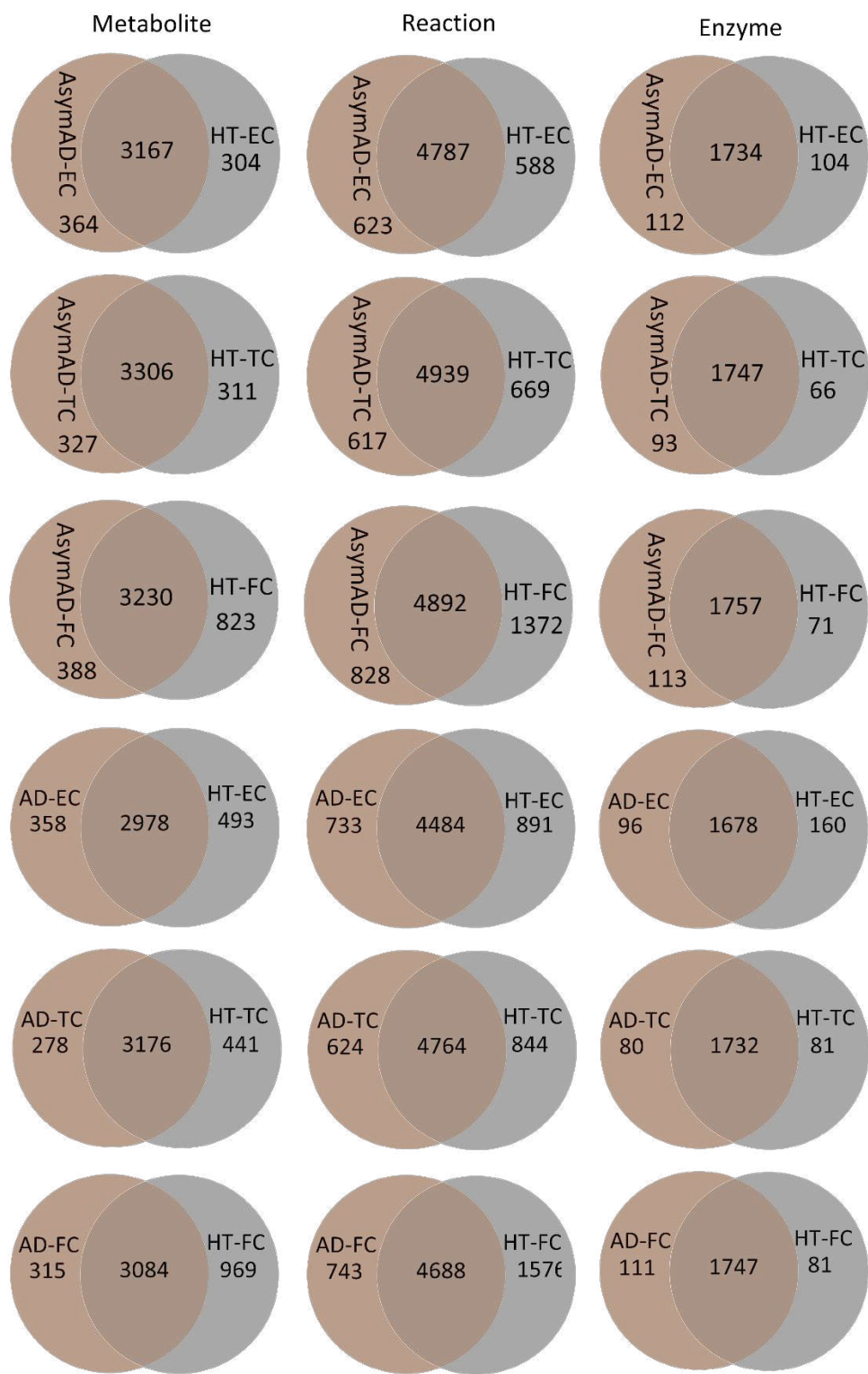


Figure 1. Comparison of metabolites, reactions, and enzyme-encoding genes between reconstructed AsymAD or AD metabolic models and their corresponding healthy-tissue models. Venn diagrams compare the numbers of compartment-specific metabolites (left column), reactions (middle column), and enzyme-encoding genes (right column) in reconstructed AsymAD (upper three rows) and symptomatic AD (lower three rows) models with their corresponding healthy-tissue (HT) models. Within each disease stage, the entorhinal cortex (EC), temporal cortex (TC), and frontal

cortex (FC) are shown from top to bottom. Numbers in the overlapping regions indicate components shared between the disease-state and corresponding HT models; orange regions indicate components unique to the AsymAD or AD model, and grey regions indicate components unique to the HT model.

