

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL

GENOME-SCALE METABOLIC MODELING OF
***DROSOPHILA MELANOGASTER* TO UNLOCK**
PARKINSON'S DISEASE-RELATED METABOLIC PROCESSES

MÜBERRA FATMA CESUR

A THESIS OF DOCTORATE
BIOENGINEERING DEPARTMENT
BIOINFORMATICS AND SYSTEMS BIOLOGY PROGRAM

ADVISOR: PROF. DR. TUNAHAN ÇAKIR

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**PARKİNSON HASTALIĞIYLA İLİŞKİLİ METABOLİK SÜREÇLERİN
AYDINLATILMASI İÇİN *DROSOPHILA MELANOGASTER*'İN GENOM
ÖLÇEKLİ METABOLİK MODELLENMESİ**

MÜBERRA FATMA CESUR

DOKTORA TEZİ
BİYOMÜHENDİSLİK ANABİLİM DALI
BİYOİNFORMATİK VE SİSTEM BİYOLOJİSİ
PROGRAMI

DANIŞMAN: PROF. DR. TUNAHAN ÇAKIR
2. DANIŞMAN: PROF. DR. KİRAN RAOSAHEB PATİL

TEMMUZ 2024

DOCTORATE JURY APPROVAL FORM

A thesis submitted by Müberra Fatma CESUR, defended on 24/07/2024 before the jury formed with the 04/07/2024 date and 2024/34 numbered decision of the GTU Graduate Administration Board, has been accepted as a DOCTOR of PHILOSOPHY thesis in the Department of Bioengineering, Bioinformatics and Systems Biology Program.

JURY

MEMBER

(THESIS ADVISOR) : Prof. Dr. Tunahan ÇAKIR

MEMBER

: Assist. Prof. Dr. Pınar PİR

MEMBER

: Assoc. Prof. Dr. Emrah NIKEREL

MEMBER

: Prof. Dr. Işıl KURNAZ

MEMBER

: Assoc. Prof. Dr. Ali ÇAKMAK

APPROVAL

Gebze Technical University Graduate Administration Board
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To my beloved family

ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide, induced by various genetic and environmental factors. Over the last decade, there has been a tremendous effort to decipher metabolic processes underlying the progression of PD. Many researchers reported that α -synuclein (α Syn) proteins have a central role in the disease process by inducing the loss of dopaminergic neurons. However, effective treatment strategies have still not been developed. The current approaches mainly aim to alleviate disease symptoms instead of focusing on the early stages of PD. On the other hand, holistic computational approaches can provide a comprehensive insight into the disease metabolism during the progression of PD. In this regard, genome-scale metabolic network models are powerful platforms that harbor whole metabolic information within the organism. Even though transgenic flies are widely utilized to experimentally investigate the PD process, to our knowledge, this thesis is the first attempt to analyze PD metabolism in *Drosophila* from different perspectives through genome-scale metabolic modeling. Here, a genome-scale metabolic network model was first reconstructed for *Drosophila* called iDrosophila1, because such resources are very limited in the literature for this organism. The predictive performance of this model was shown to be better than the available generic fly models in the literature. Then, this metabolic model was used in three different applications associated with PD. In the first iDrosophila1-guided study, PD-related vitamins and their impact on the disease metabolism were revealed, highlighting the potential of these vitamins as an effective adjuvant treatment for PD. In the second metabolic network-based study, the possible metabolic processes associated with the onset of PD were investigated. Accordingly, α Syn-induced metabolic regulations and crosstalk in a community of the enteroendocrine cell (a special midgut cell with various neural characteristics) and gastrointestinal bacteria were characterized to elaborate on the existing hypothesis about the onset of PD. In the last study, the iDrosophila1 model was used for the neurotoxicological assessment of a man-made industrial chemical, GenX. The comparison of this chemical's metabolic effect with the α Syn-induced metabolic regulations showed its possible harmful impact on the brain. As a result, the preliminary findings obtained from this thesis contribute to the literature on PD in different aspects.

Keywords: Parkinson's Disease, Genome-Scale Metabolic Modeling, Transcriptome, Vitamin Metabolism, Enteroendocrine Cell-Microbiota Interactions, GenX Toxicity.

ÖZET

Parkinson hastalığı (PH), çeşitli genetik ve çevresel faktörlerin neden olduğu, dünya çapında en yaygın ikinci nörodejeneratif hastalıktır. PH'nin ilerlemesinin altında yatan metabolik süreçleri çözmek, yıllardır sürmekte olan araştırmaların konusudur. Birçok araştırmacı, α -sinüklein (α Sin) proteinlerinin dopaminerjik nöronların kaybına neden olarak hastalık sürecinde merkezi bir role sahip olduğunu raporlamıştır. Ancak etkili tedavi stratejileri henüz geliştirilememiştir. Mevcut yaklaşımlar, temel olarak PH'nin erken evrelerine odaklanmak yerine hastalık semptomlarını hafifletmeyi amaçlamaktadır. Diğer yandan, bütünsel hesaplamalı yaklaşımlar hastalık metabolizmasına dair kapsamlı bir bakış açısı sağlayabilir. Bu bakımdan genom-ölçekli metabolik ağ modelleri, organizmadaki tüm metabolik bilgiyi barındıran güçlü platformlardır. Her ne kadar transgenik sinekler PH sürecini deneysel olarak araştırmak için yaygın olarak kullanılsa da bilgimiz dahilinde bu tez, *Drosophila*'daki PH metabolizmasını genom-ölçekli metabolik modelleme yoluyla farklı perspektiflerden analiz etmeye yönelik ilk girişimdir. Burada ilk olarak *Drosophila* için iDrosophila1 adı verilen genom-ölçekli bir metabolik ağ modeli geliştirilmiştir. Çünkü bu organizma için literatürde bu tür kaynaklar çok sınırlıdır. Bu modelin tahmin performansının literatürdeki mevcut sinek metabolik modellerinden daha iyi olduğu gösterilmiştir. Ardından, bu metabolik model, PH ile ilişkili üç farklı uygulamada kullanılmıştır. İlk iDrosophila1 odaklı çalışmada PH ile ilişkili vitaminler ve bu vitaminlerin hastalık metabolizması üzerindeki etkisi ortaya çıkartılmış ve bu vitaminlerin PH için etkili bir adjuvan tedavi olma potansiyeline vurgu yapılmıştır. İkinci metabolik ağ temelli çalışmada PH'nin başlangıcına ilişkin olası metabolik süreçlere odaklanılmıştır. Buna göre, PH'nin başlangıcı hakkındaki mevcut hipotezi detaylandırmak için enteroendokrin hücre (çeşitli sinirsel özelliklere sahip özel bir orta bağırsak hücresi) ve gastrointestinal bakterilerden oluşan bir toplulukta α Sin kaynaklı metabolik düzenlemeler ve etkileşimler karakterize edilmiştir. Son çalışmada ise, insanlar tarafından oluşturulan endüstriyel bir kimyasal olan GenX'in nörotoksikolojik değerlendirmesi için iDrosophila1 modeli kullanılmıştır. Bu kimyasalın metabolik etkisinin α Sin kaynaklı metabolik regülasyonlarla karşılaştırılması ile beyin üzerindeki olası zararlı etkileri ortaya konulmuştur. Sonuç olarak, bu tezden elde edilen ön bulgular, PH'a dair farklı yönlerden literatüre katkı sağlamaktadır.

Anahtar Kelimeler: Parkinson Hastalığı, Genom Ölçekli Metabolik Modelleme, Transkriptom, Vitamin Metabolizması, Enteroendokrin Hücre-Microbiyota Etkileşimleri, GenX Toksisitesi.

ACKNOWLEDGEMENTS

I would like to express my first sincere gratitude to my supervisors, Prof. Dr. Tunahan Çakır and Prof. Dr. Kiran Raosaheb Patil for their never-ending patience, excellent guidance, and constant encouragement. I feel very lucky to have an opportunity to work with them throughout my PhD journey. I am also very grateful to Dr. Arianna Basile and Francisco Zorrilla at Patil Group for their guiding advice and comments, particularly on community metabolic modeling. In addition, I would like to thank Assist. Prof. Dr. Souvarish Sarkar and his Research Group (Sarkar Lab) for their kind collaboration in the last section of the thesis.

My other special thanks to Assoc. Prof. Dr. Aleksej Zelezniak for accepting me as a Visiting Researcher at Chalmers University of Technology and for his valuable comments on our research. I would also like to thank Zelezniak Lab for providing me with a very friendly, respectful, and scientific working atmosphere. Additionally, I appreciate the nice conversations of Dr. Gizem Erensoy and Danish Anwer at the same university. I would also like to express my gratitude to the Turkish people in Sweden, especially Nuran Küçükdoğru, Hacı Sak, and Adem Can who did not spare their help even in the smallest difficulties I encountered.

I gratefully acknowledge TUBITAK (2214/A International Research Fellowship) and Chalmers University of Technology for their financial support when I was in Sweden.

I am so grateful to my thesis defense committee; Assist. Prof. Dr. Pınar Pir, Assoc. Prof. Dr. Emrah Nikerel, Prof. Dr. Işıl Kurnaz, and Assoc. Prof. Dr. Ali Çakmak for their recommendations about my thesis work.

I am thankful to all members of the Computational Systems Biology Group at Gebze Technical University for providing me with a friendly working atmosphere and their nice collaborations. I would like to thank especially Regan Odongo and Dilara Uzuner for their beneficial comments and suggestions during the PhD process. I also thank Nagihan Genel for the beautiful times spent together at Gebze Technical University, and my other close friend, Havva Buğda for her moral support in the PhD process.

I would like to sincerely thank my mother Zehra Cesur for all of her unbelievable support and encouragement throughout my life. I am also deeply indebted to other valuable members of my family; my father Ali Cesur, my brother Raşit Cesur, and my sister Elif Cesur for their everlasting patience and support. I would also like to thank the youngest member of the family, my 4-year-old nephew, Zeynep Yusra Cesur, for improving my morale even in the most depressive times. I am also grateful to all my other relatives and friends who have supported me with their prayers. None of this could have been possible without them.

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LIST OF SYMBOLS AND ABBREVIATIONS

α Syn	: α -Synuclein
A β	: Amyloid beta
AD	: Alzheimer's disease
BBB	: Blood-brain barrier
CNS	: Central nervous system
DA	: Dopaminergic
DMEM/F-12	: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
EBC	: Enteroblast cell
EC	: Enzyme Commission
ECC	: Enterocyte cell
EEC	: Enteroendocrine cell
ENS	: Enteric nervous system
ER	: Endoplasmic reticulum
FBA	: Flux balance analysis
FVA	: Flux variability analysis
GMN	: Genome-scale metabolic network
GO	: Gene ontology
GPR	: Gene-protein-reaction rules
GSH	: Glutathione
Gut	: Gastrointestinal tract
Hd	: Holidic diet
HD	: Huntington's disease
HFPO-DA	: Hexafluoropropylene oxide dimer acid
HTT	: Huntingtin
iMAT	: Integrative metabolic analysis tool
ISC	: Intestinal stem cell
<i>LRRK2</i>	: Leucine-rich repeat kinase 2
MFA	: Metabolic flux analysis
MOMA	: Minimization of metabolic adjustment
NGAM	: Non-growth associated maintenance
Non-DA	: Non-dopaminergic
PD	: Parkinson's disease
PFAS	: Per- and polyfluoroalkyl substances
PFOS	: Perfluorooctane sulfonate
<i>pink1</i>	: PTEN-induced novel kinase 1
RMA	: Reporter metabolite analysis
ROS	: Reactive oxygen species
SCFA	: Short-chain fatty acid
SNe	: Substantia nigra pars compacta
TCA	: Tricarboxylic acid cycle

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1. INTRODUCTION

Almost 7-10 million individuals suffer from Parkinson's disease (PD), which is the second most common age-related neurodegenerative disorder worldwide after Alzheimer's disease (AD) (Aryal & Lee, 2019; Nagoshi, 2018). Almost 1% of people over the age of 60 are affected by some motor (e.g., bradykinesia, resting tremor, and postural instability) and non-motor symptoms of this disorder (e.g., sleep disorders, depression, anxiety, impaired sense of smell, pain, and cognitive impairments) (Dung & Thao, 2018; Giguère et al., 2018; Nagoshi, 2018).

PD is a multifactorial disease that can be dramatically affected by age, microbial toxins, gender, and genetic predisposition (Bakshi et al., 2019; Chia et al., 2020). Aging is the greatest risk factor to develop sporadic (also known as idiopathic) PD, particularly characterized by the loss of dopaminergic (DA) neurons in several brain regions (e.g., substantia nigra pars compacta (SNc), ventral tegmental area, and retrorubral area) (Reeve et al., 2014). Given that the aging process leads to significant physiological changes due to the defective cellular systems (e.g., enhanced sensitivity to autophagy, mitochondrial DNA mutations, mitochondrial dysfunction, inflammation, and oxidative stress), a strong relationship was suggested between aging and metabolic dysfunctions (Avanesov et al., 2014; Laye et al., 2015). Available therapeutic strategies are limited for curing PD, whose incidence is increasing day by day. These strategies only support symptomatic relief in patients (Bakshi et al., 2019; Chia et al., 2020). One of the most commonly used PD drugs, levodopa, increases dopamine levels in patients but its long-term use can result in an elevated level of neurotoxic homocysteine and accelerate the progression of PD (Lee et al., 2005; Müller, 2009). Besides, many non-motor symptoms and some motor symptoms were reported to be resistant to the levodopa treatment (Lee & Koh, 2015).

Drosophila melanogaster is a promising model organism to interrogate metabolic alterations in age-related neurodegenerative diseases due to its short generation time, highly tractable genetics, conserved disease genes and metabolic processes with humans, and relatively small brain size (Coquin et al., 2008). Thus, this organism was selected in the current work for the comprehensive metabolic analysis of PD through metabolic modeling. Genome-scale metabolic network (GMN) models representing

all biochemical transformations within an organism or a cell are invaluable platforms to extensively investigate the differential metabolism of disrupted cellular systems. In this regard, a GMN model of *Drosophila* called *iDrosophila1* (8,230 reactions, 6,990 metabolites, and 2,388 genes) was first reconstructed in the scope of this thesis (Cesur et al., 2023). This model was used to analyze PD from three aspects: (i) the importance of vitamin metabolism in PD, (ii) the metabolic interplay between the enteroendocrine cells (EECs) and gastrointestinal tract (gut) microbiota in the PD process, and (iii) the potential neurotoxicity and α -synuclein (α Syn)-like metabolic disruptions induced by a fluorinated organic chemical, GenX.



2. PURPOSE OF THE STUDY

PD is the second most prevalent age-related neurodegenerative disorder without effective therapeutic strategies (Aryal & Lee, 2019; Nagoshi, 2018; Pardo-Moreno et al., 2023). Despite the significant experimental effort to uncover the cellular mechanisms underlying PD, to our knowledge, no metabolic modeling studies have been performed so far to analyze PD metabolism in *Drosophila*. Therefore, the main purpose of this study is to reconstruct a comprehensively curated genome-scale metabolic network model of *Drosophila* and to characterize PD from different aspects using this metabolic model. Thus, it was aimed to provide insight into (i) the therapeutic potentials of the vitamins, (ii) potential metabolic interactions at the onset of PD, and (iii) the potential neurotoxicity of GenX.

2.1. Parkinson's Disease

PD is one of the most common neurodegenerative disorders, which can be triggered by genetic and environmental factors. Familial PD with a positive family history is based on genetic factors, and it has a lower incidence in comparison with sporadic PD (also known as idiopathic PD) (Polymeropoulos et al., 1997). *SNCA* encoding α Syn protein is the first gene identified to be responsible for familial PD. This gene can also increase the risk of sporadic PD (Mizuno et al., 2011; Polymeropoulos et al., 1997). About 5% of all known PD incidences are of familial origin. Sporadic PD is a non-familial PD form that can occur in the absence of any known precise pathologic reasons (Hewitt & Whitworth, 2017; Nagoshi, 2018). Oxidative stress, mitochondrial dysfunction, protein degradation, calcium dysregulation, and inflammation are among the known molecular hallmarks of this disorder (Hewitt & Whitworth, 2017).

The α Syn protein (14 kDa) predominantly localizes in pre-synaptic terminals, and its regulatory activities in membrane trafficking and vesicular dynamics were reported. Some mutations in this protein (e.g., A30P and E46K substitutions) can trigger the formation of Lewy bodies and Lewy neurites through the aggregation of α Syn (Chia et al., 2020; Giguère et al., 2018). Although these cellular inclusions are composed of different molecules (e.g., tubulin, mitochondria, neurofilaments, and ubiquitin), α Syn

is the major component (Giguère et al., 2018). The abnormal aggregations in the DA neurons can trigger several metabolic changes (e.g., mitochondrial dysfunction, and oxidative stress) and lead to neuronal loss via apoptosis or autophagy (Grieco et al., 2019). DA neurons were reported to have a selective vulnerability for PD. This phenomenon has been attributed to several reasons, including an increased oxidative stress in the SNc due to the age-related activation of Fenton reactions, a higher susceptibility of the DA neurons to mitochondrial dysfunctions due to their large bioenergetic requirements, the toxic effect of dopamine in certain conditions, and a higher axonal arborization size of these neurons) (Giguère et al., 2018). On the other hand, PD is not associated with only dysfunctional DA neurons despite their prominent roles in this process (Giguère et al., 2018; Titova et al., 2017). α Syn is known to accumulate first in the lower medulla and the anterior olfactory bundle, and later in the midbrain. Thus, it likely affects non-DA nuclei (Braak et al., 2003). A faster and higher degeneration of distinct non-DA neurons (e.g., cholinergic pedunculopontine nucleus neurons and the neurons in the dorsal motor nucleus of the vagus) was observed in the early stages of PD (Titova et al., 2017). Therefore, the early non-motor symptoms of PD were suggested to be associated with the non-DA neurons. This is consistent with increasing interest in the investigation of different PD-related regions in both the central and peripheral nervous system (Giguère et al., 2018).

Even though α Syn pathology is a common hallmark of PD, many patients without Lewy body pathology have been reported (Nagoshi, 2018). Besides, it is unclear whether this pathology occurs as the cause of this disease or only a result of PD (Dung & Thao, 2018). Since 1997, over 20 genes carrying autosomal dominant mutations (e.g., *LRRK2*, *HtrA2*, and *VPS35*) or recessive mutations (e.g., *parkin*, *pink1*, *DJ-1*, and *ATP122*) have been linked to PD (Aryal & Lee, 2019; Urbizu & Beyer, 2020). Of these genes, *parkin* (a ubiquitin E3 ligase) and *pink1* (PTEN-induced novel kinase 1) are involved in the mitochondrial quality control process (Sekine & Youle, 2018). Mitochondrial fusion and fission are essential for the exchange of cellular components (e.g., respiratory proteins, mitochondrial genome, and various metabolic products) and the selective removal of damaged mitochondria to maintain a healthy cellular metabolism. The fission process leads to reduced mitochondrial membrane potential, and it induces the accumulation of the PINK1 proteins on the outer mitochondrial membrane by preventing their degradation. Autophosphorylation and activation of

PINK1 mediate the recruitment of the Parkin proteins from the cytosol to mitochondria via phospho-ubiquitin signals. These signals also activate the Parkin proteins. The combined action of the Parkin and PINK1 proteins promotes mitophagy-mediated degradation of the damaged mitochondria based on the phosphorylated polyubiquitin signals (Sekine & Youle, 2018) (**Figure 2.1**). Accordingly, these proteins are essential to manage healthy mitochondrial dynamics and cellular energy. PD patients carrying *parkin* mutation with/without Lewy body pathology were reported, suggesting a potential pathological heterogeneity in the familial PD induced by the *parkin* mutations (Gouider-khouja et al., 2003; Johansen et al., 2018; Sasaki et al., 2004; Suda & Mizuno, 1998). To compensate for the effect of *parkin* and *pink1* mutations, one-carbon metabolism-mediated nucleotide biogenesis was shown to be beneficial. This process allowed to handle the loss of DA neurons and impaired motor activity in *Drosophila* (Celardo et al., 2017; Tufi et al., 2014).

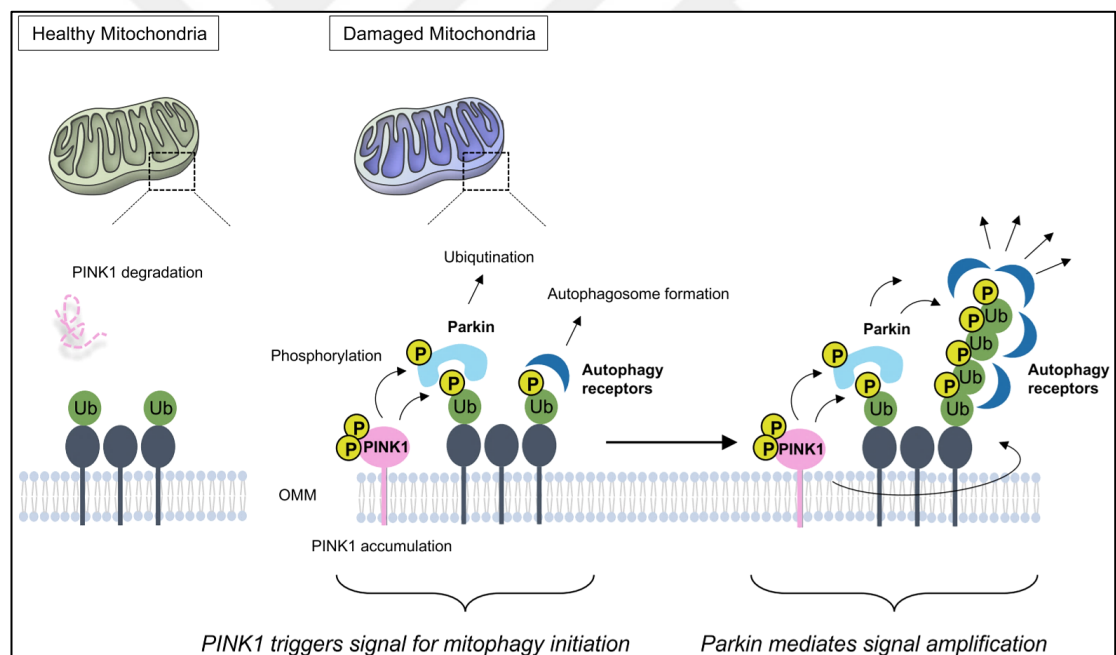


Figure 2.1: Mitochondrial quality control process managed by PINK1 and Parkin proteins (Sekine & Youle, 2018).

Protein deglycase DJ-1 is another protein associated with the familial form of PD, whose mutation can cause early onset of dyskinesia, rigidity, tremors, and psychiatric symptoms (e.g., anxiety and cognitive disorders) (Dolgacheva et al., 2019). Furthermore, the mutant form of another gene, leucine-rich repeat kinase 2 (*LRRK2*) can induce both familial and sporadic PD (Tolosa et al., 2020) because of the function

of the corresponding gene product in microtubule stability, which is essential for the energy-demanding axonal transport processes (Häbig et al., 2013).

2.2. *In vivo Drosophila* Models of Parkinson's Disease

Drosophila is a prominent model organism to get insight into the human metabolism. It includes the counterparts of various crucial human systems or structures including the central nervous system (CNS), gastrointestinal system, kidney (Malpighian tubule in *Drosophila*), liver (oenocytes in *Drosophila*), and adipose tissue (fat body in *Drosophila*) (Capo et al., 2019). A period of 10-14 days is sufficient for the transition from embryo to adulthood, and its average life span is almost 50-60 days (Mizuno et al., 2011). In addition to the short lifespan, a large number of progenies, availability of the whole genome sequence, and substantially less genetic redundancy enable easy and cost-effective use of the advanced genetic tools in this model organism (Mirzoyan et al., 2019; Mizuno et al., 2011). 60% of the *Drosophila* genes were reported to share homology with humans. 75-77% of the disease-associated genes in humans also have functional homologs in flies (e.g., *LRRK2*, *DJ-1*, *parkin*, and *pink1*) and this genetic conservation facilitates the investigation of human disorders in *Drosophila* (Mirzoyan et al., 2019; Pandey & Nichols, 2011; Reiter et al., 2001). Mutant flies can be easily obtained from public stock centers at very affordable costs (Mizuno et al., 2011).

The well-defined nervous system of *Drosophila* with conserved characteristics paves the way for PD research (Dung & Thao, 2018; Nagoshi, 2018; Xiong & Yu, 2018). As aforementioned, death of the DA neurons is one of the most prevalent symptoms of PD. *Drosophila* includes a complex DA neuron system consisting of diverse neuronal clusters. Thus, complicated DA-dependent human behaviors can be mimicked using *in vivo Drosophila* models (Xiong & Yu, 2018). A fly brain includes 21 DA neurons subdivided into seven clusters in each hemisphere at the larval stage (DM1a, DM1b, DM2, DL1a, DL1b, DL2a, and DL2b) while 200 DA neurons are subdivided into nine clusters (PAM, PAL, PPL1, PPL2ab, PPL2c, PPM1, PPM2, PPM3, and VUM) in an adult brain (**Figure 2.2**) (Dung & Thao, 2018; Xiong & Yu, 2018).

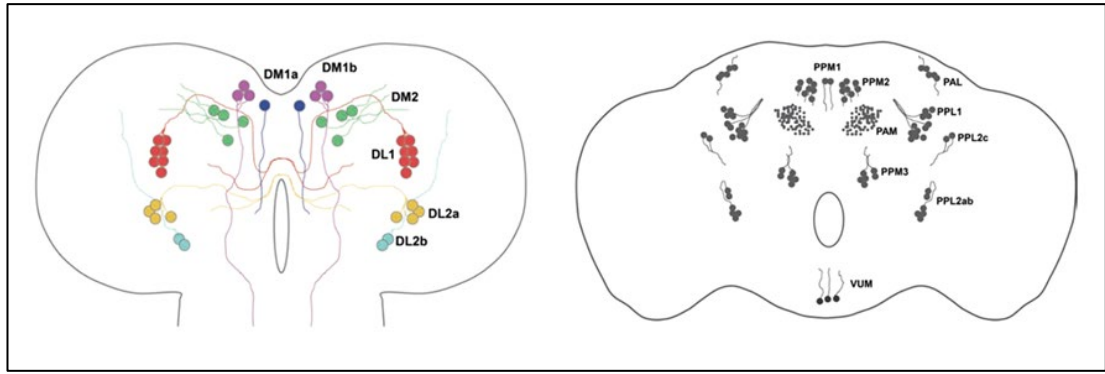


Figure 2.2: The dopaminergic neuron clusters of *Drosophila* at larval and adult stages (Dung & Thao, 2018).

Identification of the DA neurons' locations in the *Drosophila* brain and the development of powerful tools for the targeted transgene expression prompted the researchers to analyze PD-related environmental and genetic factors using fly models (Dung & Thao, 2018; Xiong & Yu, 2018). The PD models of *Drosophila* are commonly used in genome-wide screening experiments for the characterization of disease phenotypes, the investigation of putative drug candidates, and the analysis of PD-related genetic interactions (Xiong & Yu, 2018). To date, the fly models representing various pathologic phenotypes (e.g., locomotion deficiency, mitochondrial dysfunction, oxidative stress, protein aggregation, and DA neuron degeneration) have shed light on the molecular mechanism of this neurodegenerative disorder (**Table 2.1**) (Dung & Thao, 2018; Muñoz-Soriano & Paricio, 2011; Nagoshi, 2018).

PD models can be classified according to the methodology used for establishing these systems: (i) genetically induced (transgenic/knockout) and (ii) chemically induced models. The models induced by the mutant forms of *SNCA*, *pink1*, *parkin*, *DJ-1*, and *LRRK2* can be given as examples of the genetically induced models (**Table 2.1**) (Dung & Thao, 2018; Muñoz-Soriano & Paricio, 2011; Nagoshi, 2018). The chemically induced models can be developed via exposure to toxins such as rotenone and paraquat. These toxins can induce ROS production and oxidative damage through different mechanisms. Rotenone suppresses mitochondrial complex I activity while paraquat causes a changed redox cycling (Nagoshi, 2018). Thus, both toxins can induce PD-like phenotypes (e.g., the loss of DA neurons and movement disorders) (Muñoz-Soriano & Paricio, 2011). Several works about the genetically and chemically induced PD models are represented in **Table 2.1**. Further studies can be found in the

review articles of Dung, et al. (Dung & Thao, 2018), Muñoz-Soriano, et al. (Muñoz-Soriano & Paricio, 2011), and Xiong, et al. (Xiong & Yu, 2018).

Table 2.1: Experimental studies on the PD models of *Drosophila*.

Main function	<i>Drosophila</i> model	PD-like phenotype	Reference
Mitochondrial quality control	<i>parkin</i>	<i>Loss of DA neurons</i> ↑, <i>locomotor activity</i> ↓, <i>mitochondrial defects</i> ↑	(Whitworth et al., 2005)
		<i>Loss of DA neurons</i> ↑, <i>locomotor activity</i> ↓, <i>mitochondrial defects</i> ↑, <i>immune response</i> ↑	(Greene et al., 2005)
		<i>Locomotor activity</i> ↓, <i>cell mass/ size</i> ↓, <i>mitochondrial defects</i> ↑	(Pesah et al., 2004)
	<i>pink1</i>	<i>Loss of DA neurons at 30 days</i> ↑, <i>mitochondrial defects</i> ↑, <i>locomotor activity</i> ↓	(Park et al., 2006)
		<i>Loss of DA neurons</i> ↑, <i>locomotor activity</i> ↓, <i>mitochondrial defects</i> ↑	(Tufi et al., 2014)
Regulation of the membrane trafficking and vesicular dynamics	<i>SNCA</i>	<i>Loss of DA neurons</i> ↑, <i>Lewy body-like aggregations</i> ↑, <i>locomotor activity</i> ↓	(Feany & Bender, 2000)
		<i>Loss of DA neurons</i> ↑, <i>Lewy body-like aggregations</i> ↑	(Periquet et al., 2007)
Regulation of cytoskeletal dynamics	<i>LRRK2</i>	<i>Loss of DA neurons</i> ↑, <i>lifespan</i> ↑, <i>locomotor activity at 10 days</i> ↓ and <i>at 20 days</i> ↑, <i>rotenone sensitivity</i> ↑	(Venderova et al., 2009)
		<i>Loss of DA neurons</i> ↑, <i>locomotor activity</i> ↓	(Liu et al., 2008)

Table 2.1: Continue

Main function	<i>Drosophila</i> model	PD-like phenotype	Reference
Transcriptional regulations and protein folding	<i>DJ-1</i>	<i>Loss of DA neurons</i> ↑, <i>locomotor activity</i> ↓, <i>dysregulation of some synaptic vesicle proteins</i>	(Dung & Thao, 2018)
		<i>Lifespan</i> ↓, <i>locomotor activity</i> ↓, <i>sensitivity to paraquat-induced oxidative stress</i> ↑	(Lavara-culebras & Paricio, 2007)
		<i>Loss of DA neurons</i> ↑, <i>oxidative stress</i> ↑, <i>impaired PI3K/Akt signaling</i> ↑	(Yang et al., 2005)
ROS production and oxidative damage	Rotenone	<i>Loss of DA neurons</i> ↑, <i>locomotor activity</i> ↓	(Coulom & Birman, 2004)
	Paraquat	<i>Mitochondrial defects</i> ↑, <i>lifespan</i> ↓, <i>oxidative stress</i> ↑	(Hosamani, 2010)

2.3. *In silico Drosophila* Models and Computational Methods

Even if *in vivo* fly models have been widely used to characterize the PD process, experimental approaches are costly and time-consuming. On the other hand, metabolic modeling approaches can facilitate the analysis of genome-wide metabolic changes without such drawbacks. A GMN model refers to the mathematical representation of whole metabolic events within a cell or an organism through all known stoichiometry-based chemical reactions, metabolites, and their associated genes. Genes and reactions in a metabolic model are connected via logical associations called gene-protein-reaction rules, GPRs (Gu et al., 2019; Khodaei et al., 2020). Since the development of the first GMN model for *Haemophilus influenzae* in 1999 (Edwards et al., 1999), constraint-based models have been commonly used to gain insight into complicated biological systems. They are especially useful in metabolic engineering and medical fields for the identification of potential drug targets, the detection of putative biomarkers, the prediction of effective gene manipulations for the production of

valuable industrial chemicals, pan-reactome analysis, the investigation of differential metabolism underlying complex human diseases, the modeling of host-pathogen or host-microbiota interactions, and the inference of uncharacterized enzymatic functions (Gu et al., 2019; Nielsen & Keasling, 2016; Oberhardt et al., 2013).

A systems-wide analysis of impaired metabolic pathways in *Drosophila* can increase our understanding of PD. However, to our knowledge, there is no GMN modeling study on PD using the *Drosophila* model in the literature despite the presence of many *in vivo* studies (**Table 2.1**). Four different metabolic network models have been so far developed for *Drosophila* (**Table 2.2**) (Büchel et al., 2013; Feala et al., 2007; Schönborn et al., 2019; Wang et al., 2021). Of these models, BMID000000141998 stored in the BioModels database (Malik-Sheriff et al., 2020) is a computationally generated model in the scope of Path2Models project without any manual curations (Büchel et al., 2013). The remaining models were manually curated, but both of them (the flight muscle model and FlySilico) have very limited metabolic information (Feala et al., 2007; Schönborn et al., 2019).

Table 2.2: The metabolic contents of the previously published *Drosophila* models.

Model	No. of:		
	Reactions	Metabolites	Genes
BMID000000141998	6,198	4,020	2,873
Flight muscle	158	162	184
FlySilico	363	261	293
Fruitfly1	12,056	8,132	2,049

The first manually curated *Drosophila* model (the flight muscle model) includes only core metabolic processes such as glycolysis, tricarboxylic acid (TCA) cycle, and amino acid metabolism (Feala et al., 2007). This model was refined and extended in 2008 via the addition of 38 new reactions and 49 new genes to analyze age-related changes in the hypoxia tolerance of flight muscle (Coquin et al., 2008). Similarly, FlySilico is composed of only core metabolic reactions to simulate larval development (Schönborn et al., 2019). More recently, the Fruitfly1 model was developed using comprehensive metabolic knowledge in the generic human model (Human1) (Robinson et al., 2020), considering gene orthology information from the Alliance of

Genome Resources (The Alliance of Genome Resources Consortium, 2020). In the scope of this thesis, a systems-wide *Drosophila* model, called iDrosophila1 (Cesur et al., 2023) was reconstructed to close the gap in the literature associated with the *Drosophila*-mediated *in silico* PD analyses. In the GMN model reconstruction processes, different approaches can be preferred as summarized in the following subsections. Additionally, general network-based approaches and iDrosophila1-based applications covered by this thesis are discussed in the other subsections.

2.3.1. Automated Metabolic Reconstruction Tools

The reconstruction process of GMN models is based on either conventional or semi-automatic approaches. The conventional model reconstruction process includes the association of organism-specific genomic information and biochemical components in a bottom-up manner. In contrast to this time-consuming approach, semi-automatic methods use software tools and packages to retrieve metabolic information at a rapid pace (Gu et al., 2019; Thiele & Palsson, 2010). The reconstruction of a draft metabolic network is the first step. This network contains metabolic information from the genome annotation and a variety of biochemical databases (e.g., KEGG (Kanehisa & Goto, 2000), BioCyc (Caspi et al., 2016), Transport DB (Ren et al., 2004), Transport Classification Database (Saier et al., 2006), and BRENDA (Schomburg et al., 2002)). To date, many tools have been introduced to build a draft model. Of these bioinformatic tools, AutoKEEGRec generates metabolic models based on the KEGG database without biomass, transport, and exchange reactions. The main advantage of this tool is that it facilitates the reconstruction of multiple models in a single run (Karlsen et al., 2018). Another reconstruction tool, CarveMe, generates the draft model from a BiGG-based manually curated universal template network in a short time using its own gap-filling algorithm (Machado et al., 2018). RAVEN toolbox can be used for both reconstruction and the curation of metabolic networks using template models or metabolic pathway databases (i.e., KEGG and MetaCyc databases) (Wang et al., 2018). AuReMe also uses a template-based algorithm to integrate information from different databases including BiGG, ModelSEED, and MetaCyc. It also allows the visualization of the model content at any time in the reconstruction process (Chevallier et al., 2018). ModelSEED is a beneficial web source for model reconstruction and gap-filling in addition to the metabolic analyses of microbial or plant models (Henry et al.,

2010). In a study, Mendoza and colleagues compared the performances of the most prominent genome-scale network reconstruction tools including AuReMe, CarveMe, RAVEN, and ModelSEED for several microorganisms (*Lactobacillus plantarum*, *Bordetella pertussis*, and *Pseudomonas putida*). Performance-based assessments based on the available manually curated GMN models of these organisms showed that any tools did not outperform each other (Mendoza et al., 2019).

The use of the available tools considerably accelerates the reconstruction processes. However, the draft models may include inaccurate or redundant metabolic information or GPR associations as well as stoichiometrically inconsistent reactions. Therefore, these models should be refined carefully via a manual curation step (Khodae et al., 2020; Thiele & Palsson, 2010). In this step, each network component is evaluated by the researchers via iterative analyses and the use of experimental knowledge from the literature (Gu et al., 2019; Khodae et al., 2020; Mendoza et al., 2019). To accelerate the curation step, automatized approaches can be also preferred. In addition, different approaches (e.g., MEMOTE (Lieven et al., 2020) and FROG analysis (Sheriff, 2024)) can be used for the quality assessment of the reconstructed model in an organism-independent manner according to a variety of consensus tests such as annotation test, biomass consistency test, stoichiometry consistency test, and the assessment of model reproducibility.

After the manual/semi-automatic model curation steps, the predictive capacity of the models has been often assessed using organism-specific experimental datasets under certain growth conditions (e.g., phenotype microarray and gene knockout experiments) (Mendoza et al., 2019; Thiele & Palsson, 2010). Different types of omics data (e.g., metabolome and fluxome) can be also used for the curation and validation of the metabolic networks (Mendoza et al., 2019). For instance, ^{13}C metabolic flux analysis (^{13}C -MFA) enables the identification of reaction fluxes (Kim & Lun, 2014). The experimentally measured fluxes are then used as the additional model constraints to accurately predict the flux distributions of model reactions in the defined growth condition. Additionally, the experimentally detected fluxes can allow the assessment of model performance through the comparison of the predicted and measured fluxes (Cheah & Young, 2018; Volkova et al., 2020). It should be noted that the datasets of the phylogenetic neighbors can be also preferred for the model validations in the absence of organism-specific information (Thiele & Palsson, 2010).

2.3.2. Metabolic Reconstructions of Model Organisms

Model organisms have been commonly used for *in vivo* mimicking and analysis of disease phenotypes in humans. Additionally, *in silico* models of such organisms have been reconstructed since 2003. *Saccharomyces cerevisiae* has been the first eukaryotic model organism whose GMN model was reconstructed (Famili et al., 2003). For higher eukaryotic systems, orthology-based reconstruction approaches are especially intriguing. The orthology-based inference of the GMN models is based on the use of metabolic knowledge from the metabolic network of another organism, which has a high degree of genomic homology. The genes and the related metabolic knowledge in this reference network are replaced by the orthologous counterparts in the target organism (Khodae et al., 2020). Thus, only reaction sets including orthologous genes are kept in the final metabolic model. The manual curation and validation processes highlighted in the previous section are also applied to the orthology-based draft networks.

Mus musculus, *Rattus norvegicus*, and *D. melanogaster* are among the most used animals to mimic human-related metabolic processes, develop new therapeutical strategies, and explore novel biomarkers. These organisms were previously modeled through the orthology-based reconstruction approaches based on the template human models (Blais et al., 2017; Khodae et al., 2020; Sigurdsson et al., 2010; Wang et al., 2021). For instance, Sigurdsson and colleagues developed the draft metabolic reconstructions of five mammals including *M. musculus* from the human metabolic network, Recon 1 (Duarte et al., 2007). Their approach is based on the transfer of metabolic information after the identification of the gene orthologs in the Recon 1 model. They decided on the orthologous gene sets using the HomoloGene (NCBI) database. Subsequently, the validation tests defined for Recon 1 and manual curations were applied for the gap-filled draft mouse model, ultimately called iMM1415, with 2,212 enzymatic reactions and 1,514 non-gene-associated reactions (Sigurdsson et al., 2010). Brunk et al. also reconstructed a GMN model of *M. musculus* by mapping the metabolic information in a more recent human model, Recon3D (Brunk et al., 2018), based on the orthology information from the literature and several databases. Another study also reconstructed an orthology-based GMN model for *M. musculus* (Khodae et al., 2020). Following a similar approach, Blais and colleagues reconstructed the first genome-scale network reconstruction for *R. norvegicus* (called iRno) to investigate the

potential use of rats in biomarker detection studies. To this aim, orthologous genes between humans and rats were identified using five different databases (the Rat Genome Database (Shimoyama et al., 2015), Ensembl (Yates et al., 2020), KEGG, Uniprot (The Uniprot Consortium, 2017), and Homologene), and scored according to the number of the databases supporting the orthology information. After manual curation steps, the final iRno model including 2,324 genes, 8,268 reactions, and 5,620 metabolites was obtained (Blais et al., 2017). Later, Wang and colleagues reconstructed orthology-based GMN models for five major model animals containing Mouse1 (*M. musculus*), Rat1 (*R. norvegicus*), Zebrafish1 (*Danio rerio*), Fruitfly1 (*D. melanogaster*), and Worm1 (*Caenorhabditis elegans*) (Wang et al., 2021). These models have been derived from the generic human GMN model called Human1 (Robinson et al., 2020) using the gene orthology information in the Alliance of Genome Resources (The Alliance of Genome Resources Consortium, 2020). They then investigated the metabolic alterations in the reconstructed AD mouse models (Robinson et al., 2020).

To conclude, the orthology-based model reconstruction process facilitates the effective use of the conserved metabolic information among the organisms. To date, the metabolic information in different human models has been transferred to the new networks of various model organisms. Revising (gap-filling and manual curations) and confirming steps of the metabolic information, as detailed in the previous section, improved the accuracy of these orthology-based models. It should be noted that organism-specific metabolic knowledge has been often included in such models to expand the metabolic coverage of the networks.