Metabolite	AsymAD EC					Ave. AsymAD			Ave. AD		
	DMEM	HAM	HPLM	RPMI	VMH	EC	TC	FC	EC	TC	FC
L-Lysine[c]	3	3	3	3	3	3	3	3	3	3	3
L-Phenylalanine[e]	3	3	3	3	3	3	3	3	3	3	3
L-Threonine[c]	3	3	3	3	3	3	3	3	3	3	3
L-Tryptophan[c]	3	3	3	3	3	3	3	3	3	3	3
L-Tyrosine[c]	3	3	3	3	3	3	3	3	3	3	3
L-Isoleucine[c]	3	3	3	3	3	3	3	3	2.6	3	3
L-Leucine[c]	3	3	3	3	3	3	3	3	2.6	3	3
L-Valine[c]	3	3	3	3	3	3	3	3	2.6	3	3
L-Methionine[c]	3	3	3	3	3	3	3	3	2.2	3	3
L-Glutamine[c]	3	3	3	3	3	3	3	2.8	3	3	3
L-Histidine[c]	3	3	3	3	3	3	3	2.8	3	3	3
L-Asparagine[c]	3	3	3	3	3	3	3	2.6	3	3	3
Glycine[e]	3	2	3	3	3	2.8	3	2.4	1.6	3	2.4
L-Glutamate[c]	1	2	3	3	3	2.4	3	2.2	1.6	3	3
Ammonium[c]	3	3	3	3	0	2.4	2.4	2.6	1	2.4	0.8
L-Aspartate[e]	2	2	2	1	3	2	2.8	3	2.4	2.6	3
Coenzyme A[m]	-2	-3	-2	-2	-1	-2	-2.8	-2.6	-2.6	-0.4	2.2
Itaconyl Coenzyme A[m]	-2	-3	-2	-2	-1	-2	-3	-3	-2.6	-2	2.2
Itaconic Acid[m]	-2	-3	-2	-2	-1	-2	-3	-3	-2.6	-2	2.2
Inosine-5'-Diphosphate[c]	-3	-3	-3	-3	-1	-2.6	-3	-3	-2.6	-2.6	1.8
Inosine-5'-Diphosphate[m]	-3	-3	-3	-3	-1	-2.6	-3	-3	-2.6	-2.6	1.8
Inosine-5'-Triphosphate[c]	-3	-3	-3	-3	-1	-2.6	-3	-3	-2.6	-2.6	1.8
Inosine-5'-Triphosphate[m]	-3	-3	-3	-3	-1	-2.6	-3	-3	-2.6	-2.6	1.8
Diphosphate[c]	-3	-3	-3	-3	-2	-2.8	-3	-3	-2.8	-3	-0.8
Isocitric Acid[m]	0	0	0	0	0	0	0	0	3	3	3
Trans-4-Hydroxy-L-Proline[m]	-1	0	0	0	0	-0.2	0	0	2.2	3	3
L-1-Pyrroline-3-Hydroxy-5-Carboxylate[m]	-1	0	0	0	0	-0.2	0	0	2.2	3	3
Glutathione Disulfide (Oxidized Glutathione)[c]	3	3	3	3	3	3	3	-3	2.4	1.2	-1.4
3-Methoxy-4-Hydroxyphenylglycolaldehyde[m]	3	3	3	3	0	2.4	3	-3	0	0	0
3-Methoxy-4-Hydroxymandelate[m]	3	3	3	3	0	2.4	3	-3	0	0	-3
Dihydroxyacetone Phosphate[c]	-3	-3	-3	-3	-3	-3	0.8	3	-2.4	-3	2.8
D-Fructose 1,6-Bisphosphate[c]	-3	-3	-3	-3	-3	-3	0.8	3	-2.4	-3	2.8
Fumarate[c]	-3	-3	-3	-3	-3	-3	-0.6	-1.8	-3	3	-2.4

Figure 2. Predicted alterations in metabolite production-flow rates in AsymAD and AD models across brain regions and nutrient conditions. For the AsymAD-EC model, the first five score columns report predictions under the individual media; the subsequent columns report mean scores across the five media for each brain region in AsymAD and AD. These mean scores quantify the consistency of the predicted alteration with respect to nutrient availability. A metabolite production-flow rate meeting the predefined classification-score and statistical criteria in a given disease-state-HT comparison was considered a candidate medium-robust metabolic biomarker (see Methods). Metabolite identifiers followed by [c], [e], and [m] denote the cytoplasmic, extracellular, and mitochondrial compartments, respectively.