2.3.3. Applications of Metabolic Network Models

The reconstructed GMN models have been widely used to infer condition-specific metabolic alterations within the organism of interest. Two common approaches can be used to obtain condition-specific metabolic networks (Gu et al., 2019). The first approach relies on setting the growth condition to a laboratory medium or host-mimicking medium to simulate the metabolic behavior of an organism in a specific growth condition. To this aim, the boundaries of the medium components are set to the maximum uptake rates while the intakes of other exchange metabolites in the model are not allowed (Cesur et al., 2020). In this way, host- or laboratory-specific

environments can be designed to mimic *in vitro* or *in vivo* growth conditions. The second approach is the integration of omics datasets (e.g., transcriptome and proteome) to metabolic models in order to obtain contextualized reduced networks (Gu et al., 2019). In this approach, omics datasets provide information about the reaction activities in the conditions of interest. Transcriptome datasets have been predominantly used in this approach due to their higher availability, compared to metabolome and proteome datasets.

To date, many different methods have been developed for the integration of transcriptome datasets onto metabolic networks (Agren et al., 2012; Becker & Palsson, 2008; Colijn et al., 2009; Simeonidis et al., 2013; Wang et al., 2012; Zur et al., 2010). Of these methods, iMAT (Integrative Metabolic Analysis Tool) algorithm, uses two different threshold values to classify the expression data as discrete tri-valued states including low, moderate, and high. In this way, it aims to minimize the number of reactions with lowly-expressed genes retained in the model (Kim & Lun, 2014; Zur et al., 2010). Many more transcriptome integration methods are available in the literature (Kim & Lun, 2014; Machado & Herrgård, 2014), and the selection of the proper method enables correct predictions about the metabolic systems. For instance, the iMAT algorithm has been widely used for higher eukaryotic systems due to the lack of a consensus in the literature about the biological objective function of these organisms.

The condition-specific metabolic models can be then utilized to compare the reaction activities across different conditions. In this regard, binarized matrices are first created based on the active and inactive reactions in the models (represented by “1” and “0”, respectively). Fisher’s exact test or other similar methods are subsequently used to reveal the significantly differential (upregulated or downregulated) reactions associated with the imposed environmental changes or disease conditions, as detailed in (Lüleci et al., 2023). The condition-specific metabolic models can be also used to determine metabolic changes induced by genetic mutations. In this regard, the MOMA (minimization of metabolic adjustment) approach focusing on the minimization of the Euclidean distance between the wild-type and mutant flux distributions is often used for phenotypic predictions using quadratic programming (Carter et al., 2024; Segrè et al., 2002). In addition to the reconstruction and analysis of the condition-specific metabolic models, fold change-based methods such as Δ FBA (Ravi & Gunawan,

2021) are also beneficial to predict condition-specific metabolic changes through the combinatory use of constraint-based generic models and differential expression profiles. Thus, they support the elucidation of differential metabolic processes in response to environmental adaptations or disrupted cellular systems without a need for specifying a biological objective function. Differential expression profiles can be also used along with network topology information to predict the reporter metabolites around which the most significant gene expression changes occur (Çakir, 2015; Patil & Nielsen, 2005). These metabolites may be promising as potential biomarkers or dietary supplements.

Another application of the metabolic network models includes the simulation of metabolic interactions between organisms. This strategy is based on the modeling of reciprocal interactions considering the abundance, metabolic exchanges, and nutrient requirements of each member within the community (Baldini et al., 2019). It can be used for the analysis of microbial adaptation mechanisms or their interactions with the host systems. To date, several gut microbiota models such as semiautomatic AGORA reconstruction (Magnúsdóttir et al., 2017), its expanded version AGORA2 (Heinken et al., 2020), and *Drosophila*-specific microbial reconstructions (Ankrah et al., 2021a; Schönborn et al., 2021) have been developed as an effort to simulate microbial crosstalk. To simulate the microbial interactions at a large scale, numerous algorithms (e.g., the BacArena software package (Bauer et al., 2017), community flux balance analysis (FBA) (Khandelwal et al., 2013), SteadyCom (Chan et al., 2017), and Microbiome Modeling Toolbox 2.0 (Heinken & Thiele, 2022)) have been developed. Importantly, this approach can be also expanded to the simultaneous simulations of host-microbe interactions (Ankrah et al., 2021b; Esvap & Ulgen, 2021). In this process, the metabolic reconstructions of the host and microorganism(s) are connected via a joint compartment (gut lumen) considering the reciprocal exchanges of their metabolites (Baldini et al., 2019; Sen & Orešič, 2019). In such a community model, the relative abundances of the microbial species (i.e., metagenomics data) can be used to generate a microbiota biomass reaction representing the growth of the microbial community (Esvap & Ulgen, 2021). Optimization of the community model can provide significant knowledge about the condition-specific metabolic alterations within the community members and their crosstalk in a specific condition. For instance, Bhalla and colleagues demonstrated the crosstalk between microbial

dysbiosis and colon cancer through the simulations of host-microbe interactions with the Microbiome Modeling Toolbox. They showed that silver nanoparticles-treated *Enterococcus durans* exhibited a cytotoxic effect against the cancer cells using a variety of microbial metabolites (Bhalla et al., 2022). Ternes and colleagues also investigated the impact of another bacterium (*Fusobacterium nucleatum*) on colon cancer, and they showed that this bacterium promoted the development of colon cancer (Ternes et al., 2022). Together, community model-based analyses are remarkable in characterizing the disease-related metabolic interplay between the host and microbial cells.

2.3.4. iDrosophila1 Model-Based Current Applications

The orthology-based generic *Drosophila* model reconstructed in the scope of the current thesis, iDrosophila1 (Cesur et al., 2023), was used to uncover neurodegeneration-associated metabolic processes in the following three studies. To our knowledge, this is the first attempt to use a GMN model of *Drosophila* in PD-related research.

- Investigating the Potentials of Vitamins as Dietary Supplements

Vitamins are bioactive substances that assist many cellular processes and act as natural antioxidants. There are two primary groups of vitamins: fat-soluble vitamins (A, D, E, and K) and water-soluble vitamins (B and C), almost all of which are essentially obtained via diet in humans (Zhao et al., 2019). In the past few decades, a growing interest has been seen in the use of vitamin-rich functional foods or supplements in conjunction with medication therapy (Ciulla et al., 2019; Zhao et al., 2019). Accordingly, the size of the worldwide market for dietary supplements was estimated at \$133 billion in 2016 and it is expected to reach \$300 billion by 2028 (Ciulla et al., 2019; Djaoudene et al., 2023). Vitamin-rich dietary supplements are promising in supporting health by either preventing or improving age-related diseases such as Huntington's disease (HD), PD, and AD (Ciulla et al., 2019; Zhao et al., 2019). It has been shown that vitamin D₃, a well-known nutraceutical vitamin, regulates the overproduction of oxidized macromolecules and redox imbalance. As a result, PD, AD, and HD were linked to low serum levels of this vitamin, which led to cognitive and motor deficits (SKV et al., 2024). Moreover, its deficiency lowers dopamine levels and promotes α Syn proteins to accumulate in the PD process. Similarly, deficiencies

in vitamins A, B₁, and B₁₂ were previously connected to the accumulation of amyloid beta (A β) proteins in AD (Kumar et al., 2022). Consistently, Lam and colleagues determined that feeding the A β -expressing *Caenorhabditis* with dietary vitamin B₁₂ decreased bioenergetic deficits and oxidative stress by controlling the methionine/S-adenosylmethionine cycle (Lam et al., 2021). Furthermore, vitamin B₅ and C deficiencies were found to be associated with metabolic disturbances in HD, including increased oxidative stress and defective synthesis of certain hormones (Kumar et al., 2022). Thus, based on the relationship of vitamins with neurodegeneration, their appropriate supplementation may slow the progression of these disorders and help the improvement of disease symptoms. In this regard, a genome-wide examination of differential vitamin metabolism and corresponding pathways in the *Drosophila* brain expressing human α Syn was the main focus of this part of the thesis. To this end, PD-related vitamins were first identified using the iDrosophila1 model, and their effects on brain metabolism in the PD process were investigated in our network-based study.

- Characterizing α Syn-Induced Differential Metabolism in the EEC-Microbiota Communities

Drosophila gut consists of three main parts (foregut, midgut, and hindgut) including conserved functions with the human counterparts (Bonfini et al., 2016; Capo et al., 2019; Lemaitre & Miguel-Aliaga, 2013). The foregut with the oral cavity (analogous to the esophagus) and crop (analogous to the stomach) are responsible for the digestion of foods, while the anterior and posterior midguts are functionally analogous to the human small intestine (Chiang et al., 2022; Douglas, 2018; Kuraishi et al., 2013). The hindgut (analogous to the large intestine) provides a selective absorption of cellular substances (e.g., water, ions, and nutrients), which are released by Malpighian tubules (analogous to the kidney) (Chiang et al., 2022; Douglas, 2018). Similar to humans, the midgut epithelium of *Drosophila* has proliferative intestinal stem cells (ISCs) and their post-mitotic and intermediate differentiating daughter cells (enteroblasts, EBs), absorptive enterocytes (ECs), and secretory EECs surrounded by visceral muscles and nerves (Capo et al., 2019; Kuraishi et al., 2013; Zhai et al., 2017). The microbial content resides in the gut lumen (**Figure 2.3**) (Capo et al., 2019; Kuraishi et al., 2013). Gut microbiota has been proposed to play a prominent role in the misfolding and aggregation of α Syn proteins in enteric nerves at the onset of PD (Hill et al., 2021). According to this hypothesis, the early stages of PD may be associated with an

infection induced by the unproven pathogen(s) or toxin(s), which destroys the integrity of the intestinal mucosal barrier and causes microbial dysbiosis (Dong et al., 2022; Hill et al., 2021). Thus, the gut-brain-axis has been increasingly investigated by researchers using different animal models of PD (Gorecki et al., 2019; Parker-Character et al., 2021; Romano et al., 2021; Xu et al., 2020; Zhang et al., 2020). Holmqvist and colleagues have provided the first evidence for the gut-to-brain transmission of different α Syn forms (monomeric, oligomeric, and fibrillar) through the vagal nerve after the injection of these proteins into the wall of rat gut (Holmqvist et al., 2014). Similarly, Kim et al. demonstrated the reduced propagation of α -synucleinopathy in a mouse model after truncal vagotomy (Kim et al., 2019). More recently, the potential role of intestinal α Syn in dysbiosis and PD pathology was shown through the overexpression of human α Syn proteins in the *Drosophila* midgut. The intestinal α -syn proteins were found to accelerate the progression of PD by inducing oxidative stress (Liu et al., 2022). Taken together, there may be a possible link between microbiota, ENS, and CNS at the onset of PD, and importantly EECs are among the potential members of this circuit.

Even though EECs represent only ~1% of the intestinal cell population in mice and humans, they can produce over 20 types of hormones responsible for significant physiological processes (e.g., lipid metabolism, feeding behavior, and stem cell division). They also exhibit their chemosensory functions by detecting nutrients and microorganisms in the gut lumen and regulating the insulin metabolism of the brain via hormones (Hung et al., 2020; Mulak, 2020). Based on their hormones, these cells are divided into 12 subtypes with regional diversity such as anterior midgut (regions R1 and R2), middle midgut (the gastric region, R3), and posterior midgut (regions R4 and R5) (Guo et al., 2019). In addition to hormone secretion, EECs have several neuron-like characteristics such as the inclusion of axon-like structures (known as neuropods), electrical excitability, the use of neurotrophin receptors, and the synthesis of synaptic proteins (Bohórquez et al., 2014; Chandra et al., 2017; Mulak, 2020). In 2017, α Syn expression in these extraordinary cells was observed by Chandra and colleagues (Chandra et al., 2017), suggesting the transfer of misfolded α Syn proteins produced by EECs from the gut lumen to the enteric nerves in the early stages of PD (Chandra et al., 2017; Hill et al., 2021). In line with this observation, mouse EECs were shown to synapse with vagal neurons, and the interactions of the α Syn-expressing

EECs with the neuronal cells were determined (Chandra et al., 2017; Kaelberer et al., 2018). In addition, *Akkermansia*, whose abundance was previously reported to be increased in PD patients (Baldini et al., 2020), caused mitochondrial calcium overload, increased ROS, and the elevated level of aggregated α Syn proteins in EECs (Dionísio Pedro Amorim Neto et al., 2022).

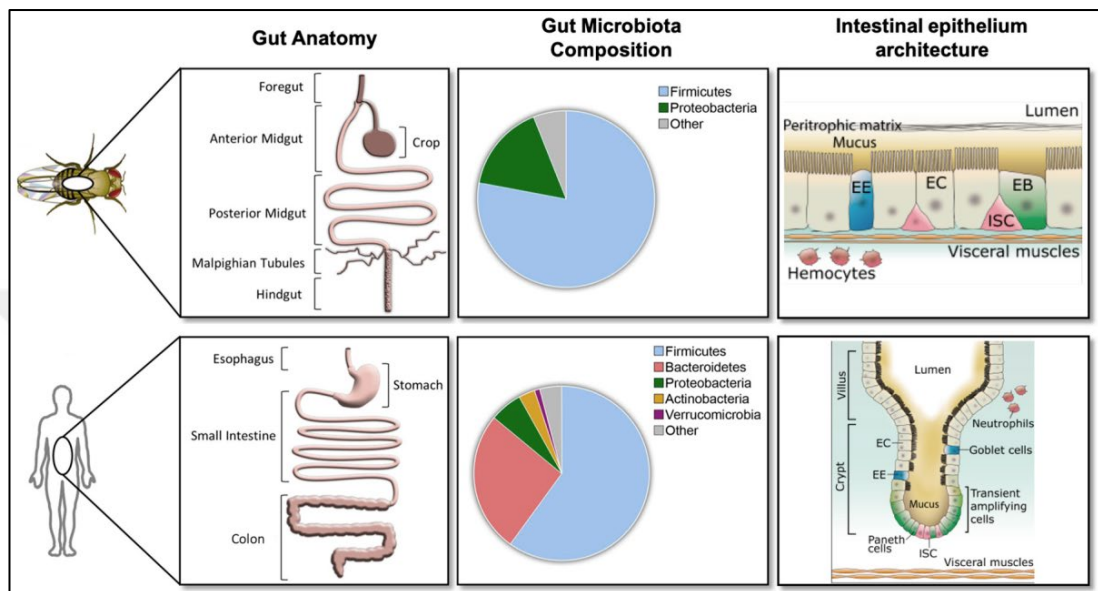


Figure 2.3: *Drosophila* gut including 3 main regions; foregut, midgut, and hindgut. The midgut has conserved epithelial cell types (ISC: intestinal stem cell, EB: enteroblast, EC: enterocyte, and EE: enteroendocrine) (Capo et al., 2019).

Hence, the second iDrosophila1-based study in this thesis focused on the hypothesis assuming that misfolding and aggregation of α Syn proteins in EECs under the influence of various factors (e.g., toxin or pathogen exposure) may be related to the early stages of PD. In this context, the α Syn-induced differential metabolic processes in the EEC-microbiota models were analyzed. It is important to note that the mammalian microbiome is composed of hundreds to thousands of different bacterial species unlike *Drosophila*, which has a simple microbial composition (only 5-20 leading bacterial species) (Bonfini et al., 2016; Dobson et al., 2016; Lee & Lee, 2014). The most abundant microbes in the *Drosophila* gut are *Lactobacilli* (e.g., *L. plantarum* and *L. brevis*) and *Acetobacter* genera, which can be culturable under laboratory conditions. In the current study, the use of *Drosophila* was well-suited to study host-microbiota interactions in a simplified manner.

- Analyzing the Neurotoxic Effect of GenX on the *Drosophila* Brain

A man-made industrial chemical, hexafluoropropylene oxide dimer acid (HFPO-DA; also known as GenX), has been marketed as a replacement for perfluorooctanoic acid (PFOA), which has been widely used in numerous industrial manufacturing fields (e.g., textile, paper industry, packaging, and the production of surfactants) (Heidari et al., 2021; Supreeyasunthorn et al., 2016). PFOA and perfluorooctane sulfonate (PFOS) are the most detected PFAS (per- and polyfluoroalkyl substances) in the environment, and they are known as “forever chemicals” because of their persistency in nature and bioaccumulation potentials (Wee & Aris, 2023). The toxic effects of PFAS (e.g., hepatotoxicity, carcinogenicity, developmental toxicity, and immunotoxicity) caused the emergence of a global contamination crisis (particularly the contamination of drinking waters) (Fenton et al., 2021; Wee & Aris, 2023). Even if GenX has been developed due to the well-known harmful effects of PFAS, its potential toxicity was also reported (Supreeyasunthorn et al., 2016). In addition, the US Environmental Protection Agency confirmed that GenX exposure is related to pancreatic and liver cancers, and it has negative effects on the various tissues including the liver, kidneys, and blood of rats or mice. Moreover, it has been regarded by the European Chemicals Agency as a substance with significant environmental mobility and persistence that may cause cancer in humans (Vakili et al., 2021). On the other hand, there is limited research about the impact of GenX on the nervous system. In the third iDrosophila1-based study in this thesis, this prompted us to characterize the GenX-induced metabolic alterations in the *Drosophila* brain and their relationship with α Syn-related metabolic impairments.

3. METHODS

In the scope of the thesis, a GMN model of *Drosophila melanogaster* called iDrosophila1 was first reconstructed. Thus, the first section includes the methodological details of the reconstruction of this fly model and the evaluation of its predictive performance. The other sections consist of the methods used in different iDrosophila1-based applications to investigate the relationship between vitamin metabolism and PD, α Syn-induced metabolic changes in the gut-microbiota-brain axis, and the neurotoxic impact of GenX on the brain (**Figure 3.1**).

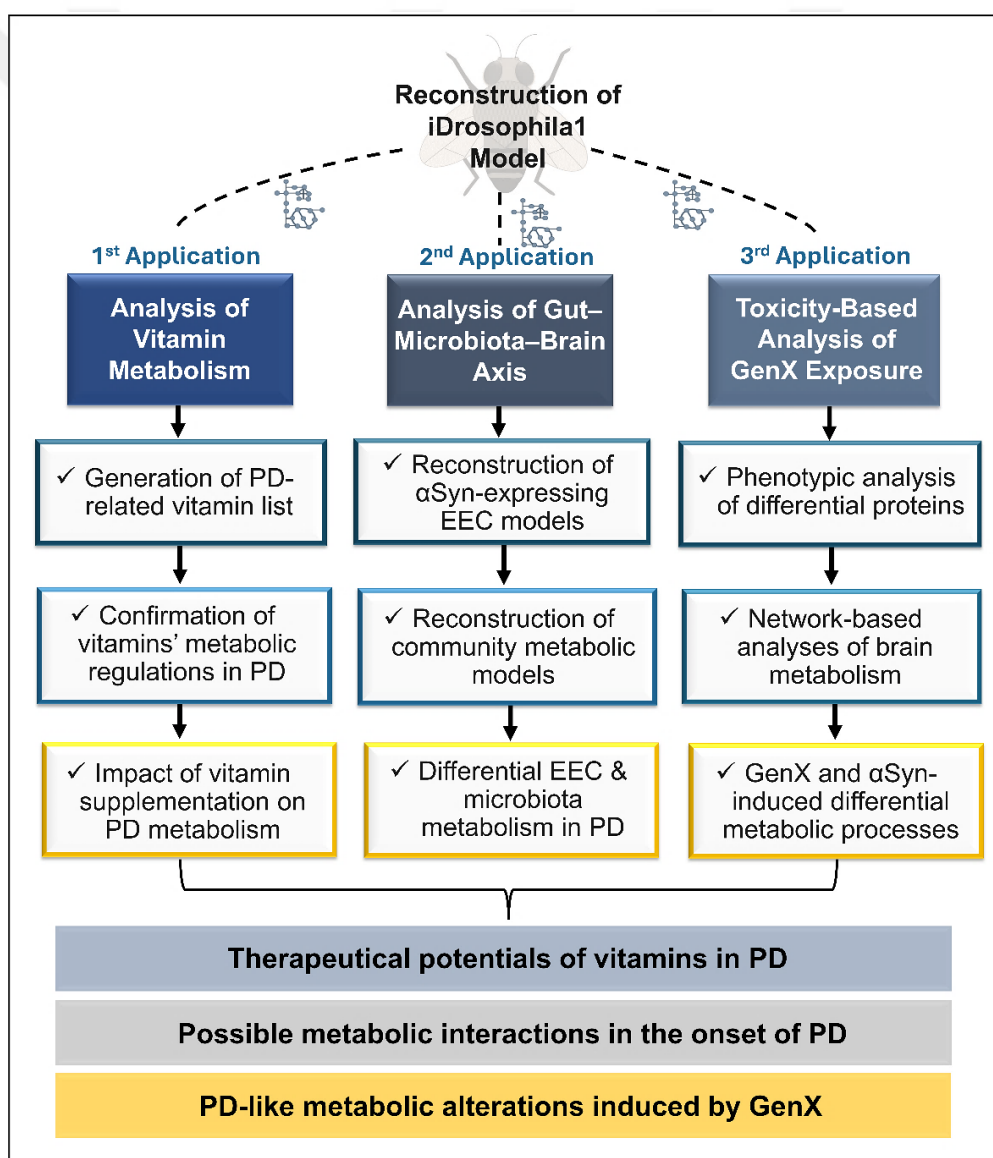


Figure 3.1: A schematic depiction of the methodology covers the reconstruction of the fly metabolic model and its utilization in three diverse research.

Several optimization strategies were preferred in the iDrosophila1-based applications to elucidate differential metabolism in diverse conditions. In the first and second research, the MOMA approach (Segrè et al., 2002) was used to investigate the effects of the increasing vitamin levels in the growth medium or α Syn synthesis in the EECs on the PD metabolism, by assuming that a minimum change should occur in the reference conditions for the altered vitamin levels or α Syn synthesis. Accordingly, new flux distributions were estimated, and differential pathways were uncovered through the comparison of these fluxes with the reference flux distributions. Furthermore, the Δ FBA approach (Ravi & Gunawan, 2021) was used in the first and third applications to determine the regulated pathways between the control flies and the mutant or GenX-treated flies by maximizing consistency between flux changes and differential gene expressions/protein abundances mapped to the reactions. In addition to the fold change-based Δ FBA approach, iMAT-based reaction activity analysis is based on the use of omics datasets (e.g., transcriptome and proteome data), unlike the MOMA approach. This method was used in the scope of the first and third applications to identify the differential reactions and the associated pathways, by the reconstruction of iMAT models and the comparison of reaction activities between the control flies and the mutant or GenX-treated flies.

3.1. Orthology-Based Model Reconstruction for *Drosophila*

Using an orthology-based approach, a GMN model for *D. melanogaster* was reconstructed in the first part of the thesis. This approach is based on the transfer of metabolic information from a template model of a phylogenetically close organism to the fly model. Considering the high conservation of especially disease-associated human genes in *Drosophila* (Mirzoyan et al., 2019; Pandey & Nichols, 2011; Reiter et al., 2001), a draft *Drosophila* model was reconstructed using a flux-consistent version of the human model, HMR2 (7,518 reactions, 5,426 metabolites, and 2,479 genes) as the template (Zirngibl, 2021). Subsequently, the metabolic information in the draft model was expanded and semi-automatically curated. The final model was validated based on phenotypic analyses, and its performance was comparatively assessed. All steps were explained in detail in the following subsections.

3.1.1. Metabolic Reconstruction Process

In the first step of the reconstruction process, the GPR associations in the HMR2-derived template model (kindly provided by Prof. Kiran R. Patil, University of Cambridge) (Zirngibl, 2021) were curated by improving the protein complex information based on another well-curated human model (iHsa) (Blais et al., 2017). For the orthology-based reconstruction of the *Drosophila* model, the genes in the GPR rules of the template human model were replaced with their *Drosophila* orthologs. To this end, the “human orthologs” list was retrieved from the FlyBase database (last accessed: 02/02/2021) (Larkin et al., 2021). This list contains evidence scores (DIOPT scores), *Drosophila* genes (FlyBase gene IDs), their human orthologs, and the associated disease phenotypes. The DIOPT score represents the number of tools supporting the corresponding orthology prediction (Hu et al., 2011). The scores ranging from 3 to 15 were considered in orthology-based gene mapping to raise the degree of confidence. In this method, two criteria were used: (1) When a human gene has several *Drosophila* orthologs with different DIOPT scores, only the ortholog(s) with the highest DIOPT score were taken into consideration. (2) Given that the orthology association of the human genes with over nine *Drosophila* orthologs is not specific (Blais et al., 2017), such gene associations were excluded. On the other hand, the related reactions were retained in the model. This is because the presence of *Drosophila* orthologs for at least one human gene shows that the relevant reaction is likely present in *Drosophila*. Finally, the human genes without any *Drosophila* orthologs were removed from the model. Thus, the GPR rules of the template human model were modified by replacing the human genes with their high-confidence-level orthologs in *Drosophila*. All non-enzymatic reactions of the human model were also transferred to the draft *Drosophila* model.

To increase the gene and reaction coverage in the draft model, another metabolic network of *Drosophila* was generated using the RAVEN toolbox (Wang et al., 2018), based on the protein homology against KEGG (Kanehisa & Goto, 2000) and MetaCyc (Caspi et al., 2016) databases. In light of the study of Wang and colleagues (Wang et al., 2018), a KEGG-based metabolic network was first created by combining two distinct networks: (1) KEGG organism identifier (dme) was used in the reconstruction of the first network, and (2) the subsequent network was created by comparing the *Drosophila* protein sequences from the FlyBase database (version r6.37) to the latest

pre-trained hidden Markov model (euk100_kegg94) via the BLAST algorithm. The same parameters (default cut-off: 10^{-50} , minScoreRatioG: 0.95, and minScoreRatioKO: 0.7) were used for the generation of both KEGG-based networks. In this process, incomplete information or undefined stoichiometries were excluded from the networks. Using the RAVEN toolbox, a MetaCyc-based metabolic network was also generated by following a similar reconstruction process. In this process, default parameters (bit-score ≥ 100 and positives $\geq 45\%$) were selected, and the unbalanced/undetermined reactions were removed from the MetaCyc-based network. The KEGG- and MetaCyc-based metabolic networks were then merged into a single network (hereinafter referred to as the KEGG-MetaCyc network).

The KEGG-MetaCyc-specific genes that were not found in the HMR2-based draft *Drosophila* model were determined and functionally characterized based on the significantly enriched KEGG pathways through the g:Profiler web server (Raudvere et al., 2019) for false discovery rate (FDR) < 0.05 . The reactions associated with the KEGG-MetaCyc-specific genes were included in the draft *Drosophila* model. It is important to note that any KEGG-MetaCyc reactions were not compartmentalized by the RAVEN toolbox. Therefore, an additional step was applied for assigning the compartment information to reactions. To do so, the subcellular locations of the proteins in the KEGG-MetaCyc network were first retrieved from several sources: COMPARTMENTS (Binder et al., 2014), FlyBase (Larkin et al., 2021), UniProt (The UniProt Consortium, 2017), GLAD (Hu et al., 2015), Reactome (Fabregat et al., 2016), AmiGO 2 (The Gene Ontology Consortium, 2015), QuickGO (Huntley et al., 2009), CELLO2GO web server (Yu et al., 2014), and a mass-spectrometry-based study (Tan et al., 2009). For an accurate gene-compartment mapping, gene ID consistency is crucial. Therefore, the FlyBase ID Validator tool (Larkin et al., 2021) was used to update and standardize the gene IDs derived from the subcellular localization databases and tools. In this way, all genes were converted to the current versions of FlyBase gene IDs. Next, a compartment dictionary was generated by mapping the subcellular locations to the KEGG-MetaCyc-specific genes according to the Gene Ontology (GO) terms (cellular components). When creating this dictionary, only the compartments present in the draft *Drosophila* model were taken into account. The dictionary-mediated assigned compartments were ultimately transferred from the genes to the KEGG-MetaCyc reactions.

To merge the metabolic information in the KEGG-MetaCyc network and the HMR2-based draft *Drosophila* model, the *addReaction* function in the COBRA toolbox was used (Heirendt et al., 2019). Furthermore, the biomass formation equation obtained from the template human model was curated as explained in the following section. With the use of the COBRA *biomassPrecursorCheck* function, the biomass components, which could not be synthesized by the merged model, were determined. Subsequently, the reactions required for the synthesis of these components were added to the model using the gap-filling algorithm, *fillGaps* in the RAVEN toolbox. While executing this algorithm, the lower bound of the biomass formation reaction was set to 0.1 to guarantee the production of all biomass components. After setting the “useModelConstraints” parameter to true, both the reference human model and the merged fly model were set to a chemically defined medium (Holidic diet, Hd) with free uptake rates ($1000 \text{ mmol g}^{-1} \text{ h}^{-1}$). This diet was originally designed for the growth of *Drosophila* in laboratory conditions (Piper et al., 2014; Schönborn et al., 2019), and several extra vitamin derivatives were herein added to the Hd medium in order to ensure biomass formation in the model. After constraining the models by mimicking this expanded Hd condition (**Table A1.1**) (Cesur et al., 2023), the HMR2-derived human model was used as the template to fill in the metabolic gaps in the merged *Drosophila* model with the gap-filling procedure. Thus, new reactions were added from the human model without their gene information.

Several curation steps were applied to the gap-filled model. One of them is the curation of cholesterol metabolism to represent cholesterol auxotrophy in *Drosophila*. To revise the cholesterol synthesis pathway in the fly model, the human reactions that are not conserved in flies were identified based on the literature (Santos & Lehmann, 2004; Zhang et al., 2019) and removed from the model. In the second curation step, the GPR rules in the fly model were updated based on a network of 556 *Drosophila* protein complexes generated by Guruharsha and colleagues, the list of FlyBase gene groups, and the literature (Allan et al., 2005; Attrill et al., 2016; Avval & Holmgren, 2009; Grant et al., 2010; Guruharsha et al., 2011; Kay et al., 2020; Kemppainen et al., 2014; Kovacs et al., 2018; Marygold et al., 2020a; Pavlovic & Bakovic, 2013; Santos & Lehmann, 2004; Tang & Zhou, 2013; Van den Berghe et al., 1997; Wahl et al., 2005). In the next curation step of the fly model, the presence of leaking energy metabolites, which can be synthesized even without any nutrient uptakes, was investigated. The

presence of leaking ATP metabolite in the model can cause a higher growth rate. Therefore, the production of each charging energy metabolite listed by Fritzemeier and colleagues (e.g., ATP, NADH, NADPH, FADH₂, GTP, and H⁺) (Fritzemeier et al., 2017) was investigated by blocking all uptake reactions in the model and only allowing the secretions of exchange metabolites. If necessary, a new dissipation reaction was added to the model for an energy metabolite. Subsequently, the rate of each dissipation reaction was maximized to decide whether the corresponding energy metabolite was leaking or not. If the rate of a dissipation reaction is nonzero in the lack of any dietary uptakes, the corresponding energy metabolite was classified as the leaking metabolite. To address this issue, the reactions causing the leaking of the identified metabolite were investigated by applying double reaction deletion simulations in the same nutritional condition, where all the uptake reactions were constrained to zero flux. Thus, the incorrectly compartmentalized or redundant reaction pairs, whose deletions prevent the production of a leaking metabolite without any nutritional inputs, were determined and revised. Another curation step included revising the incorrect link between heme and cytochrome-C metabolites by updating the reaction “HMR_4762”, which is catalyzed by cytochrome-C heme lyase (FBgn0038925). This reaction is responsible for the conversion of apocytochrome-C and heme to cytochrome-C reversibly. To revise the incorrect conversion reaction in the template human model, apocytochrome-C was added to the corresponding reaction (HMR_4762) in the *Drosophila* model and the missing reactions of the apocytochrome-C metabolism were added from the original HMR2 model. These changes influenced the growth rate due to the presence of cytochrome-C in the “cofactor and vitamin” composition of the biomass equation.

Furthermore, each component of the metabolic networks (reactions, metabolites, and genes) was curated in terms of metabolic redundancy, name standardization, and missing/incomplete components in order to increase the accuracy of each metabolic model, which contributes to the reconstruction of final *Drosophila* model. For the removal of redundant reactions, the duplicated reactions in each network were identified by pairwise comparisons iteratively, and common currency metabolites (H⁺, H₂O, and P_i) were ignored in this process. When searching duplicated reactions derived from the KEGG-MetaCyc-specific model and template human model, compartment information was also excluded due to the flexible compartmentalization

process employed for the KEGG-MetaCyc-specific model. Thus, compartmental inconsistencies between the duplicated reactions with the same gene contents were also determined and eliminated after revising the reaction redundancy. To avoid any gene loss, the curation step of the redundant reactions was performed in a tightly coupled manner with the curation of GPR rules. In the removal of gene-related redundancy, gene annotations were first standardized for each network by converting the FlyBase protein and annotation IDs in the KEGG- and MetaCyc-based networks into the FlyBase gene IDs. In addition, Ensembl IDs in the template model were replaced by the FlyBase IDs in the aforementioned orthology-based mapping step. Collectively, these gene replacement steps caused redundancy in the GPR rules. Therefore, each model was curated in such a way that it includes unique associations in each GPR rule. In the curation of redundant metabolites, a metabolite dictionary-based approach was applied. In this regard, the synonymous metabolites were identified by creating a dictionary including compound names (metabolite name and BioCyc name) and IDs (KEGG (Kanehisa & Goto, 2000), ChEBI (Degtyarenko et al., 2008), PubChem (Kim et al., 2021), and LIPID MAPS (Fahy et al., 2007)). To this aim, the compound information was collected from available metabolic networks and several web servers including Metabolite Translation Service (Caspi et al., 2016), MBROLE 2.0 (López-Ibáñez et al., 2016), MetaboAnalyst (Chong et al., 2018), and Chemical Translation Service (Wohlgemuth et al., 2010). It should be noted that only three-star ChEBI IDs (parent IDs), which were manually curated by the ChEBI team, were kept in the compound list by excluding less reliable ChEBI IDs. Metabolic redundancy can emerge due to the presence of duplicated (synonymous) metabolites within a single network and our first approach investigated such redundant metabolites for each network using the network-specific metabolite dictionary. In addition, merging different models (e.g., combining draft *Drosophila* and KEGG-MetaCyc network) can also result in metabolic redundancy. In the second approach, the dictionaries of such networks were compared to decide the synonymous metabolites. The matched metabolites with the highest number of identifiers (compound names and IDs) were accepted to be synonymous. If the same number of identifiers was detected for multiple metabolites in the dictionary comparison step, such hits were further investigated to decide the most possible synonymous metabolites using several additional sources (BioCyc (Karp et al., 2018), ModelSEED (Seaver et al., 2021), ChemSpider (Pence & Williams, 2010), MetaNetX (Moretti et al., 2021), and the

literature). For each metabolic match, one of the duplicated metabolites was removed from the model after the assembly of their stoichiometric coefficients. This final non-redundant model is called iDrosophila1 including 8,230 reactions, 6,990 metabolites, and 2,388 genes (Cesur et al., 2023).

The iDrosophila1 model was annotated through a variety of sources (HMR 2.0, Metabolic Atlas (Pornputtapong et al., 2015), FlyBase, MetaNetX (Moretti et al., 2021), ModelSEED (Seaver et al., 2021), BiGG Models (King et al., 2016), Metabolite Translation Service (Caspi et al., 2016), MBROLE 2.0 (López-Ibáñez et al., 2016), MetaboAnalyst (Xia et al., 2009), Chemical Translation Service, MetaBridge (Hinshaw et al., 2018), and SBOannotator (Leonidou et al., 2023)), and it is available in MATLAB (MAT), XML (SBML), and JSON formats in the GitHub repository (<https://github.com/SysBioGTU/iDrosophila>). The quality of this model was assessed via the metabolic model test suite, MEMOTE (version 0.13.0) using the Gurobi solver (Gurobi Optimization, LLC).

3.1.2. Curation of Biomass Formation Reaction

The biomass equation of the draft *Drosophila* model derived from the reference human model was modified based on the FlySilico model (Schönborn et al., 2019) and the literature. When the biomass composition of the draft model was compared to the biomass formation reaction in the FlySilico model, the majority of metabolites (amino acids, glycogen, triglycerides, and cholesterol) were found to be shared. Using the stoichiometric coefficients in the FlySilico, the missing growth-associated ATP maintenance reaction was added to our GMN model. Moreover, cardiolipin was removed from the biomass reaction because *Drosophila* does not require this metabolite for growth (Kubota-sakashita, 2020). Similarly, vitamin D3 was removed from the biomass reaction in the curation of cholesterol metabolism. This is because the synthesis reaction of 7-dehydrocholesterol (a cholesterol precursor) overlaps with the synthesis of vitamin D, and the gene encoding the enzyme responsible for the synthesis of 7-dehydrocholesterol was reported to be absent in the fly genome (Santos & Lehmann, 2004). Thus, vitamin D metabolism was also modified while updating the cholesterol metabolism in the model.

3.1.3. Analysis of Growth Phenotypes

The predictive performance of the reconstructed fly model (iDrosophila1) was first evaluated by analyzing cholesterol auxotrophy, aspartate non-essentiality, and the impact of essential amino acids (**Figure A1.1**) on growth in the expanded Hd condition (**Table A1.1**). A flexible intake of 47 dietary compounds was allowed by setting the maximum sucrose and vitamin uptake rates to $\sim 2.212 \text{ mmol g}^{-1}\text{h}^{-1}$ (Schönborn et al., 2019) and 1/100 of the sucrose uptake rate, respectively. The maximum consumption rate of the remaining Hd components (apart from salts and water) was limited to 1/10 of the sucrose uptake rate. The maximum oxygen uptake rate was set to $24 \text{ mmol g}^{-1}\text{h}^{-1}$ by estimating the amount of oxygen needed to consume all the sucrose by aerobic respiration. In this growth condition, the effect of raising cholesterol and amino acid levels on growth rate was investigated *in silico*, as described in the study of Schönborn and colleagues (Schönborn et al., 2019). Accordingly, growth rates were predicted using the flux balance analysis (FBA) approach (Orth et al., 2010) with the Gurobi solver under the objective of biomass formation for each growth condition.

3.1.4. Gene Essentiality Analysis

Gene essentiality analysis was carried out under the expanded Hd condition by permitting the infinite consumption of all diet ingredients to predict essential genes in the iDrosophila1 model. For the deletion of each gene, the associated reactions were blocked by setting their boundaries to zero and maximizing the growth rate with the Gurobi solver using the *singleGeneDeletion* function in the COBRA toolbox (Schellenberger et al., 2011). If the deletion of a gene resulted in a 30% reduction in the growth rate (Khodae et al., 2020), this gene was assumed to be essential. Thus, the essential and non-essential gene sets were determined. Subsequently, the genes that are involved in only inactive (blocked) reactions were removed from the gene set list since they do not affect biomass production. Under the expanded Hd condition, the blocked reactions were determined using flux variability analysis (FVA) (Mahadevan & Schilling, 2003). Reactions were considered inactive if their FVA-predicted total absolute minimum and maximum fluxes were less than 10^{-5} . Following the identification of essential and non-essential gene sets in the active reactions, the g:Profiler web server was used for FDR at 0.05 level to characterize the essential genes

through the enriched KEGG pathways and biological processes. Next, the prediction power of iDrosophila1 was evaluated based on the experimental gene essentiality dataset, retrieved from the Online GENE Essentiality (OGEE) database (Gurumayum et al., 2020). In the OGEE dataset, the conditionally essential genes were also categorized as essential, and then several metrics (sensitivity, specificity, accuracy, precision, F1 score, and Matthew's correlation coefficient (MCC)) were calculated for the comparison of the gene essentiality predictions with the experimental results to assess the model performance.

3.1.5. iDrosophila1-Mediated Analysis of Parkinson's Disease

The capacity of the iDrosophila1 model in phenotypic predictions was also evaluated by examining age- and PD-dependent differential pathways in *Drosophila*. To this aim, a microarray dataset (Agilent) from ArrayExpress (accession number: E-MTAB-1406) was used. It contains the samples from the heads of male flies harboring *pink1*^{B9} (*pink1*) and *park*²⁵ (*parkin*) mutations at different age groups: young flies (3-day-old) and middle-aged flies (21-day-old (*parkin*) and 30-day-old (*pink1*)) (Celardo et al., 2017). For each age group, three biological replicates of the *pink1* and six biological replicates of *parkin* mutant flies are present, respectively. The data was processed, normalized, and analyzed using the limma package (Law et al., 2014) for R version 4.1.0. In the data processing step, the intensity data was read via the *read.maimages* function, and the *backgroundCorrect* function was used to eliminate the impacts of the non-specific signals in the dataset. The consistency between different arrays was achieved with the *normalizeBetweenArrays* function.

Differential expression analysis was applied using the normalized log-transformed transcriptome dataset to find significant fold change values for each mutant group relative to the control group (i.e., the age-matched wild-type fly). To ensure the consistency of the genes with those in the iDrosophila1 model, all gene annotation IDs in the transcriptome dataset were transformed into the current versions of FlyBase gene IDs through the FlyBase ID Validator tool (Larkin et al., 2021). Multiple hits were manually revised in this step. The *limma-trend* function in the limma package was subsequently employed by setting *robust = TRUE* to uncover the differentially expressed genes (*p*-value < 0.01). If a gene has multiple probe measurements, the fold change values of this gene were filtered according to several criteria: (1) If at least one

probe of a gene has a fold change ≥ 1.5 , the highest fold change value of these probes was taken into consideration by presuming the upregulation of this gene. (2) Contrary, the minimum fold change value was chosen for the fold change values of any probe(s) ≤ 0.67 ($\sim 1/1.5$), by assuming the downregulation of this gene. (3) If all probes of a gene have moderate ($0.67 < \text{fold change} < 1.5$) or ambiguous fold change values (i.e., the presence of both upregulated and downregulated probes), the average fold change value of these probes was assigned to the related gene.

On the metabolic network side, no dietary constraints were applied for the exchange metabolites. Furthermore, the flux of the non-growth-associated ATP maintenance was presumed to remain constant across the wild-type and mutant groups. Using the COBRA function *mapExpressionToReactions*, the filtered fold change values were mapped to the reactions in the iDrosophila1 model. In the mapping procedure, GPR associations in the model were used. If the genes of a reaction were linked with the “AND” operator, the minimum fold change value was assigned to this reaction. On the other hand, the maximum fold change value was selected as the reaction expression if a reaction has the genes linked with the “OR” operator. Next, the differential reaction expression levels were used to determine PD-induced differential metabolism through the Δ FBA approach (Ravi & Gunawan, 2021) with default parameters. The Δ FBA algorithm uses a two-step optimization process to maximize consistency and minimize inconsistency between the flux changes (Δv) and differential reaction expressions in computing Δv between two different cases. In this way, the upregulated and downregulated reaction sets with differential fluxes above the selected threshold ($\varepsilon = 1$) were identified. The genes involved in the regulated reactions were determined based on the GPR associations, and then the significantly enriched KEGG pathways ($\text{FDR} < 0.05$), and disease terms ($p\text{-value} < 0.01$) associated with these genes were uncovered using the g:Profiler (Raudvere et al., 2019) and FlyEnrichr (Kuleshov et al., 2016) web servers.

3.2. Vitamin Metabolism in Parkinson’s Disease

The first iDrosophila1-based application herein focused on characterizing the roles of vitamins during PD. Proper supplementation of the neuroprotective vitamins is a potential strategy to improve PD symptoms as an adjuvant treatment. Briefly, a PD-related vitamin list was created through metabolic network-based approaches, and

their conservation was tested by comparing these lists with the predicted AD- and HD-related vitamins. The regulations in the reactions consuming or producing several PD-related vitamins were confirmed by a reaction activity-based approach. Lastly, the impact of these vitamins on PD metabolism was investigated. Overall, the relationship of the vitamins with PD was extensively examined in this section, as explained below.

3.2.1. Pre-processing and Normalization of RNA-seq Data

Two different RNA-seq datasets used in the current work include gene expression levels for human α Syn expression in the *Drosophila* brain at the bulk tissue level (accession number: syn34767280) or the cell level (accession number: GSE128120). The first dataset obtained from the female *Drosophila* brain represents three types of neurodegenerative disorders (PD, AD, HD) caused by aggregation-prone mutant human proteins: 1) α Syn, 2) A β 42 and tau, and 3) HTT^{NT231[Q128]} (fragment) and HTT^{FL[Q200]} (full length) (Wan, 2022). Initially, zero or low-count values were filtered out from the raw data, which included three replicates per age group (each replicate from 100 heads). The dataset was then normalized by converting the raw counts into log₂-counts per million (logCPM) values using the *cpm* function within the edgeR package (R version 4.1.0) (Law et al., 2014). It should be noted that five age groups (2-, 5-, 7-, 10-, and 14-day-old) were considered in the study, and the age-matched elav-Gal4/+ flies were used as the controls for each disease group. Covariate adjustment analysis was applied to the normalized data to eliminate the impact of age covariate. This adjustment is based on the generation of multiple linear regression models for each gene to exclude the effects of the specified covariate. The covariate-adjusted counts were compared to the normalized, non-adjusted data based on the Pearson correlation coefficients, and the correlation values were found to range from 0.97 to 0.98 (**Figure A1.2**). Principal component analysis (PCA) using the *prcomp* function in R was applied to the adjusted data to check out the presence of any outlier samples, and no samples were removed from the dataset.

The second dataset contains the raw sequencing reads of 10-day-old male and female fly brains expressing the human α Syn protein in the neuronal cells, glial cells, or both cell types. Syb-QF2 and repo-GAL4/+ flies without α Syn expression served as the control groups (Olsen & Feany, 2019). The raw reads retrieved from the NCBI Sequence Read Archive with the SRA toolkit (version 3.0.1) include four biological

replicates per genotype, each obtained from six fly heads (three male and three female flies) (Leinonen et al., 2011). A rapid pre-processing of the dataset was conducted using the Kallisto tool (version 0.46.1) (Bray et al., 2016), involving the creation of the transcriptome index (*kallisto index*) and pseudoaligning the paired-end sequencing reads to the *D. melanogaster* genome (BDGP Release 6) to generate count data with the *kallisto quant* function. The quality of the data was assessed with the FASTQC tool (version 0.12.1) (Andrews, 2010). Gene-level counts were obtained by summing the associated transcript levels through the tximport package in R (Soneson et al., 2016). Subsequently, the counts were normalized using the DESeq2 R package, and outlier samples were checked using PCA via the DESeq2 *plotPCA* function (**Figure A1.3.a**) (Love et al., 2014). After excluding the outlier sample in the neuronal group (Sample ID: SRR8708105), hierarchical clustering was performed with the *hclust* function in R utilizing Ward's method and Spearman distance (**Figure A1.3.b**).

3.2.2. Δ FBA-Mediated Identification of the Regulated Pathways

Differential expression profiles for the covariate-adjusted dataset (syn34767280) were determined using the R *limma-trend* function of the limma package by setting `robust = TRUE` (Law et al., 2014). For the second dataset (GSE128120), differential expression analysis was carried out for the gene-level raw counts using the Wald test from the DESeq2 package (Love et al., 2014). In this test, outlier genes were detected through Cook's distance cut-off, and lowly expressed genes were filtered out automatically using the default independent filtering method. The same methodology was followed in the next analysis steps for both datasets. Accordingly, significant fold changes ($p\text{-value} < 0.05$) were first identified based on the differential expression analyses for each disease group: PD (α Syn expression at tissue or cell level), AD ($A\beta_{42}$ or tau expression), and HD ($HTT^{NT231[Q128]}$ or $HTT^{FL[Q200]}$ expression). The fold changes were mapped to the reactions in iDrosophila1 (Cesur et al., 2023) through the *mapExpressionToReactions* function within the COBRA toolbox (Heirendt et al., 2019) to determine differential reaction expressions.

While no constraints were imposed for the exchange reactions in the GMN model, the flux of the non-growth associated maintenance (NGAM) reaction was assumed to be unchanged between the disease and control groups. The next step involved the use of a method called Δ FBA (Ravi & Gunawan, 2021) to predict a regulated reaction set by

optimizing consistency between the flux changes (Δv) and differential reaction expressions. For this analysis, different threshold values ($\varepsilon = 0.5$ and $\varepsilon = 0.1$, respectively) were selected for both datasets based on the number of differentially expressed genes. The reactions whose flux differences were above the specified threshold value were accepted to be significantly regulated. The genes involved in these reactions were determined based on the GPR associations in the iDrosophila1 model. These genes were characterized based on the enriched terms (GO biological processes, KEGG pathways, and Reactome pathways) using the FLAME web tool (version 2.0) (Karatzas et al., 2023) with default parameters (FDR < 0.05 or g:SCS threshold < 0.05). This tool combines metabolic information from aGOTool (Schölz et al., 2015), g:Profiler (Raudvere et al., 2019), and EnrichR (Kuleshov et al., 2016). Overall, the enriched terms associated with the conserved metabolic pathways and vitamin metabolism were selected for all disease groups to illustrate the enrichment results via the Sankey diagram with the *SSankey* function in MATLAB (Liu, 2023) and bubble plots with the ggplot2 package in R. A regulated vitamin list was ultimately generated based on the significantly enriched metabolic pathways of the vitamins in PD.

3.2.3. Reporter Metabolite Analysis

Reporter metabolite analysis (RMA) systematically combines genome-wide expression data and network topology information to predict reporter metabolites (Çakır, 2015; Patil & Nielsen, 2005). Thus, this approach facilitates the identification of key metabolic compounds, which are adjacent to the differentially expressed genes in the network. Such metabolites are potential biomarkers or nutritional supplements. For each neurodegenerative disorder, RMA was applied by creating two files: 1) a list of the genes and their *p*-values, and 2) a network of the associations between metabolites and neighboring genes in the iDrosophila1 model. The significant reporter metabolites (*p*-value < 0.05) were identified using these files as the inputs of the RMA web tool (Çakır, 2015; Çakır, 2019). The vitamins with at least two neighbors were chosen for each disease group (i.e., PD: bulk tissue-level or cell type-specific α Syn expression; AD: A β 42 or tau expression; HD: HTT^{NT231[Q128]} or HTT^{FL[Q200]} expression). The vitamins predicted as the reporter metabolites were added to the previously compiled PD-related vitamin list.

3.2.4. Metabolic Reconstruction of GMN Models

To mimic the metabolism of healthy and PD brains in *Drosophila*, sample-based context-specific GMN models were reconstructed using the iDrosophila1 model. To this aim, all reversible reactions in this generic model were first made irreversible via the *convertToIrreversible* function in the COBRA toolbox. Medium constraints and enzymatic constraints were then applied to the irreversible model. The medium constraints were provided to represent an expanded version of Hd with additional essential vitamins reported in (Cesur et al., 2023) in addition to the listed PD-related vitamins (A, B₁, B₂, B₃, B₆, B₉, C, and D). The free intakes of 49 dietary compounds were allowed to mimic this growth condition (**Table A1.1**). In addition, the lower bounds of several crucial reactions were set to a small value to ensure that these reactions were kept in the contextualized models. Accordingly, a small flux boundary value (10^{-5} mmol g⁻¹h⁻¹) was used for the minimum uptake rates of the regulated vitamins while the minimum uptake rates of glucose and oxygen were set to 0.01 mmol g⁻¹h⁻¹. In a similar vein, the lower bounds of the NGAM reactions and biomass formation were set to a small value (10^{-4} mmol g⁻¹h⁻¹).

The solution space was further narrowed down by applying enzymatic constraints to the irreversible fly model. Normalized protein abundance data from the α Syn transgenic brains of 10-day-old male and female flies (kindly provided by Prof. Souvarish Sarkar, University of Rochester Medical Center) was utilized to mimic enzyme concentrations in the calculations. The data contains five and six replicates for the control and PD cases, respectively (Sarkar et al., 2020). PCA identified two transgenic brain samples to be outliers, and they were removed from the dataset (**Figure A1.4.a**). Hierarchical clustering with Ward's method and Spearman distance was employed for the remaining samples using the *hclust* function (**Figure A1.4.b**). For compatibility with the iDrosophila1 model, the UniProt protein IDs in the normalized relative protein abundance dataset were replaced by the FlyBase gene IDs through the annotation tables in the UniProt (The UniProt Consortium, 2017) and FlyBase databases (Larkin et al., 2021). The *mapExpressionToReactions* function was then used to map the protein abundances onto the model reactions. Subsequently, the BRENDA database (Chang et al., 2021) was accessed through the GECKO toolbox (Domenzain et al., 2022) to obtain the *kcat* values (turnover numbers, s⁻¹) of the enzymes from *Drosophila* species and humans. Enzyme Commission (EC) numbers

and substrate information were used to match the *kcat* values with the reactions in the irreversible model. In this step, a compound ID-based mapping approach was used to match the substrate information between the iDrosophila1 model and the BRENDA data. Thus, the metabolites were matched based on their names and compound IDs from different sources (i.e., KEGG (Kanehisa & Goto, 2000), ChEBI (Degtyarenko et al., 2008), PubChem (Kim et al., 2021), HMDB (Wishart et al., 2007), ModelSEED (Seaver et al., 2021), BiGG Models (King et al., 2016), and MetaNetX (Moretti et al., 2021) IDs determined by Metabolite Translation Service in MetaCyc (Caspi et al., 2016) and MBROLE 2.0 (López-Ibáñez et al., 2016))). The details of this approach are available in Section 3.1.1. Lastly, the mapped average *kcat* values and protein abundance values were multiplied to obtain maximum flux rates (Li et al., 2022), and the calculated values were used as the upper flux bounds of the corresponding intracellular reactions in the irreversible model.

Control and PD models were reconstructed with the iMAT algorithm (Zur et al., 2010) using the constrained irreversible iDrosophila1 model. This step involved the mapping of the normalized covariate-adjusted RNA-seq dataset of PD (syn34767280) to the model reactions using the *mapExpressionToReactions* function, and the generation of a reduced model for each sample (i.e., a total of 30 models; 15 for each group) by maximizing the consistency between the reaction fluxes and the expression states to keep the reactions with highly expressed genes (Walakira et al., 2021). Threshold values were selected as the 25th and 75th percentiles of the transcriptome data to decide the activity of the reactions based on the expression levels of the associated genes; low, moderate, or high.

3.2.5. Analyses of the Parkinson's Disease Models

Using two distinct approaches, the metabolic relationship between PD and the listed PD-related vitamins (A, B₁, B₂, B₃, B₆, B₉, C, and D) was examined for the reconstructed control and PD models subjected to the medium and enzymatic constraints. In the first approach, the regulation of several PD-related vitamins was confirmed based on differential reaction activities, as described in (Lüleci et al., 2023). To this end, “1” (kept) or “0” (removed) was assigned to the reactions in the iDrosophila1 model based on their existence in the sample-based iMAT models to generate binary matrices. Using the binary matrices, the reaction activities were

compared between the control and PD cases by performing Fisher's exact test with the *fishertest* function in MATLAB. Thus, the significantly upregulated (odds ratio > 2) and downregulated (odds ratio < 0.5) reactions were uncovered for the p -value < 0.01. In this way, the vitamins involved in the affected reactions, and the corresponding subsystems (pathways) were determined through the iDrosophila1 model.

In the second approach, a multiple-stage optimization strategy was used to investigate the effects of varying uptake levels of the regulated vitamins on PD metabolism. To this end, the maximum glucose uptake rates of 15 sample-based PD models were limited to $\sim 0.186 \mu\text{mol g (wet) brain}^{-1} \text{ min}^{-1}$, which was calculated according to the experimentally detected average of 8.5% reduction in the glucose uptake level of the PD brain relative to the healthy controls (Supandi & van Beek, 2018). In a similar vein, the maximum oxygen uptake rate ($\sim 0.987 \mu\text{mol g (wet) brain}^{-1} \text{ min}^{-1}$) was calculated taking into account a 19% average decrease in the oxygen consumption level in the human PD brain (Supandi & van Beek, 2018). The maximum uptake rates of all vitamins in the Hd medium (**Table A1.1**) were limited to 1/100 of the maximum glucose uptake rate considering the lower tendency of organisms for vitamin consumption compared to other nutritional compounds. In addition, 1/10 of the maximum glucose uptake rate was assigned to the maximum uptake rates of the remaining Hd compounds (except for free salts and water). Since the brain consumes up to almost 20% of the body's energy in an adult human (Padamsey & Rochefort, 2023), the maximum rate of the NGAM reaction in the *Drosophila* brain was estimated as the 20% of the reported ATP consumption flux for the generic *Drosophila* model (Schönborn et al., 2019), allowing its 50% reduction. The reference flux distributions in this growth condition were computed by addressing a two-stage optimization problem. In the first optimization step, the maximum growth rates of the 15 PD models were identified via the FBA approach under a steady-state assumption (Orth et al., 2010). In the second optimization step, the minimum growth rate was defined as 20% of the FBA-predicted maximum growth rate, and minimization of the sum of absolute intracellular fluxes was used as the secondary objective to mimic minimal enzyme usage in the biological systems and to narrow down the solution space (Cesur et al., 2022; Tarlak et al., 2014). This allowed the prediction of a reference flux distribution for each model.

In the last optimization step, the maximum possible uptake rate of each PD-related vitamin was gradually elevated by fixing its upper boundary to 1/50, 1/20, and 1/10 of the maximum glucose uptake rate while setting their minimum possible flux to 90% of the maximum possible uptake rate. For each nutritional modification (24 in total; three different uptake levels for each of 8 vitamins), a new flux distribution was estimated via the linear version of the MOMA approach (Segrè et al., 2002) by minimizing the difference between the absolute value of the reference flux distribution and the flux distribution of the modified nutritional condition. In this way, it was assumed that a small flux change would occur when the consumption level of each PD-related vitamin was changed. Note that two out of 15 PD models were excluded from the analyses for vitamin B₂ because an optimal solution could not be obtained with these models (i.e., obtaining suboptimal solutions for two models). Thus, MOMA-predicted flux distributions (n = 15) were obtained per uptake ratio of each vitamin except for vitamin B₂ (n = 13 for each uptake ratio of this vitamin). Finally, using MATLAB's *signrank* function, the Wilcoxon signed rank test was applied for the reciprocal comparison of the reference and the MOMA-predicted flux distributions of each model reaction for the modified level of each vitamin. In other words, for each reaction, 15 reference flux values were compared to 15 MOMA-predicted flux values obtained by each new vitamin uptake constraint (13 flux distributions in each group for vitamin B₂). This allowed us to determine the significantly affected reactions (p -value < 0.05) and the associated subsystems (pathways) in the PD models. The changes in the key pathways are represented through the heatmap generated using the *SHeatmap* (special heatmap) function in MATLAB (Liu, 2024). Note that the optimization problems were solved via Gurobi solver (Gurobi Optimization, LLC; version 8.0.1), and MATLAB R2023a was used in all model-based analyses unless otherwise stated.

3.3. EEC-Gut Microbiota Interactions in Early PD

In the second *iDrosophila*1-based research, the α Syn-mediated differential metabolic processes in the EECs and their metabolic interactions with gut microbiota were investigated. Such interactions may be collectively associated with the initiation of the PD process in the gut. To mimic the presumed early stages of PD, EEC models with the ability of α Syn synthesis were generated for the midgut regions R1-R5 and the

whole midgut. Community metabolic models were generated by combining each EEC model with the microbial models. The α Syn-induced differential metabolism of the community was simulated as detailed in the following subsections.

3.3.1. Pre-processing and Normalization of FACS-Sorted RNA-seq Data

A FACS-sorted RNA-seq dataset of 7- and 10-day-old female flies (accession number: GSE61361) (Dutta et al., 2015) was used to reconstruct the six GMN models of EECs for five different midgut regions (R1-R5) and the whole midgut. The raw single-end sequencing reads of each sample with two replicates were accessed through the NCBI Sequence Read Archive with the SRA toolkit (version 3.0.1) (Leinonen et al., 2011), and a pre-processing step was applied to the raw counts in order to obtain gene-level estimated counts, as described in Section 3.2.1. Subsequently, the gene counts were normalized by applying the Gene length corrected trimmed mean of M-values (GeTMM) normalization technique (Smid et al., 2018). PCA was applied to the normalized data via the *prcomp* function in R, and no samples were labeled as outliers due to the insufficient number of replicates (only two replicates per midgut region) to decide the outlier samples. Additionally, hierarchical clustering was carried out with the *hclust* function in R using the average linkage algorithm and Euclidean distance to identify the samples exhibiting similar or diverse expression patterns.

3.3.2. Identification of Region-Specific Metabolic Patterns in EECs

The pathways of the genes exhibiting unique expression patterns in the region-specific EECs were investigated using the GeTMM-normalized RNA-seq counts. The normalized counts were first mapped to the iDrosophila1 reactions by the *mapExpressionToReactions* function. For each subsystem (pathway) in the model, the associated reaction expression values were summed to calculate pathway scores. It should be noted that the pathways of exchange and transport reactions in the model were ignored in these calculations due to their high prevalence. The pathway scores were then transformed into z-scores and illustrated through the hierarchical clustering-based heatmap and dendrogram (using Euclidean distance), obtained by the *clustergram* function in MATLAB.

3.3.3. Addition of α Syn Synthesis Reaction

A protein pool reaction was generated by separating the amino acid composition in the biomass reaction of the generic iDrosophila1 model (Cesur et al., 2023). An artificial α Syn synthesis reaction was subsequently created based on the molar level of each amino acid in this protein. This information was obtained from the sequence of human α Syn (GenBank accession number: AAL15443.1) due to the lack of *SNCA* ortholog in *Drosophila*, and α Syn synthesis was defined as the weighted linear combinations of amino acids depending on their molar levels. The protein pool and α Syn were also represented in the biomass formation reaction at the metabolite levels, and only a small fraction of the α Syn protein was assumed to contribute to the biomass formation, considering its low cytosolic abundance relative to the whole cytosolic proteins of humans (Benskey et al., 2016; Wang et al., 2019). Together, α Syn was added to the biomass reaction with a coefficient of 10^{-3} .

3.3.4. Reconstruction of EEC Models

In the contextualization process of the generic fly model, a minimum nonzero flux bound was first assigned to several crucial reactions (10^{-2} for the minimum biomass formation reaction and 10^{-5} for the α Syn synthesis, glucose uptake, and NGAM reactions) to ensure the inclusion of these reactions in the EEC models. Additionally, the oxygen uptake rate was fixed at the rate of $0.1 \text{ mmol g}^{-1}\text{h}^{-1}$ to mimic the microaerobic environment (Schönborn et al., 2021), which is required for the growth of aerotolerant anaerobic *Lactobacillus* species in the community modeling step (see the next section). Subsequently, the GeTMM-normalized FACS-sorted RNA-seq data of EECs were mapped to iDrosophila1 reactions via the *mapExpressionToReactions* function. In the mapping process, the average values of the replicates were used for each midgut region. EEC models were then reconstructed using the iMAT algorithm (Zur et al., 2010) by maximizing the number of flux-carrying reactions with highly expressed genes in the models based on the defined threshold values (i.e., 25th and 75th percentiles of all RNA-seq data). For each EEC model, the inactive reactions defined by iMAT were kept in the iDrosophila1 model by assigning small lower and upper bound values (-10^{-9} and 10^{-9}) to block these reactions. This step is beneficial to avoid infeasibility in the model simulations. In this way, a total number of six EEC models

were reconstructed. The growth of each iMAT-based mutant EEC model under the expanded Hd condition (**Table A1.1**) was investigated by applying flexible medium constraints. In this step, the maximum glucose intake was limited to $\sim 2 \text{ mmol g}^{-1}\text{h}^{-1}$ (Schönborn et al., 2019). The maximum uptake rates of all vitamins in the Hd medium were set at 1/100 of the maximum glucose uptake rate, while the maximum uptake rates of the remaining dietary components (apart from free salts and water) were fixed at 1/10 of the maximum glucose uptake rate. In this growth condition, the optimal growth rates were predicted using the FBA approach (Orth et al., 2010). Similarly, growth simulations were repeated in another medium, DMEM/F-12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) (Lewis et al., 2018) (**Table A1.1** and **Figure A1.5**), which was previously used by Bohórquez and colleagues to culture the intestinal organoids of mice (Bohórquez et al., 2014).

3.3.5. Modification of Microbial Models

The metabolic network models of the leading gut bacteria in laboratory-reared *Drosophila* (i.e., *Lactobacillus plantarum* A2, *Lactobacillus plantarum* B2, and *Acetobacter pasteurianus* B5), developed by Schönborn and colleagues (Schönborn et al., 2021), were herein used to reconstruct community metabolic network models representing the simplified midgut of *Drosophila*. Transport and exchange reactions in the microbial models were revised based on the metabolic information from the literature and several databases (Ankrah et al., 2021a; Kanehisa & Goto, 2000; Norsigian et al., 2020; Pelicaen et al., 2019; Saier et al., 2006; The UniProt Consortium, 2017). Furthermore, the nomenclatures of the microbial exchange metabolites were standardized. In this step, synonymous microbial metabolites shared by the iDrosophila1 model were determined using a compound ID-based approach, detailed in Section 3.1.1, and these microbial metabolites were replaced by their counterparts in iDrosophila1. Similarly, compartment representations in the microbial models were updated based on the iDrosophila1 model for compatibility between the models. The microbial models had also the trivial uptake reactions of some essential cytosolic macromolecules (protein, DNA, and RNA) and they were removed from the models. The optimal growth rates of the modified microbial models were subsequently investigated under the Hd and DMEM/F-12 media using the same constraints as the previous section. It should be noted that the uptake for several additional metal ions

(Mg^{2+} , Mn^{2+} , Fe^{3+} , and Cu^{2+}) had to be allowed for *Lactobacillus* and *Acetobacter* species since they did not grow otherwise. Under this growth condition, the optimal growth rate of each modified model was predicted using the FBA approach. Given that the use of DMEM/F-12 (**Figure A1.6**) did not considerably influence the optimal simulation results of the EEC and bacteria in comparison with the Hd condition due to their compositional similarity, only Hd medium was used in the simulations in the next sections.

3.3.6. Reconstruction of Community Metabolic Models

For the wild-type and αSyn -expressing mutant cases, community models were developed by combining the metabolic information in the region-specific EEC (the regions R1-R5 or whole midgut) and microbial models, obtaining 12 community models in total (6 models for each group). In the reconstruction process, the metabolic nomenclature in each model was updated by adding the corresponding cell type information (i.e., the names of bacterial strains and regional EECs) to the reactions and metabolites as a suffix. Then, the modified strain-level bacterial models were merged with each EEC model one by one using the *mergeTwoModels* function in the COBRA toolbox. In each community, the members were connected via a common compartment (gut lumen). The gut lumen-mediated metabolic transports were allowed between the community members. The original exchange reactions were removed from the community model before defining the dietary exchange and fecal excretion compartments. These compartments facilitate the intake of nutritional compounds and the discharging of wastes (Thiele et al., 2020). According to this community design, nutritional compounds in the dietary compartment were transferred to the gut lumen via transport reactions for their consumption by the community members. The metabolites secreted by each community member were also transported to the lumen for utilization by the other members. Ultimately, the metabolic wastes and dying cells in the lumen were transferred to the fecal compartment for excretion. Thus, this metabolic crosstalk in the lumen regulates the growth and metabolic capacity of the cells. To further control the growth of the bacterial cells, a microbial community biomass equation (hereafter called microbiota biomass equation) representing a weighted linear combination of the strain-specific biomasses was created. The relative bacterial abundances (sum is equal to 1) derived from 16S rRNA gene sequencing

experiments of the 28-day-old female fly midguts were used as the weights (i.e., stoichiometric coefficients) in the equation (Liu et al., 2022). The relative abundance of *Lactobacillus pentosus* was used instead of *L. plantarum* considering their phylogenetic closeness (Zheng et al., 2020), due to the lack of information about *L. plantarum* in the dataset (Liu et al., 2022). In conclusion, two diverse community models were developed for each midgut region by considering the relative bacterial abundances to mimic the varying microbial prevalence in the wild-type and PD midguts. Transport and secretion reactions were also added for the microbiota and EEC biomasses for the clearance of the dying cells from the midgut. Another difference between the wild-type and mutant models is that the α Syn synthesis reaction was not included in the wild-type EEC models due to the lack of *SNCA* expression in the wild-type *Drosophila*. The maximum α Syn synthesis capacity of each mutant community model was analyzed in the expanded Hd condition (**Table A1.1**) with the above-stated flexible medium constraints (Section 3.3.4) using the FBA approach by setting the minimum growth rates of the EEC and microbiota to 10^{-4} to ensure their biomass productions.

3.3.7. Pareto Optimality Analysis

Pareto optimality analysis is a multi-objective optimization approach for the assessment of the trade-offs among a set of biological objectives. According to this approach, an improvement in a biological task can lead to the worsening of other objectives (Griesemer & Navid, 2023). In the current study, this approach was used to predict the microbial growth rates and/or α Syn synthesis rate in the wild-type and mutant community models based on the points on the Pareto front (i.e., a set of Pareto-optimal solutions). To this end, a similar rationale behind the MultiMetEval tool (Zakrzewski et al., 2012), which optimizes an objective by iteratively decreasing the maximum value of another objective at each step until reaching zero (Griesemer & Navid, 2023), was herein used to identify the Pareto-optimal solutions.

First, the expanded Hd (**Table A1.1**) was set as the nutritional condition of each community by allowing the intake of only medium compounds from the dietary compartment. The flexible dietary constraints defined in Section 3.3.4 were used to limit the maximum uptakes of the dietary compounds. The rate of NGAM reaction was fixed at the measured value (Schönborn et al., 2019) by allowing its 50% reduction. In

this growth condition, the FBA approach allowed us to predict the maximum growth rate of the EEC for each group of the regional wild-type and mutant community models at a steady state, as represented in equations (3.1)-(3.3).

$$\sum_{j=1}^n (S_{ij}V_j) = 0 \quad \text{for } i = 1, 2, \dots, m \quad (3.1)$$

where the mass balance equation includes the stoichiometric matrix (S_{ij}) with the coefficient of the metabolite i (within a total of m metabolites) and the rate of the j^{th} reaction (V_j) within a total of n reactions, respectively, as formulated in equation (3.1).

$$\text{lb}_j \leq V_j \leq \text{ub}_j \quad \text{for } j = 1, 2, \dots, n \quad (3.2)$$

$$\text{optimize the growth rate of EEC: } V_{eec_max} \quad (3.3)$$

In equation (3.2), lb_j and ub_j are the lower and upper bounds for the j^{th} reaction flux. Based on the mass balance and boundary constraints, FBA revealed the maximum growth rate of wild-type and mutant EEC (V_{eec_max}) for each midgut region (equation 3.3). These optimal growth rates were used to calculate maximum and minimum Pareto-optimal points. Accordingly, the maximum point on the Pareto front (V_{eec_maxP}) was assumed to be equal to 20% of the FBA-predicted optimal growth rate (V_{eec_max}) for each EEC because the EECs are terminally differentiated cells under the control of Notch signaling pathways despite their some degree of cellular plasticity (Guo et al., 2024; Ohlstein & Spradling, 2006). Thus, their suboptimal growth was presumed under the defined growth condition. In addition, the minimum point on the Pareto front (V_{eec_minP}) was estimated as 5% of the FBA-predicted optimal growth rate (V_{eec_max}). For each community model, ten flux points (V_{eec_P}) were identified on the Pareto front between V_{eec_maxP} and V_{eec_minP} , as formulated in equations (3.4)-(3.6).

$$\Delta = V_{eec_maxP} - V_{eec_minP} \quad (3.4)$$

$$V_{eec_P} = \left[V_{eec_maxP}, V_{eec_maxP} - \frac{p \times \Delta}{P-1} \right] \quad (3.5)$$

$$p = \{1, 2, 3, \dots, P-1\} \quad (3.6)$$

where Δ shows the difference between the maximum (V_{eec_maxP}) and minimum Pareto-optimal points (V_{eec_minP}) in equation (3.4). Each point on the Pareto front

(V_{eec_P}) was calculated by iteratively decreasing the $V_{eec_{maxP}}$. The level of reduction was calculated by multiplying Δ by the point p and then dividing it by the total number of points minus one ($P - 1$), as represented in equation (3.5) and equation (3.6).

In the final step, the maximum growth rates of the wild-type microbial community and the maximum α Syn production rates of the mutant EEC were determined in the Hd condition using FBA by fixing each point on the Pareto front to constrain the growth of EEC in the defined interval. In the calculation of the maximum biomass formation rate in the mutant microbial community, 5% of the FBA-predicted maximum α Syn production rate was assigned as the lower flux boundary to ensure nonzero α Syn production for each fixed point on the Pareto front. Under these constraints, the maximum growth rates of the mutant microbial community were calculated through the FBA approach. This optimization step was repeated for each midgut region to determine the region-specific growth rates of the gut microbiota (**Figure 3.2**).

3.3.8. Prediction of Flux Distributions

To identify the flux distributions of the wild-type communities, each Pareto optimal point and the predicted growth rate of gut microbiota were used to constrain each model. The solution space was further narrowed down by setting the expanded Hd condition (**Table A1.1**) with flexible uptake rates, as described in Section 3.3.4. Importantly, the maximum uptake rate of oxygen was set to $0.1 \text{ mmol g}^{-1}\text{h}^{-1}$ (Schönborn et al., 2021) to mimic the microaerobic environment required for the survival of *L. plantarum* strains. The sum of squares of the intracellular fluxes was then minimized as the secondary objective function. Thus, the flux distributions were estimated for a total of six wild-type community models (regions R1-R5 and the whole midgut) for a total of ten Pareto optimal solutions (**Figure 3.2**).

In the prediction of mutant flux distributions, the linear version of MOMA (Segrè et al., 2002) was used by assuming that the metabolic state of mutant *Drosophila* should be similar to the wild-type due to the robustness of metabolic systems and the reprogramming of various metabolic processes to compensate the overexpression of α Syn. On the other hand, a set of reactions expected to change by the α Syn synthesis in the EECs was excluded from the MOMA optimization. These reactions were determined based on the list of differentially expressed genes (p -value < 0.01) in the

Drosophila midgut expressing α Syn, defined in the study of Liu and colleagues (Liu et al., 2022). A list of 247 EEC reactions possessing at least 50% of the differentially expressed genes was created. These EEC reactions are involved in the metabolism of fundamental biomolecules (i.e., nucleotides, amino acids, lipids), carnitine shuttle, TCA cycle and glyoxylate/dicarboxylate metabolism, pentose phosphate pathway, vitamin metabolism (i.e., retinol (vitamin A) metabolism, pantothenate (vitamin B₅) and CoA biosynthesis, folate (vitamin B₉) metabolism), and glutathione (GSH) metabolism. Since the intracellular ROS production promoted by the elevated uptake levels of mitochondrial Ca^{2+} was reported to be highly correlated with the phosphorylation and aggregation of α Syn proteins in the EECs (Neto et al., 2022), the regulated reaction list was extended by including the additional reactions of GSH metabolism and ROS detoxification. Thus, the final list contained a total of 255 reactions (**Table A1.2**). As aforementioned, fluxes of the listed reactions were allowed to freely change in the MOMA simulations by presuming that the fly EECs should try to cope with the overexpression of human α Syn through the regulation of these reactions. Ultimately, ten flux distributions were also obtained for each mutant community in a specific midgut region or the whole midgut (**Figure 3.2**).

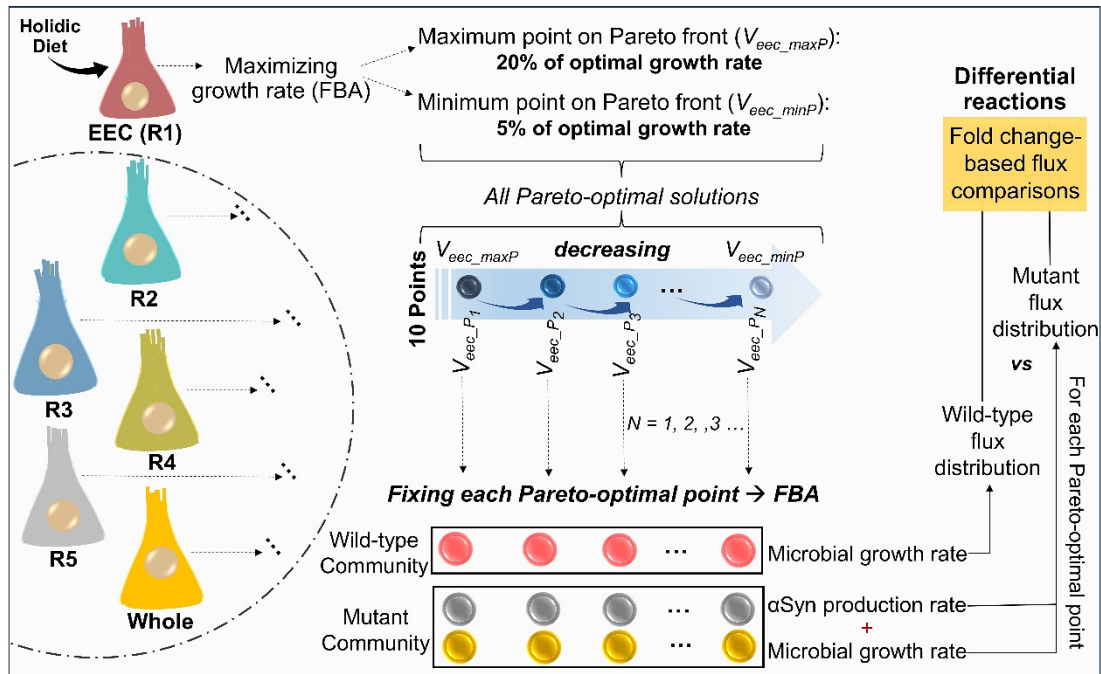


Figure 3.2: Illustrating the method followed to determine the α Syn-induced differential reactions in the community models. For simplicity, it is represented for only one midgut region (R1), and microbial interactions in the community of EEC-gut microbiota are not included in the figure. The same calculations

were also applied to the other midgut regions. Accordingly, 10 Pareto-optimal points were calculated in a decreasing flux interval based on the FBA-mediated growth simulation of the EEC in the midgut region R1. The maximum growth rate of microbiota and the maximum α Syn production rate were predicted using each Pareto-optimal point as the model constraint. Finally, 10 wild-type and 10 mutant flux distributions were estimated by constraining the model with all these FBA-predicted fluxes and Pareto-optimal points for the region R1. Fold change-based comparisons enabled the determination of differential reactions induced by the α Syn synthesis in *Drosophila* EEC.

3.3.9. Analysis of Differential Pathways

Differential EEC reactions in each midgut region were determined based on the fold change (FC) values calculated by dividing the mutant flux distribution by the wild-type fluxes for each community. If a reaction has FC higher than 2 or less than 0.5, it was accepted to be regulated by the expression of α Syn in the EEC. Metabolites and genes in the differential EEC reactions, which were obtained for at least 50% of 10 Pareto-optimal points, were listed for further characterization. In this regard, a gene-metabolite network was created by the MetaboAnalyst web server for each midgut region-dependent list, and it was analyzed to elucidate the significantly enriched KEGG pathways (p -value < 0.05) (Xia et al., 2009). The enrichment results were finally illustrated via a Sankey diagram created with the *SSankey* function in MATLAB (Liu, 2023). Furthermore, the microbial transport reactions (luminal transport) affected by the α Syn synthesis in the EECs were examined to characterize differential metabolic interactions between EECs and gut microbiota.

3.4. Impact of GenX on Neurodegeneration

The last application of the iDrosophila1 model in this thesis study includes neurotoxicity-based research about the impact of a member within the per- and polyfluoroalkyl substances (PFAS) chemical class, GenX (hexafluoropropylene oxide dimer acid, HFPO-DA), on the *Drosophila* brain. In this section, genes and metabolites in the reactions, which were affected by the exposure to GenX, were identified through two network-based approaches: 1) Δ FBA approach and 2) iMAT-based reaction activity analysis. Then, the associated biological processes and metabolic pathways were uncovered to analyze whether there is a relationship between the GenX and α Syn-

induced neurotoxicity-associated metabolic signatures, as described in the following subsections.

3.4.1. Δ FBA-Based Network Analysis

A normalized proteome dataset of 7-day-old female *Drosophila* brains (unpublished dataset, kindly provided by Prof. Souvarish Sarkar, University of Rochester Medical Center) with three biological replicates for each group (GenX exposure or control case) was used in the current study. The gene annotations of this dataset were converted to FlyBase gene IDs using the FlyBase (Larkin et al., 2021) and UniProt (The UniProt Consortium, 2017) databases to identify the metabolic gene coverage of the proteome data. After ensuring its high gene coverage through the comparison of the iDrosophila1 genes (Cesur et al., 2023) with those in the proteome dataset, differential proteomic analysis was carried out using the R (version 4.3.0) *limma-trend* function under the limma package by setting robust = TRUE (Law et al., 2014). To assign a single fold change value to each protein, the fold changes of the differentially abundant proteins (p -value < 0.05) with multiple measurements were filtered based on the approach outlined in Section 3.1.5. As explained before, differential reaction expressions were determined using the Δ FBA approach (Ravi & Gunawan, 2021). To this end, the filtered fold change values were mapped to iDrosophila1 reactions via the *mapExpressionToReactions* function, and the flux of the NGAM reaction was assumed to be the same between GenX exposure and control cases. The Δ FBA approach was used with a flexible threshold value ($\epsilon = 0.5$) and the differential reactions in the metabolic model were uncovered. Subsequently, a list of associated metabolic information (genes and metabolites) was compiled.

3.4.2. Reaction Activity Analysis

As an alternative approach, reaction activity analysis was applied to determine the reactions affected by the GenX exposure. First, a context-specific metabolic model was reconstructed for each sample. In the reconstruction step, small flux boundaries (i.e., 10^{-3} h^{-1} and $10^{-4} \text{ mmol g}^{-1} \text{ h}^{-1}$) were assigned for the minimum nonzero production or uptake of crucial model components (i.e., the reactions of the biomass formation, glucose uptake, and oxygen uptake) to prevent their removal from the models. For the

NGAM reaction, a 50% reduction was allowed for its experimentally measured value (8.55 mmol ATP g⁻¹ h⁻¹) (Schönborn et al., 2019). For each protein, the duplicate measurements in the normalized proteome dataset derived from the protein isoforms were filtered by choosing the maximum value in the sample-based average measurements, and the filtered data was mapped to the iDrosophila1 reactions via the *mapExpressionToReactions* function. The iMAT algorithm enabled the reconstruction of six sample-specific metabolic models for control and GenX treatment groups (three models for each group). In this step, the algorithm identified active and inactive reactions using the 25th and 75th percentiles of the whole proteomics dataset as the lower and upper threshold values. iMAT runs a mixed-integer linear programming-based optimization algorithm to keep the reactions associated with high-level proteins in the fly model while removing the reactions associated with low-level proteins, subject to mass-balance constraints around the intracellular metabolites (Walakira et al., 2021). The reconstructed control and GenX models were subsequently used to elucidate differential reaction activities, as detailed in (Lüleci et al., 2023). In this method, binary vectors were generated by assigning “0” and “1” for the active (kept) and inactive (removed) reactions in the control and GenX models. If a reaction was active in all three replicates of the GenX models and inactive in all three control models (or vice versa), it was referred to as a differential reaction. A list of the genes and metabolites involved in these reactions was created. Furthermore, these binary vectors were also used to examine the separation of control and GenX groups via the PCA approach with the *prcomp* function of R. In this step, the reactions that were inactive in all samples of both control and GenX groups were discarded from the vectors.

3.4.3. Analysis of Biological Processes and Metabolic Pathways

To integrate the predictive power of both techniques, the GenX-induced significantly regulated genes and metabolites derived from different network-based approaches (Δ FBA and iMAT-based reaction activity analysis) were merged. Using the recently developed *Drosophila*-specific web tool PANGEA (Pathway, Network and Gene-set Enrichment Analysis) (Hu et al., 2023), the significantly enriched GO biological processes and KEGG pathways were identified for FDR < 0.05. In a similar vein, the KEGG pathways associated with the GenX-induced differential metabolism were investigated for the merged metabolites using the MBROLE3 (Metabolites Biological

Role) server (Lopez-Ibanez et al., 2023). Thus, the significantly enriched KEGG pathways ($\text{FDR} < 0.05$) with at least four overlapping metabolites were determined across *Homo sapiens*. Note that MATLAB R2019a was herein used in the metabolic network-based analyses unless otherwise stated.



4. RESULTS AND DISCUSSION

4.1. Reconstruction of GMN Model of *Drosophila*

In the current section, a genome-scale metabolic network model was reconstructed for *D. melanogaster* using metabolic knowledge from different sources. To increase the accuracy of metabolic information in the model, a variety of common curation steps (revising missing or incomplete components, the standardization of gene and metabolite names, and the curation of metabolic redundancy) were carried out during the reconstruction process, as detailed in (Cesur et al., 2023). The curation processes were applied to each network covered in the study. In this regard, each metabolic component (reactions, metabolites, and genes) in the networks was revised to avoid any inconsistencies and redundancies. The general reconstruction pipeline is illustrated in **Figure 4.1**.