Exchange Reaction		AsymAD EC					Ave. AsymAD			Ave. AD		
		DMEM	HAM	HPLM	RPMI	VMH	EC	TC	FC	EC	TC	FC
Secretion	Ammonium	3	3	3	3	0	2.4	2.4	2.6	1	2.4	0.8
	1D-Myo-Inositol 1-Phosphate	0	0	0	0	0	0	3	3	0	3	1.8
	Phosphoenolpyruvate	3	3	0	3	0	1.8	3	0	1.8	3	0
	(S)-Glycerate	1	1	-1	0	0	0.2	3	0	0.6	3	0.6
Uptake	D-Galactose	0	0	0	0	0	0	-3	-3	0	-3	-1.2
	L-Glutamine	3	3	3	3	3	3	3	3	3	3	3
	L-Isoleucine	3	3	3	3	3	3	3	3	3	3	3
	L-Leucine	3	3	3	3	3	3	3	3	3	3	3
	L-Lysine	3	3	3	3	3	3	3	3	3	3	3
	L-Methionine	3	3	3	3	3	3	3	3	3	3	3
	L-Phenylalanine	3	3	3	3	3	3	3	3	3	3	2.4
	L-Proline	3	3	3	3	3	3	3	3	2.6	3	3
	L-Threonine	3	3	3	3	3	3	3	3	3	3	3
	L-Tryptophan	3	3	3	3	3	3	3	3	3	3	3
	L-Tyrosine	3	3	3	3	3	3	3	3	3	3	3
	L-Valine	3	3	3	3	3	3	3	3	3	3	3
	L-Asparagine	3	3	3	3	3	3	3	2.6	3	3	3
	L-Histidine	3	3	3	3	3	3	3	2.6	3	3	3
	L-Glutamate	2	3	3	3	3	2.8	3	2.2	1.8	3	1.2
	Glycine	3	2	3	3	3	2.8	3	2.2	1.6	3	1.2
	L-Alanine	0	0	3	3	3	1.8	3	3	1.4	3	3
	L-Aspartate	2	2	2	1	3	2	2.8	3	2.4	2.6	3
	L-Arginine	3	3	3	3	3	3	0.6	3	2.6	-0.2	3
	L-Cystine	3	0	3	0	3	1.8	2.4	1.8	1.2	2.4	1.2
	L-Serine	1	-3	1	1	1	0.2	2.2	3	0.2	2.4	3
	Hydrogen Peroxide	0	0	0	0	0	0	-3	0	-0.6	-3	-2.4
	Hypoxanthine	-3	-3	-3	0	0	-1.8	-1.2	-1.8	-1.8	-1.2	-1.2

Figure 3. Predicted alterations in metabolite secretion and uptake fluxes in AsymAD and AD models across brain regions and nutrient conditions. For the AsymAD-EC model, the first five score columns report predictions under the individual media; the subsequent columns report mean scores across the five media for each brain region in AsymAD and AD. A secretion or uptake flux meeting the predefined classification-score and statistical criteria in a given disease-state–HT comparison was considered a candidate medium-robust metabolic biomarker.