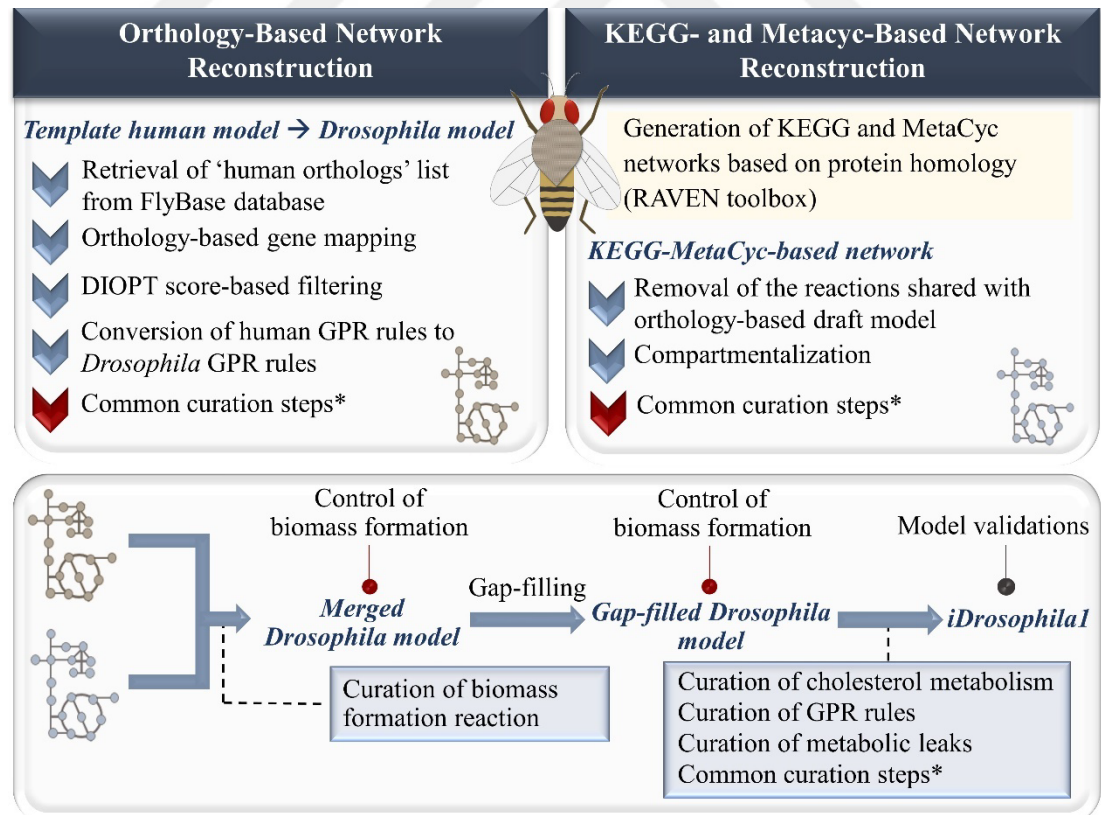


Figure 4.1: The general reconstruction process of the genome-scale metabolic network model for *Drosophila melanogaster*.

4.1.1. Reconstruction of Draft Metabolic Networks

Based on an orthology-based reconstruction strategy, a draft *Drosophila* model was first developed using a recent HMR2-derived human model (Zirngibl, 2021) as the template. The template human model has several improvements compared to the HMR2 (an updated version of the human metabolic reaction (HMR) database) (Zur et al., 2010) such as the addition of mitochondrial intramembrane space, the curation of the reactions in the beta-oxidation pathway, the elimination of atomically unbalanced processes, and the curation of GPR associations. The GPR associations were further revised in this human model based on the protein complex information in another highly curated human model, iHsa (Blais et al., 2017). This step enabled the curation of more than 300 GPR associations. In the reconstruction process of the draft fly model, the metabolic information in the template human model was transferred to the draft *Drosophila* model via the orthology-based gene mapping approach. Accordingly, at least one *Drosophila* ortholog for 1,986 out of 2,479 human model genes was detected while a single fly ortholog was assigned for around 90% of the human genes in the template model. Since the same *Drosophila* orthologs are present for some human genes, duplicated genes in the updated GPR rules were determined. After revising this metabolic redundancy, the reactions without orthologous *Drosophila* genes (except for non-enzymatic reactions) were excluded from the draft model. Furthermore, compartment information (cytosol, nucleus, lysosome, Golgi apparatus, endoplasmic reticulum (ER), mitochondrial intermembrane space, and peroxisome) along with their Gene Ontology IDs in the template model was included in the draft *Drosophila* model. Thus, this model contained a total of 6,873 reactions, 4,856 metabolites, and 1,321 genes.

To extend the metabolic information (e.g., gene coverage) in the draft model, a *Drosophila*-specific KEGG-MetaCyc network was created using the metabolic knowledge in the KEGG and MetaCyc databases. Two main issues were encountered with this network: (1) the names of the metabolites needed to be standardized and (2) there was no compartmentalization in this network. The standardization of the metabolite names was crucial for the proper merging of this metabolic network with the draft model and for preventing metabolic redundancy. Therefore, the synonymous metabolites were uncovered between both networks based on the compound names and IDs as explained in (Cesur et al., 2023), and those in the KEGG-MetaCyc network

were replaced by their synonyms in the draft *Drosophila* model. 811 KEGG-MetaCyc genes shared by the draft model were also removed to prevent metabolic redundancy. Additionally, 1,197 KEGG-MetaCyc-specific genes were identified that are predominantly associated with the fundamental pathways (e.g., the metabolism of carbohydrates, amino acids, fatty acids, and cofactors) and the metabolism of drugs and xenobiotics. Any exogenous, harmful, and life-threatening substances (e.g., pesticides, pharmaceuticals, and pollutants) are collectively referred to as xenobiotics (Misra et al., 2011; Trinder et al., 2017). According to previous studies, xenobiotics have a significant role in neurodegenerative disorders such as AD and PD (Bjørklund et al., 2020; Chin-Chan et al., 2015). Of such toxic compounds, rotenone and paraquat are frequently employed to cause oxidative stress in *Drosophila*, which in turn causes PD-like phenotypes such as the loss of DA neurons and motor abnormalities (Coulom & Birman, 2004; Hosamani, 2010; Muñoz-Soriano & Paricio, 2011; Nagoshi, 2018). Together, xenobiotics are crucial for modeling the PD phenotype in flies, and the inclusion of this information from the KEGG-MetaCyc network may support future analyses on the metabolism of neurodegenerative disorders and xenobiotics.

Another issue about the KEGG-MetaCyc-specific metabolic network is the lack of subcellular locations of the reactions. Therefore, the KEGG-MetaCyc-specific metabolic network was compartmentalized based on the Gene Ontology IDs in the draft *Drosophila* model. To this aim, a dictionary of gene-compartment pairs was created using the available sources (COMPARTMENTS, FlyBase, GLAD, QuickGO, AmiGO 2, UniProt, Reactome, CELLO2GO (cut-off of 10^{-5}), and a mass-spectrometry-based analysis). Based on the GPR associations, subcellular localizations were assigned to the reactions in the KEGG-MetaCyc network. If a gene product had multiple compartments, the corresponding reaction was repeated in the model for each compartment. If necessary, new reactions and metabolites were also included in the network. Multiple compartments were also assigned to a reaction if the associated gene products were involved in diverse compartments, and ultimately these genes were distributed to the repeated reactions in a compartment-specific manner (**Figure 4.2**). Collectively, 2,015 enzymatic reactions with 1,077 genes and 3,511 metabolites were involved in the compartmentalized KEGG-MetaCyc-specific network.

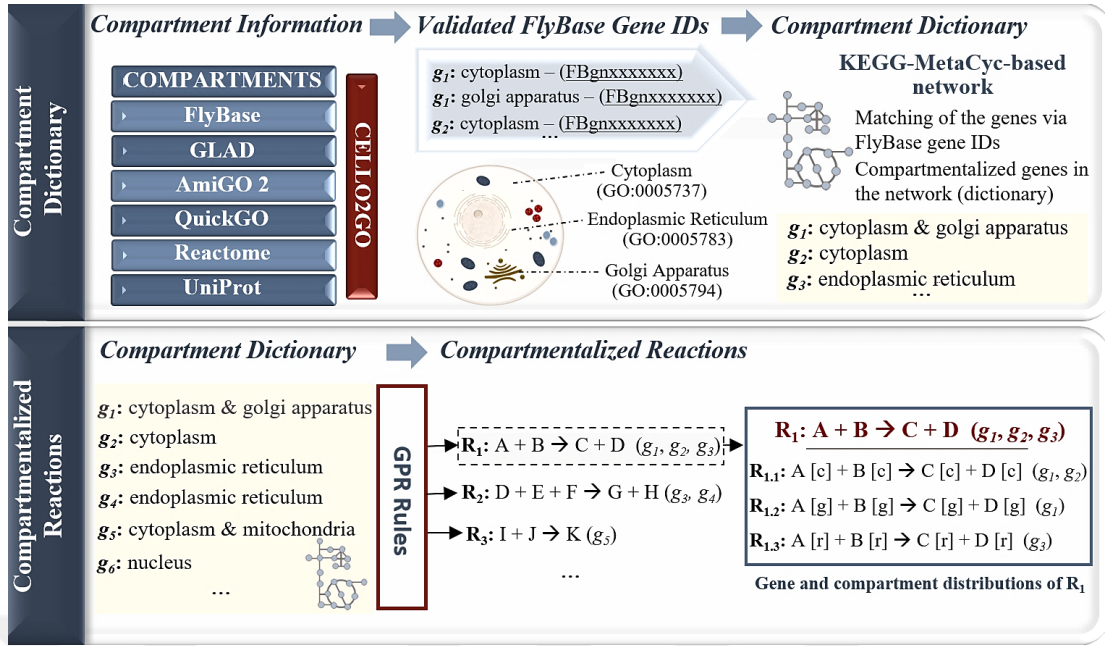


Figure 4.2: Methodology followed for the compartmentalization of the KEGG-MetaCyc network. First, compartment information along with validated FlyBase gene IDs is retrieved from different sources, and a compartment dictionary is generated by mapping the compartments to the KEGG-MetaCyc genes. This information is transferred to the reactions as illustrated in the toy network. In the compartmentalization of the example reaction (R_1), this reaction is repeated for each gene compartment (c: cytosol, g: Golgi apparatus, and r: endoplasmic reticulum), and the genes are distributed to the reactions derived from R_1 ($R_{1.1}$, $R_{1.2}$, and $R_{1.3}$).

4.1.2. Additional Curations for the Merged *Drosophila* Model

The gene coverage of the orthology-based draft *Drosophila* model was elevated to 2,398 by merging this model with the compartmentalized KEGG-MetaCyc network. On the other hand, this merged model was found to be unable to produce many cofactors and vitamins. Therefore, 29 new reactions were added to the merged model in the gap-filling algorithm step to achieve biomass production. This gap-filled model was further revised in terms of cholesterol and apocytochrome-C metabolism, GPR rules, and metabolic leaks in addition to the common curation steps, outlined in (Cesur et al., 2023).

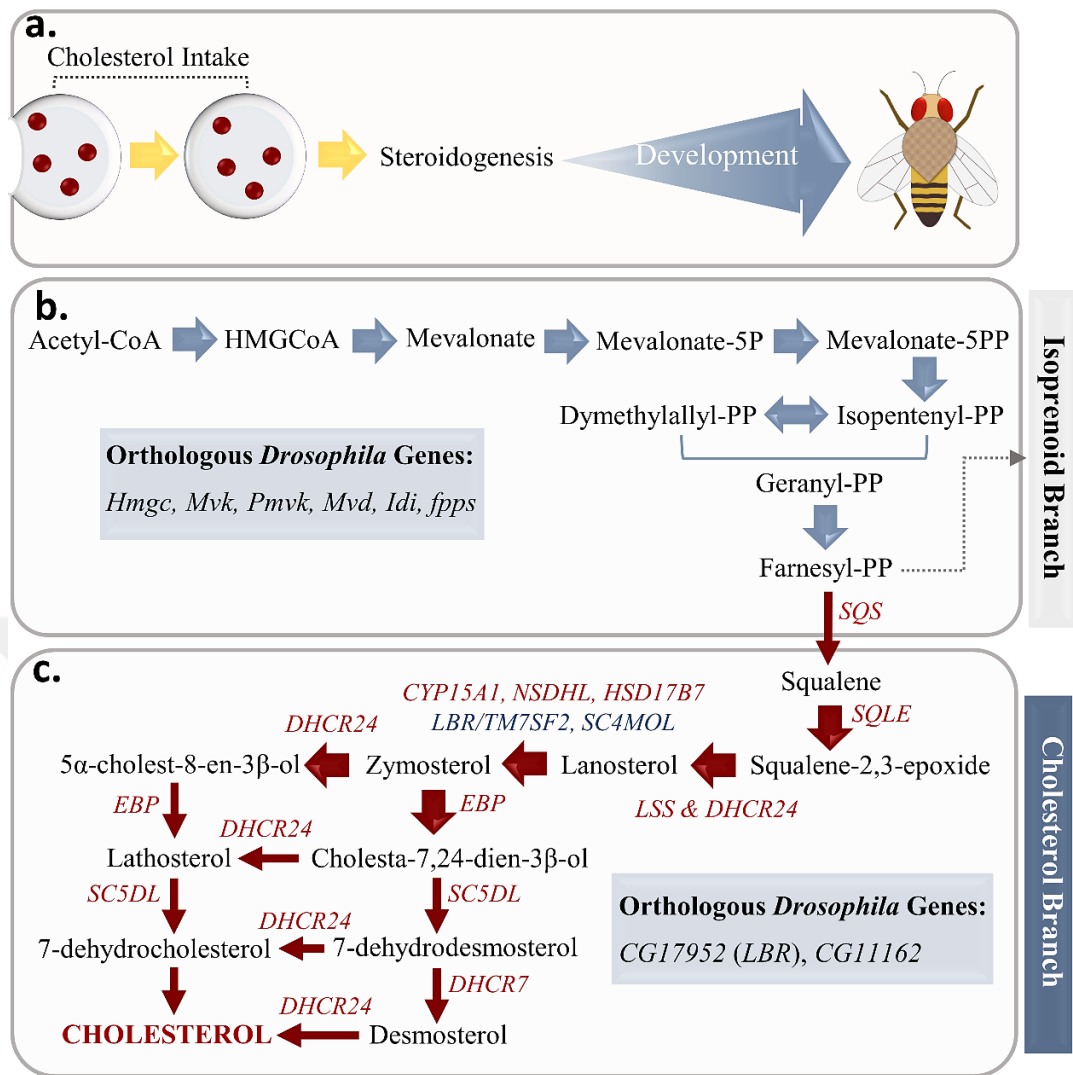


Figure 4.3: Cholesterol metabolism in *Drosophila melanogaster*. a. This organism needs cholesterol to produce steroid hormones for the regulation of developmental processes. b. The first step of the cholesterol metabolic pathway including farnesyl-PP production is conserved in all animals. c. While mammals can metabolize farnesyl-PP through two primary pathways: the cholesterol branch and the isoprenoid branch, *Drosophila* lacks many genes in the cholesterol branch (shown in red).

Cholesterol is one of the main structural elements of the *Drosophila* membrane that also serves as a precursor for steroid hormones. Steroid production is important for the generation of an adult organism (Danielsen et al., 2016; Niwa & Niwa, 2011) (**Figure 4.3.a**). However, *D. melanogaster* has been reported to be a cholesterol auxotroph due to its incomplete cholesterol biosynthesis pathway (Knittelfelder et al., 2020; Vinci et al., 2008). The mammalian 3-hydroxy-3-methylglutaryl CoA reductase enzyme produces mevalonate from 3-hydroxy-3-methylglutaryl CoA, and it is then converted to farnesyl pyrophosphate by a group of enzymes. Several fly orthologs catalyzing this

pathway, which is branched to the isoprenoid synthesis process, were reported by Santos et al. (Santos & Lehmann, 2004) (**Figure 4.3.b**). Another branch supports the synthesis of cholesterol from farnesyl pyrophosphate, and most human genes associated with this branch are not conserved in flies (Santos & Lehmann, 2004; Zhang et al., 2019) (**Figure 4.3.c**). Therefore, *Drosophila* must obtain sterols from diets (Danielsen et al., 2016; Knittelfelder et al., 2020; Vinci et al., 2008). Even though two cholesterol synthesis genes in humans (*SC4MOL* and *LBR/TM7SF2*) contain *Drosophila* orthologs, fly orthologs were not reported for a majority of the human genes such as *SQS*, *SQLE*, *LSS*, *CYP51A1*, *NSDHL*, *ERG27*, *DHCR24*, *EBP*, *SC5DL*, and *DHCR7* (Zhang et al., 2019) (**Figure 4.3.c**).

To present the cholesterol auxotrophy, *Drosophila* genes that were mistakenly defined as human orthologs in the draft model were determined (**Table 4.1**). Of these genes, *CG7724* (FBgn0036698) associated with steroid biosynthesis was assigned as the ortholog of *NSDHL* (ENSG00000147383) encoding sterol-4- α -carboxylate 3-dehydrogenase (decarboxylating) at the maximum DIOPT score of 3. Since Zhang et al. reported the lack of *NSDHL* ortholog in *Drosophila*, the related reactions responsible for the synthesis of 3-keto-4-methylzymosterol, 5 α -cholesta-8,24-dien-3-one, 4 α -methyl-5 α -cholesta-8-en-3-one, and 5 α -cholesta-8-en-3-one compounds were excluded from the model. Similarly, the reactions “HMR_1519” and “HMR_1565” were removed since *LBR* (FBgn0034657) encoding lamin B receptor was mistakenly assigned as the ortholog of *DHCR7* (ENSG00000172893) at the maximum DIOPT score of 5. The reaction “HMR_1519” produces desmosterol from 7-dehydrodesmosterol while the reaction “HMR_1565” is responsible for the conversion of provitamin D3 (7-dehydrocholesterol) to cholesterol. Additionally, eight non-conserved cholesterol biosynthesis reactions, which were included in the model in the gap-filling step, were excluded from the draft *Drosophila* model. All curations regarding cholesterol metabolism are summarized in **Table 4.1**. In addition to the cholesterol metabolism, the apocytochrome-C metabolism was curated via the addition of apocytochrome-C to the porphyrin metabolism-related reaction “HMR_4762” and the inclusion of the missing reactions associated with the apocytochrome-C metabolism in the draft model.

Table 4.1: Literature-based curations of the non-conserved cholesterol biosynthesis reactions that were incorrectly included in the draft *Drosophila* model via the gap-filling step or orthology-based gene mapping step.

Cholesterol Biosynthesis Gene	Incorrectly Matched <i>Drosophila</i> Gene	Corresponding <i>Drosophila</i> Reaction
<i>SQLE</i> (ENSG000000104549)	NA Gap-filling-aided addition	HMR_1470: squalene 2,3-oxide (squalene 2,3-epoxide) synthesis from squalene
<i>LSS</i> (ENSG000000160285)	NA Gap-filling-aided addition	HMR_1473: conversion of squalene 2,3-oxide (squalene 2,3-epoxide) to lanosterol HMR_1477: 4,4-dimethyl-14 α -hydroxymethyl-5 α -cholesta-8,24-dien-3 β -ol synthesis from lanosterol
<i>CYP51A1</i> (ENSG000000001630)	NA Gap-filling-aided addition	HMR_1478: 4,4-dimethyl-14 α -formyl-5 α -cholesta-8,24-dien-3 β -ol synthesis HMR_1479: 4,4-dimethyl-5 α -cholesta-8,14,24-trien-3 β -ol synthesis HMR_1495: 3-keto-4-methylzymosterol synthesis HMR_1496: 3-keto-4-methylzymosterol synthesis
<i>NSDHL</i> (ENSG000000147383)	<i>CG7724</i> (FBgn0036698) DIOPT score: 3 Orthology-aided addition	HMR_1505: 5 α -cholesta-8,24-dien-3-one synthesis HMR_1546: 4 α -methyl-5 α -cholesta-8-en-3-one synthesis HMR_1551: 5 α -cholesta-8-en-3-one synthesis

Table 4.1: Continue

Cholesterol Biosynthesis Gene	Incorrectly Matched <i>Drosophila</i> Gene	Corresponding <i>Drosophila</i> Reaction
<i>DHCR24</i> (ENSG00000116133)	NA Gap-filling-aided addition	HMR_1570: cholestenol synthesis from zymosterol
EBP (ENSG00000147155)	NA Gap-filling-aided addition	HMR_1553: conversion of cholestenol to lathosterol
<i>SC5DL</i> (ENSG00000109929)	NA Gap-filling-aided addition	HMR_1557: provitamin D3 (7-dehydrocholesterol) synthesis from lathosterol
<i>DHCR7</i> (ENSG00000172893)	<i>LBR</i> (FBgn0034657) DIOPT score: 5 Orthology-aided addition	HMR_1519: desmosterol synthesis from 7-dehydrodesmosterol HMR_1565: cholesterol synthesis from provitamin D3 (7-dehydrocholesterol)

The GPR rules were also revised to increase the accuracy of gene-based analyses. This was accomplished using a *Drosophila* network of 556 protein complexes (Guruharsha et al., 2011). If the fly genes were present in the same protein complex, they were connected with “AND” operators. In addition, gene associations, specifically those related to energy metabolism, were curated based on the FlyBase gene group list and previous studies (Allan et al., 2005; Attrill et al., 2016; Avval & Holmgren, 2009; Grant et al., 2010; Kay et al., 2020; Kemppainen et al., 2014; Kovacs et al., 2018; Marygold et al., 2020a; Pavlovic & Bakovic, 2013; Santos & Lehmann, 2004; Tang & Zhou, 2013; Van den Berghe et al., 1997; Wahl et al., 2005). The FlyBase gene groups contain manually curated members of distinct gene families, the subunits of protein complexes, and other functional gene sets (Attrill et al., 2016; Marygold et al., 2016). Apart from the paralogous genes, the genes involved in a protein complex were linked with “AND” operators. Thus, protein complex information was curated, and the missing genes were added to the protein complexes in the metabolic model.

In the scope of the common curation steps, the *Drosophila* model was further revised by the removal of the duplicated reactions. Such reactions were determined by

ignoring H^+ , H_2O , and free inorganic phosphate (P_i) during the reaction comparisons. The compartment information was also excluded in these comparisons because a relatively flexible compartmentation procedure was applied for assigning subcellular compartments to the KEGG-MetaCyc network. In this way, improperly compartmentalized KEGG-MetaCyc reactions were carefully determined and removed from the draft *Drosophila* model. This step also eliminated the leaking ATP problem by removing the improperly compartmentalized reactions of succinate dehydrogenase (ubiquinone), uridine 5'-monophosphate phosphohydrolase, and NADH dehydrogenase. The presence of other energy-leaking metabolites was also investigated, and several metabolites (NADH, NADPH, $FADH_2$, and H^+) were found as the leaking metabolites. The reactions associated with the metabolic leaks were determined based on the FBA-mediated double-reaction knockout simulations. They were incorrectly compartmentalized reactions or redundant reactions. One of each duplicated reaction pair was excluded from the model to prevent metabolite leakage. Ultimately, the final *Drosophila* model derived from the orthology-based draft model and KEGG-MetaCyc network has been called iDrosophila1 and it contains 8,230 reactions (5,787 enzymatic and 2,443 non-enzymatic), 6,990 metabolites, and 2,388 genes. The quality of this model was evaluated via the MEMOTE test suite. Accordingly, the iDrosophila1 model has a comparable score with the 108 models in the BiGG Models database examined by Norsigian and colleagues (Norsigian et al., 2020).

To further improve the reconstructed model, the iDrosophila1 reactions lacking subsystem (pathway) information were examined and the missing pathways in the model were included if this information was available in the KEGG database. Furthermore, the pathways derived from the KEGG-MetaCyc network were called "KM pathways" for a future revision. A high prevalence of the fundamental biological processes (e.g., cholesterol ester metabolism and the metabolisms of lipids, amino acids, and nucleotides) in the model was revealed (**Figure 4.4.a**). Triacylglycerol and cholesterol ester accumulated in the *Drosophila* fat body cells serve as the storage lipids (Liu & Huang, 2013). Under normal feeding conditions, these dietary lipids are transformed into free fatty acids, sterols, and monoacylglycerols, which are absorbed by the intestinal cells of flies. Subsequently, the resynthesized triacylglycerols are packaged into lipoproteins along with carrier proteins, cholesterol, and cholesterol

esters, and they can be utilized or stored by other tissues like adipose and liver. Thus, their excess amounts can be consumed as energy-supplying fuels (Sieber & Thummel, 2012). Another prominent pathway, xenobiotic metabolism is crucial to cope with the xenobiotics that are natural or synthetic life-threatening compounds detoxified by the animal cells via sequestration or metabolic degradation by the enzymes such as cytochrome P450 monooxygenase and esterases (Amichot & Tarès, 2021; Misra et al., 2011; Trinder et al., 2017). In addition to the iDrosophila1 pathways, the compartment distribution was examined, and a similar distribution was determined between the template human model (**Figure 4.4.b**) and the iDrosophila1 model (**Figure 4.4.c**). In both models, a high frequency of cytosolic and transport reactions was found, followed by a mitochondrial distribution (**Figure 4.4.b** and **4.4.c**).

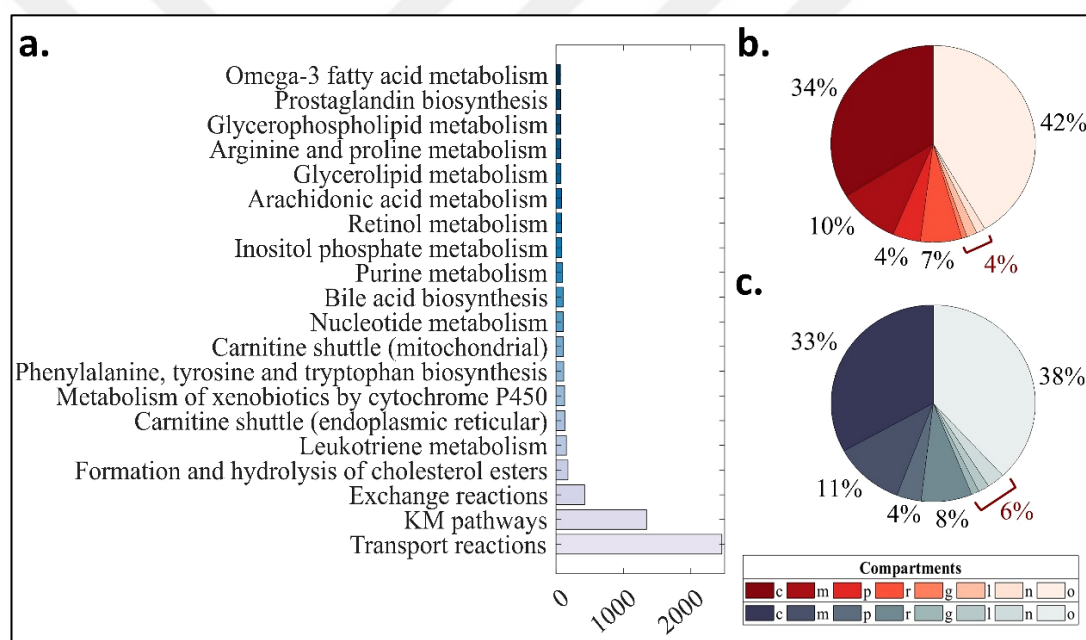


Figure 4.4: The distribution of the pathway and compartment information in the final model, iDrosophila1. a. The top ten pathways of the HMR2-based metabolic pathways in the model based on their frequencies. The percentage of compartments in b. template human model and c. iDrosophila1 model (Abbreviations: c, cytosol; m, mitochondria; p, peroxisome; r, endoplasmic reticulum; g, Golgi apparatus; l, lysosome; n, nucleus; o, others (exchange and transport reactions)).

4.1.3. Prediction of Growth Phenotypes by iDrosophila1

Drosophila consumes fermenting fruits that are high in organic acids and ethanol in nature. In a laboratory setting, this organism was shown to grow with a basic diet consisting of sucrose, lyophilized yeast, and mild organic acids. In this regard, Piper

and colleagues designed the Hd (Holidic diet) medium combining vitamins, cholesterol, sugar, essential and nonessential amino acids, several metal ions, and lipid precursors for the development of fruit flies under this dietary restriction (Piper et al., 2014). Schönborn and colleagues used this medium to mimic the growth of larval flies in laboratory conditions, and they found the optimum growth rate of the FlySilico model in this condition to be 0.088 h^{-1} (Schönborn et al., 2019). Using the same experimental constraints in the expanded Hd condition (**Table A1.1**), the maximum growth rate of iDrosophila1 was also investigated and predicted as 0.040 h^{-1} .

As previously indicated, flies are unable to produce cholesterol, even though it plays critical functions in membrane structure and signaling pathways (Vinci et al., 2008). The capacity of iDrosophila1 was evaluated to predict this phenotypic property. By limiting the maximum uptake rate of sucrose to $2.212 \text{ mmol g}^{-1}\text{h}^{-1}$ and providing the other metabolites at a flexible ratio, the fly growth was able to be simulated in the expanded Hd. In this growth condition, the maximum growth rate was estimated to be approximately 0.774 h^{-1} . Next, the growth profiles were examined at different cholesterol levels from zero to $\sim 1/10$ of the sucrose uptake rate. As expected, *Drosophila* could not grow without cholesterol in the medium while the increasing cholesterol level promoted its growth until reaching the optimal growth rate (**Figure 4.5.a**). This simulation was repeated with the Fruitfly1 and FlySilico models by applying the same constraints to limit the consumption of the Hd compounds. Three extra substances (lipoic acid, linoleate, and linolenate) were provided to allow biomass formation in the Fruitfly1 model. In the right-hand side of the biomass formation reaction in this model (version 1.1.0), ADP is missing despite its importance in establishing the balance between ATP and ADP. Therefore, this metabolite was incorporated into the biomass production reaction of Fruitfly1 for all simulations in the current study. It should be noted that the Fruitfly1 model did not produce biomass when experimentally detected uptake rates in the study of Schönborn et al. were imposed (Schönborn et al., 2019). However, the growth rate of 0.450 h^{-1} was obtained using flexible boundary constraints (see Section 3.1.3). For the flexible uptake rates, the FlySilico simulation yielded the maximum growth rate of 0.057 h^{-1} . Consistent with iDrosophila1, increasing cholesterol consumption led to a faster growth rate in FlySilico (**Figure 4.5.b**), whereas Fruitfly1 (**Figure 4.5.c**) was unable to represent this phenotypic feature.

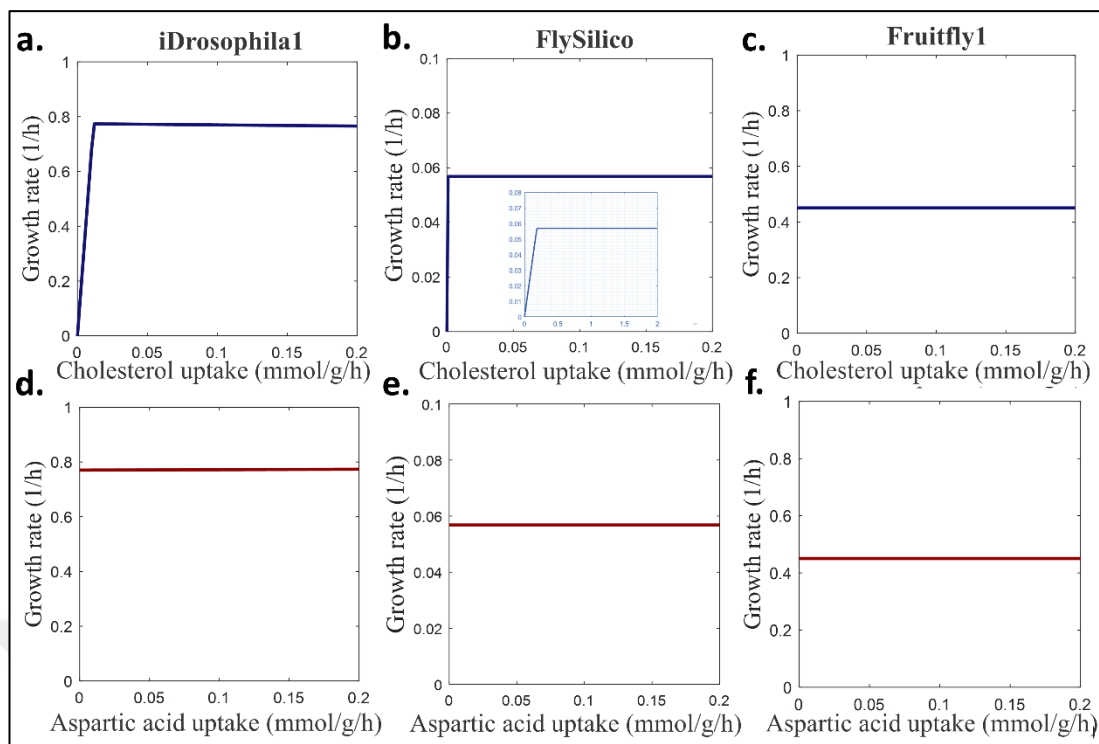


Figure 4.5: The growth profiles of *Drosophila* under expanded Hd conditions using the models iDrosophila1, FlySilico, and Fruitfly1 for increasing cholesterol intakes (a., b., and c.) and elevating aspartate uptake rates (d., e., and f.)

According to Piper et al. (Piper et al., 2014), the deficiency of the non-essential amino acid, aspartate, has no negative impact on the longevity of *Drosophila*. Similarly, it was shown that aspartate depletion did not affect biomass production due to the inherent aspartate biosynthesis system of *Drosophila*. In addition, growth rates were not influenced by the elevating level of aspartate amino acid in the iDrosophila1 (Figure 4.5.d), FlySilico (Figure 4.5.e), and Fruitfly1 (Figure 4.5.f) simulations. Conversely, ten essential amino acids (Croset et al., 2016; Manière et al., 2020) significantly supported biomass formation in the iDrosophila1 model (Figure A1.1). Collectively, iDrosophila1 could correctly predict the growth profiles of *Drosophila* across the varying levels of cholesterol and amino acids.

4.1.4. Prediction of Gene Essentiality Profile by iDrosophila1

Gene essentiality represents the requirement of a gene for survival under specific growth conditions. Even if it is especially useful to characterize cell-type-specific vital genes, the predictive capabilities of the reconstructed generic metabolic models have

been commonly assessed through gene essentiality analyses due to the lack of sufficient cell-type-specific information for many eukaryotic organisms. The prediction performance of the generic iDrosophila1 model was also evaluated via the *in silico* single-gene deletion analysis by blocking all associated reactions for each gene to be deleted if the reaction does not have any associated isoenzyme. After the deletion of each gene, optimal growth rate was predicted and the genes whose deletions caused a significant reduction in the growth rate were assumed to be essential. In this way, 128 essential genes were determined in the iDrosophila1 model for the free intake of the expanded Hd components. GO and pathway enrichment analyses of these genes showed that they are predominantly responsible for the biosynthetic processes of crucial cellular substances (e.g., nucleotides, amino acids, aminoacyl-tRNAs, cofactors, and lipids). The over-represented KEGG pathways also supported these results.

To further evaluate the performance of iDrosophila1, gene essentiality analysis was also applied to the Fruitfly1 and FlySilico models. Before these analyses, two modifications were introduced to the Fruitfly1 model. Two different cytosolic RNA synthesis reactions are available in iDrosophila1 (HMR_7161 and HMR_7162) and Fruitfly1 (MAR07161 and MAR07162). These reactions need nucleoside triphosphates (NTPs) and nucleoside diphosphates (NDPs) as substrates, respectively, and both metabolites are significant energy sources due to harboring phosphoanhydride bonds. NTPs (ATP, GTP, UTP, and CTP) are required for nucleic acid biosynthesis by DNA-directed RNA polymerase (RNAP) enzymes, and each RNAP catalyzes the production of distinct RNA classes from ribosomal RNAs to non-coding RNAs (Gottesman & Mustaev, 2019; Marygold et al., 2020b). Both the nuclear and mitochondrial genes encoding RNAP subunits are present in the Fruitfly1 and iDrosophila1 models. On the other hand, polynucleotide phosphorylase (PNPase) catalyzes another RNA metabolism-related reaction in the presence of NDPs (Gottesman & Mustaev, 2019; Pajak et al., 2019). These conserved enzymes manage RNA turnover through the degradation of mitochondrial RNAs (Das et al., 2011; Pajak et al., 2019). It was recently suggested that PNPase and the ATP-dependent RNA helicase SUV3 form RNA degradosome complex in the fly mitochondria for mRNA decay (Pajak et al., 2019). It has been shown that PNPases (EC 2.7.7.8) are involved in RNA polymerization (non-template-encoded RNA synthesis), which results in a

high Pi level and can also facilitate RNA phosphorolysis (Gasteiger et al., 2003; Gottesman & Mustaev, 2019). On the other hand, RNAPs can irreversibly polymerize NTP. While only the PNPase-encoding *Drosophila* gene (FBgn0039846/CG11337) is involved in the catalysis of NDP polymerization (HMR_7162) in the iDrosophila1 model, RNAP genes were detected in the GPR rules of both NDP polymerization (MAR07162) and NTP polymerization (MAR07161) reactions in the Fruitfly1 model. The NDP polymerization driven by only PNPase in the iDrosophila1 model is compatible with both the iHsa model (Blais et al., 2017) and our template human model (Zirngibl, 2021). Pajak and colleagues also determined that the “CG11337” gene is the fly ortholog of human PNPase (Pajak et al., 2019). Together, the GPR rule of the Fruitfly1 reaction (MAR07162) was updated by replacing the RNAPs with the PNPase. Furthermore, the reversibility of this reaction was changed in both the Fruitfly1 and iDrosophila1 models to represent its reversible characteristics.

The second modification in the Fruitfly1 model is related to the GPR association of the nucleo-cytoplasmic DNA transport process in Fruitfly1 (MAR08639), where nuclear pore complex genes were assigned. Nuclear pore complexes are composed of 30 diverse proteins known as nucleoporins and facilitate the free diffusion of water, sugars, and ions in addition to their functions in macromolecular trafficking (such as the transport of RNAs, proteins, and ribosomal subunits) between the cytoplasm and nucleus (Ibarra & Hetzer, 2015; Kuhn & Capelson, 2018; Wente & Rout, 2010). The *Drosophila* nucleoporins, such as Nup98 and Nup62, exhibit dynamic chromatin binding behavior in the nucleoplasm; nevertheless, their function is limited to mediating transcriptional controls (Kuhn & Capelson, 2018). Moreover, the artificial DNA transport reactions in the iDrosophila1 and Fruitfly1 models only represent the contribution of DNA to cytosolic biomass formation. Therefore, nucleoporin genes were discarded from the artificial nucleo-cytoplasmic DNA transport reaction of the Fruitfly1 model. The iDrosophila1 model lacks any genes in the corresponding transport reaction (HMR_8639) in agreement with the template human model (Zirngibl, 2021) and the iHsa model (Blais et al., 2017). According to the updated GPR rules, essential and non-essential gene sets in the expanded Hd were identified for Fruitfly1 and FlySilico.

Table 4.2: Predictive metrics to assess the performances of the generic fly models.

Metrics	Fly Metabolic Network Models		
	iDrosophila1 (This study)	FruitFly1	FlySilico
Sensitivity	0.16	0.09	0.03
Specificity	0.96	0.96	0.97
Precision	0.74	0.72	0.50
Accuracy	0.61	0.52	0.52
F1 score	0.26	0.16	0.05
MCC	0.20	0.11	0.01

The iDrosophila1-specific essential genes ($n = 64$ out of 128 genes) were predominantly found to be associated with the metabolism of nucleotides, cofactors, lipids, amino acids, and aminoacyl-tRNAs. Metabolic processes linked to vitamins, cofactors, amino acids, aminoacyl-tRNAs, and nucleotide sugars were shown to be significantly enriched for the Fruitfly1-specific essential genes ($n = 33$) while only six vital genes related to the metabolism of amino acids and carbohydrates were predicted by the FlySilico model. The essential gene sets derived from each generic fly model were then compared to the essential gene list obtained from the OGEE database (Gurumayum et al., 2020). Using four confusion matrix categories (true positive (TP), false positive (FN), true negative (TN), and false negative (FN)), the ratio of correct essentiality predictions to incorrect results was assessed. Based on these categories, several metrics (sensitivity, specificity, accuracy, precision F1 score, and MCC) were estimated. For evaluating the binary classification of extremely imbalanced datasets (e.g., many TNs but few TPs, or vice versa), accuracy and F1 score (two of the most popularly adopted metrics) are especially useful (Chicco & Jurman, 2020). Since MCC is a powerful metric in all confusion matrix categories for predictions with imbalanced datasets (Chicco et al., 2021; Chicco & Jurman, 2020), this metric was also included in the comparisons of model performances, represented in **Table 4.2**. The sensitivity of iDrosophila1 predictions was determined to be considerably higher than the other fly models. Unsurprisingly, the FlySilico model had the worst sensitivity score due to the lack of protein complex information. Unlike the low sensitivity values, the specificity results showed that the fly models could properly categorize up to ~96-97% of the non-essential genes. The higher precision values were obtained for the iDrosophila1 (TP: 90 and FP: 32) and Fruitfly1 (TP: 64 and FP: 25) models compared

to FlySilico (TP: 3 and FP: 3), representing a higher ratio of correctly detected essential genes within all predicted essential gene sets. It should be noted that ten essential genes predicted by Fruitfly1, and six essential genes predicted by iDrosophila1 were not included in any confusion matrix categories due to a lack of evidence. Since only two categories were taken into account by the binary metrics, additional metrics relying on at least three confusion matrix categories were employed. According to three adopted measures (accuracy, F1 score, and MCC), iDrosophila1 outperformed the other models.

The F1 score was identified to be dramatically low for the FlySilico predictions, whereas there was a better compromise between sensitivity and precision for the iDrosophila1 predictions. One disadvantage of the F1 score is the tendency of this value to converge into smaller values for low sensitivity or precision. The misclassification potential of this metric for the datasets with imbalanced prevalence is another issue with this metric (Hand et al., 2021). Since the correctly predicted non-essential genes (TN) were prevalent in the predicted essential/non-essential gene sets for all fly models, MCC scores (ranging from -1 to +1) were calculated for a better classification of this unbalanced data. Based on this metric, iDrosophila1 exhibited a better predictive performance than the other models. To examine the effect of the medium constraints on the model performances, the analyses were repeated in the expanded Hd medium with flexible uptake rates. In this growth condition, 12 additional true essential genes were predicted using iDrosophila1, which enhanced sensitivity (0.18) and F1 score (0.29). On the other hand, limiting the uptake rates did not affect the prediction scores determined by the Fruitfly1 and FlySilico models. Taken together, iDrosophila1 was shown to be superior or comparable to the other models for all metrics regardless of the uptake levels of the nutrients.

4.1.5. iDrosophila1-Mediated Metabolic Profiling of PD

To assess the ability of iDrosophila1 for omics data-based predictions, differential pathways in the second most prevalent age-related neurodegenerative disorder, PD, were analyzed (Aryal & Lee, 2019; Nagoshi, 2018). Mitochondrial dysfunction is among the main factors contributing to this disorder. For proper mitochondrial homeostasis and neuroprotection, mitochondrial fusion and fission processes enable the exchange of respiratory proteins and the elimination of damaged mitochondria

(Gouider-khouja et al., 2003; Johansen et al., 2018; Sasaki et al., 2004; Suda & Mizuno, 1998). A decrease in mitochondrial membrane potential during the fission process supports the accumulation of PINK1 (PTEN-induced new kinase 1) proteins on the outer mitochondrial membrane, and the Parkin (a ubiquitin E3 ligase) proteins are also recruited from the cytosol to mitochondria (Sekine & Youle, 2018). Thus, their combined action supports the mitophagy-mediated degradation of the defective mitochondria (Sekine & Youle, 2018) to prevent a PD-like phenotype in *Drosophila* such as DA neuron loss, locomotor abnormalities, and reduced lifespan (Parker-Character et al., 2021; Xu et al., 2020).

Using transcriptome datasets from the young and middle-aged *Drosophila* models of PD (Celardo et al., 2017), the metabolic changes triggered by *pink1* and *parkin* mutations were examined. Based on the significant fold changes induced by *pink1* and *parkin* mutations, differential iDrosophila1 reactions were identified using Δ FBA (**Figure 4.6.a**). The key enriched metabolic pathways of the genes involved in these reactions (**Figure 4.6.b**) are mainly associated with fundamental pathways (lipid metabolism, nucleotide metabolism, and amino acid metabolism). Glycine and serine metabolism is especially important for *de novo* nucleotide biosynthesis, which uses a variety of substrates (glycine, glutamine, and 10-formyl-tetrahydrofolate). The *de novo* nucleotide biosynthesis is the main strategy for providing DNA precursors for proliferating cells (Holland et al., 2011; Legent et al., 2006; Tufi et al., 2014). Folate supplementation therefore shows a stimulatory effect in *de novo* nucleotide production and has a protective role in reducing mitochondrial abnormalities (Celardo et al., 2017; Tufi et al., 2014; Villa et al., 2019). For both *pink1* and *parkin* mutants in different age groups, “purine and pyrimidine metabolism” and “folate biosynthesis” were among the enriched KEGG pathways. Importantly, a significant decline in folate production among PD patients was shown to be correlated with the drop in bacterial folate (vitamin B₉) production. According to another previous study, a diet supplemented with folate was found to help *pink1* mutant flies avoid losing their DA neurons (Tufi et al., 2014). The common metabolic over-representation of another vitamin, riboflavin (vitamin B₂) was also determined in all mutant groups. This vitamin supports a healthy folate cycle and energy metabolism in addition to its neuroprotective functions by reducing glutamate excitotoxicity, mitochondrial dysfunction, oxidative stress, and NF- κ B-induced neuroinflammation (Wong et al.,

2014). Similarly, Parkin is important for stable glutamatergic synapses, and its mutation leads to an increased susceptibility to glutamate neurotoxicity, which is associated with the onset of PD (Marashly & Bohlega, 2017). This may be proof of the relationship between the mitochondrial quality control mechanism and riboflavin metabolism.

The “selenocompound metabolism” was another enriched process for the 30-day-old *pink1* mutant (**Figure 4.6.b**). Selenium modifies the gut flora and combats oxidative stress and inflammation to promote neuroprotection. The supplementation of this essential micronutrient has been shown to result in an elevated level of *Akkermansia* in rodents. This bacterium plays a role in immune modulation, host metabolic regulations, and gut barrier protection. Human selenium levels decrease with age, and in recent years, there has been an increase in interest in nutraceuticals and functional foods including selenium (Arias-Borrego et al., 2019; Callejón-Leblic et al., 2021). “β-Alanine metabolism” is also associated with neuroprotection (**Figure 4.6.b**) and this non-proteinogenic amino acid serves as a precursor of carnosine dipeptide, responsible for muscular contraction, metal chelation, proton buffering, and antioxidation. Carnosine dipeptide supplementation showed its therapeutic potential to ameliorate decreased motor activity in PD (Rezende et al., 2020). In agreement with these observations, there is a correlation between the physiopathology of PD and the changed amount of β-alanine (Solana-manrique et al., 2022). More precisely, this substance increases the levels of extracellular GABA and dopamine levels in SNc (Allman et al., 2018). Besides, it has been demonstrated that taurine and β-alanine supplementation enhanced fatigue resistance in the muscles of mice and reduced contraction-induced oxidative stress (Horvath et al., 2016). Another β-amino acid, taurine, is implicated in Ca²⁺ homeostasis, neuromodulation, and the regulation of antioxidant processes (**Figure 4.6.b**). It can regulate dopamine release and DA neuron activity when present at high levels in SNc. Additionally, “nicotinate and nicotinamide metabolism” was significantly enriched for the genes of the differential reactions in the *pink1* mutant flies (**Figure 4.6.b**). Nicotinamide riboside has been recently shown to exhibit neuroprotective properties and elevate cerebral NAD levels in PD patients through the regulation of gene expressions associated with the oxidative stress response, inflammatory response, histone acetylation, mitochondrial respiration, and proteasomal metabolism (Brakedal et al., 2022).

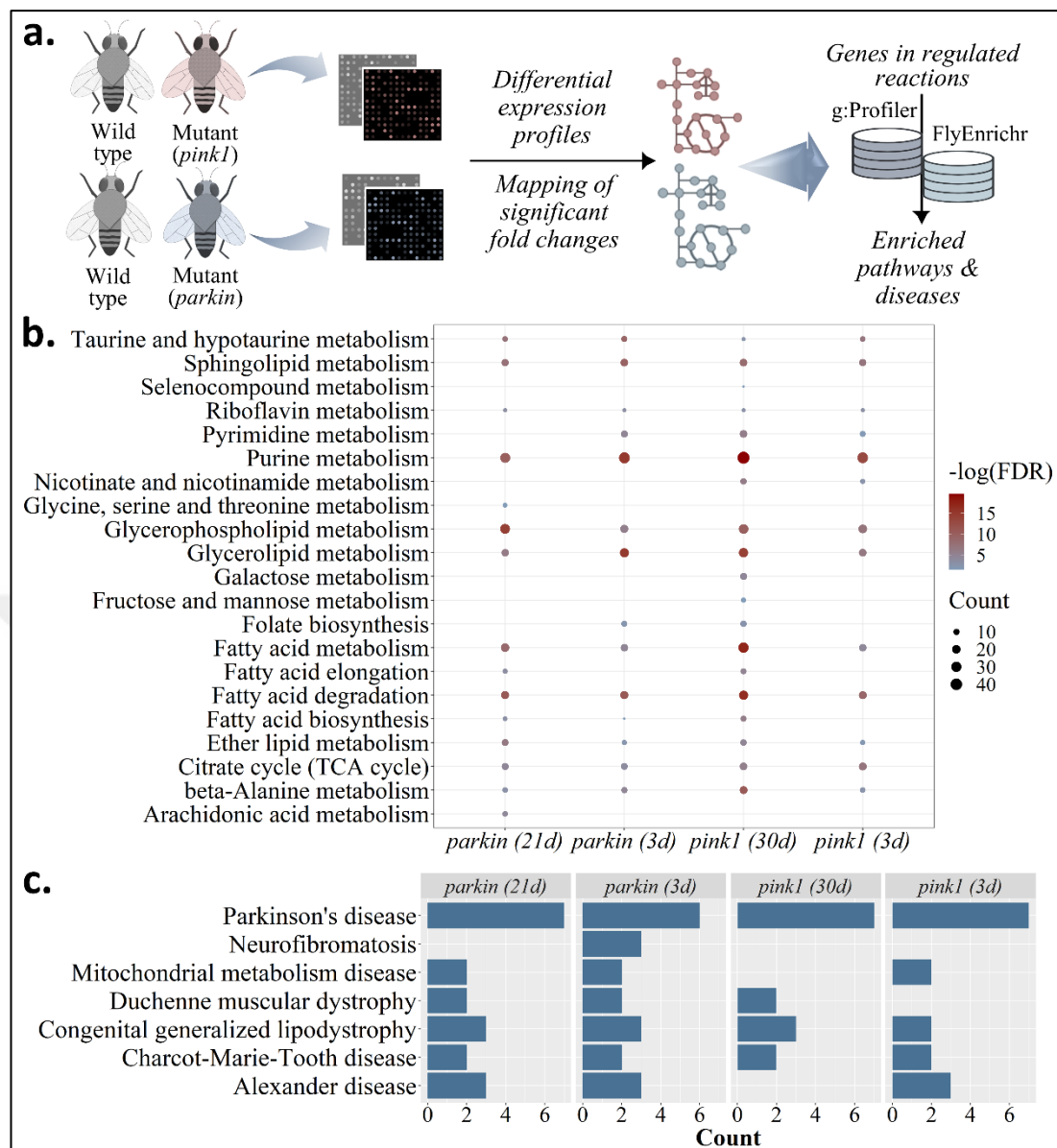


Figure 4.6: Enriched pathway and disease terms associated with *pink1* and *parkin* mutations. a. Identification of the genes in the differential reactions based on the gene expression fold changes to determine b. enriched pathways and c. diseases in different age groups (3d: 3-day-old, 30d: 30-day-old, and 21d: 21-day-old).

The genes affected by *pink1* and *parkin* mutations were also found to be linked to lipid metabolic pathways, which include “fatty acid metabolism”, “fatty acid degradation”, “glycerolipid metabolism”, “arachidonic acid metabolism”, “sphingolipid metabolism”, “glycerophospholipid metabolism”, and “ether lipid metabolism” (Figure 4.6.b). PINK1 and Parkin proteins accumulated at the ER-mitochondria contact sites regulate inter-organelle communication to modulate lipid metabolism, Ca^{2+} signaling, and mitophagy (Barazzuol et al., 2020; Fais et al., 2021; Gómez-Suaga

et al., 2018). Lipid metabolism and mitochondrial activity are closely related, so *parkin* knock-out mice were shown to possess changed lipidomic compositions in their mitochondrial membrane (Fais et al., 2021). Furthermore, fatty acid oxidation plays a role in the synthesis of acetyl-CoA in the TCA cycle. The *pink1* mutant flies were observed to have downregulation in many tricarboxylic acid molecules (Tufi et al., 2014). Likewise, differential TCA cycle and carbon metabolism were identified in the *pink1* and *parkin* mutants (**Figure 4.6.b**). According to the study of Requejo-Aguilar and colleagues, *pink1* mutation induces the reprogramming of glucose metabolism to support metabolic adaptability in agreement with our findings (Requejo-Aguilar et al., 2014). Furthermore, an increase was observed in the fructose levels of PD patients, indicating a possible protective effect of this metabolite against oxidative stress via a respiratory shift in the early stages of the disease. Besides, carbon sources are important for protein modifications by glycation or glycosylation (Videira & Castro-Caldas, 2018). Accordingly, the enrichment of metabolic pathways associated with several glycating agents (e.g., fructose, mannose, and galactose) was determined in 30-day-old *pink1* mutants (**Figure 4.6.b**).

For further confirmation of the model's predictive ability, the enriched disease terms of the genes involved in the regulated reactions were identified. To this aim, these genes were compared to the gene set library in FlyEnrichr that was derived from "Human Disease data from FlyBase (2017)". The genes affected by *pink1* or *parkin* mutation were found to be linked to neurogenerative disorders, muscle diseases, and mitochondrial diseases. Thus, these terms further supported the accuracy of the transcriptome-guided iDrosophila1 predictions (**Figure 4.6.c**). Among these diseases, "Duchenne muscular dystrophy" is distinguished by mitophagy and malfunctioning mitochondria. Defective mitochondria can lead to increased inflammation, which triggers disease pathophysiology (increased fibrosis and muscle damage) (Reid & Alexander, 2021). In a similar vein, "Charcot-Marie-Tooth" disease is also linked to abnormal mitochondrial processes, causing significant muscle impairments (Nandini et al., 2019; Schiavon et al., 2021). Consistently, "mitochondrial metabolism disease" and "Parkinson's disease" were found to be over-represented (**Figure 4.6.c**). Collectively, the transcriptome-based network analysis confirmed the power of iDrosophila1 to gain insight into complicated human diseases. Thus, this metabolic network was used in three different applications to better understand (i) the connection

of vitamin metabolism with PD, (ii) the role of metabolic interplay between the EECs and gut microbiota at the onset of PD, and (iii) the neurotoxic potential of GenX in *Drosophila*. These network-based studies are detailed in the following sections.

4.2. Vitamin Metabolism in Parkinson's Disease

In this thesis study, the reconstructed iDrosophila1 model was first applied to the analysis of vitamin metabolism in the PD process. To this end, differential biological processes and metabolic pathways in PD brains were first determined using network-based analyses. In this step, the RNA-seq datasets obtained by the expression of α Syn in the bulk brain tissue or specific brain cells (neuronal, glial, or both) of *D. melanogaster* were used. The effect of age was excluded from the bulk tissue-level samples through the covariate adjustment analysis without a significant modification in the dataset (**Figure A1.2**).

Significantly affected metabolic patterns associated with the α Syn pathology were comprehensively characterized using these datasets. Based on the PD-associated over-represented terms, a regulated vitamin list was created. Using the same strategy, the conservation of various PD-related biological processes and metabolic pathways in AD and HD was determined, which emphasizes the importance of vitamin metabolism among different neurodegenerative disorders. In the next step, the effect of α Syn production on vitamin metabolism was further characterized by uncovering reporter metabolites. These initial analyses are important to confirm and extend the regulated vitamin list (**Figure 4.7.a**). An additional approach was then used for the validation of the differential vitamin metabolism in PD through the identification of regulated reactions and the associated vitamins via the reaction activity analysis. The results supported the downregulation of some vitamins in the PD process (**Figure 4.7.b**). In the last step, the metabolic pathways affected by an increasing level of each PD-related vitamin were determined to characterize their metabolic roles and significance in PD (**Figure 4.7.c**). All steps covered in this section are summarized in **Figure 4.7**.

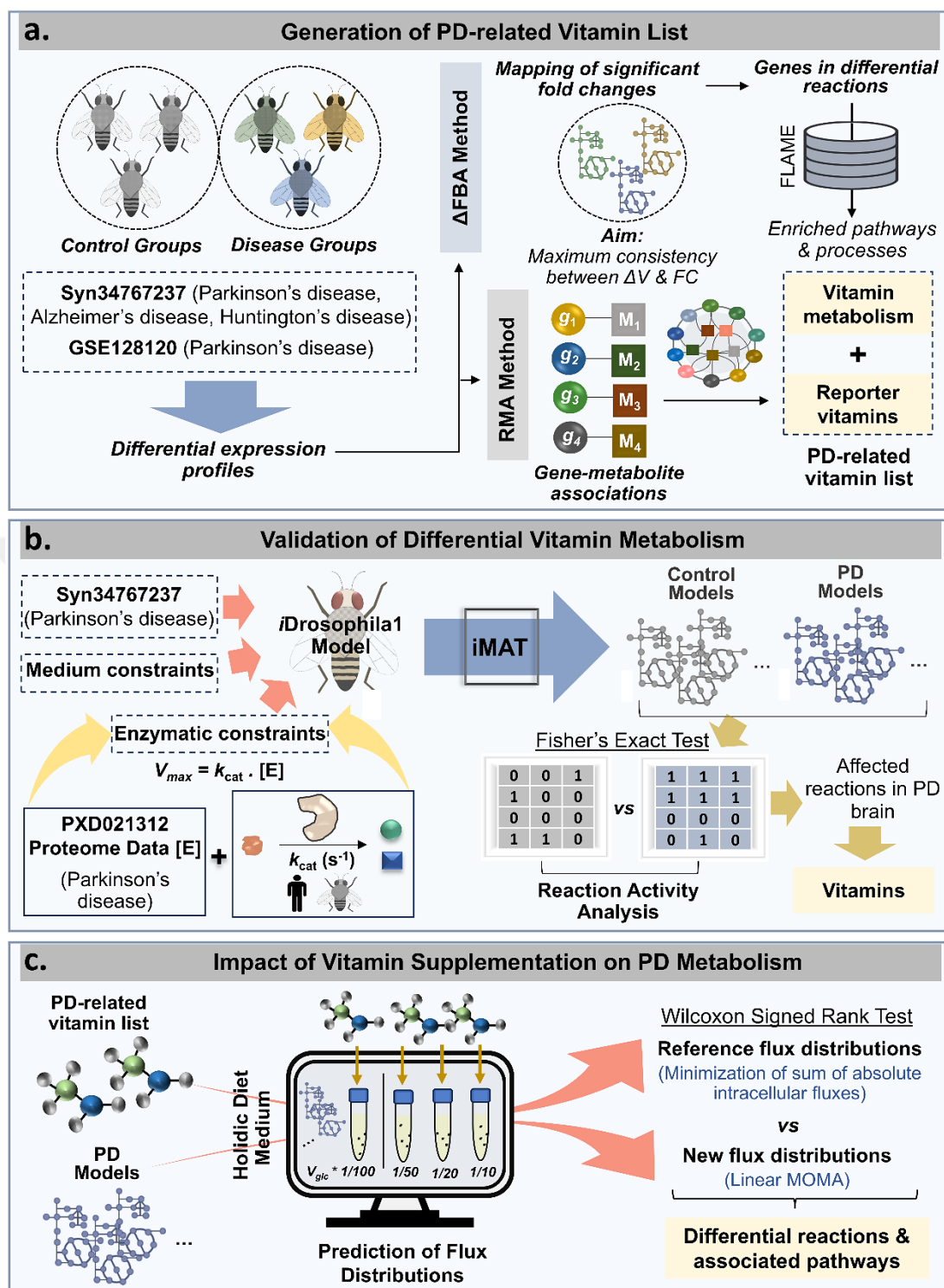


Figure 4.7: The general methodology followed to characterize the vitamin metabolism in the PD brain consists of a. the generation of a PD-related regulated vitamin list, b. validation of the regulations of these vitamins, and c. elucidation of their effects on PD metabolism.

4.2.1. Differential Metabolic Patterns in Neurodegenerative Diseases

Given that PD causes significant metabolic dysfunctions in patients, PD-related metabolic patterns were examined by determining regulated reactions and the associated genes for tissue-level and cell type-specific expression of α Syn in the *Drosophila* brain (**Figure 4.7.a-c**). In this way, a list of PD-related vitamins was identified. For the glial expression of this protein, a lower number of regulated reactions were determined relative to the neuronal expression (**Table 4.3**). The PCA plot and dendrogram of the transcriptomics data also showed that the samples with glial α Syn expression were clustered with the controls while the samples expressing the α Syn protein in only neuronal cells or both cell types clustered together (**Figure A1.3a** and **Figure A1.3b**). The genes in the regulated reactions were subsequently characterized via the significantly enriched biological processes and metabolic pathways (**Figure 4.8.a**).

Table 4.3: The numbers of Δ FBA-predicted regulated reactions, the associated unique compartment-free metabolites, and genes for each disease group.

Disease Group	No. of:		
	Reactions	Metabolites	Genes
PD (α Syn - Bulk)	178	223	75
PD (α Syn - Neuron)	112	249	172
PD (α Syn - Glia)	75	208	135
PD (α Syn - Both)	93	121	99
AD (A β 42)	57	121	35
AD (Tau)	373	332	352
HD (HTT)	186	238	154
HD (HTT, FL)	387	341	385

The glial α Syn expression regulated additional signaling pathways (e.g., “signaling by G-protein-coupled receptor (GPCR)” and “Hedgehog off state”) compared to the neuronal expression (**Figure 4.8.b**). *Drosophila* has a conserved repertoire of the G-protein subunits, and these proteins can be expressed by astrocytes to trigger enzymes responsible for calcium efflux from ER for neurotransmitter secretion, synaptic plasticity, and glial activity (Wong et al., 2023). The regulation of synaptic plasticity by astrocytic Ca^{2+} dynamics regulates memory formation and retrieval (Escalada et al., 2024). In agreement with this phenomenon, “olfactory learning”, “associative

learning”, and “short-/medium-term memory” were enriched for the glial group (**Figure 4.8.b**). In addition, Hedgehog signaling pathway was also among the enriched pathways, and it is another evolutionarily conserved pathway in flies. The ageing-dependent depletion of the Hedgehog was previously reported to reduce lifespan, locomotor activity, and DA neuron integrity in *Drosophila* while its glial overexpression improved longevity, proteostasis, locomotion, and defects in the DA neurons. Moreover, activation of this signaling pathway in the *Drosophila* AD model rescued the harmful effects of A β (1-42) through the downstream chaperone targets (Rallis et al., 2020). Collectively, the AD-related intellectual defects also seen in the early PD stages may be associated with glial α Syn expression.

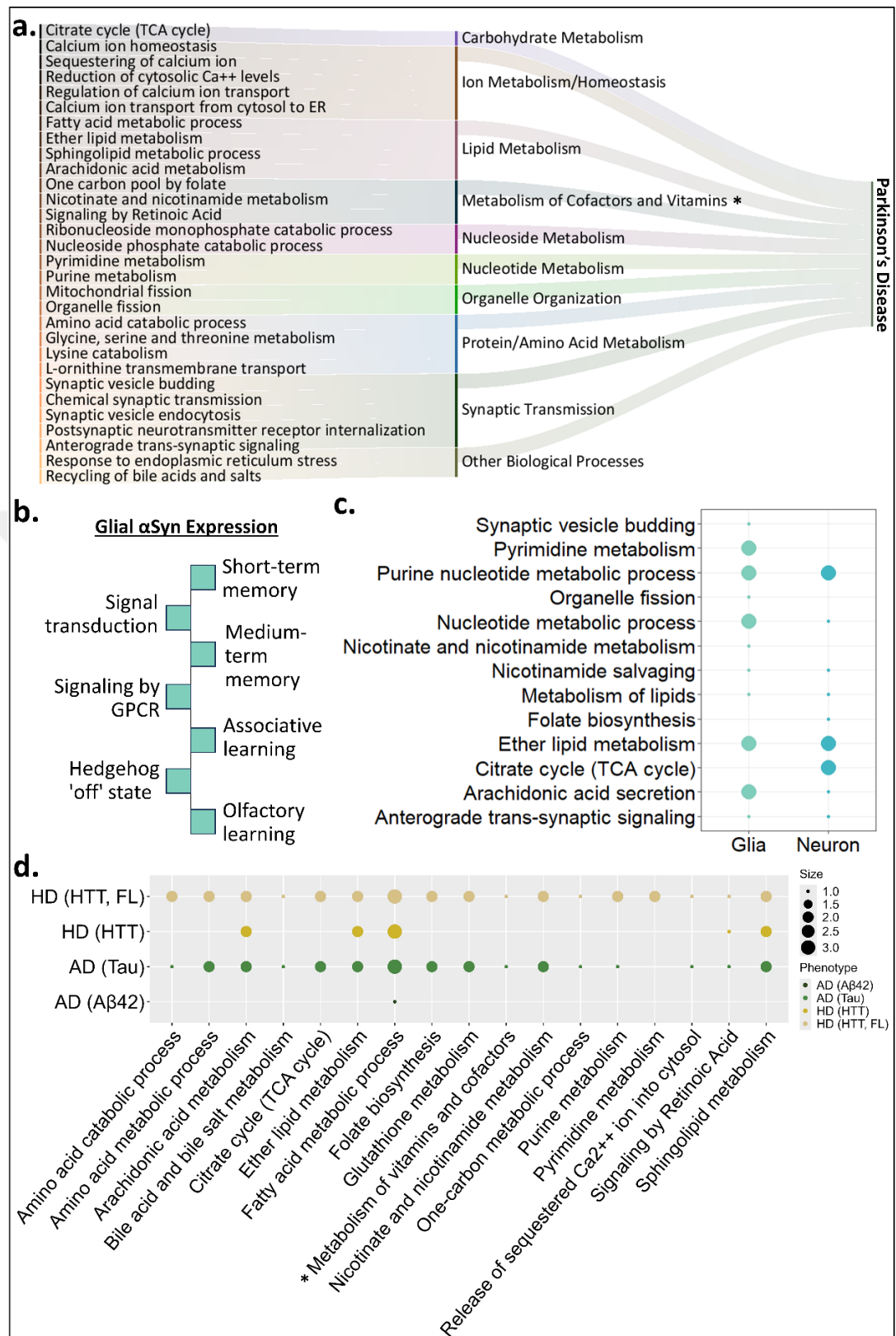
The bulk tissue-level and cell type-specific α Syn expression also exhibited common metabolic changes in the fly brain. The significantly enriched pathways of lipid metabolism were found to be the most prevalent terms for all PD groups. Eight different classes of lipids (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides) are present in the human brain, and defective lipid metabolism is linked with numerous neurodegenerative disorders (Shamim et al., 2018; van Kruining et al., 2020). Lipid composition is among the crucial factors determining the interactions of α Syn proteins with the cellular membrane. These interactions modulate the oligomerization of these proteins, and a protective role of arachidonic acid against the production of toxic α Syn forms was reported (Alecú & Bennett, 2019; Iljina et al., 2016). Arachidonic acid metabolism was also found to be changed in PD regardless of the α Syn-expressing cell types (**Figure 4.8.a** and **Figure 4.8.c**).

Nucleic acids are other macromolecules that were significantly affected in all PD groups. In other words, metabolic processes of the purines or pyrimidines were enriched by both tissue-level and cell type-specific expression of α Syn. The *de novo* nucleotide biosynthesis is based on the production of necessary DNA precursors to drive essential metabolic processes in the organism (Holland et al., 2011; Legent et al., 2006). Consistently, the regulation of “one-carbon pool” and/or “folate (vitamin B₉) metabolism” were shown for all PD groups except for the glial α Syn expression (**Figure 4.8.a** and **Figure 4.8.c**). Dietary folate supports mitochondrial activity for protection against elevated oxidative stress in the *Drosophila* model of PD. Ageing process considerably affects folate metabolism in different organisms including

Drosophila (Escalada et al., 2024). The pathways of other neuroprotective vitamins including vitamin A and vitamin B₃ were also over-represented for at least one PD group, suggesting their potential as nutritional supplements.

Structures of the macromolecules (e.g., lipids and nucleic acids) and energy metabolism can be damaged by mitochondrial dysfunction and oxidative stress in the PD process (Maszka et al., 2023; Yan et al., 2020). The “tricarboxylic acid cycle (TCA)” was found to be significantly enriched for the tissue-level and neuronal expression of the α Syn protein (**Figure 4.8.a** and **Figure 4.8.c**). Similarly, the over-representation of “mitochondrial fission” was observed in the PD samples (**Figure 4.8.a**). Mitochondrial activity is regulated by the mitochondrial fusion and fission processes in a healthy cell, and unfolded α Syn monomers can regulate this cellular process. On the other hand, the neurotoxic α Syn oligomers lead to the maintenance of defected mitochondria and concomitant elevated ROS levels (Maszka et al., 2023; Risiglione et al., 2021). In PD patients, the selective depletion of GSH has been shown (Martin & Teismann, 2009; Segura-Aguilar & Mannervik, 2023; Smeyne & Smeyne, 2013). Together, the enrichment of “glutathione metabolism” for the cell type-specific α Syn expression confirmed the altered ROS process in PD (**Figure 4.8.a**).

In addition to the oxidative stress, a relationship was identified between the expression of α Syn in the brain tissue and ER stress and calcium homeostasis based on the significantly enriched terms (e.g., “reduction of cytosolic Ca⁺⁺ levels”, “regulation of calcium ion transport”, “calcium ion transport from cytosol to ER”, and “response to endoplasmic reticulum stress”) (**Figure 4.8.a**). The α Syn expression can affect Ca²⁺ homeostasis and the accompanying stimulation of ER stress. The loss of DA neurons was reported to be recovered by the overexpression of stress response genes (Colla, 2019). Ca²⁺ ions can also influence the interactions between α Syn and small synaptic vesicles, which are likely associated with the trafficking and recycling of the synaptic vesicles (Cai et al., 2020). “Synaptic vesicle endocytosis”, “synaptic vesicle budding”, “chemical synaptic transmission”, and “anterograde trans-synaptic signaling” were found to be commonly enriched for all PD groups (**Figure 4.8.a** and **Figure 4.8.c**).



The conservation of PD-associated metabolic pathways including vitamin metabolism was also investigated comparatively with other neurodegenerative disorders. In this regard, regulated reactions were determined for the AD (A β 42 and tau) and HD groups (HTT^{FL}[Q200] and HTT^{NT231}[Q128]) through Δ FBA (**Figure 4.7.a**). A lower number of affected reactions were detected for the AD and HD groups harboring mutant A β 42 or HTT^{NT231}[Q128] proteins compared to mutant tau or HTT^{FL}[Q200] proteins (**Table 4.3**). The differential PD profile was compared to the AD- and HD-associated metabolic regulations. Based on the enrichment analyses, many PD-related biological processes and metabolic pathways (e.g., “citrate cycle (TCA cycle)”, “purine metabolism”, “pyrimidine metabolism”, “fatty acid metabolic process”, “arachidonic acid metabolism”, “bile acid and bile salt metabolism”) were identified to be conserved in AD and/or HD (**Figure 4.8.d**). Of the enriched terms, conservation of the regulations in the vitamin metabolism (e.g., “metabolism of vitamins and cofactors”, “signaling by retinoic acid (vitamin A)”, “thiamin (vitamin B₁) metabolism”, riboflavin (vitamin B₂) metabolism, “nicotinate (vitamin B₃) and nicotinamide metabolism”, “folate (vitamin B₉) biosynthesis”, “ascorbate (vitamin C) metabolism”, and “calciferol (vitamin D) metabolism”) are especially promising considering their potentials as dietary supplements. Therefore, a list of the PD-related conserved vitamins was compiled by combining the compounds obtained for all PD groups.

4.2.2. PD-related Vitamins as Reporter Metabolites

To extend the list of PD-related vitamins predicted in the previous section, the RMA approach was applied (**Figure 4.7.a**). In this way, several vitamins in the iDrosophila1 model were defined as reporter metabolites in the PD case. Some of these vitamins (A, B₁, B₃, and B₉) were commonly predicted with Δ FBA while vitamin B₆ was only predicted by RMA and added to the Δ FBA-predicted regulated vitamin list (**Figure 4.9.a**). Thus, a final list of PD-related vitamins (A, B₁, B₂, B₃, B₆, B₉, C, and D) was obtained. This approach was also expanded to investigate the reporter metabolites in AD and HD. Based on the Δ FBA and/or RMA approaches, vitamin A, all the B family of vitamins (except for vitamins B₅-B₇), and vitamin C were found to be commonly affected in PD, AD, and HD. For the HD case, only vitamin B₉ was classified as the reporter metabolite while some additional vitamins (E, K₁, and K₂) were determined for the AD phenotypes by RMA in comparison with PD. The Δ FBA- and RMA-

predicted vitamins derived from at least one phenotype of each disease were combined and all results are represented in **Figure 4.9.a**. The therapeutic potentials of these vitamins were also evaluated based on several key characteristics (recommended daily allowance (RDA) values, ability to cross the BBB, and lethal dose (LD₅₀) values) (**Figure 4.9.a**).

The especially elderly population suffers from vitamin deficiency, and the gender-specific RDA values of appropriate vitamin combinations are important to improve the quality of life in the population, maintain the homeostasis of neural metabolism, and avoid neurodegenerative disorders including PD, AD, and HD (Rai et al., 2021; Zhao et al., 2019). Therefore, these values have been herein represented to guide scientists in determining appropriate doses of disease-related vitamins (NIH, 2024). Additionally, knowledge about the permeability of BBB for these vitamins was provided based on the literature (Anjum et al., 2018; Est & Murphy, 2023; Feng et al., 2023; Gasperi et al., 2019; Greenwood et al., 1982; Hulme et al., 2022; Jadhav et al., 2022; P. Lee & Ulatowski, 2019; Patel et al., 2012; Segarra et al., 2021; Tiani et al., 2019). This is because these vitamins may be useful as nutritional supplements to improve brain pathologies (Rai et al., 2021; Zhao et al., 2019). Thus, it has been confirmed that all PD-related vitamins can cross the BBB.

The toxicity levels of these vitamins were also investigated via databases (PubChem and HMDB) and the literature (EL-Hak et al., 2019; Gupta, 2007) (**Figure 4.9.a**). Accordingly, the safety degrees of the vitamins were assessed using the LD₅₀ values for oral toxicity based on GHS (the Globally Harmonized System of Classification and Labeling of Chemicals) classification systems, including five hazard categories from the category I (the highest toxicity: LD₅₀ ≤ 0.005 g/kg) to category V (the lowest toxicity: 2 g/kg < LD₅₀ ≤ 5 g/kg) (Gadaleta et al., 2019). Except for vitamin D (LD₅₀ of 0.04; category II), the LD₅₀ values of all vitamins are higher than 2, representing the lowest toxicity (category V).

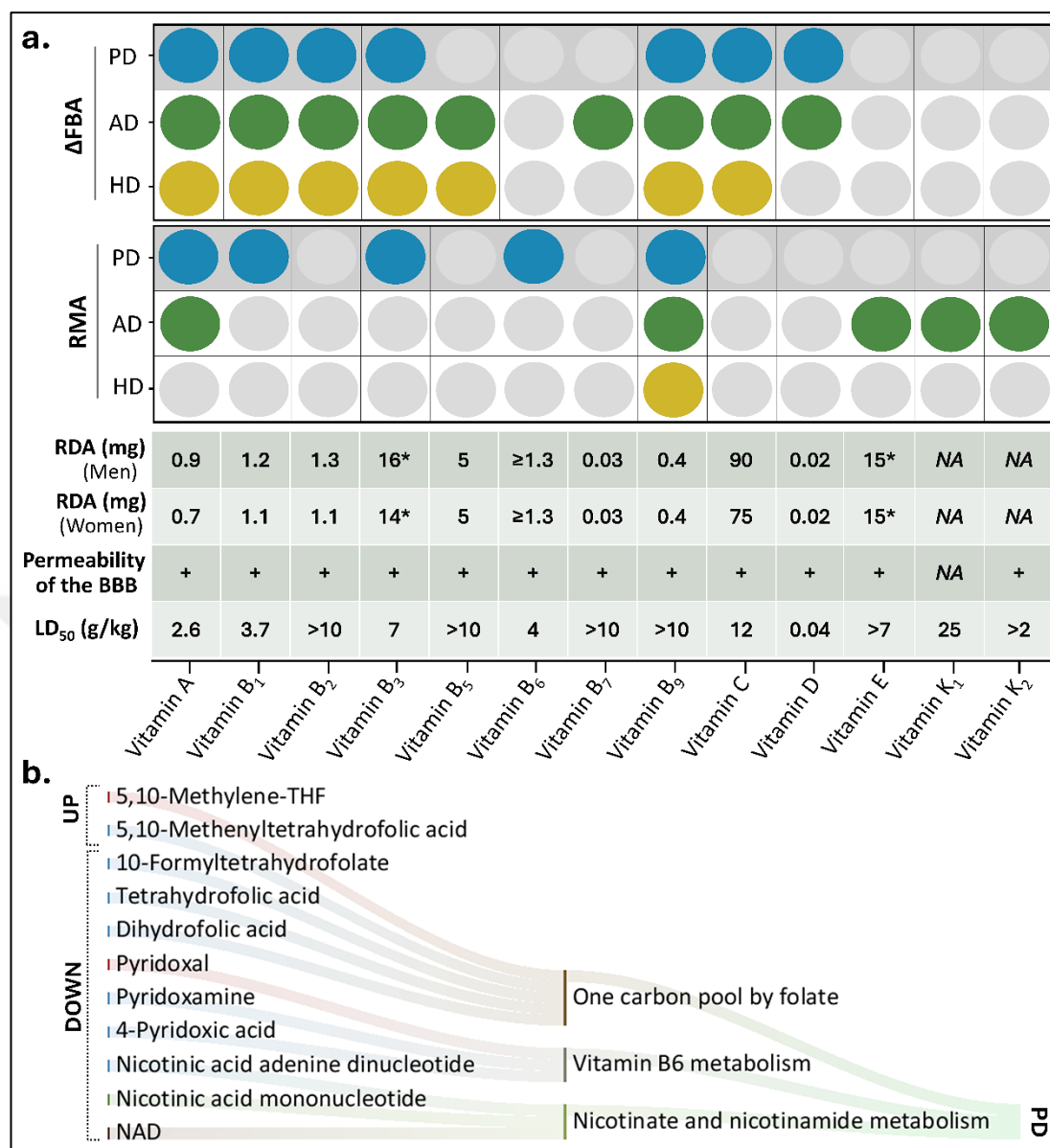


Figure 4.9: Illustrating the conservation and metabolic dysregulation of the PD-related vitamins a. by applying the Δ FBA and/or RMA approaches for AD and HD, and b. a reaction activity-based analysis indicating a predominant downregulation of the vitamin metabolism. Several significant properties of these vitamins (RDA, permeability of the BBB, and LD₅₀) are also represented in the given table. Note that the RDA values for adults (19 years and older) are given for the vitamins (except for the vitamin E). The RDA values of the vitamin E, denoted by an asterisk (*), were estimated for those at the age of 14 years or older. The unit of RDA values is mg NE (niacin equivalents) for vitamin B₃, also denoted by an asterisk.

A reaction activity-based analysis was then applied for further analysis and validation of the regulations in the metabolism of PD-related vitamins by comparing the activity status of each reaction between the control (n = 15) and disease (n = 15) cases (**Figure 4.7.b** and **Figure 4.9.b**). Thus, 38 significantly upregulated and 100 significantly

downregulated reactions were identified based on the iMAT-mediated contextualized metabolic models. According to this analysis, the reactions associated with vitamin B₃, vitamin B₆, and vitamin B₉ were predominantly downregulated (inactive) in PD (**Figure 4.9.b**). The blood levels of these vitamins confirmed their reduction in PD patients, supporting the downregulation of their metabolism (**Table 4.4**). The B family vitamins mainly act as cofactors in the nervous system, and they have been linked to PD in previous studies. These vitamins are usually obtained from dietary sources (except for vitamin B₃ (niacin)) (Sandeep et al., 2023; Zhao et al., 2019). The dietary supplementation of an active form of vitamin B₃ (nicotinamide) or NAD⁺ in appropriate doses was reported to improve PD-associated mitochondrial defects, neurotoxicity, and the loss of DA neurons (Lehmann et al., 2017; Lu et al., 2014; Sandeep et al., 2023; Zhao et al., 2019). Similarly, the dietary intake of vitamin B₆ (pyridoxine) was shown to be useful for a significant reduction in the risk of PD by a population-based cohort study on 5,289 participants aged 55 years or older (Lau et al., 2006). This vitamin can be also obtained by means of gut microbiota, and its reduced level was reported in various neurodegenerative disorders including PD, AD, autism, schizophrenia, and epilepsy (Sandeep et al., 2023).

Vitamin B₉ (folate) is another vitamin that can be supplied by dietary sources or gut microbiota, and its reduced levels were reported in PD patients, coupled with a decrease in bacterial folate biosynthesis (Rosario et al., 2021). Dietary supplementation of this vitamin was shown to be useful for reducing blood homocysteine levels, which cause neurotoxicity and oxidative stress in various disorders such as PD and AD (Ayshi, 2024; Homocysteine Lowering Trialists' Collaboration, 1998; Kaye et al., 2020; Nguyen et al., 2023; Rosario et al., 2021). Since levodopa increases homocysteine levels, the effect of vitamin B₉ on the PD patients treated with levodopa has been analyzed in an ongoing phase II clinical trial (ClinicalTrials.gov ID: NCT05959044) (Ayshi, 2024). The impact of this nutritional substance on the progression of PD has also been clinically investigated by different researchers (ClinicalTrials.gov IDs: NCT00853879, NCT01238926, and NCT05959044) (Flores-Torres et al., 2023; Lau et al., 2006; New York Institute of Technology, 2010; Northwell Health, 2015). Taken together, vitamin supplementation may be beneficial to compensate for the reduction in their levels or decrease the risk of PD. Thus, identification of the pathways regulated by the altered PD-related vitamin

levels may provide a deep understanding of the design of efficient therapeutical cocktails of the vitamins.

Table 4.4: Literature-based validation of the reduced levels of the vitamins B₃, B₆, and B₉ in PD patients.

Vitamin Type	Sample Type	Sample Size	Vitamin Level	Year	Reference
B ₃	Plasma	9 PD patients 9 Healthy controls	Reduced	2014	(Wakade et al., 2014)
B ₆	Plasma	24 PD patients	Reduced (19/24)	2020	(Rojo-Sebastián et al., 2020)
B ₉	Plasma	2,359 PD patients 2,330 Healthy controls	Reduced	2023	(Quan et al., 2023)

4.2.3. Differential Metabolic Pathways Regulated by Vitamins

Following the characterization of the regulations in vitamin metabolism, the effect of PD-related vitamins (A, B₁, B₂, B₃, B₆, B₉, C, and D) on metabolic pathways in the brains of PD flies was investigated. Briefly, the uptake rate of each vitamin was gradually increased and the significantly affected reactions were determined for each level of the selected vitamin. To this aim, sample-based reference flux distributions were first determined via the PD models (n = 15) by setting the maximum uptake levels of all vitamins in the growth medium to 1/100 of the maximum glucose uptake rate.

The uptake rate of each PD-related vitamin was set to 1/50, 1/20, and 1/10 of the maximum glucose uptake rate one by one to predict a new flux distribution based on the corresponding reference flux distribution for each modified condition. Significantly affected reactions were then identified by comparing the flux distributions between the PD conditions with low and modified vitamin uptake rates (**Figure 4.7.c**). The total numbers of the differential reactions are represented in

Figure 4.10.a for each ratio (i.e., 1/50, 1/20, or 1/10), regardless of the vitamin types (considering all vitamins). Increasing uptake ratio caused the regulation of a higher number of reactions ($n = 1,192$ for the ratio of 1/50, $n = 1,321$ for the ratio of 1/20, and $n = 1,357$ for the ratio of 1/10). The numbers of differential reactions are also listed in a vitamin-specific manner (**Figure 4.10.a**), and in general, more reactions were determined at the ratios 1/20 and 1/10 than the ratio of 1/50 for each vitamin.