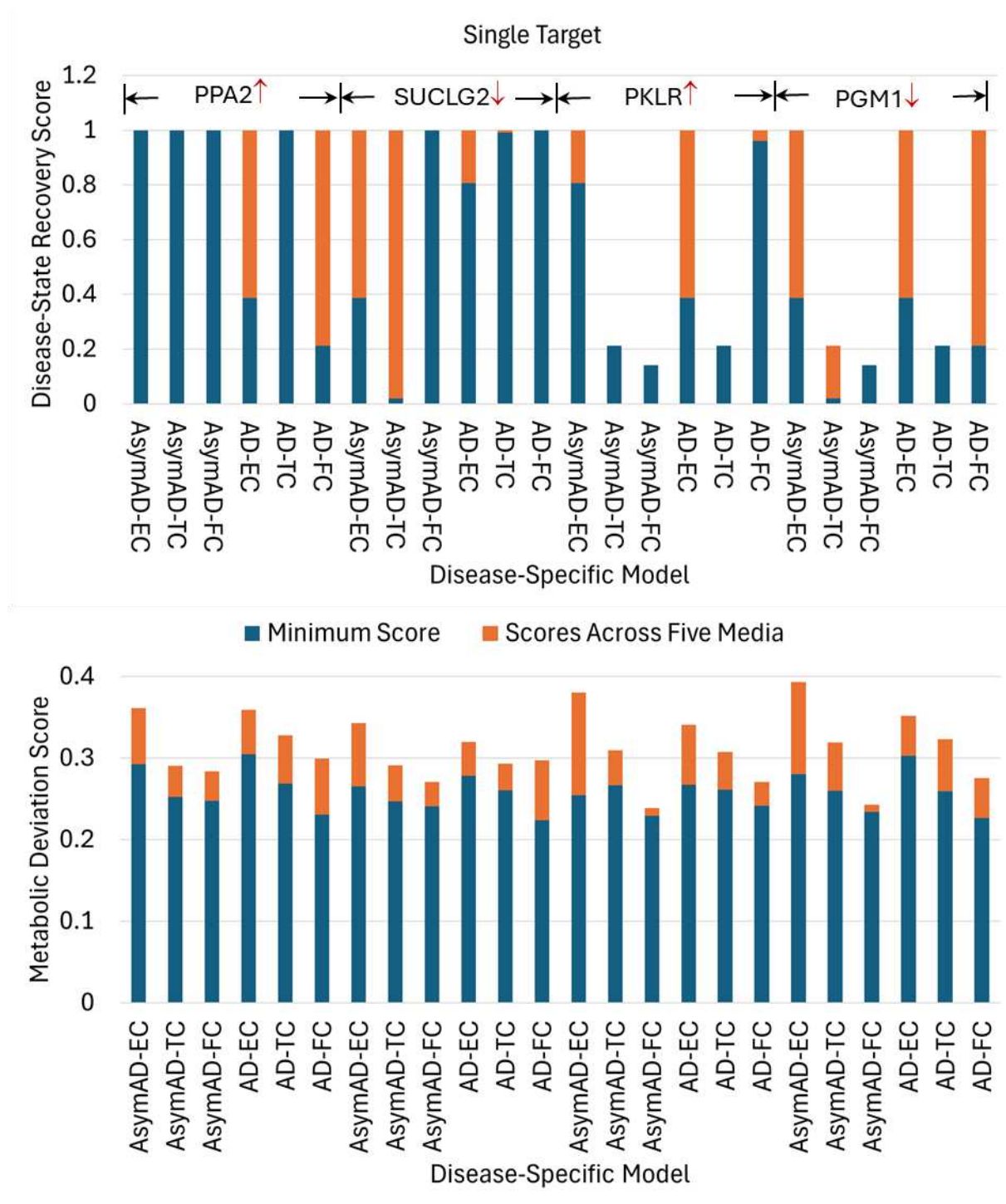


Figure 4. Predicted disease-state recovery and metabolic-deviation scores associated for single-enzyme perturbations across six disease-specific models and five nutrient media. A higher disease-state recovery score indicates a more favorable predicted response of the treated disease-state model, characterized by stronger suppression of tau, A β , IL-6, and TNF- α synthesis while maintaining the prescribed energetic objective. The metabolic-deviation score evaluates global metabolic reprogramming; larger values indicate that the treated disease-state pattern shifts away from the untreated disease-state (DS) template while the perturbed HT pattern remains closer to its healthy reference. Blue bars indicate the minimum score across the five nutrient media, and orange

segments indicate the range from the minimum to the maximum score; a short orange segment indicates consistent effects across media. Symbols $\uparrow$ and $\downarrow$ denote enzyme upregulation and downregulation, respectively.

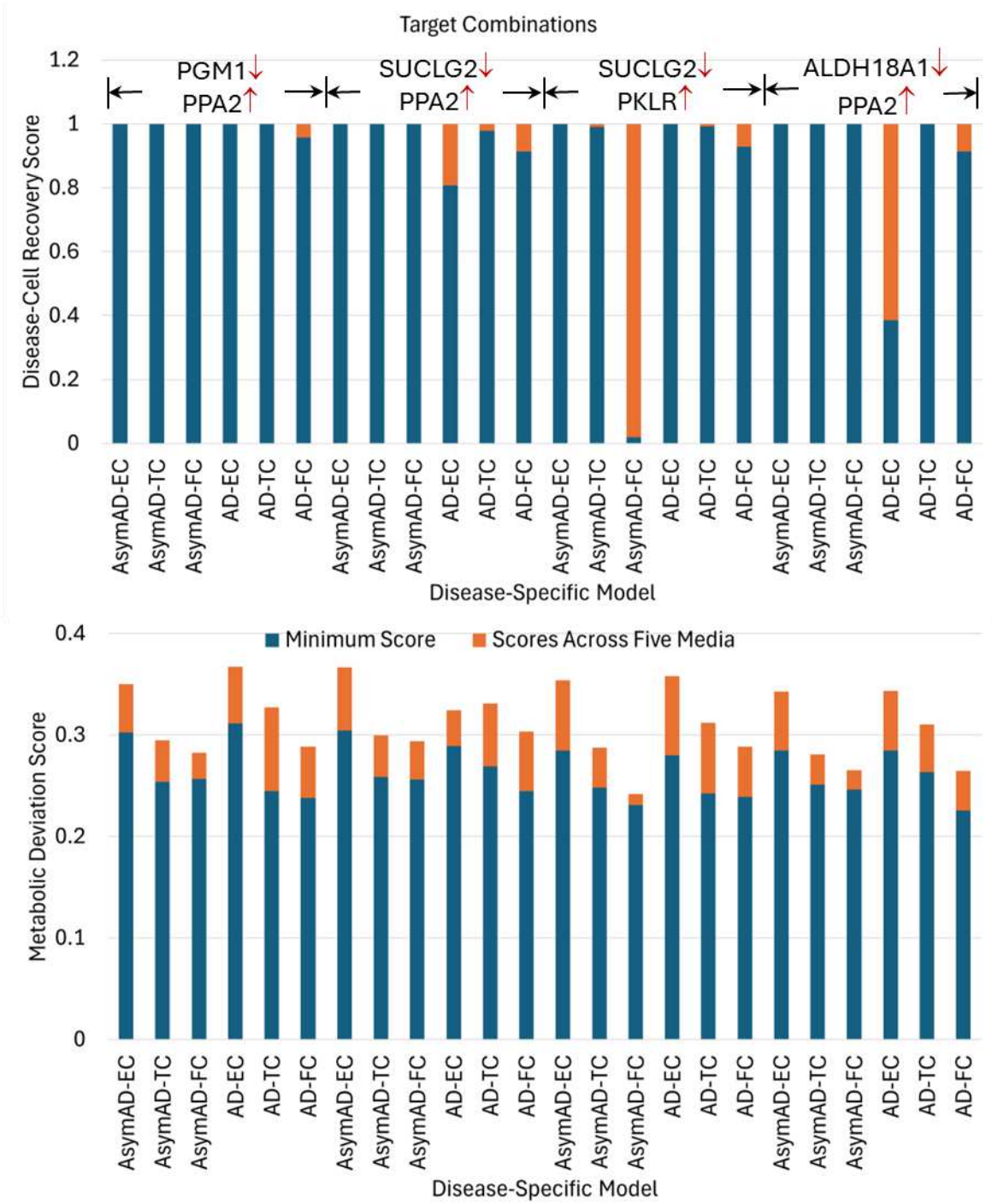


Figure 5. Predicted disease-state recovery and metabolic-deviation scores for two-target combinations across six disease-specific models and five nutrient media. Blue bars indicate the minimum score across the five nutrient media, and orange segments indicate the range from the

minimum to the maximum score. Symbols $\uparrow$ and $\downarrow$ denote enzyme upregulation and downregulation, respectively.