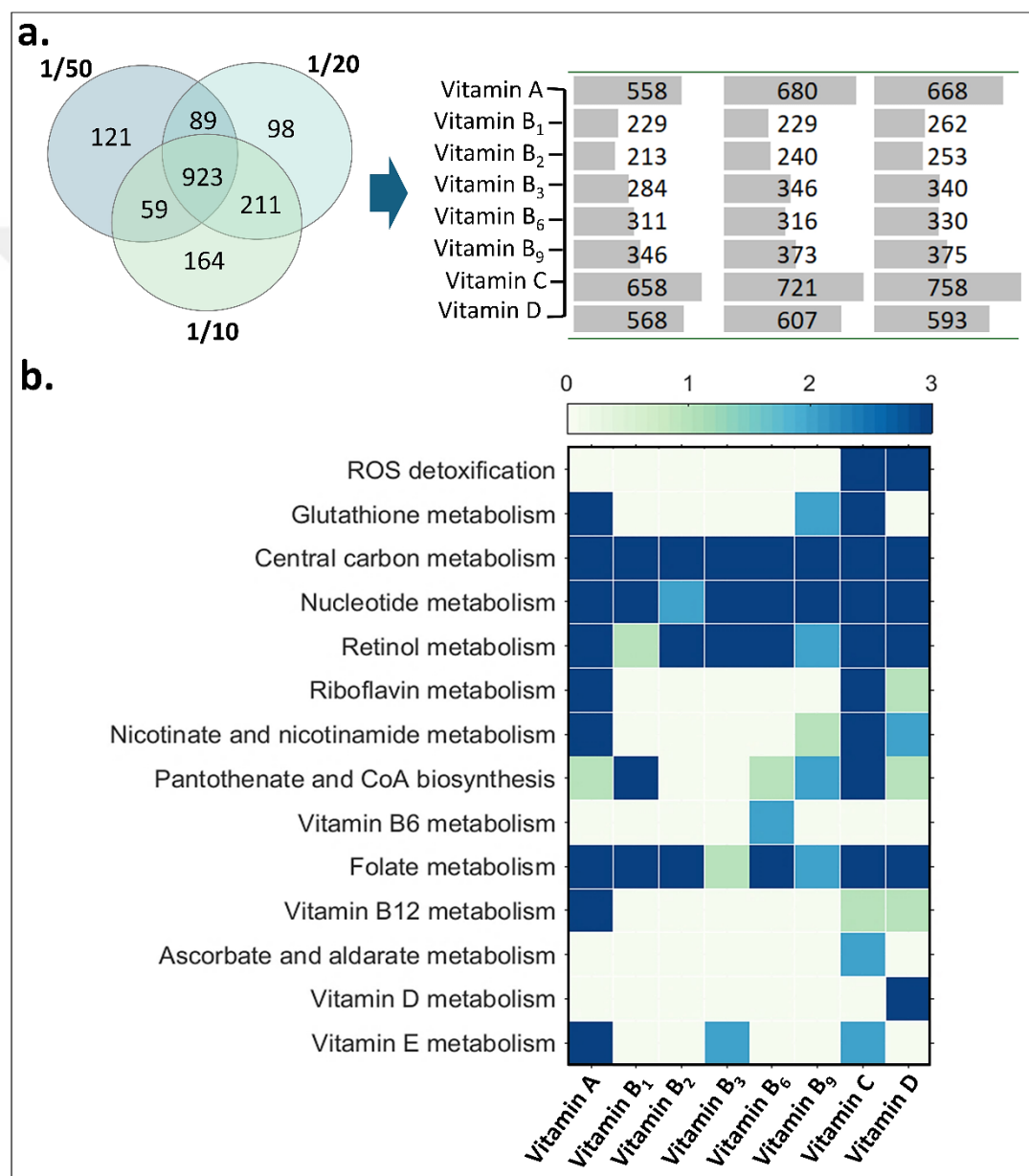


Figure 4.10: The effect of the PD-related vitamins in the differential brain metabolism. For the increasing vitamin uptake rates, a. the total or vitamin-specific numbers of the regulated reactions and b. major pathways. The changed colors from green to blue on the heatmap indicate the number of uptake-ratio simulations (1/50, 1/20, or 1/10) where a pathway was identified.

For each modified condition, the pathways associated with the differential reactions were identified (**Figure 4.10.b**). Central carbon metabolism (i.e., “glycolysis/gluconeogenesis”, “pentose phosphate pathway”, “pentose and glucuronate interconversions”, “tricarboxylic acid cycle, and glyoxylate/dicarboxylate metabolism”, and “oxidative phosphorylation”) was among the significantly affected pathways by the varying levels of the PD-related vitamins (**Figure 4.10.b**). The water-soluble B and C vitamins are crucial, as cofactors, for the regulation of fundamental biochemical pathways (Zhao et al., 2019). The vitamins B₁, B₂, B₃, B₅, and C were reported to be involved in glycolysis, PPP, glycogenolysis, and gluconeogenesis. Most of them (vitamins B₁, B₂, B₃, and B₅) and vitamin B₇ also function in the TCA cycle and oxidative phosphorylation (Godoy-Parejo et al., 2020; Tardy et al., 2020) (**Figure 4.11**). Consistently, glycolysis and/or PPP were also found to be affected by an increasing level of vitamin B₁, B₂, B₃, or C while TCA was directly or indirectly regulated by almost all PD-related vitamins (except for vitamin B₁). Thus, the significance of these vitamins in the central carbon metabolism of the PD flies was shown (**Figure 4.10.b**). The function of phosphorylated vitamin B₁ (thiamine pyrophosphate) as a cofactor in the central carbon metabolism is based on the activation of pyruvate dehydrogenase complex, oxoglutarate dehydrogenase complex, and the transketolase of the non-oxidative branch in PPP (Jeong & Vacanti, 2020). In a similar vein, vitamin B₂ serves in many metabolic pathways in the form of FMN or FAD for electron transfer such as the oxidation-reduction reactions of the TCA cycle and oxidative phosphorylation (Marashly & Bohlega, 2017; Tardy et al., 2020; Xin et al., 2017). Another well-known redox cofactor, NAD, is derived from vitamin B₃ and functions in the respiration system (Tardy et al., 2020). Vitamin B₅ is required for the synthesis of coenzyme A, which is used to catalyze the oxidative decarboxylation of pyruvate dehydrogenase complex, oxoglutarate dehydrogenase complex, and branched chain keto acid dehydrogenase complex. Vitamin C is another essential cofactor required by prolyl and lysyl hydroxylases to produce the hydroxylated amino acid residue and succinate from oxygen and α -ketoglutarate (Jeong & Vacanti, 2020). Additionally, it is crucial for the synthesis of carnitine and the β -oxidation of fatty acids (Johnston et al., 2006) (**Figure 4.11**). Taken together, the listed vitamins, whose metabolism was significantly regulated in the PD process, have prominent roles in the energy homeostasis of the fly brain. It is important to note that the impact of some vitamins on energy metabolism may be indirectly predicted in the simulations due to

the lack of representations about their regulatory functions in the metabolic network. In this regard, their contributions to biomass formation may also affect the central carbon metabolism through the cofactor pool.

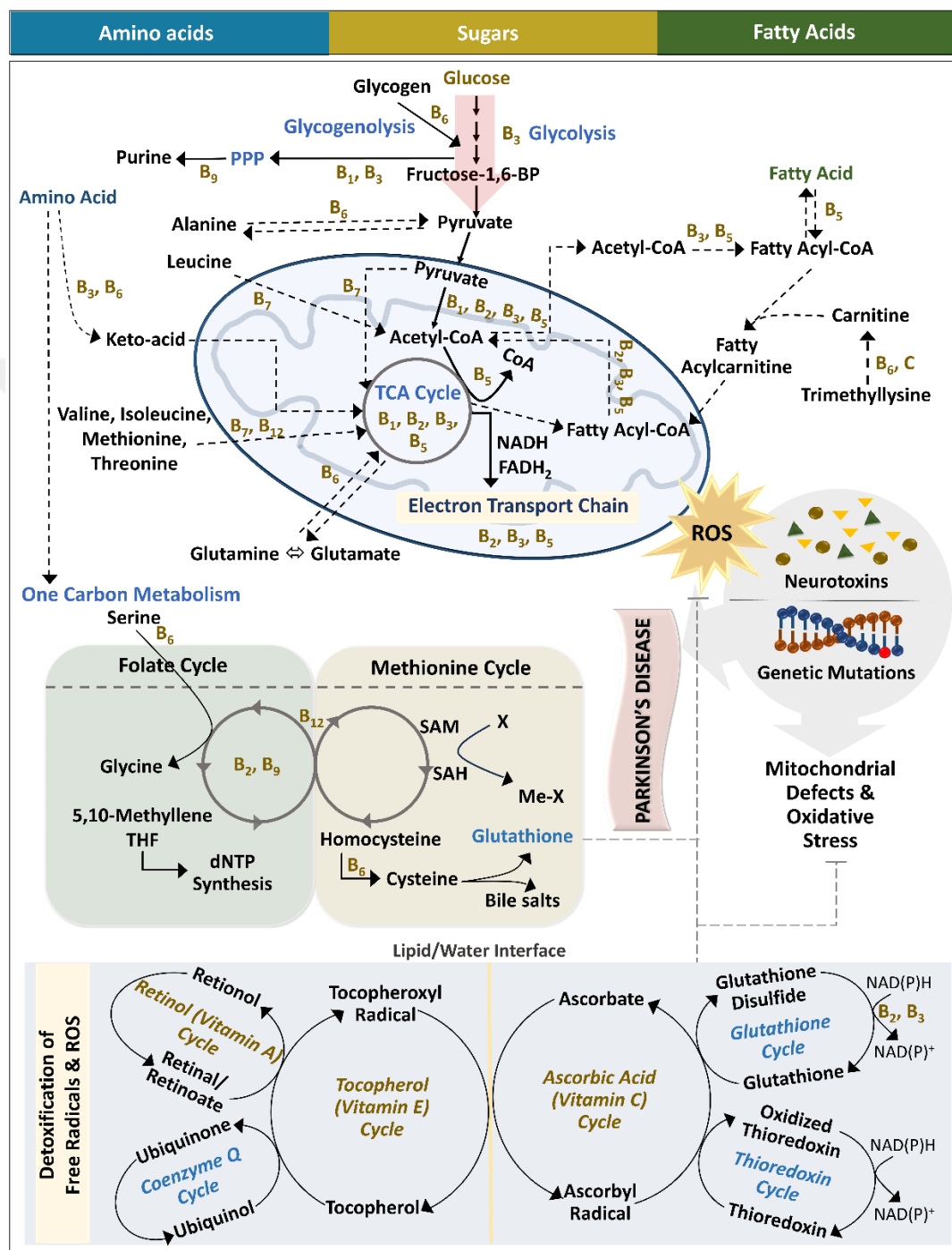


Figure 4.11: Central carbon metabolism, one-carbon metabolism, and metabolic detoxification pathways are among the major metabolic pathways associated with vitamins, linked to PD.

Vitamin C also exhibits antioxidant features via the regeneration of other antioxidants including vitamin E and GSH without changing the activities of glutathione peroxidase and glutathione reductase enzymes (Gęgotek & Skrzydlewska, 2022; Jeong & Vacanti, 2020; Pullar et al., 2017; Sandeep et al., 2023; Shi et al., 2020). In the PD process, mitochondrial malfunctions can damage energy metabolism and enhance oxidative stress (**Figure 4.11**). Thus, the antioxidative functions of such vitamins may be beneficial to reduce PD symptoms as an adjuvant treatment. Accordingly, ROS detoxification and GSH metabolism were affected by the increasing level of vitamin C and/or vitamin D. According to the iDrosophila1 network, ascorbate metabolism is connected to hydrogen peroxide metabolism, GSH production, and vitamin E metabolism. This relationship supports the function of vitamin C in ROS detoxification. Similarly, hydrogen peroxide production is linked to the metabolism of vitamin D in iDrosophila1. This vitamin was reported to be crucial in coping with oxidative stress and systemic inflammation (Wimalawansa, 2019). Additionally, GSH metabolism was found to be affected across the varying uptake rates of vitamins A and B₉ (**Figure 4.10.b**). The administration of vitamin A supports GSH metabolism while vitamin B₉ is indirectly linked to this antioxidant process via methionine (Cha et al., 2016; Raghubeer & Matsha, 2021). Folate and methionine metabolisms collectively establish one-carbon metabolism (Lyon et al., 2020; Raghubeer & Matsha, 2021). In the folate cycle, vitamin B₉ is converted into 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) using a series of enzymatic reactions for nucleotide synthesis. Vitamin B₂ is utilized as a cofactor for the synthesis of 5-methylTHF from 5,10-methyleneTHF. The 5-methylTHF and vitamin B₁₂ are involved in the regeneration of methionine from homocysteine (Jeong & Vacanti, 2020; Lyon et al., 2020; Raghubeer & Matsha, 2021). The neurotoxic homocysteine is converted to cystathionine and cysteine using vitamin B₆ as the cofactor, and cysteine is used for the synthesis of GSH (Lyon et al., 2020; Raghubeer & Matsha, 2021). Thus, this metabolic process is linked to diverse crucial pathways associated with amino acid metabolism, ROS response, and nucleotide synthesis (**Figure 4.11**). Here, the results showed that varying levels of all PD-related vitamins significantly affected folate and nucleotide metabolism, and this effect may be direct or indirect by regulating the metabolism of related amino acids (e.g., glycine and serine). In this regard, the metabolic regulations of fundamental biomolecules (e.g., lipids, short-chain fatty acids (SCFAs), and amino acids) were determined for the increasing uptake levels of the PD-related vitamins (**Figure 4.11**). In accordance

with these results, the vitamin B family has been known to function in fatty acid metabolism (vitamins B₂, B₃, and B₅) and amino acid metabolism (vitamins B₃, B₆, B₉, and B₁₂) (Godoy-Parejo et al., 2020; Tardy et al., 2020).

The modified uptake level of a vitamin may also influence the metabolism of other vitamins based on their metabolic relationship (**Figure 4.10.b**). Therefore, the bioavailability of vitamins may be also important in the design of functional foods with certain vitamin contents. Bioavailability shows nutritional efficacy, which is the available fraction of the vitamins that can be consumed by the body (Rein et al., 2013). The bioavailability in animal-sourced sources was reported as 74% for preformed vitamin A, 82% for vitamin B₁, 61% for vitamin B₂, 67% for vitamin B₃, 80% for vitamin B₅, 83% for vitamin B₆, 89% for vitamin B₇, 67% for vitamin B₉, and 65% for vitamin B₁₂ while bioavailability in plant-based foods is 15.6% for provitamin A carotenoid β -carotene, 81% for vitamin B₁, 65% for vitamin B₂, 76% for vitamin C, and 16.5% for vitamin K. (Chungchunlam & Moughan, 2023). Taken together, different factors should be taken into account in designing vitamin supplements to efficiently use them as an adjuvant treatment strategy in PD.

4.3. Metabolism of Gut Microbiota-ECC in PD

In the second *Drosophila*-based study, the main focus was to analyze the potential relationship among a special midgut cell (EEC), gut microbiota, and PD. Given that α Syn-expressing EECs can interact with both microbial metabolites in the gut lumen and α Syn-containing nerves, the microbial activation of the EECs may induce the transmission of misfolded α Syn proteins from these cells to the brain in the early stages of PD (**Figure 4.12.a** and **Figure 4.12.b**) (Chandra et al., 2017). To characterize these metabolic interactions, community metabolic models were reconstructed with the host and microbial populations (i.e., EEC and gut bacteria) for the midgut regions R₁-R₅ and the whole midgut, considering the region-specific metabolic patterns of the EECs (**Figure 4.12.c** and **Figure 4.12.d**). For each midgut region, the flux distributions of the wild-type and α Syn-expressing mutant community models were predicted based on the gradually decreased growth rates of the EECs on the Pareto front (**Figure 4.12e**). The metabolites of differential reactions, which were obtained by comparing the wild-type and mutant flux distributions, were further characterized and significantly enriched pathways were elucidated.

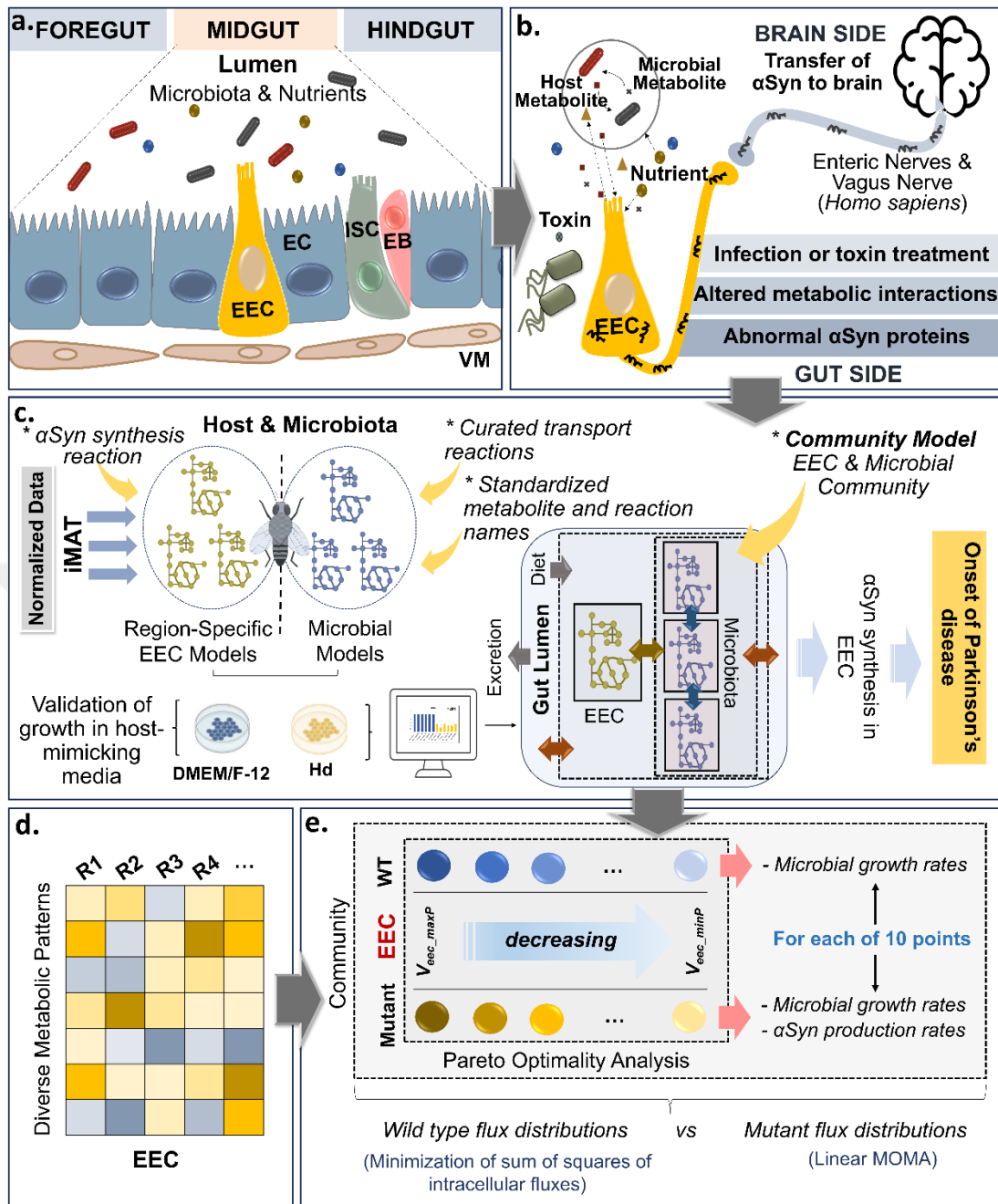


Figure 4.12: General overview of the community modeling approach. a. *Drosophila* gut consists of three main regions (foregut, midgut, and hindgut), and the midgut has conserved epithelial cells including EECs. b. The EECs likely trigger the initiation of PD by the synthesis and transfer of α Syn aggregates to the brain. c. Illustrating the reconstruction of wild-type (WT) and mutant community metabolic networks for each midgut region, d. considering the region-specific metabolic patterns in the EECs. e. Identification of the differential reactions by comparing WT and mutant fluxes for each point on the Pareto front.

4.3.1. Region-Specific Metabolic Patterns of ECCs

The midgut regions of *Drosophila* are specialized based on their functions, morphology, and transcriptional profiles (Dutta et al., 2015). The distribution of EEC-specific RNA-seq samples obtained from the midgut regions R1-R5 and the whole midgut of female flies was first examined using the PCA approach. All replicates except for one of the R3 samples were shown to be grouped together in the plot. The R3 sample was not excluded from the dataset due to the presence of insufficient replicates (only two replicates per sample) (**Figure 4.13.a**). Hierarchical clustering supported the region-specific distributions of the EEC samples. Accordingly, the regions R2 and R4 were clustered together, and the region R5 was found to have a close expression profile to this group compared to the other samples. On the other hand, the region R1 and the whole midgut have a lower degree of similarity to the regions R2, R4, and R5 in terms of expression patterns. Particularly, the expression profile of the whole midgut was clearly separated from the other samples (**Figure 4.13.a**).

EEC samples were also characterized according to the similarity of the upregulated pathways (subsystems) by mapping the average RNA-seq counts to the iDrosophila1 reactions and calculating pathway scores. Five different pathway groups were identified based on the clustering patterns in terms of the pathway scores. Accordingly, each group is predominantly upregulated for a region or the whole midgut (i.e., G1: R2, G2: R1, G3: R3, G4: whole, G5: R5). The regions R2 and R4 were found to be clustered together based on the metabolic patterns (**Figure 4.13.b**) in agreement with **Figure 4.13.a**, whereas the upregulated pathway profile of the whole midgut behaved independently (**Figure 4.13.b**). The prominent region-specific pathways are illustrated in **Figure 4.13.c**, colored based on the groups. In this regard, upregulated pathways in the region R5 relative to the other regions were mainly shown to be associated with lipid metabolism. Similarly, a variety of pathways regarding lipid metabolism (e.g., “steroid metabolism”, “fatty acid biosynthesis”, “arachidonic acid metabolism”, and “glycerolipid metabolism”) were also identified for the region R1 and the whole midgut. For the regions R1, R3, and the whole midgut, energy metabolism-associated pathways including highly expressed genes (e.g., “glycolysis/gluconeogenesis”, “pentose phosphate pathway”, “tricarboxylic acid cycle and glyoxylate/dicarboxylate metabolism”, and “oxidative phosphorylation”) were identified. Consistent with the

differential expression profiles in (Dutta et al., 2015), amino acid metabolism was determined to be upregulated for the regions R3 and R5 in addition to the whole midgut (e.g., “alanine, aspartate, and glutamate metabolism”, “valine, leucine, and isoleucine metabolism”, and “cysteine and methionine metabolism”). The samples of the region R3 were also shown to have highly expressed genes linked to nucleotide metabolism.

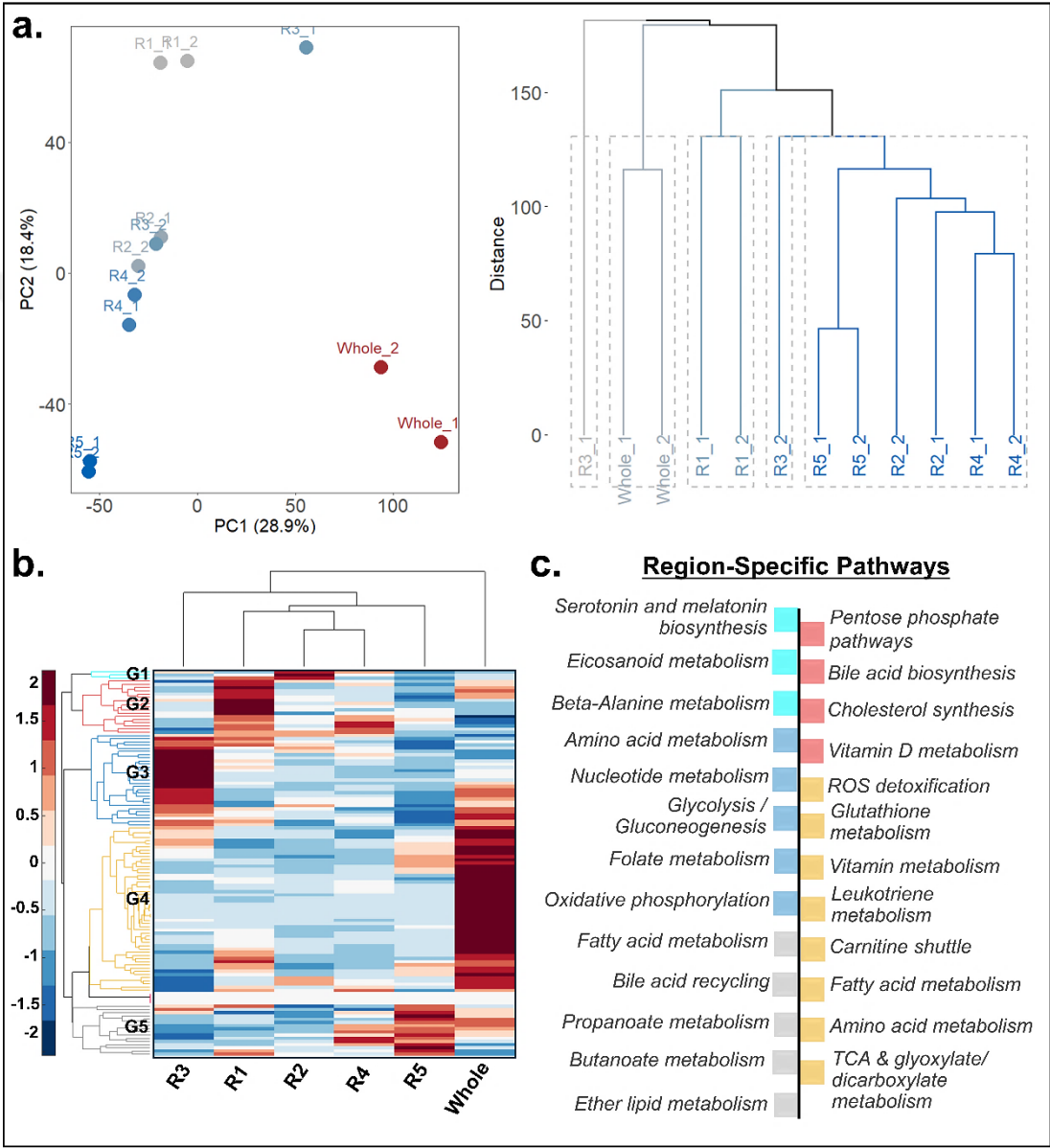


Figure 4.13: Region-specific or whole-midgut metabolic patterns in EECs. Applying PCA and hierarchical clustering (a) for grouping the EEC samples from different regions (R1-R5) and whole midgut. b. Using heatmap with dendrogram to determine the region-specific regulated pathways colored based on the group information (c).

4.3.2. Community Models of EEC and Gut Microbiota

Wild-type and α Syn expressing mutant community models including EEC (the regions R1-R5 or whole midgut) and prevalent gut bacteria (*L. plantarum* A2, *L. plantarum* B2, and *A. pasteurianus* B5) were reconstructed to characterize differential EEC metabolism and EEC-microbiota interactions. To represent the mutant models, the α Syn synthesis reaction was added to the generic iDrosophila1 model as the linear combinations of the molar amino acid levels (**Table 4.5**). Using the iMAT algorithm, an EEC model was generated for each midgut region and the whole midgut. In this way, six EEC models in total were obtained for the mutant case. The wild-type models were obtained by discarding all reactions associated with the α Syn metabolism from the mutant models. Thus, the number of reactions in the wild-type and mutant models ranged from ~2,500 to ~3,000 on average while the number of metabolites varied between ~1,900 and ~2,000. The metabolic model of the whole midgut was the largest, with 3,104 reactions and 2,210 metabolites (**Figure 4.14.a**). To ensure the biomass formation in the expanded Hd and DMEM/F-12 growth media (**Table A1.1**), growth optimization was applied to each α Syn-expressing EEC model by setting the α Syn production rate to 10^{-5} to ensure the synthesis of this protein. As a result of the growth simulations, a significant difference could not be determined in the growth rates between the expanded Hd (**Figure 4.14.b**) and DMEM/F-12 media (**Figure A1.5**) because of the high similarity of their dietary contents. In addition, the growth rates of the mutant models were not considerably different from those predicted by wild-type models due to the low production rates of α Syn.

Table 4.5: Molar level of each amino acid in the structure of α Syn protein.

Amino acid	1-Letter Code	Molecular Formula	Molar Level (mol)
Glycine	G	$C_2H_5NO_2$	18
Glutamine	Q	$C_5H_{10}N_2O_3$	6
Tyrosine	Y	$C_9H_{11}NO_3$	4
Isoleucine	I	$C_6H_{13}NO_2$	2
Proline	P	$C_5H_9NO_2$	5
Leucine	L	$C_6H_{13}NO_2$	4
Threonine	T	$C_4H_9NO_3$	10
Methionine	M	$C_5H_{11}NO_2S$	4

Table 4.5: Continue

Amino acid	1-Letter Code	Molecular Formula	Molar Level (mol)
Valine	V	C ₅ H ₁₁ NO ₂	19
Glutamic acid	E	C ₅ H ₉ NO ₄	18
Asparagine	N	C ₄ H ₈ N ₂ O ₃	3
Alanine	A	C ₃ H ₇ NO ₂	19
Histidine	H	C ₆ H ₉ N ₃ O ₂	1
Serine	S	C ₃ H ₇ NO ₃	4
Lysine	K	C ₆ H ₁₄ N ₂ O ₂	15
Phenylalanine	F	C ₉ H ₁₁ NO ₂	2
Aspartic acid	D	C ₄ H ₇ NO ₄	6

On the microbial side, the metabolic network models of *L. plantarum* A2, *L. plantarum* B2, and *A. pasteurianus* B5 (Schönborn et al., 2021) were used to represent a simplified *Drosophila* midgut. These bacterial models were first modified by curating transport reactions based on several sources (Ankrah et al., 2021a; Kanehisa & Goto, 2000; Norsigian et al., 2020; Pelicaen et al., 2019; Saier et al., 2006; The UniProt Consortium, 2017). After applying a variety of additional modifications explained in Section 3.3.5, growth simulations were also performed for these updated models including *L. plantarum* A2 (1,817 reactions and 1,566 metabolites), *L. plantarum* B2 (1,815 reactions and 1,565 metabolites), and *A. pasteurianus* B5 (1,802 reactions and 1,674 metabolites) in the expanded Hd and DMEM/F-12 media with additional metal ions (Mg²⁺, Mn²⁺, Fe³⁺, and Cu²⁺). The microbial growth rates were found to be close in both microaerobic growth conditions (**Figure 4.14.c** and **Figure A1.6**). Therefore, the microaerobic expanded Hd condition was preferred in all remaining analyses covered by this study.

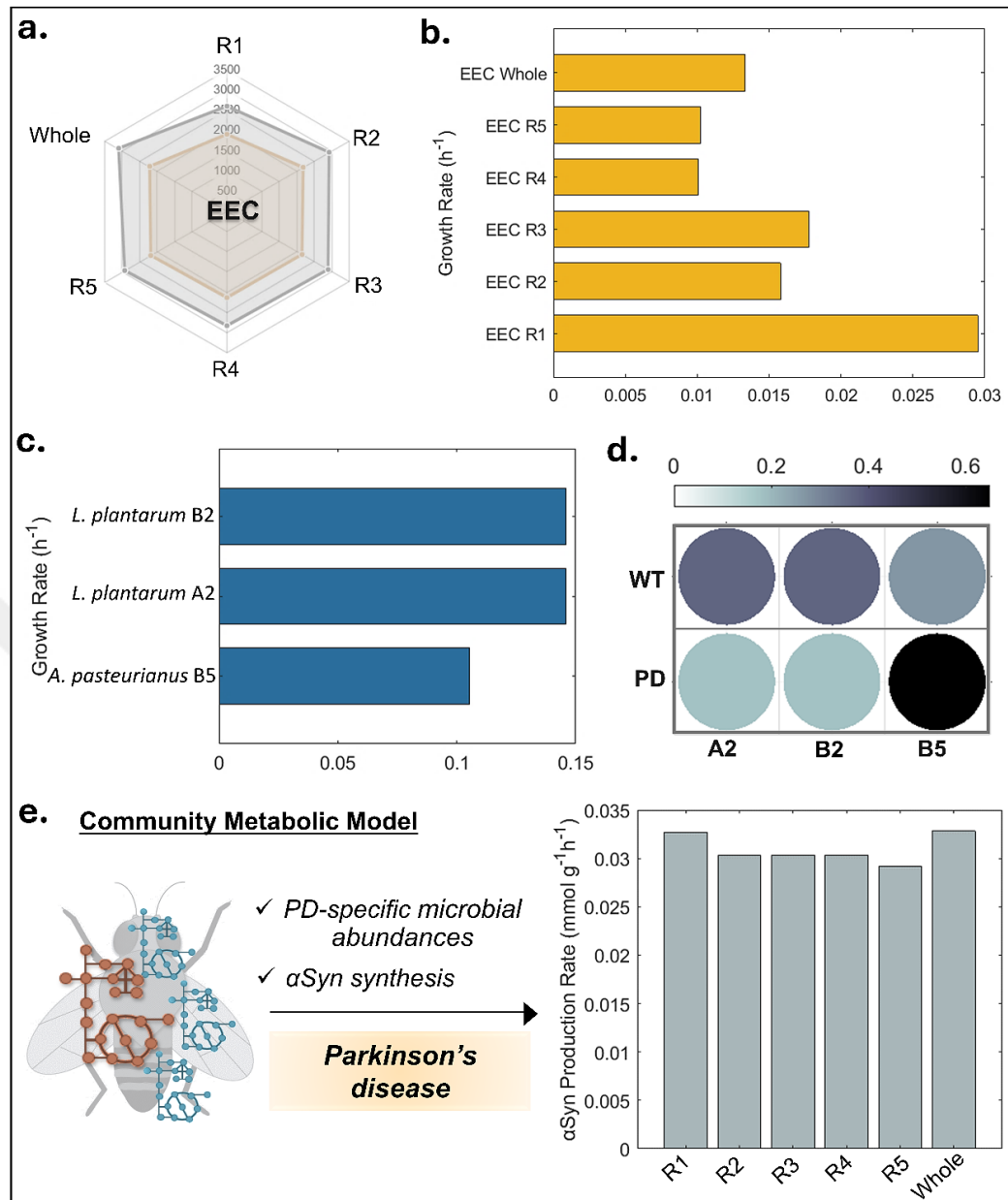


Figure 4.14: Reconstruction of the community metabolic models starts with a. the generation of EEC models, whose reactions and metabolite numbers are displayed by gray and orange lines on the radar plots (mutant case), validation of biomass formation rates for b. each mutant EEC model, and c. modified bacterial models. After merging the EEC and microbial models, a microbiota biomass equation is created based on d. the relative abundance levels of bacteria in the wild-type and mutant midguts. e. Optimizing the αSyn production rates in the mutant community metabolic model.

A total of 12 community metabolic models were reconstructed by connecting each region-specific or whole-midgut EEC model to the modified metabolic networks of the *L. plantarum* and *A. pasteurianus* strains via the gut lumen. To mimic the varying microbial composition in the PD process, microbiota biomass equations were created

based on the microbial abundances in the wild-type midgut and the mutant midgut overexpressing the human α Syn in *Drosophila*. Based on the 16S rRNA gene sequencing experiments of Liu et al., it was shown that the relative abundance of *A. pasteurianus* significantly increased (almost 140% increase) by the intestinal expression of α Syn in *Drosophila* despite a reduction in the abundance of *Lactobacillus* (almost 50% decrease) (**Figure 4.14.d**). These bacteria stand for ~70% of the wild-type and ~96% of the mutant microbial community in the *Drosophila* midgut (Liu et al., 2022). As highlighted before, α Syn production was allowed by the EECs of only mutant communities, in agreement with the *in vivo* studies of this organism. Hence, the maximum α Syn production capacity was tested in the expanded Hd by exposing the mutant community models to flexible dietary constraints. The maximum α Syn synthesis rates of the EECs in the region R1 and the whole midgut were estimated to be slightly higher than the other regions (**Figure 4.14.e**).

4.3.3. PD-Related Differential Community Metabolism

The flux distributions of the wild-type and mutant communities, representing the simplified midgut of *Drosophila*, were compared to investigate the hypothesis on the role of α Syn synthesis by the EECs in the initiation of PD. In this process, a set of Pareto-optimal solutions was determined by optimizing the growth rate of each region-specific EEC model and gradually decreasing the maximum point (20% of the optimal growth rate) on the Pareto front in the specified interval. For each growth rate of the EEC, the α Syn production rate was calculated by the mutant community model, and the growth rate of microbiota was predicted in such a manner that the minimum α Syn production rate was equal to 5% of its optimal value. The biomass formation rate of wild-type microbiota was determined by fixing the growth rate of EEC to each point on the Pareto front without α Syn synthesis. In the wild-type communities, these predicted fluxes were used to limit the growth rates of EEC and microbiota in the expanded Hd condition. The wild-type flux distributions were then estimated using a secondary objective function while the flux distributions of the mutant communities were predicted by minimizing their differences from the wild-type case. In this step, flux changes were allowed for only certain reactions that were expected to be regulated in the fly midgut by the α Syn expression (**Figure 3.2** and **Figure 4.12e**; also see Section 3.3.8).

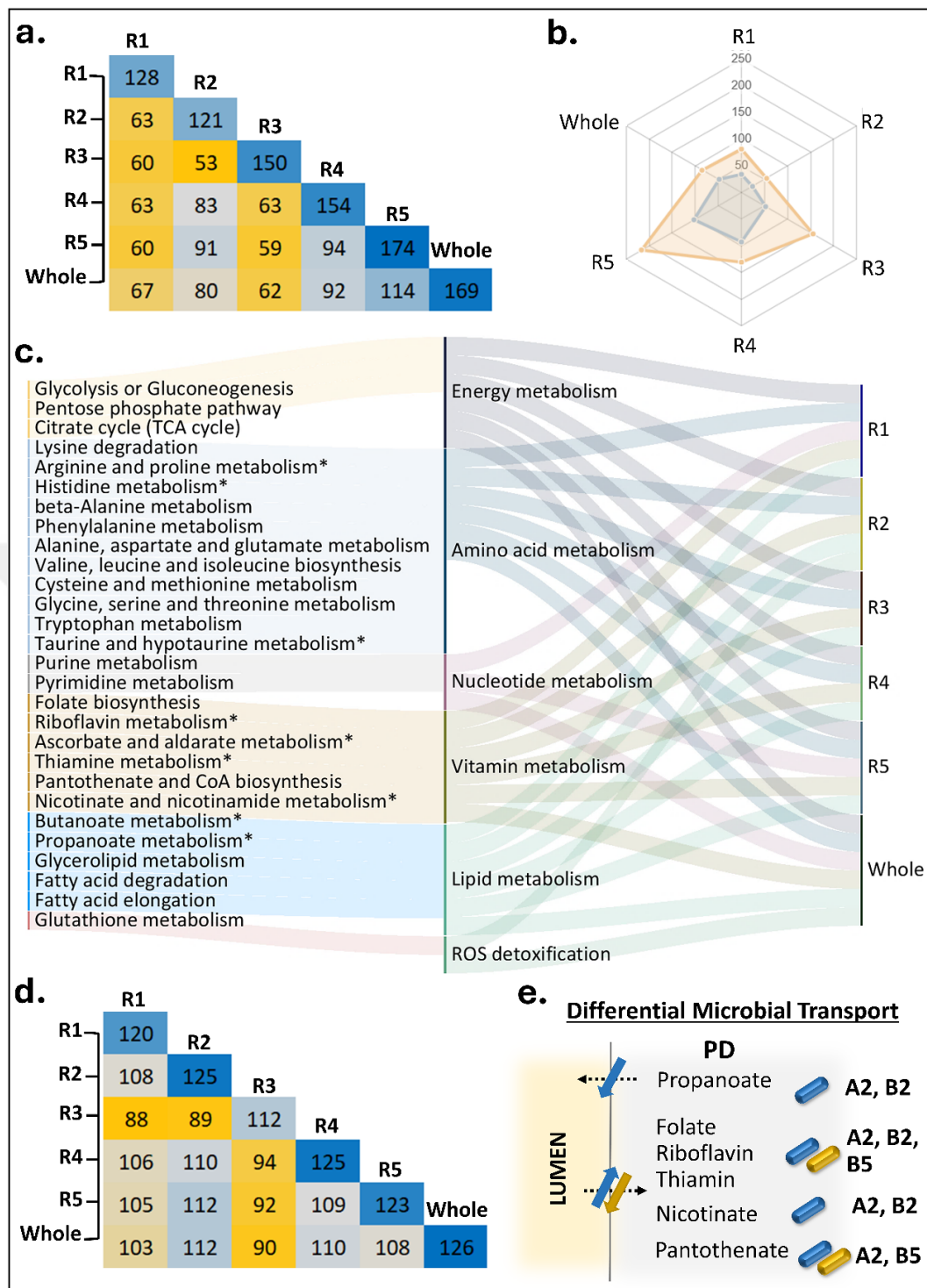


Figure 4.15: Characterizing the region-specific differential metabolism in the EECs and gut microbiota induced by α Syn synthesis. a. The comparisons of differential EEC reactions identified for the regions R1-R5 and the whole midgut represented the highest reaction numbers in dark blue and the lowest reaction numbers in orange. b. The numbers of filtered EEC metabolites and genes involved in the differential reactions and c. enriched pathways of the gene-metabolite networks. d. The comparisons of differential microbial transport reactions and e. several prominent metabolites transported via these reactions.

The affected metabolism depending on the α Syn synthesis was separately characterized for the EECs and the microbial cells. On the EEC side, the FC-based comparisons of the wild-type and α Syn-induced mutant flux distributions resulted in the elucidation of the differential reactions ($FC > 2$ or $FC < 0.5$). The number of these reactions was first compared in a region-specific manner. Accordingly, a higher number of reactions was found to be affected in the region R5 ($n = 174$) by the α Syn synthesis, followed by the whole midgut ($n = 169$) (**Figure 4.15.a**). The metabolites and genes involved in the differential reactions were also determined and filtered based on their detection for at least 50% of 10 Pareto-optimal points. In agreement with the high reaction frequency, the numbers of metabolites and genes were also determined to be the highest for the region R5, as illustrated in the radar plot (**Figure 4.15.b**). To characterize the connected genes and metabolites, a gene-metabolite network was created using the MetaboAnalyst web server for each midgut region, and these networks were analyzed in terms of the significantly enriched KEGG pathways. The prominent pathway terms were then grouped for each midgut region (**Figure 4.15.c**).

Of the enriched terms, “amino acid biosynthesis”, “ROS metabolism” and “energy metabolism” were also identified by Liu and colleagues among the top 20 significantly perturbed pathways induced by the α Syn expression in the midgut of *Drosophila* (Liu et al., 2022). ROS can be normally produced through mitochondrial electron transfer chains or redox reactions in healthy cells for the regulation of cellular homeostasis. However, their increasing levels trigger oxidative stress, causing defective cellular structures (e.g., nucleotides, proteins, and lipids) in the PD brain. The accumulation of α Syn proteins can also contribute to ROS production (Puspita et al., 2017). In this regard, α Syn aggregates are responsible for the impaired energy metabolism due to the increased production of ROS by the maintenance of defected mitochondria despite the regulatory roles of the monomeric α Syn forms in mitochondrial dynamics (fusion and fission processes) (Maszka et al., 2023; Risiglione et al., 2021). Consistently, the significant enrichment of “glycolysis or gluconeogenesis” and “pentose phosphate pathway” was identified for the synthesis of α Syn in the EEC cells from the region R1 while “Citrate cycle (TCA cycle)” was over-represented for all EEC groups. Furthermore “glutathione metabolism” was significantly enriched by the analysis of the EECs in the region R2 and whole midgut (**Figure 4.15.c**). Organisms can cope with excessive ROS using antioxidant enzymes such as glutathione peroxidase,

cytochrome P450, and superoxide dismutase. This phenomenon is important in preventing apoptosis as a result of lipid peroxidation, protein damage, and dysfunctional enzymatic activities (Zhang et al., 2018). Selective depletion of GSH has been reported in PD patients (Martin & Teismann, 2009; Segura-Aguilar & Mannervik, 2023; Smeyne & Smeyne, 2013). Furthermore, the metabolism of various vitamins including “thiamine (vitamin B₁)”, “riboflavin (vitamin B₂)”, “folate (vitamin B₉)”, and “ascorbate (vitamin C)” were also determined to be enriched for all EEC groups. As discussed in Section 4.2, vitamin metabolism is promising to improve the PD phenotypes as an adjuvant treatment. Taken together, the α Syn-mediated regulation of many common pathways with PD brains was demonstrated, suggesting that α Syn may induce some similar metabolic patterns in both brain and intestinal tissues during the progression of PD.

The FC-based analyses were also repeated on the microbial side. Thus, the differential transport reactions in microbiota were uncovered and compared between the midgut regions in **Figure 4.15.d**. Accordingly, a higher number of microbial transport reactions were predicted to be affected by α Syn synthesis in EECs for the regions R2, R4, and whole midgut, followed by the region R5. Of these reactions, the rate of propionate transport was shown to be decreased in the mutant community by indicating the downregulation of the transfer of propionate produced by *Lactobacillus* strains to the gut lumen for region R1 (**Figure 4.15.e**). In agreement with this result, the supplementation of probiotic *L. plantarum* was reported to increase SCFAs (acetate, propionate, and butyrate) in women suffering from functional constipation (Dyah et al., 2019). Thus, the reduced propionate transport levels in the current study may be associated with the significantly decreased abundance of *L. plantarum* in the mutant communities (**Figure 4.14.d**). On the other hand, Hurley and colleagues have recently investigated the impact of SCFAs on the α Syn expression of the mouse EECs, and they identified an increased level of α Syn mRNAs by 2 mM butyrate and 4 mM propionate. Conversely, 4 mM acetate supplementation for 48 h significantly reduced the α Syn expression (Hurley et al., 2023). Here, an increase was identified for acetate levels transported to the lumen for the region R1 (**Figure 4.15.e**), along with the elevated abundance of *Acetobacter* with the intestinal overexpression of α Syn in *Drosophila* (**Figure 4.14.d**). More recently, the elevated level of intestinal propionate in a *C. elegans* PD model was shown to rescue neurodegeneration through the gut-brain

interplay by reversing the α Syn-induced transcriptomic changes in the intestine (e.g., rescuing intestinal energy production) without reducing neural α Syn aggregation (Wang et al., 2024). Additionally, the enrichment of “propanoate metabolism” and “butanoate metabolism” was observed for the EECs of different midgut regions (**Figure 4.15.c**). Taken together, these preliminary findings suggest that the SCFAs may play crucial roles in the crosstalk between EECs and microbiota. Thus, reprogramming of numerous metabolic processes may drive intestinal α Syn toxicity in the early stages of PD. Moreover, a variety of additional alterations in the microbial transports of numerous metabolites including amino acids (e.g., methionine, serine, tryptophan, and tyrosine), ions (e.g., Fe^{3+} , Mg^{2+} , and K^+), and vitamins (e.g., folate, riboflavin, nicotinate) were commonly found regardless of the midgut region. In a recent study, de Ora et al. demonstrated that nitrite produced by the nitrate dissimilatory metabolism of *Escherichia coli* K-12 led to the oxidation of Fe^{2+} to Fe^{3+} , which induced a cascade of oxidation reactions and the accompanying α Syn aggregation in the EECs (STC-1 cells) (Ortiz de Ora et al., 2024). Collectively, the preliminary work in this thesis study provided a comprehensive insight into the α Syn-induced metabolic alterations in the EECs and their metabolic interactions with gut microbiota likely in the early stages of PD. Further research is needed for the validation of our community modeling-based predictions.

4.4. Potential Role of GenX in Neurodegeneration

As a third application of iDrosophila1, differential metabolism in the *Drosophila* brain was investigated in response to GenX treatment (**Figure 4.16**). To this end, the effect of the differentially abundant proteins on the fly phenotype (p -value < 0.05) was first analyzed by identifying significantly enriched FlyBase phenotypes (FDR < 0.05) via the PANGEA web tool. In this way, the over-representation of several motor (abnormal locomotor behavior and abnormal flight) and non-motor (abnormal oxidative stress response, abnormal neurophysiology, short living, and abnormal learning) properties linked to the differentially abundant proteins were identified (**Figure 4.17.a**). These phenotypic properties have been also reported in various neurodegenerative illnesses including PD (Dung & Thao, 2018; Nagoshi, 2018; Xiong & Yu, 2018). Using two different network-based approaches, Δ FBA (Ravi & Gunawan, 2021) and iMAT-based reaction activity analysis (Lülecı et al., 2023), the

metabolic alterations induced by GenX were determined, as summarized in **Figure 4.16**.

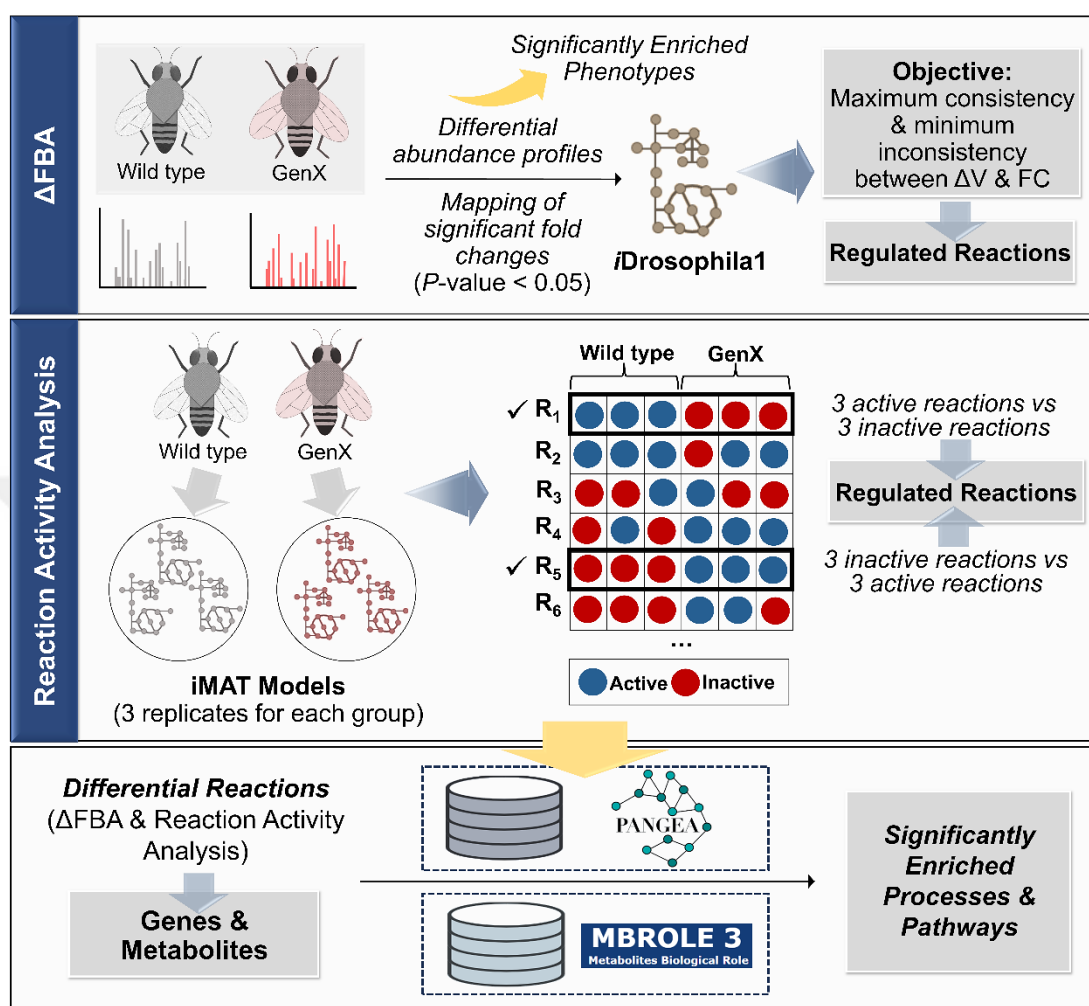


Figure 4.16: General workflow of the metabolic network analyses of GenX toxicity.

4.4.1. GenX Exposure and Neurodegeneration in *Drosophila*

Using the ΔFBA and iMAT-based reaction activity analysis, a total of 155 genes were determined from 85 differential reactions, which were influenced by GenX exposure (**Figure 4.17.b**). Since ΔFBA relies on fold changes and only a small number of genes exhibiting high fold changes were found with GenX exposure, this method predicted fewer genes regarding neurotoxicity. Even though both methods identified only three common genes, a majority of the enriched biological processes and metabolic pathways were shown to be common. Moreover, it has been clearly demonstrated that combining these complementary techniques is advantageous to increase the power of each method. For example, it has been previously reported that GenX stimulates the

generation of ROS (Ivantsova et al., 2023; Yoo et al., 2021). This term was not directly determined by Δ FBA while “regulation of reactive oxygen species metabolic process” was over-represented for the genes (the *WWOX* gene encoding WW domain-containing oxidoreductase and the *fdh* gene encoding formaldehyde dehydrogenase) derived from the reaction activity analysis. Another pathway is “fatty acid metabolism”, which was reported to be affected by the GenX treatment (Guo et al., 2021). Here, more genes (eight genes) associated with this term were determined by the Δ FBA method in comparison with those predicted by iMAT-based reaction activity analysis (five genes). Taken together, the merged gene list and the merged metabolite list associated with the differential reactions were used in the enrichment analyses considering the superiority of the use of both methods together.

For the gene-based analyses, pathways such as “carbon metabolism”, “fatty acid metabolism”, “steroid metabolic process”, “arachidonic acid metabolism”, “biosynthesis of amino acids”, “beta-alanine metabolism”, “purine metabolism”, and “pyrimidine metabolism”, which metabolize essential biomolecules, were found to be over-represented (**Figure 4.17.c**). In the previous studies, GenX-induced changes in the glucose, lipid, and amino acid metabolism were shown in rats (Conley et al., 2021) and mice (Blake et al., 2020) by analyzing the metabolomic composition of maternal and offspring liver and/or serum. Furthermore, it was demonstrated that GenX and its unique analogs impaired the ability of mice to metabolize fatty acids (Guo et al., 2021). More recently, Rashid and colleagues examined how GenX affected the liver metabolomic profile of mice. They explored significantly perturbed pathways dedicated to the metabolism of nitrogen, bile acids, amino acids, lipids, and steroidogenesis. The researchers also determined alterations in the microbial composition and metabolic pathways in the small intestine and colon, induced by the GenX exposure. In this regard, an increasing level of GenX regulated the production of aromatic/branched-chain amino acids, purine degradation, cobalamin (vitamin B₁₂) synthesis, and glutamate and glutamine biosynthesis in the small intestine (Rashid et al., 2023). In agreement with the varying microbial abundances in the GenX-treated mice, the enrichment of SCFA metabolism was herein identified for the *Drosophila* brain, including “butanoate (butyrate) metabolism” and “propanoate (propionic acid) metabolism” (**Figure 4.17.c**). These BBB-crossing microbiota-derived products are crucial for controlling the crosstalk in the microbiota-gut-brain axis and they play

important roles in age-related neurodegenerative diseases (Arora et al., 2021; Guo et al., 2023; Mirzaei et al., 2021). Vitamin metabolism was also affected by GenX exposure since “vitamin transport”, “folate (vitamin B₉) biosynthesis”, and “nicotinate and nicotinamide metabolism (vitamin B₃ derivatives)” were found to be enriched (**Figure 4.17.c**). As highlighted before, vitamin B₉ is involved in *de novo* nucleotide synthesis (Li et al., 2021), and the studies on neurodegenerative disorders (e.g., PD, AD, and HD) have shown a decrease in the vitamin B₉ level dependent on these diseases (Pradhan et al., 2023; Rosario et al., 2021; Zhang et al., 2021). Since vitamin B₉ deficiency leads to an increase in homocysteine levels and accompanying oxidative stress, this vitamin improves the α Syn-induced locomotor defects in PD (Li et al., 2021; Sarkar et al., 2020). Additionally, the promising role of vitamin B₃ in the treatment of several neurodegenerative disorders was also reported (Maiese, 2021; Wuerch et al., 2023).

The structures of the abovementioned biomolecules like lipids and proteins can be disrupted in an oxidative or nitrosative manner by oxidative stress (Maszka et al., 2023). Consistently, the enrichments of the “regulation of reactive oxygen species (ROS) metabolic process”, “response to toxic substance”, and “xenobiotic metabolic process”, supporting the molecular toxicity of GenX in the *Drosophila* brain were identified in the current study (**Figure 4.17.c**). Yoo and colleagues also showed that GenX increased intracellular ROS levels in the human-derived liver cells, which in turn triggered oxidative stress and apoptosis in a dose-dependent manner (Yoo et al., 2021). A toxicological evaluation was also conducted for the early stages of zebrafish development in a recent study. At a high concentration of GenX, the researchers found transcriptional regulations linked to neurotoxicity and an elevated ROS level (Ivantsova et al., 2023). It has also been demonstrated that GenX has triggered oxidative stress in aquatic organisms (Liu et al., 2021). Importantly, numerous neurodegenerative disorders are connected with oxidative stress resulting from mitochondrial malfunction and abnormal ROS generation (Chen et al., 2012; Jadiya et al., 2021; Landolfo et al., 2022; Ryan et al., 2014). Furthermore, the intracellular calcium and ROS signaling pathways interact with each other, so the calcium signal can regulate a wide range of pathways that involve ROS-generating enzymes. Thus, any abnormalities in this crosstalk may result in neurodegenerative disorders such as AD and PD (Görlach et al., 2015). “Cellular ion homeostasis” and “regulation of

sequestering of calcium ion” were found to be among the enriched terms for the GenX treatment (**Figure 4.17.c**). As a result, the oxidative stress-associated differential pathways supported the enriched FlyBase phenotypes illustrated in **Figure 4.17.a** and the connection between GenX exposure and neurodegeneration.

To provide additional information regarding the role of GenX in neurodegeneration, Gong et al. studied the differential expression profiles in zebrafish embryos exposed to varying concentrations of this chemical substance. In this way, they determined that the differentially expressed genes were associated with visual, neural, and vascular systems, cellular metabolism, transcriptional regulation, and developmental processes (Gong et al., 2023). The significant enrichment of “phototransduction – fly” was found to be consistent with the effects of GenX on the visual system (**Figure 4.17.c**). The developmental neurotoxicity of GenX was also examined in a recent study (Wasel et al., 2023). In contrast to the results of a related earlier study (Gaballah et al., 2020), Wasel et al. reported altered locomotor activity and dopamine levels in zebrafish (Wasel et al., 2023). Rericha and colleagues also demonstrated the neurodevelopmental toxicity of GenX in the zebrafish (Rericha et al., 2021). In this organism, the *gad1b* gene encoding glutamate decarboxylase, which catalyzes the conversion of glutamate to γ -aminobutyric acid (GABA), was previously reported to be downregulated via a transcriptomic study (Gong et al., 2023). Based on the differential proteomic analysis in this thesis study, the glutamate decarboxylase encoded by the *gad1* gene was not significantly regulated (p -value = ~ 0.3) at the proteomic level. The changes in the transcript levels showing only a proxy of differential protein abundances may be compensated at the protein levels (Smirnov et al., 2023). Thus, this transcriptional alteration may be compensated by the organism. On the other hand, a variety of GABA pathways ("GABA receptor activation", "GABA metabolism", and "GABA shunt") were determined to have differentially abundant proteins or the proteins within the GenX-mediated differential reactions (**Figure 4.17.c** and **Figure 4.17.d**). The chemically stimulated locomotor activity was shown to be reduced in rodents and humans by the GABA_B receptor agonist baclofen (Lees et al., 1978; Lhuillier et al., 2007; Woo et al., 2001). Consistently, it was induced in mice by GABA_B receptor antagonists (SCH 50911, CGP 46381, and CGP 52432) likely through the removal of GABA_B receptor-mediated tonic inhibition of dopaminergic neurons (Colombo et al., 2001) despite the availability of some

contradictory results in the literature for *Drosophila* due to the differences between the mammalian and *Drosophila* GABA_B receptors (Hillman et al., 2012; Mezler et al., 2001). It was also reported that the accumulation of extracellular GABA and the activation of GABA receptors by the inhibition of GABA reuptake transporters in adult female flies reduced locomotor activity (Leal & Neckameyer, 2002). Similarly, Roberts et al. showed the crucial function of astrocytes in limiting the tonic GABAergic inhibition of DA release due to their GABA intake by the GABA transporters (Roberts et al., 2020). We identified a significant upregulation of the GABA_B receptor 1 (GABA-B-R1) in the GenX-treated *Drosophila* brain (**Figure 4.17.d**). Hence, the relationship of GABA receptors with the dopaminergic system may contribute to several common phenotypic changes (e.g., abnormal locomotor behavior and abnormal flight) and differential metabolic patterns between GenX treatment and various neurodegenerative disorders, highlighting a need for further investigation of this circuit.

The results of the gene-based study were supported by the metabolite-based analysis. Using the Δ FBA and iMAT-based reaction activity analysis, 343 metabolites in the differential reactions were identified for the GenX exposure (**Figure 4.17.b**). The gene- and metabolite-based analyses result in many common metabolic pathways including “biosynthesis of amino acids”, “beta-alanine metabolism”, “purine metabolism”, “pyrimidine metabolism”, “fatty acid metabolism”, “propanoate metabolism”, and “nicotinate and nicotinamide metabolism”. Additionally, the metabolite-based analysis revealed the over-representation of the terms “oxidative phosphorylation” and “citrate cycle (TCA cycle)” (**Figure 4.17.e**). Compared to other cell types, neurons consume more energy due to their high-energy-consuming processes like synaptic transmission. They therefore favor the pathways linked to oxidative respiration (Cleland et al., 2021; Jia et al., 2023). The GenX-mediated disruption of energy metabolism and the elevated oxidative stress may be strongly associated with neurodegenerative disorders and the activation of apoptotic signaling (Hajam et al., 2022; Jia et al., 2023; Maszka et al., 2023). A recent transcriptomics study showed that apoptosis and autophagy were significantly enriched in the liver of mice treated with GenX (Thompson et al., 2023). In the scope of the thesis, the presence of ferroptosis-associated metabolites in the significantly affected reactions of the GenX-treated *Drosophila* was shown although the iron-dependent cell death,

ferroptosis, was not over-represented in the transcriptomics analysis of Thompson and colleagues (Thompson et al., 2023).

Furthermore, the “synaptic vesicle cycle”, “cholinergic synapse”, and “GABAergic synapse” were found to be significantly over-represented using the metabolite-based network analysis. Even if its *p*-value is less than 0.05, the GABAergic synapse was not significantly enriched for the specified FDR cut-off, so it was not included in **Figure 4.17.e**. Together, the enrichment results agree with the aforementioned possible involvement of GenX in neurotransmitter regulation. Similarly, Sammi et al. reported that this chemical compound caused the loss of DA neurons in *C. elegans*. Per- and polyfluoroalkyl compounds also affected non-DA neurons (GABAergic, serotonergic, and cholinergic neurons); however, the impact of GenX on these neurons was not examined (Sammi et al., 2019). Thus, more research is needed to determine whether GenX may have an impact on the metabolism of neurotransmitters.

Lastly, “pathways of neurodegeneration – multiple diseases” and “Parkinson’s disease” were enriched, confirming our hypothesis on the relationship between GenX and neurodegeneration (**Figure 4.17.e**). This prompted us to investigate the metabolic patterns regulated by the expression of the human α Syn protein in the *Drosophila* brain. Aberrant α Syn protein aggregation has been linked to PD and other neurodegenerative disorders, collectively known as synucleopathies (e.g., dementia with Lewy bodies, multiple system atrophy, rapid eye movement sleep behavior disorder, and pure autonomic failure) exhibiting several common clinical features and pathologies (e.g., mitochondrial impairment, disrupted calcium homeostasis, lysosomal dysfunction, and neuronal loss) (Bernal-Conde et al., 2020; Calabresi et al., 2023).

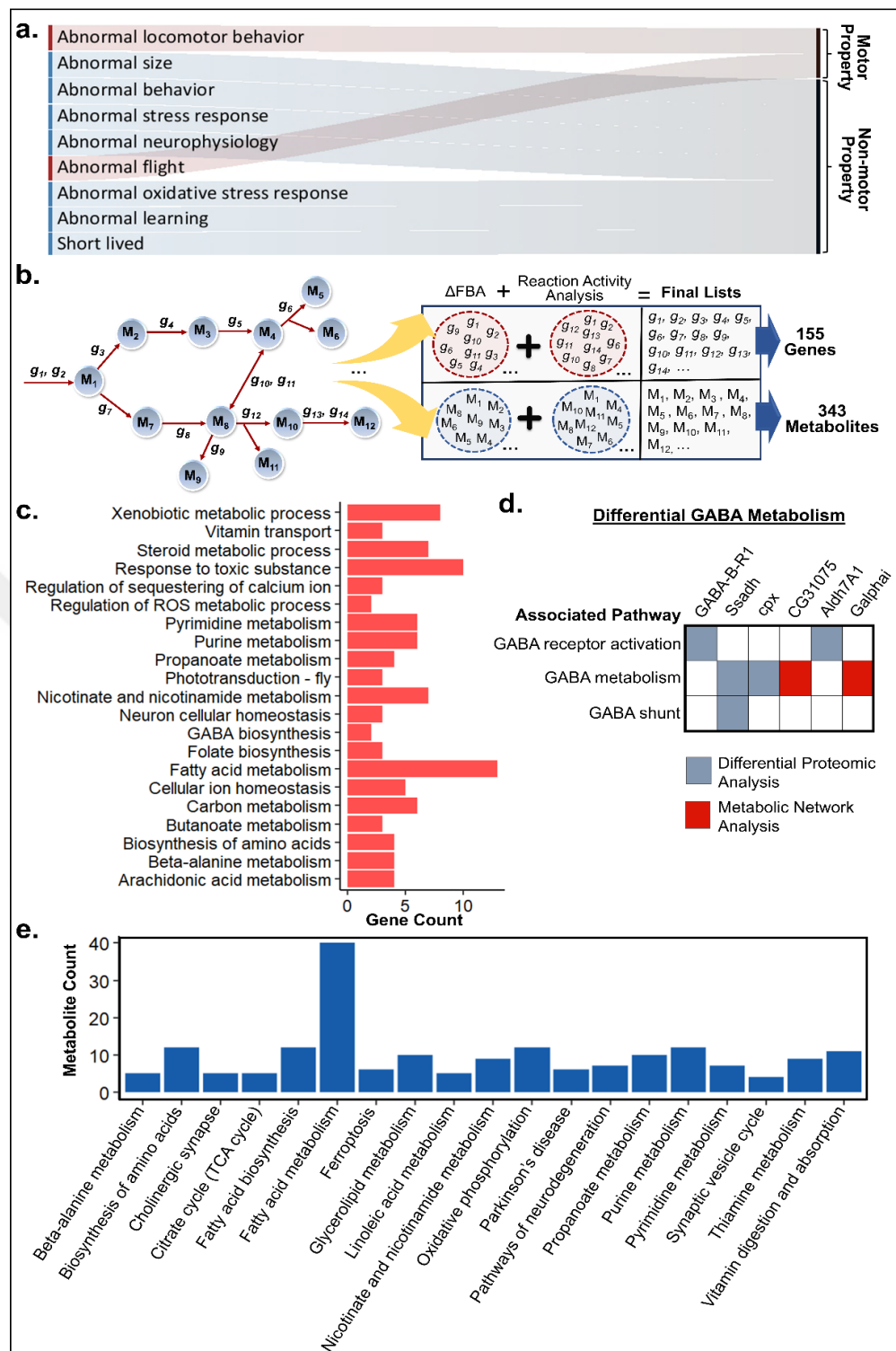


Figure 4.17: Analysis of the GenX-associated cellular regulations in the *Drosophila* brain include a. proteomics-based phenotypic predictions and b. network-based analyses to determine the regulated genes and metabolites as in the simplified illustration. Characterization of the GenX-associated regulated genes in terms of c. the significantly enriched biological processes and metabolic pathways as well as d. the GABA-related differential pathways predicted by differential proteomic analysis and network-based analyses. Enrichment analysis of e. the regulated metabolites.

The proteome data of the α Syn group was analyzed using the same methods as the gene- and metabolite-based network analyses of the GenX group. Accordingly, gene and metabolite lists in the differential reactions were compiled and the associated biological processes and metabolic pathways were determined for the 7-day-old PD model flies expressing human α Syn. The terms related to cellular respiration, oxidative stress, neurotransmission, and the metabolism of essential biomolecules were commonly found to be enriched with GenX exposure and α Syn expression despite the separation of the α Syn samples from GenX and control samples in the PCA plot in terms of the significantly affected reaction profiles (**Figure A1.7**). Collectively, further studies may provide more insight into the currently predicted roles of GenX in neurotoxicity and impaired neurobehavior in *Drosophila*.



5. CONCLUSION

PD is the second most common neurodegenerative disorder, so there is a remarkable effort to understand the metabolic processes behind this disease. On the other hand, the complex nature of this disease is the primary obstacle to the development of effective treatments. A system-level analysis of the metabolic pathways may be promising for deciphering the complicated disease-associated metabolic alterations and developing effective therapeutic strategies. Even if *Drosophila* is a workhorse in experimental studies focusing on PD, there has been still a gap in the use of this organism in PD-associated genome-scale metabolic modeling studies. Therefore, a new genome-scale metabolic network model called iDrosophila1 was first reconstructed in the current study and its superior predictive capability was shown comparatively to other generic fly models. Given the increasing amounts of multi-omics data, this model has a prominent potential to contribute to systems medicine from different perspectives. In this regard, it was used in three different research in the scope of this thesis.

In the first research, the differential PD-related vitamins were determined and their regulatory effects on PD metabolism were characterized. More specifically, the impact of the vitamins on the metabolism of other vitamins was also reported in this work. According to our findings, vitamins may be useful in the design of therapeutic functional foods to reduce the PD risk or alleviate the disease symptoms. However, their combinations and dosages in the functional foods should be carefully determined by taking into account the common and distinct PD-related pathways they regulate (e.g., central carbon metabolism, oxidative stress, and nucleotide metabolism), and their effects on each other's metabolism. In the second iDrosophila1-based research covered by the thesis, the differential metabolic pathways associated with the possible onset of PD were investigated to elaborate the hypothesis presuming that dysbiosis may cause the accumulation of α Syn proteins in EECs, and these epithelial cells may play an active role in the transfer the α Syn aggregates from the gut to the brain through their interactions with enteric nerves or vagus nerve. In this regard, α Syn-induced metabolic alterations and differential metabolic interactions between the EECs and gut microbiota were uncovered. The α Syn-induced differential metabolic pathways were shown to have similar metabolic patterns with the brain. Therefore, many PD-related

metabolic changes reported by brain-specific studies may not be the main cause of the disease, and they may also emerge in the gut in the earlier stages of PD. Furthermore, SCFA metabolism in the EECs was found to be altered by the synthesis of α Syn protein in these cells. Similarly, the transport of microbial propanoate to the gut lumen was affected in this process. This observation may be proof of the roles of the microorganisms in the PD process, as stated in the Braak hypothesis. To confirm these preliminary results, the effects of SCFAs and differential vitamins on the PD brains may be investigated in detail for flies and/or humans by following a similar methodology to Section 3.2. Additionally, the production of multi-omics datasets may be beneficial. Importantly, the interactions between only gut microbiota and EECs were characterized in the current study whereas the communication of the EECs with the enteric nerves is also promising to further characterize the potential PD-related circuit. In the third research, the iDrosophila1 model was used to assess the toxic effects of GenX on the metabolic pathways of the fly brain in a comparative manner with α Syn. This study also supported the predictive capacity of this fly model in the studies of toxicity assessment.

Even if this thesis provides an important insight into PD metabolism from diverse perspectives and the use of *Drosophila* in such studies has many advantages, it has also several drawbacks (e.g., the lack of an adaptive immune system and vagus nerve, the absence of an *SNCA* ortholog, and possessing conserved but simplified nervous and gastrointestinal systems compared to humans). Therefore, some analyses may be repeated using human models to further validate the suitability of fly models in the analysis of human metabolism from different perspectives. Furthermore, the development of multi-cell or multi-tissue genome-scale metabolic models may facilitate a deeper characterization of the complicated systems in PD such as the metabolic interactions among the gut cells, microbial cells, enteric nerves, and brain in the disease process. Despite the limited use of machine learning and deep learning algorithms in metabolic modeling, these approaches may also be further incorporated into this field to increase the accuracy of model predictions. Taken together, the iDrosophila1 model may be a promising starting point for developing complicated models and different applications in future studies.

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BIOGRAPHY

Müberra Fatma CESUR graduated from the Department of Molecular Biology and Genetics at Gebze Technical University in 2015. At the same university, she received MSc degrees from the Department of Molecular Biology and Genetics in 2017 and the Bioinformatics and Systems Biology Program under the Bioengineering Department in 2018. In the same year, she started the Bioinformatics and Systems Biology PhD program. Thus, she is currently a PhD candidate at Gebze Technical University. In the scope of her PhD thesis, she was awarded a research scholarship by TUBITAK (2214/A International Research Program) and worked as a Visiting Researcher at Chalmers University of Technology for almost 1 year. In addition, she worked as a Research Assistant at Gebze Technical University between 2015 and 2024. Since March 2024, she has been working as a Bioinformatician at the PhiTech Bioinformatics.



PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Cesur M. F., Basile A., Patil K. R., Çakır, T. (2023), “A new metabolic model of *Drosophila melanogaster* and the integrative analysis of Parkinson's disease”, Life Science Alliance, 6(8).

Abu-Salah A., Cesur M. F., Anchan A., Ay M., Langley M. R., Shah A., Reina-Gonzalez P., Çakır T., Sarkar S. (2024), “Comparative proteomics highlights that GenX exposure leads to metabolic defects and inflammation in astrocytes”, Environmental Science & Technology (*under review*).

Cesur M. F., Çakır T., Patil K. R. (2022), “iDrosophila1: An orthology-based reconstruction of genome-scale metabolic network model for *Drosophila melanogaster*”, International Symposium on Health Informatics and Bioinformatics (HIBIT 2022), Mersin/Turkey, 20-21 October (*poster presentation*).

APPENDICES

APPENDIX-A: Supporting Information

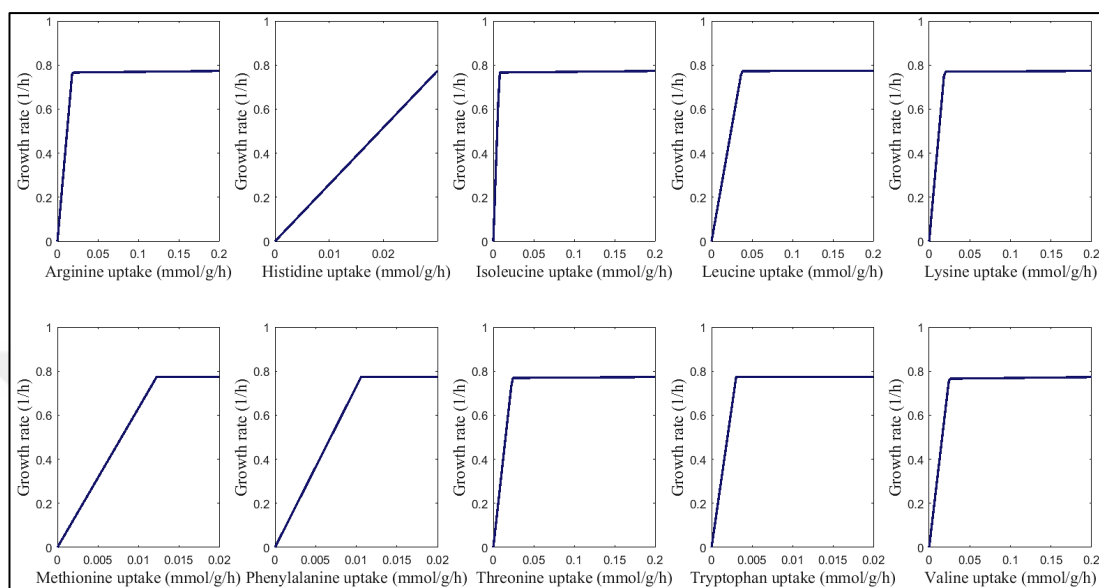


Figure A1.1: Effect of essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in the expanded Hd on the growth rates of *Drosophila* by increasing their uptake rates in the range of 0-0.2 mmol g⁻¹h⁻¹.

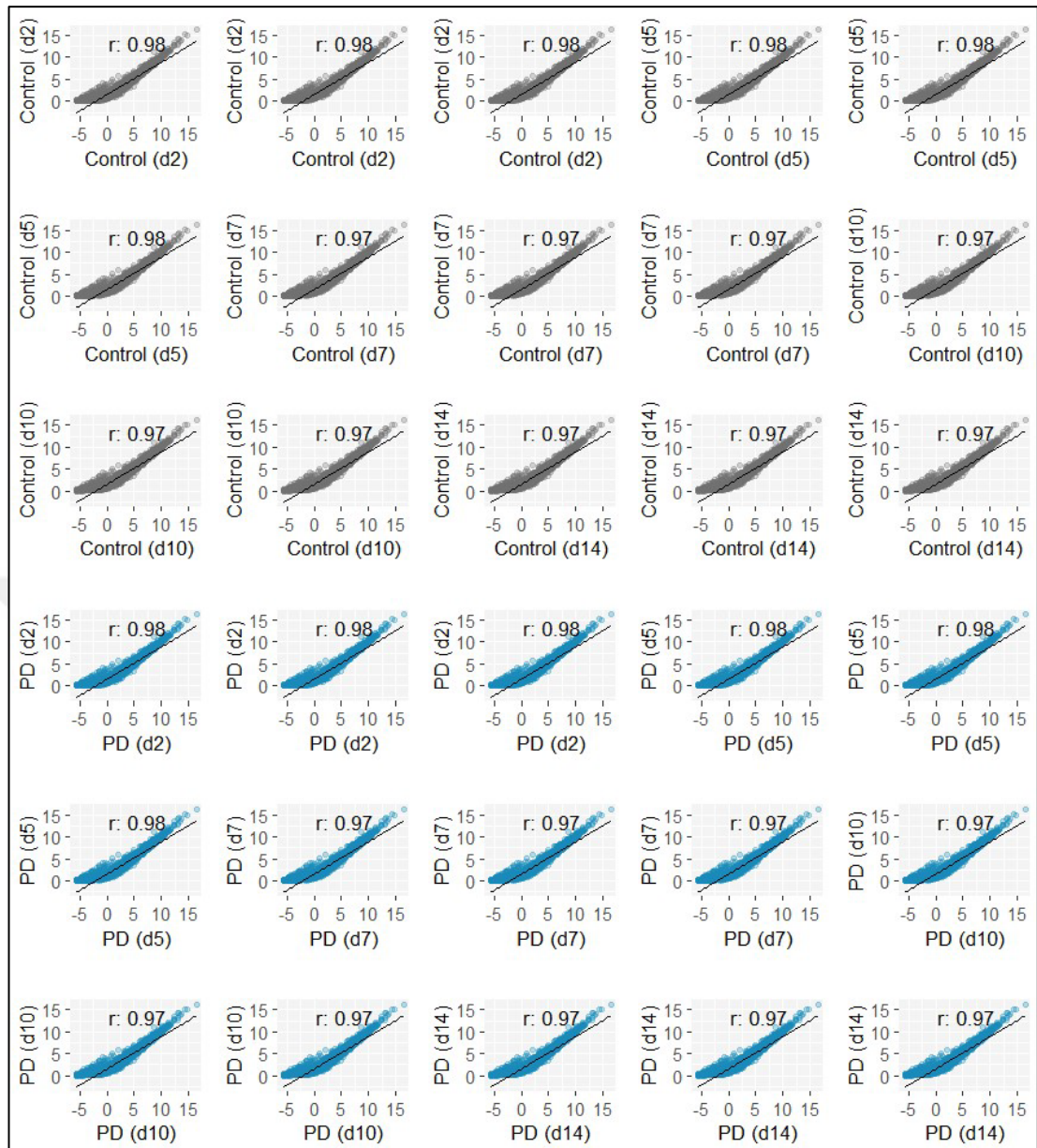


Figure A1.2: Comparison of the covariate-adjusted RNA-seq counts with the normalized (but unadjusted) counts based on the Pearson correlation coefficients.

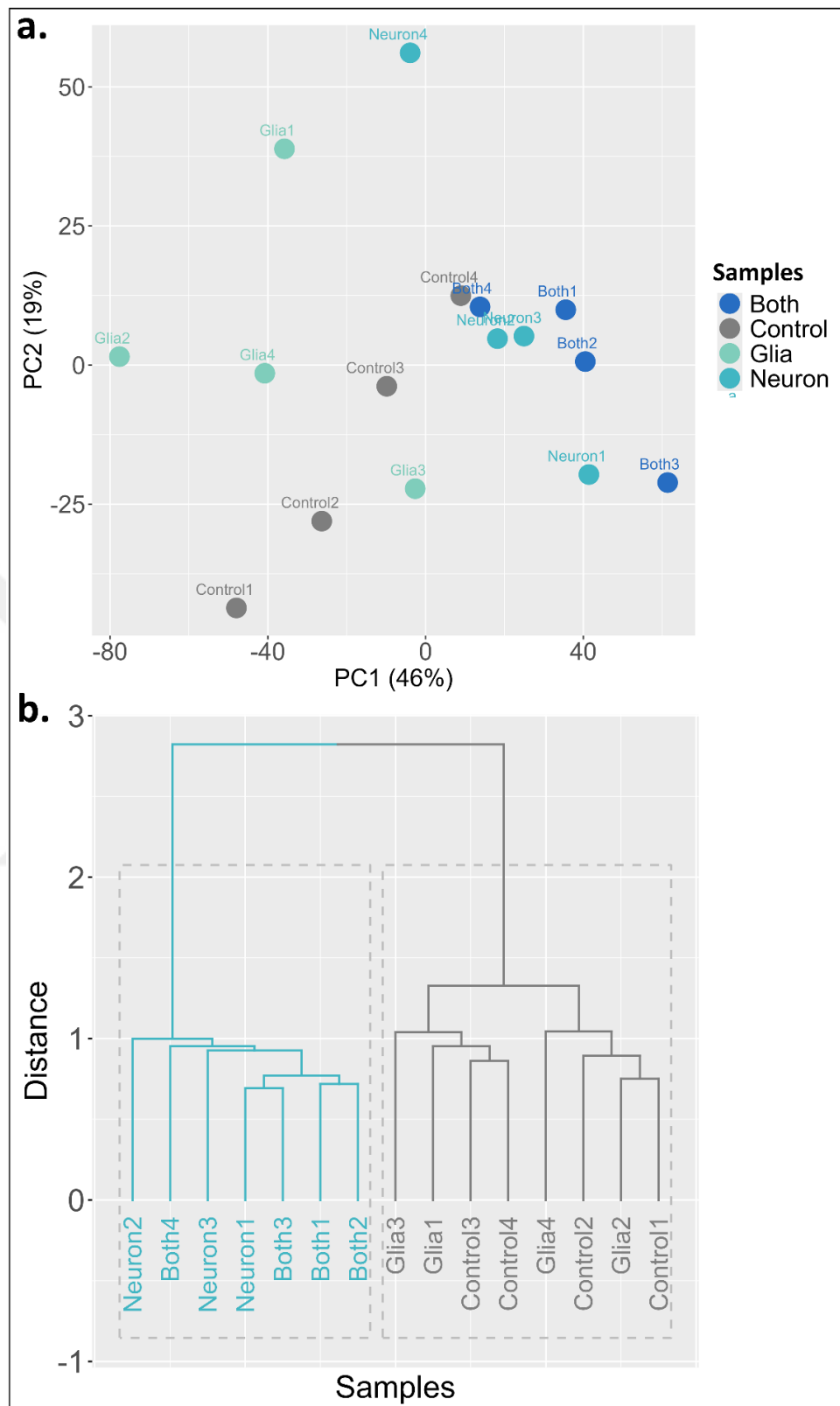


Figure A1.3: Cell type-specific distributions and clustering of the RNA-seq samples. a. Investigation of the outlier samples using PCA resulted in the identification of one outlier sample (Neuron4). b. Hierarchical clustering of samples after the removal of the outlier sample.