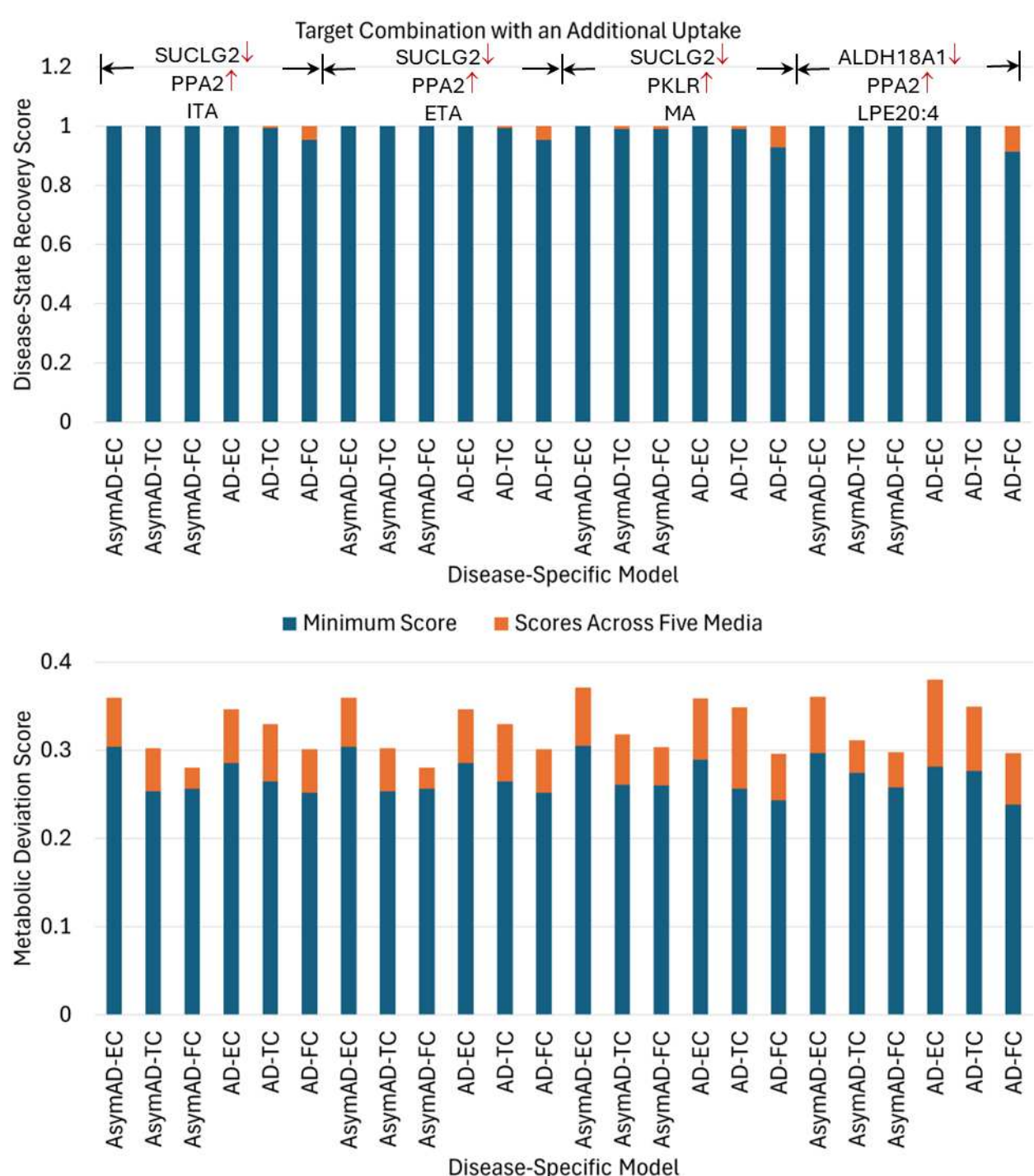


Figure 6. Predicted disease-cell recovery and metabolic-deviation scores for two-target combinations with an additional metabolite uptake across six disease-specific models and five nutrient media. Blue bars indicate the minimum score across the five nutrient media, and orange segments indicate the range from the minimum to the maximum score. Symbols $\uparrow$ and $\downarrow$ denote target

upregulation and downregulation, respectively. Uptake interventions: ITA, itaconic acid; ETA, omega-3 eicosatetraenoic acid (20:4n-3); MA, Mead acid (5Z,8Z,11Z-eicosatrienoic acid; 20:3n-9); LPE20:4, 1-arachidonoyl-sn-glycero-3-phosphoethanolamine.

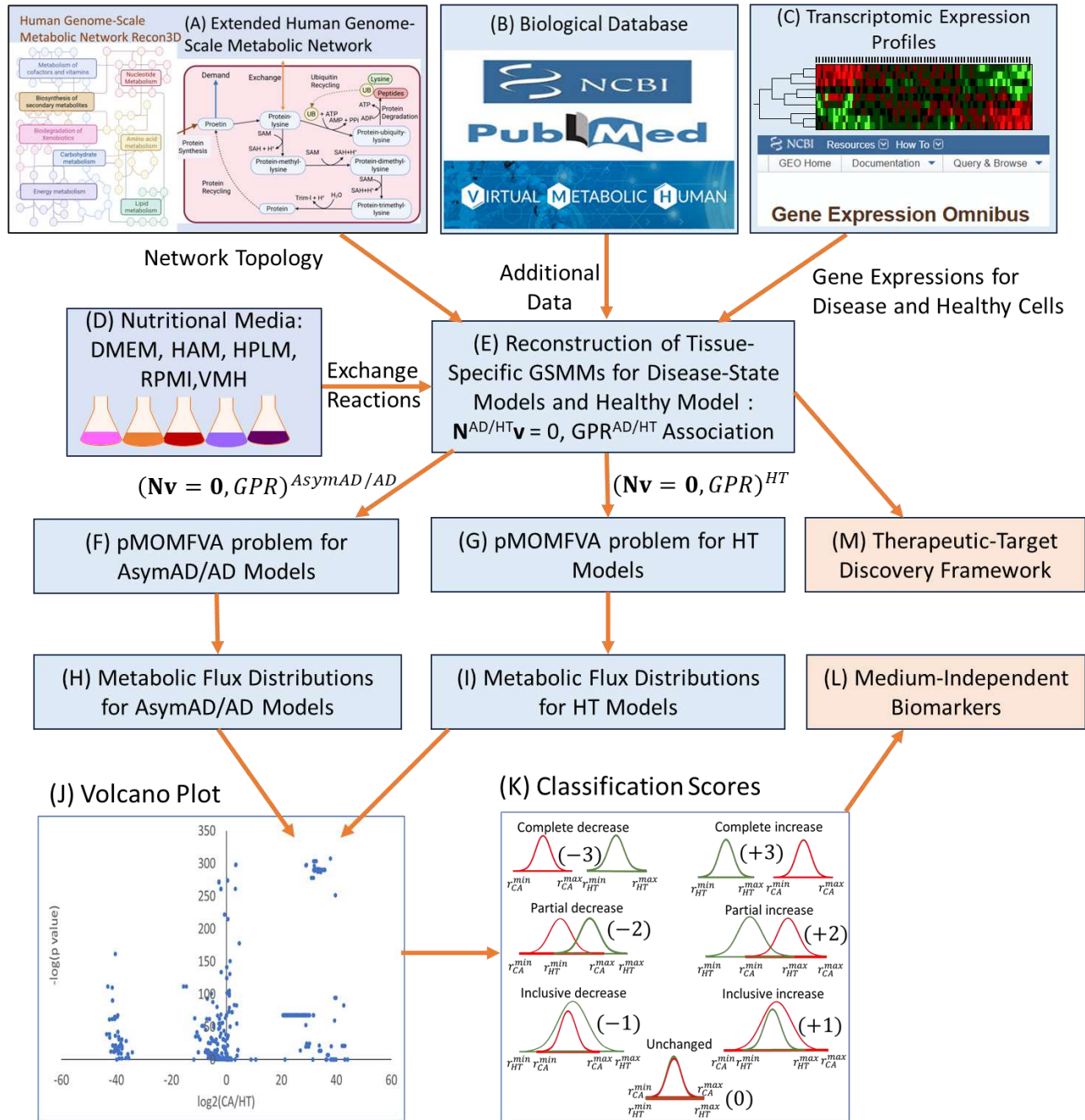


Figure 7. Workflow for identifying medium-robust metabolic biomarkers and therapeutic targets in Alzheimer's disease. (A) An extended human genome-scale metabolic network, constructed by augmenting Recon3D with reactions for the synthesis, degradation, and recycling of AD-associated proteins and inflammatory mediators, serves as the topological template for tissue-specific model reconstruction. (B) Biological databases provide complementary context-specific information on nutrient availability, metabolic pathways, and molecular functions. (C)

Transcriptomic profiles from AsymAD, symptomatic AD, and corresponding healthy tissue (HT) across brain regions are integrated with the extended network. (D) Five nutritional media are applied to evaluate the robustness of predicted alterations to nutrient availability. (E) Tissue-specific GSMMs and their GPR relationships are reconstructed for AsymAD, AD, and HT states. (F) pMOMFVA is formulated for each AsymAD and AD model to determine feasible ranges of metabolic fluxes and metabolite production-flow rates. (G) The corresponding problems are formulated for the matched HT models. (H, I) Flux and production-flow distributions are obtained for the AsymAD/AD and HT models, respectively. (J) For each metabolite or exchange flux, the log2 ratio between the mean values predicted for the disease and HT models, with the corresponding p value, quantifies the magnitude and statistical significance of alterations. (K) Feasible ranges in the disease-state and HT models are compared and classified into seven categories according to their relative positions and overlap; an ordinal score is assigned to each category. (L) Candidate medium-robust biomarkers are identified from classification scores that remain consistently altered across the five media. (M) The reconstructed GSMMs and GPR relationships are incorporated into the therapeutic-target discovery framework to identify and prioritize candidate metabolic interventions.

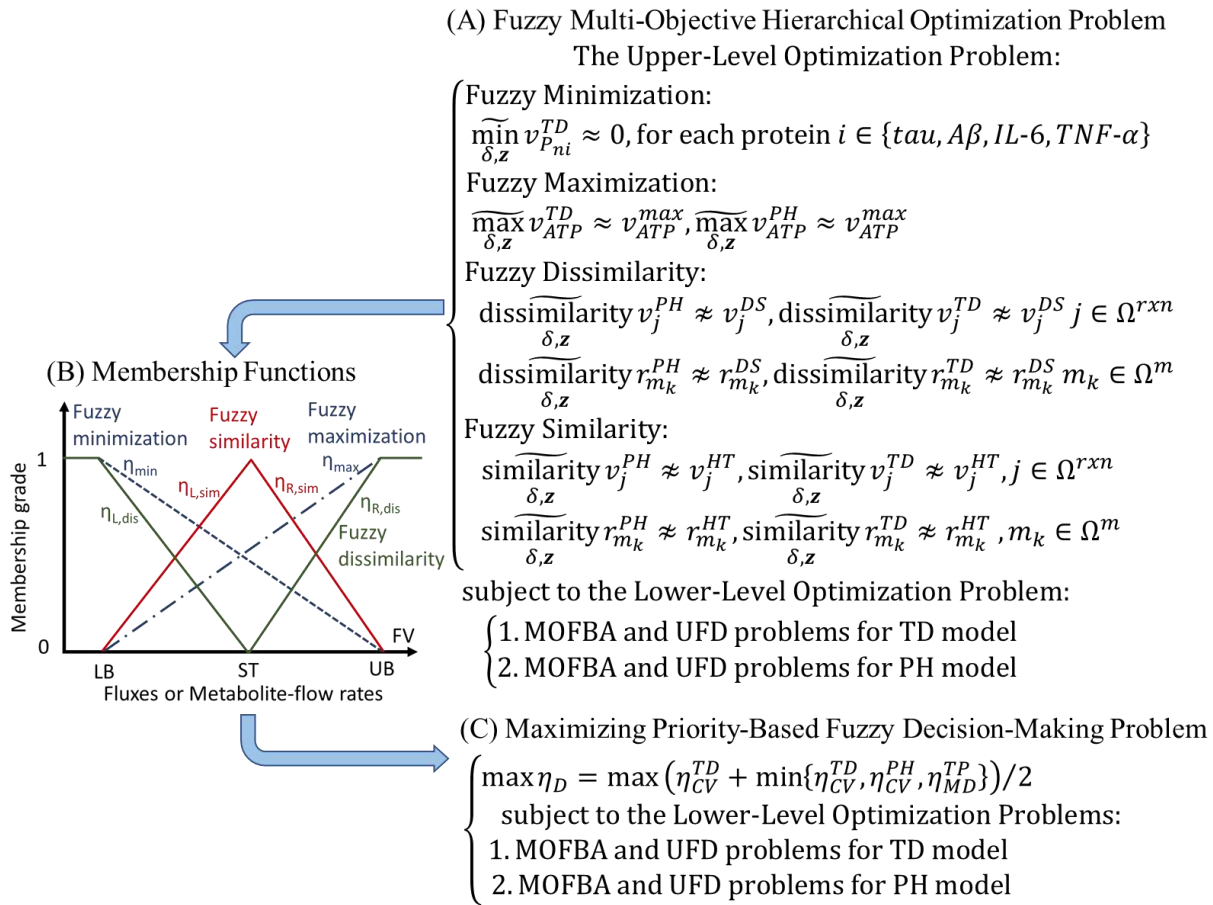


Figure 8. Transformation of a fuzzy multi-objective hierarchical therapeutic-target discovery problem into a priority-based maximizing fuzzy decision problem using fuzzy membership

functions. (A) Fuzzy multi-objective hierarchical optimization framework incorporating fuzzy minimization, maximization, similarity, and dissimilarity objectives for evaluating candidate interventions. (B) Fuzzy membership functions transform heterogeneous objective values into normalized satisfaction grades from 0 to 1. The lower bound (LB), upper bound (UB), and standard value (ST) are predefined reference parameters obtained from available experimental or clinical data or, when such data are unavailable, estimated from metabolic templates derived from the disease-state (DS) and healthy-tissue (HT) models. One-sided linear membership functions are used for fuzzy minimization and maximization objectives (dashed and dot-dashed lines, respectively); two-sided linear membership functions are used for fuzzy similarity and dissimilarity objectives (red and green lines, respectively). (C) The resulting membership grades are integrated into a priority-based maximizing fuzzy decision problem that simultaneously accounts for recovery of the disease-state metabolic phenotype toward the healthy state, preservation of healthy-tissue metabolic function, and treatment-induced metabolic deviation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryNote1.pdf](#)
- [SupplementaryData1.xlsx](#)
- [SupplementaryTable1.xlsx](#)