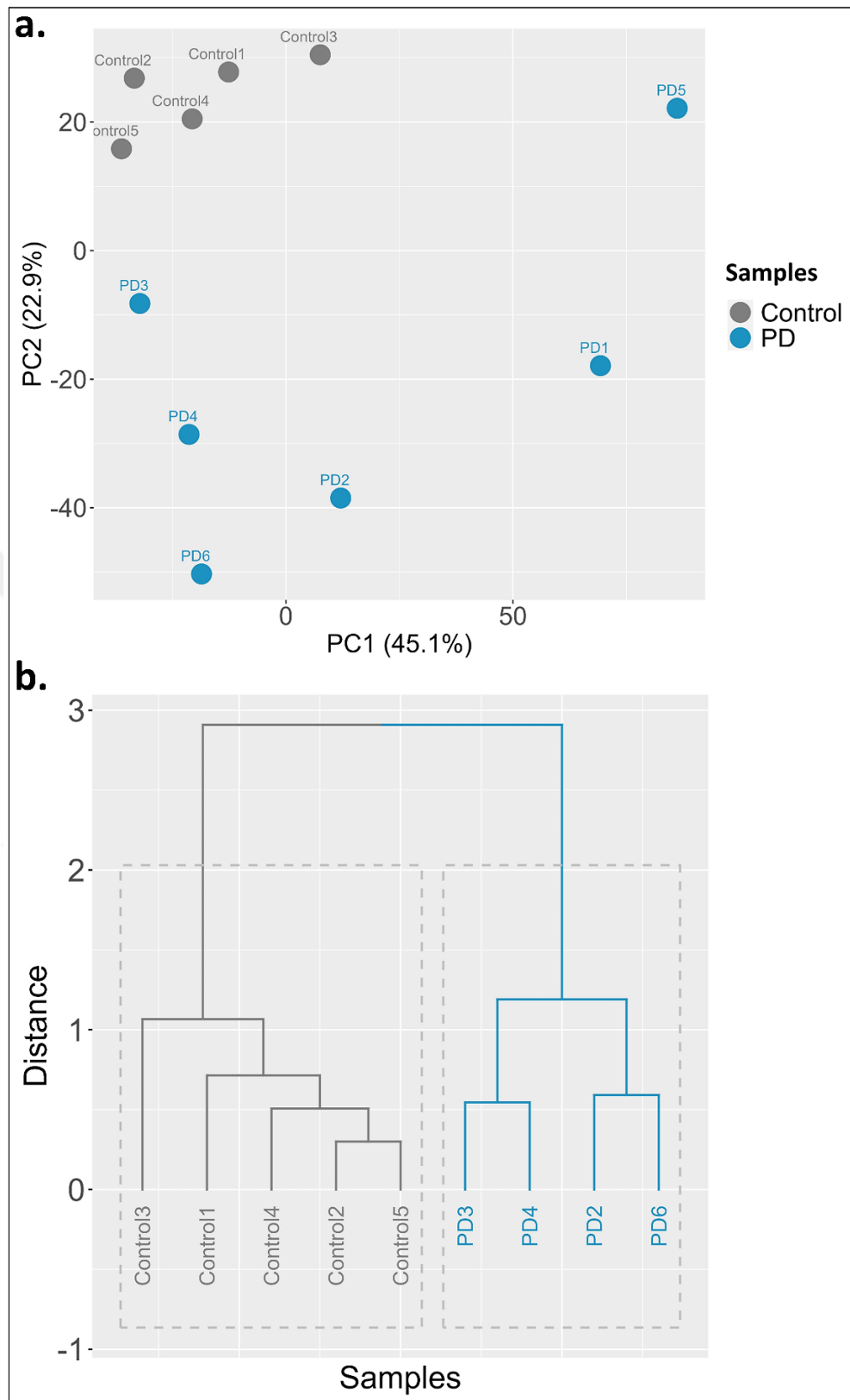


Figure A1.4: Distribution and clustering of the proteome samples. a. Two samples (PD1 and PD5) were identified as the outliers using PCA. b. Hierarchical clustering of the samples after excluding the outlier samples.

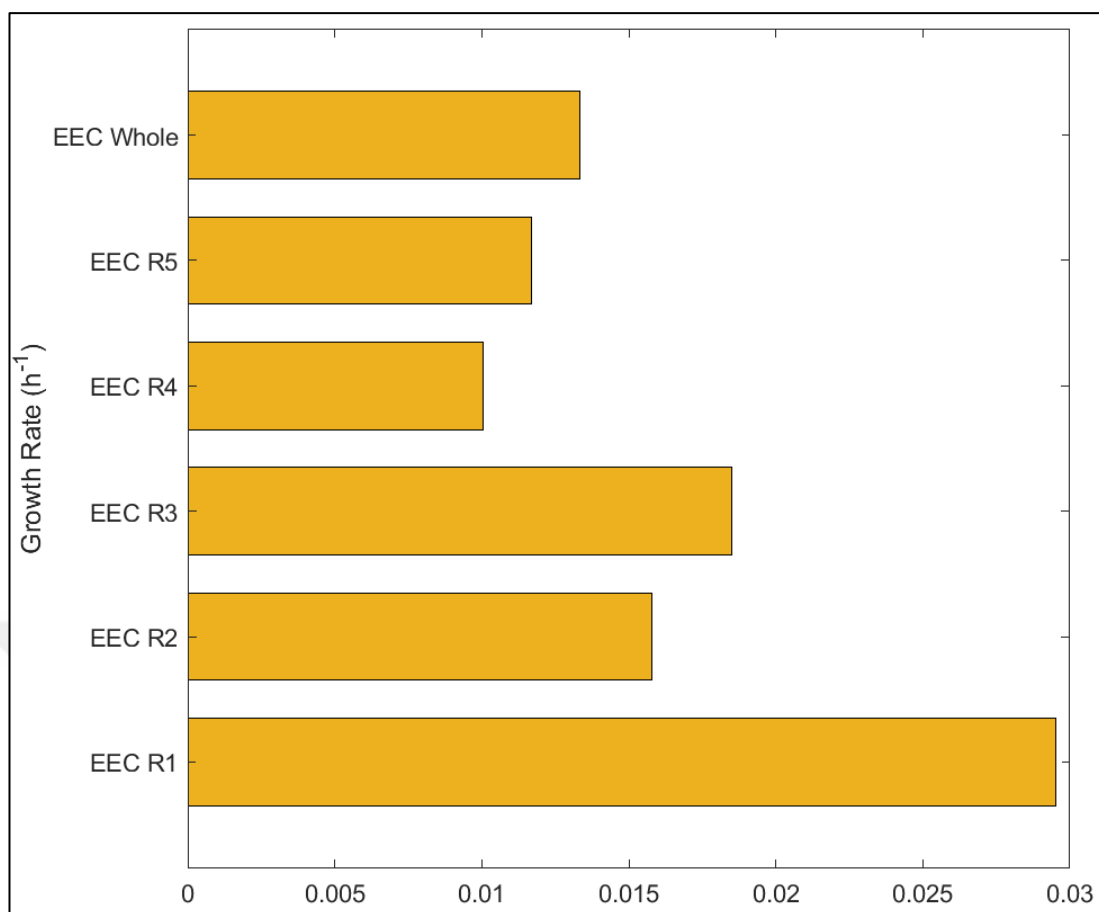


Figure A1.5: Optimal growth rates of the EECs in the DMEM/F-12 medium.

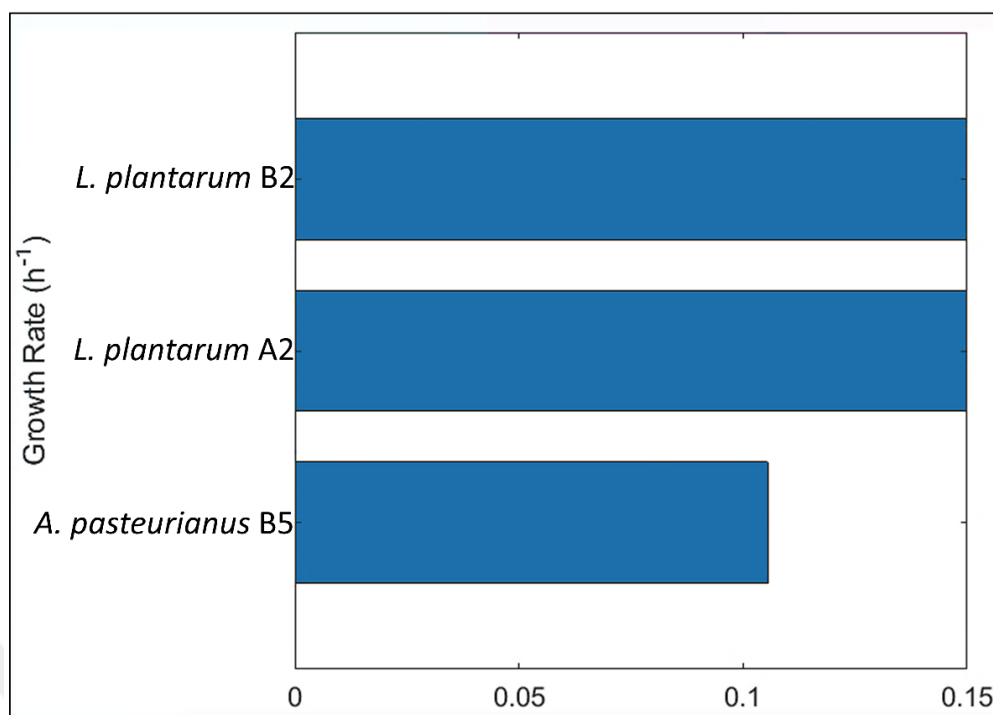


Figure A1.6: Optimal growth rates of the bacterial strains in the DMEM/F-12 medium.

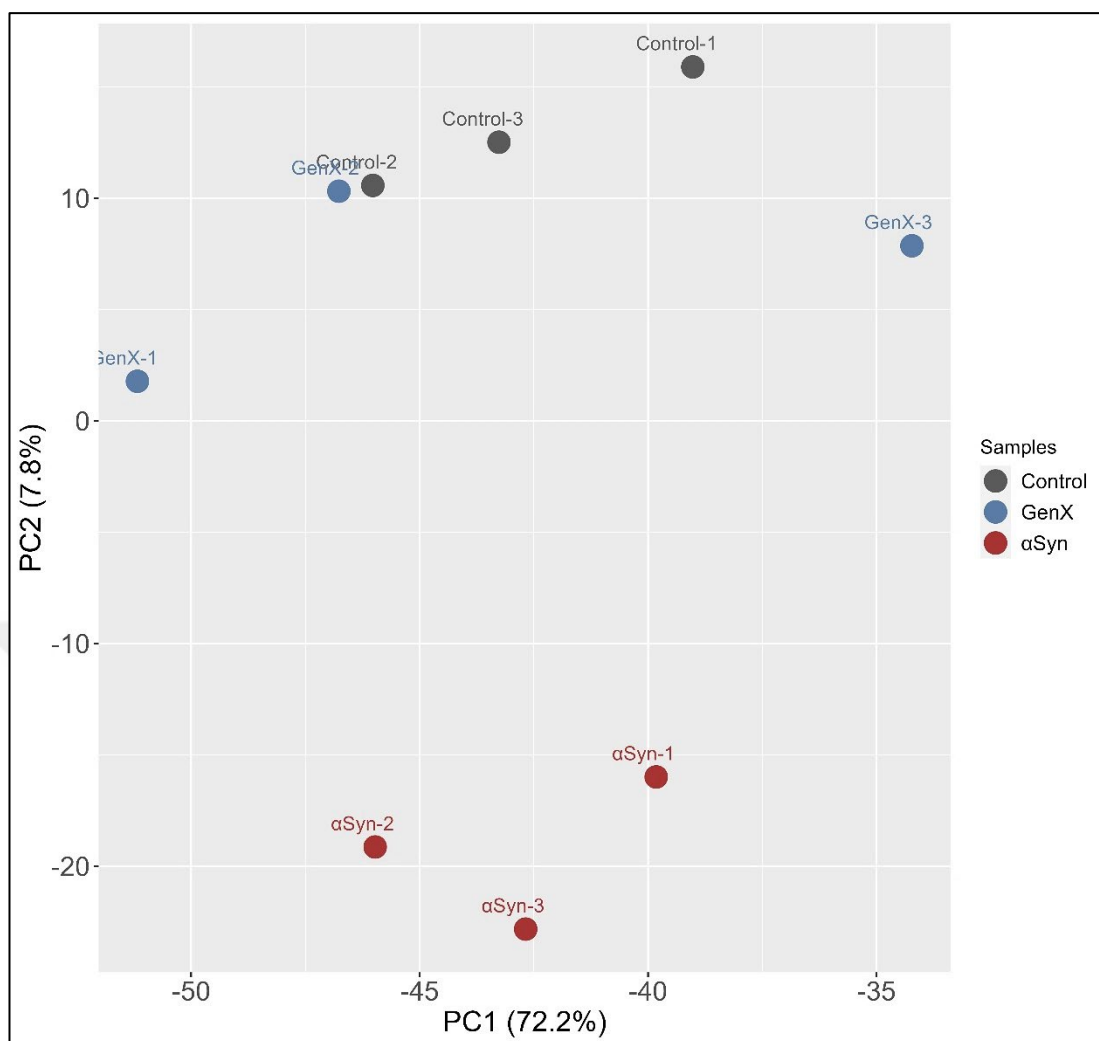


Figure A1.7: Reaction activity-based distribution of the α Syn, GenX, and control samples using the PCA approach.

Table A1.1: The recipes of the Hd and DMEM/F-12 media used in different research in the scope of this thesis. Notes were added about the modifications in their compositions based on the studies. Accordingly, expanded media obtained by the addition of several extra metabolites to the original medium. All vitamins added to the Hd medium in vitamin-based analyses are highlighted by the blue color.

Exchange Metabolite	Medium	Note
Alanine	Hd DMEM/F-12	Present in the original medium
Arginine	Hd DMEM/F-12	Present in the original medium
Asparagine	Hd DMEM/F-12	Present in the original medium
Aspartate	Hd DMEM/F-12	Present in the original medium
Cysteine	Hd DMEM/F-12	Present in the original medium
Glutamate	Hd DMEM/F-12	Present in the original medium
Glutamine	Hd DMEM/F-12	Present in the original medium
Glycine	Hd DMEM/F-12	Present in the original medium
Histidine	Hd DMEM/F-12	Present in the original medium
Isoleucine	Hd DMEM/F-12	Present in the original medium
Leucine	Hd DMEM/F-12	Present in the original medium
Lysine	Hd DMEM/F-12	Present in the original medium
Methionine	Hd DMEM/F-12	Present in the original medium
Phenylalanine	Hd DMEM/F-12	Present in the original medium
Proline	Hd DMEM/F-12	Present in the original medium
Serine	Hd DMEM/F-12	Present in the original medium
Threonine	Hd DMEM/F-12	Present in the original medium
Tryptophan	Hd DMEM/F-12	Present in the original medium
Tyrosine	Hd DMEM/F-12	Present in the original medium

Table A1.1: Continue

Exchange Metabolite	Medium	Note
Valine	Hd DMEM/F-12	Present in the original medium
Cystine	Hd DMEM/F-12	Present in the original medium
Acetate	Hd	Present in the original medium
Inositol	Hd DMEM/F-12	Present in the original medium
Glucose (or sucrose)	Hd DMEM/F-12	Present in the original medium
Pyruvate	DMEM/F-12	Present in the original medium
Ca ₂₊	Hd DMEM/F-12	Present in the original medium
Fe ₂₊	Hd DMEM/F-12	Present in the original medium
Sulfate	Hd DMEM/F-12	Present in the original medium
K ⁺	Hd DMEM/F-12	Present in the original medium
P _i	Hd DMEM/F-12	Present in the original medium
Chloride	Hd DMEM/F-12	Present in the original medium
H ₂ O	Hd DMEM/F-12	Present in the original medium
Magnesium	Hd DMEM/F-12	Added to the expanded medium for bacterial growth
Mn ²⁺	Hd DMEM/F-12	Added to the expanded medium for bacterial growth
Fe ³⁺	Hd DMEM/F-12	Present in the original medium
HCO ₃ ⁻	Hd DMEM/F-12	Present in the original medium
Na ⁺	Hd	Present in the original medium
Zinc	Hd DMEM/F-12	Present in the original medium
Cu ²⁺	Hd DMEM/F-12	Added to the expanded medium for bacterial growth
Cholesterol	Hd DMEM/F-12	Present/added to the expanded medium for fly growth

Table A1.1: Continue

Exchange Metabolite	Medium	Note
Linoleate	Hd	Present/added to the expanded medium for fly growth
Lipoic acid	Hd DMEM/F-12	Present/added to the expanded medium for fly growth
Inosine	Hd DMEM/F-12	Present in the original medium
Hypoxanthine	Hd DMEM/F-12	Present/added to the expanded medium for fly growth
Uridine	Hd DMEM/F-12	Present/added to the expanded medium for fly growth
Putrescine	DMEM/F-12	Present in the original medium
Choline	Hd DMEM/F-12	Present in the original medium
Thymidine	Hd DMEM/F-12	Present in the original medium
Nicotinamide	Hd DMEM/F-12	Present in the original medium
Oxidized form of vitamin A (retinoate)	Hd DMEM/F-12	Added to the expanded medium for fly growth
Vitamin B ₁ (thiamin)	Hd DMEM/F-12	Present in the original medium
Vitamin B ₂ (riboflavin)	Hd DMEM/F-12	Present in the original medium
Vitamin B ₃ (nicotinate)	Hd DMEM/F-12	Present/added to the expanded medium for fly growth
Vitamin B ₅ (pantothenate)	Hd DMEM/F-12	Present in the original medium
Vitamin B ₆ (pyridoxine)	Hd	Present in the original medium
Vitamin B ₇ (biotin)	Hd DMEM/F-12	Present in the original medium
Vitamin B ₉ (folate)	Hd DMEM/F-12	Present in the original medium
Vitamin C (ascorbate)	Hd	Added to the expanded medium for only vitamin analyses
Vitamin D ₂	Hd	Added to the expanded medium for only vitamin analyses
Vitamin E (gamma-tocopherol)	Hd DMEM/F-12	Added to the expanded medium for fly growth
Vitamin E (alpha-tocopherol)	Hd DMEM/F-12	Added to the expanded medium for fly growth
Vitamin B _{12a} (aquacob(III)alamin)	Hd DMEM/F-12	Added to the expanded medium for fly growth

Table A1.2: The α Syn-mediated regulated reaction list excluded from the MOMA optimization.

Reaction	Equation	Pathway
HMR_0013	HDL -> HDL remnant	Pool reactions
HMR_0459	H ₂ O + NAD ⁺ + glyceraldehyde -> H ⁺ + NADH + glycerate	Glycerolipid metabolism
HMR_0486	sn-glycerol-3-phosphate + lauroyl-CoA -> 1-acylglycerol-3P-laur + CoA	Glycerolipid metabolism
HMR_0487	tridecanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-tridec + CoA	Glycerolipid metabolism
HMR_0488	sn-glycerol-3-phosphate + myristoyl-CoA -> 1-acylglycerol-3P-myrist + CoA	Glycerolipid metabolism
HMR_0489	(9E)-tetradecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-9-tetrad + CoA	Glycerolipid metabolism
HMR_0490	(7Z)-tetradecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-7-tetrad + CoA	Glycerolipid metabolism
HMR_0491	5-tetradecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-5-tetrad + CoA	Glycerolipid metabolism
HMR_0492	pentadecanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-pentade + CoA	Glycerolipid metabolism
HMR_0493	sn-glycerol-3-phosphate + palmitoyl-CoA -> 1-acylglycerol-3P-palm + CoA	Glycerolipid metabolism
HMR_0494	sn-glycerol-3-phosphate + palmitoleoyl-CoA -> 1-acylglycerol-3P-palmn + CoA	Glycerolipid metabolism
HMR_0495	7-hexadecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-7-hexade + CoA	Glycerolipid metabolism
HMR_0496	heptadecanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-heptade + CoA	Glycerolipid metabolism
HMR_0497	(10Z)-heptadecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-10-heptade + CoA	Glycerolipid metabolism
HMR_0498	9-heptadecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-9-heptade + CoA	Glycerolipid metabolism
HMR_0499	sn-glycerol-3-phosphate + stearoyl-CoA -> 1-acylglycerol-3P-stea + CoA	Glycerolipid metabolism
HMR_0500	(13Z)-octadecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-13-octade + CoA	Glycerolipid metabolism
HMR_0501	cis-vaccenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-cis-vac + CoA	Glycerolipid metabolism
HMR_0502	sn-glycerol-3-phosphate + oleoyl-CoA -> 1-acylglycerol-3P-ol + CoA	Glycerolipid metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_0503	(9E)-octadecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-9-octade + CoA	Glycerolipid metabolism
HMR_0504	(7Z)-octadecenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-7-octade + CoA	Glycerolipid metabolism
HMR_0505	(6Z,9Z)-octadecadienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-6,9-octa + CoA	Glycerolipid metabolism
HMR_0506	nonadecanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-nanode + CoA	Glycerolipid metabolism
HMR_0507	eicosanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-eico + CoA	Glycerolipid metabolism
HMR_0508	(13Z)-eicosenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-13-eicose + CoA	Glycerolipid metabolism
HMR_0509	(11Z)-eicosenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-11-eico + CoA	Glycerolipid metabolism
HMR_0510	9-eicosenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-9-eicose + CoA	Glycerolipid metabolism
HMR_0511	(8Z,11Z)-eicosadienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-8,11-eico + CoA	Glycerolipid metabolism
HMR_0512	(5Z,8Z,11Z)-eicosatrienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-5,8,11-eico + CoA	Glycerolipid metabolism
HMR_0513	heneicosanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-heneico + CoA	Glycerolipid metabolism
HMR_0514	docosanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-docosa + CoA	Glycerolipid metabolism
HMR_0515	(13Z)-docosenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-13-docose + CoA	Glycerolipid metabolism
HMR_0516	(11Z)-docosenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-11-docose + CoA	Glycerolipid metabolism
HMR_0517	tricosanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-trico + CoA	Glycerolipid metabolism
HMR_0518	tetracosanoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-tetraco + CoA	Glycerolipid metabolism
HMR_0519	sn-glycerol-3-phosphate + (15Z)-tetracosenoyl-CoA -> 1-acylglycerol-3P-15-tetra + CoA	Glycerolipid metabolism
HMR_0520	sn-glycerol-3-phosphate + hexacosanoyl-CoA -> 1-acylglycerol-3P-hexacosa + CoA	Glycerolipid metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_0521	hexacosenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-hexecose + CoA	Glycerolipid metabolism
HMR_0522	sn-glycerol-3-phosphate + linolenoyl-CoA -> 1-acylglycerol-3P-linolen + CoA	Glycerolipid metabolism
HMR_0523	(6Z,9Z,12Z,15Z)-octadecatetraenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-6,9,12,15-octa + CoA	Glycerolipid metabolism
HMR_0524	(8Z,11Z,14Z,17Z)-eicosatetraenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-8,11,14,17-eico + CoA	Glycerolipid metabolism
HMR_0525	(5Z,8Z,11Z,14Z,17Z)-eicosapentaenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-5,8,11,14,17-eico + CoA	Glycerolipid metabolism
HMR_0526	(7Z,10Z,13Z,16Z,19Z)-docosapentaenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-7,10,13,16,19-docosa + CoA	Glycerolipid metabolism
HMR_0527	(9Z,12Z,15Z,18Z,21Z)-tetracosapentaenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-9,12,15,18,21-tetra + CoA	Glycerolipid metabolism
HMR_0528	sn-glycerol-3-phosphate + (6Z,9Z,12Z,15Z,18Z,21Z)-tetracosahexaenoyl-CoA -> 1-acylglycerol-3P-6,9,12,15,18,21-tetra + CoA	Glycerolipid metabolism
HMR_0529	sn-glycerol-3-phosphate + (4Z,7Z,10Z,13Z,16Z,19Z)-docosahexaenoyl-CoA -> 1-acylglycerol-3P-4,7,10,13,16,19-doco + CoA	Glycerolipid metabolism
HMR_0530	(11Z,14Z,17Z)-eicosatrienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-11,14,17-eico + CoA	Glycerolipid metabolism
HMR_0531	13,16,19-docosatrienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-13,16,19-doco + CoA	Glycerolipid metabolism
HMR_0532	10,13,16,19-docosatetraenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-10,13,16,19-doco + CoA	Glycerolipid metabolism
HMR_0533	12,15,18,21-tetracosatetraenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-12,15,18,21-tetra + CoA	Glycerolipid metabolism
HMR_0534	sn-glycerol-3-phosphate + linoleoyl-CoA -> 1-acylglycerol-3P-lin + CoA	Glycerolipid metabolism
HMR_0535	sn-glycerol-3-phosphate + gamma-linolenoyl-CoA -> 1-acylglycerol-3P-gamma-lin + CoA	Glycerolipid metabolism
HMR_0536	dihomo-gamma-linolenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-dihomo-gamma + CoA	Glycerolipid metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_0537	sn-glycerol-3-phosphate + arachidonyl-CoA -> 1-acylglycerol-3P-arach + CoA	Glycerolipid metabolism
HMR_0538	(7Z,10Z,13Z,16Z)-docosatetraenoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-7,10,13,16-docosa + CoA	Glycerolipid metabolism
HMR_0539	sn-glycerol-3-phosphate + (9Z,12Z,15Z,18Z)-tetracosatetraenoyl-CoA -> 1-acylglycerol-3P-9,12,15,18-tetraco + CoA	Glycerolipid metabolism
HMR_0540	sn-glycerol-3-phosphate + (6Z,9Z,12Z,15Z,18Z)-tetracosapentaenoyl-CoA -> 1-acylglycerol-3P-6,9,12,15,18-tetraco + CoA	Glycerolipid metabolism
HMR_0541	sn-glycerol-3-phosphate + (4Z,7Z,10Z,13Z,16Z)-docosapentaenoyl-CoA -> 1-acylglycerol-3P-4,7,10,13,16-docosa + CoA	Glycerolipid metabolism
HMR_0542	(11Z,14Z)-eicosadienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-11,14-eicosa + CoA	Glycerolipid metabolism
HMR_0543	(13Z,16Z)-docosadienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-13,16-docosa + CoA	Glycerolipid metabolism
HMR_0544	10,13,16-docosatrienoyl-CoA + sn-glycerol-3-phosphate -> 1-acylglycerol-3P-10,13,16-docosa + CoA	Glycerolipid metabolism
HMR_0605	1,2-diacylglycerol-LD-TAG pool + acyl-CoA-LD-TG3 pool -> TAG-LD pool + CoA	Acylglycerides metabolism
HMR_0651	ethanolamine-phosphate + CTP -> PPi + CDP-ethanolamine	Glycerophospholipid metabolism
HMR_0667	1,2-diacylglycerol-LD-TAG pool + H2O -> 1-acylglycerol-LD-TG1 pool + fatty acid-LD-TG2 pool	Acylglycerides metabolism
HMR_0668	1,2-diacylglycerol-LD-PI pool + H2O -> 1-acylglycerol-LD-PI pool + fatty acid-LD-PI pool	Acylglycerides metabolism
HMR_0669	1,2-diacylglycerol-LD-PC pool + H2O -> 1-acylglycerol-LD-PC pool + fatty acid-LD-PC pool	Acylglycerides metabolism
HMR_0670	1,2-diacylglycerol-LD-PE pool + H2O -> 1-acylglycerol-LD-PE pool + fatty acid-LD-PE pool	Acylglycerides metabolism
HMR_0671	1,2-diacylglycerol-LD-PS pool + H2O -> 1-acylglycerol-LD-PS pool + fatty acid-LD-PS pool	Acylglycerides metabolism
HMR_0672	1,2-diacylglycerol-LD-SM pool + H2O -> 1-acylglycerol-LD-SM pool + fatty acid-LD-SM pool	Acylglycerides metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_0786	H ₂ O + LacCer pool -> galactose + glucosylceramide pool	Sphingolipid metabolism
HMR_1325	prostaglandin F ₂ alpha + NAD ⁺ -> prostaglandin H ₂ + H ⁺ + NADH	Prostaglandin biosynthesis
HMR_1326	prostaglandin F ₂ alpha + NADP ⁺ -> prostaglandin H ₂ + H ⁺ + NADPH	Prostaglandin biosynthesis
HMR_1329	prostaglandin D ₂ + H ⁺ + NADPH <=> prostaglandin F ₂ alpha + NADP ⁺	Prostaglandin biosynthesis
HMR_1457	isopentenyl-pPP + dimethylallyl-PP -> geranyl-PP + PPi	Cholesterol metabolism
HMR_1460	isopentenyl-pPP + geranyl-PP -> PPi + farnesyl-PP	Cholesterol metabolism
HMR_2003	allopregnanolone + NADP ⁺ <=> 5alpha-pregnane-3,20-dione + H ⁺ + NADPH	Steroid metabolism
HMR_2004	5alpha-pregnane-3,20-dione + H ⁺ + NADH <=> allopregnanolone + NAD ⁺	Steroid metabolism
HMR_2592	L-carnitine + O-acetylcarnitine -> O-acetylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2596	O-propanoylcarnitine + L-carnitine <=> O-propanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2598	O-propanoylcarnitine + CoA <=> propanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2600	malonyl-carnitin + L-carnitine -> malonyl-carnitin + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2601	malonyl-carnitin + CoA <=> malonyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2603	dodecanoylcarnitine + L-carnitine <=> dodecanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2604	dodecanoylcarnitine + CoA <=> lauroyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2606	tridecanoylcarnitine + L-carnitine <=> tridecanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2607	tridecanoylcarnitine + CoA <=> tridecanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2609	tetradecanoylcarnitine + L-carnitine <=> tetradecanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2610	tetradecanoylcarnitine + CoA <=> myristoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2616	tetradecenoylcarnitine(7) + L-carnitine <=> tetradecenoylcarnitine(7) + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2618	(7Z)-tetradecenoyl-CoA + L-carnitine <=> tetradecenoylcarnitine(7) + CoA	Carnitine shuttle (mitochondrial)
HMR_2624	pentadecanoylcarnitine + L-carnitine <=> pentadecanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2625	pentadecanoylcarnitine + CoA <=> pentadecanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2629	L-palmitoylcarnitine + L-carnitine <=> L-palmitoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2630	L-palmitoylcarnitine + CoA <=> palmitoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_2634	hexadecenoylcarnitine(9) + L-carnitine $\rightleftharpoons$ hexadecenoylcarnitine(9) + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2635	7-hexadecenoyl-CoA + L-carnitine $\rightleftharpoons$ hexadecenoylcarnitine(9) + CoA	Carnitine shuttle (mitochondrial)
HMR_2638	palmitoleoyl-carnitine + L-carnitine $\rightleftharpoons$ palmitoleoyl-carnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2640	palmitoleoyl-carnitine + CoA $\rightleftharpoons$ palmitoleoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2644	heptadecanoylcarnitine + L-carnitine $\rightleftharpoons$ heptadecanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2646	heptadecanoylcarnitine + CoA $\rightleftharpoons$ heptadecanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2655	stearoylcarnitine + L-carnitine $\rightleftharpoons$ stearoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2656	stearoylcarnitine + CoA $\rightleftharpoons$ stearoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2658	octadecenoylcarnitine(5) + L-carnitine $\rightleftharpoons$ octadecenoylcarnitine(5) + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2659	(13Z)-octadecenoyl-CoA + L-carnitine $\rightleftharpoons$ octadecenoylcarnitine(5) + CoA	Carnitine shuttle (mitochondrial)
HMR_2661	octadecenoylcarnitine(7) + L-carnitine $\rightleftharpoons$ octadecenoylcarnitine(7) + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2662	octadecenoylcarnitine(7) + CoA $\rightleftharpoons$ cis- vaccenoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2664	L-oleoylcarnitine + L-carnitine $\rightleftharpoons$ L- oleoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2665	L-oleoylcarnitine + CoA $\rightleftharpoons$ oleoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2676	nonadecanoylcarnitine + L-carnitine $\rightleftharpoons$ nonadecanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2677	nonadecanoylcarnitine + CoA $\rightleftharpoons$ nonadecanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2679	eicosanoylcarnitine + L-carnitine $\rightleftharpoons$ eicosanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2680	eicosanoylcarnitine + CoA $\rightleftharpoons$ eicosanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2682	eicosenoylcarnitine(7) + L-carnitine $\rightleftharpoons$ eicosenoylcarnitine(7) + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2683	(13Z)-eicosenoyl-CoA + L-carnitine $\rightleftharpoons$ eicosenoylcarnitine(7) + CoA	Carnitine shuttle (mitochondrial)
HMR_2685	eicosenoylcarnitine(9) + L-carnitine $\rightleftharpoons$ eicosenoylcarnitine(9) + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2686	(11Z)-eicosenoyl-CoA + L-carnitine $\rightleftharpoons$ eicosenoylcarnitine(9) + CoA	Carnitine shuttle (mitochondrial)
HMR_2700	heneicosanoylcarnitine + L-carnitine $\rightleftharpoons$ heneicosanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2701	heneicosanoylcarnitine + CoA $\rightleftharpoons$ heneicosanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2703	docosanoylcarnitine + L-carnitine $\rightleftharpoons$ docosanoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_2704	docosanoylcarnitine + CoA $\rightleftharpoons$ docosanoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2706	docosenoylcarnitine + L-carnitine $\rightleftharpoons$ docosenoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2707	(13Z)-docosenoyl-CoA + L-carnitine $\rightleftharpoons$ docosenoylcarnitine + CoA	Carnitine shuttle (mitochondrial)
HMR_2748	gamma-linolenoylcarnitine + L-carnitine $\rightleftharpoons$ gamma-linolenoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2750	gamma-linolenoylcarnitine + CoA $\rightleftharpoons$ gamma-linolenoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2753	eicosatrienoylcarnitine + L-carnitine $\rightleftharpoons$ eicosatrienoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2754	eicosatrienoylcarnitine + CoA $\rightleftharpoons$ dihomogamma-linolenoyl-CoA + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2760	ocosatetraenoylcarnitine + L-carnitine $\rightleftharpoons$ ocosatetraenoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2761	(7Z,10Z,13Z,16Z)-docosatetraenoyl-CoA + L-carnitine $\rightleftharpoons$ ocosatetraenoylcarnitine + CoA	Carnitine shuttle (mitochondrial)
HMR_2775	10,13,16-docosatrienoylcarnitine + L-carnitine $\rightleftharpoons$ 10,13,16-docosatrienoylcarnitine + L-carnitine	Carnitine shuttle (mitochondrial)
HMR_2776	10,13,16-docosatrienoyl-CoA + L-carnitine $\rightleftharpoons$ 10,13,16-docosatrienoylcarnitine + CoA	Carnitine shuttle (mitochondrial)
HMR_2803	L-palmitoylcarnitine + CoA $\rightleftharpoons$ palmitoyl-CoA + L-carnitine	Carnitine shuttle (endoplasmic reticular)
HMR_2866	(5Z,8Z,11Z,14Z,17Z)-eicosapentaenoyl-CoA + L-carnitine $\rightleftharpoons$ (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoylcarnitine + CoA	Carnitine shuttle (endoplasmic reticular)
HMR_3035	O-propanoylcarnitine + L-carnitine $\rightleftharpoons$ O-propanoylcarnitine + L-carnitine	Carnitine shuttle (peroxisomal)
HMR_3037	O-propanoylcarnitine + CoA $\rightleftharpoons$ propanoyl-CoA + L-carnitine	Carnitine shuttle (peroxisomal)
HMR_3136	(2E)-dodecenoyl-CoA + H ₂ O $\rightarrow$ (S)-3-hydroxydodecanoyl-CoA	Beta oxidation of even-chain fatty acids (mitochondrial)
HMR_3143	(2E)-decenoyl-CoA + H ₂ O $\rightarrow$ (S)-hydroxydecanoyl-CoA	Beta oxidation of even-chain fatty acids (mitochondrial)
HMR_3150	(2E)-octenoyl-CoA + H ₂ O $\rightarrow$ (S)-hydroxyoctanoyl-CoA	Beta oxidation of even-chain fatty acids (mitochondrial)
HMR_3157	H ₂ O + (2E)-hexenoyl-CoA $\rightarrow$ (S)-hydroxyhexanoyl-CoA	Beta oxidation of even-chain fatty acids (mitochondrial)

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_3164	$\text{H}_2\text{O} + \text{crotonyl-CoA} \rightarrow (\text{S})\text{-3-hydroxybutyryl-CoA}$	Tryptophan metabolism
HMR_3179	$(2\text{E})\text{-heptadecenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxyheptadecanoyl-CoA}$	Beta oxidation of odd-chain fatty acids (mitochondrial)
HMR_3183	$(2\text{E})\text{-pentadecenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxypentadecanoyl-CoA}$	Beta oxidation of odd-chain fatty acids (mitochondrial)
HMR_3187	$(2\text{E})\text{-tridecenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxytridecanoyl-CoA}$	Beta oxidation of odd-chain fatty acids (mitochondrial)
HMR_3191	$(2\text{E})\text{-undecenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxyundecanoyl-CoA}$	Beta oxidation of odd-chain fatty acids (mitochondrial)
HMR_3195	$(2\text{E})\text{-nonenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxynonanoyl-CoA}$	Beta oxidation of odd-chain fatty acids (mitochondrial)
HMR_3199	$(2\text{E})\text{-heptenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxyheptanoyl-CoA}$	Beta oxidation of odd-chain fatty acids (mitochondrial)
HMR_3203	$(2\text{E})\text{-pentenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxypentanoyl-CoA}$	Beta oxidation of odd-chain fatty acids (mitochondrial)
HMR_3219	$\text{trans,cis-2,13-docosadienoyl-CoA} + \text{H}_2\text{O} \rightarrow 3(\text{S})\text{-hydroxy-13-cis-docosenoyl-CoA}$	Beta oxidation of unsaturated fatty acids (n-9) (mitochondrial)
HMR_3223	$\text{trans,cis-2,11-eicosadienoyl-CoA} + \text{H}_2\text{O} \rightarrow 3(\text{S})\text{-hydroxy-11-cis-eicosenoyl-CoA}$	Beta oxidation of unsaturated fatty acids (n-9) (mitochondrial)
HMR_3227	$\text{trans,cis-octadeca-2,9-dienoyl-CoA} + \text{H}_2\text{O} \rightarrow (\text{S})\text{-3-hydroxyoleyleoyl-CoA}$	Beta oxidation of unsaturated fatty acids (n-9) (mitochondrial)
HMR_3241	$\text{trans,cis-2,13-eicosadienoyl-CoA} + \text{H}_2\text{O} \rightarrow 3(\text{S})\text{-hydroxy-13-cis-eicosenoyl-CoA}$	Beta oxidation of unsaturated fatty acids (n-7) (mitochondrial)
HMR_3252	$\text{trans,cis-hexadeca-2,9-dienoyl-CoA} + \text{H}_2\text{O} \rightarrow (\text{S})\text{-3-hydroxypalmitoleoyl-CoA}$	Beta oxidation of unsaturated fatty acids (n-7) (mitochondrial)
HMR_3260	$\text{trans,cis-myristo-2,7-dienoyl-CoA} + \text{H}_2\text{O} \rightarrow \text{cis-(3S)-hydroxytetradec-7-enoyl-CoA}$	Beta oxidation of unsaturated fatty acids (n-7) (mitochondrial)
HMR_3272	$(2\text{E})\text{-dodecenoyl-CoA} \rightleftharpoons (3\text{E})\text{-dodecenoyl-CoA}$	Beta oxidation of unsaturated fatty acids (n-7) (mitochondrial)
HMR_3449	$\text{trans-2-cis,cis,cis-8,11,14-eicosatetraenoyl-CoA} + \text{H}_2\text{O} \rightarrow 3(\text{S})\text{-hydroxy-dihomo-gamma-linolenoyl-CoA}$	Omega-6 fatty acid metabolism
HMR_3747	$\text{AKG} + \text{valine} \rightarrow 3\text{-methyl-2-oxobutyrate} + \text{glutamate}$	Valine, leucine, and isoleucine metabolism
HMR_3752	$\text{methacrylyl-CoA} + \text{FADH}_2 \rightleftharpoons \text{isobutyryl-CoA} + \text{FAD}$	Glycine, serine and threonine metabolism
HMR_3753	$\text{methacrylyl-CoA} + \text{H}_2\text{O} \rightarrow 3\text{-hydroxyisobutyryl-CoA}$	Valine, leucine, and isoleucine metabolism
HMR_3757	$3\text{-hydroxyisobutyrate} + \text{NAD}^+ \rightarrow \text{H}^+ + 2\text{-methyl-3-oxopropanoate} + \text{NADH}$	Valine, leucine, and isoleucine metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_3770	3-methylcrotonyl-CoA + FADH ₂ $\rightleftharpoons$ isovaleryl-CoA + FAD	Glycine, serine and threonine metabolism
HMR_3783	2-methylbutyryl-CoA + ubiquinone $\rightleftharpoons$ ubiquinol + tiglyl-CoA	Valine, leucine, and isoleucine metabolism
HMR_3784	2-methylbutyryl-CoA + FAD $\rightleftharpoons$ FADH ₂ + tiglyl-CoA	Glycine, serine and threonine metabolism
HMR_3785	H ₂ O + tiglyl-CoA $\rightarrow$ (S)-3-hydroxy-2-methylbutyryl-CoA	Valine, leucine, and isoleucine metabolism
HMR_3792	glutamate + 2-methyl-3-oxopropanoate $\rightleftharpoons$ D-3-amino-isobutanoate + AKG	Valine, leucine, and isoleucine metabolism
HMR_3795	CoA + 2-methyl-3-oxopropanoate + NAD ⁺ $\rightarrow$ H ⁺ + NADH + CO ₂ + propanoyl-CoA	Valine, leucine, and isoleucine metabolism
HMR_3827	AKG + aspartate $\rightleftharpoons$ glutamate + OAA	Alanine, aspartate and glutamate metabolism
HMR_3881	L-cystathionine + H ₂ O $\rightarrow$ cysteine + NH ₃ + 2-oxobutyrate	Cysteine and methionine metabolism
HMR_3885	acetoacetyl-CoA + CoA $\rightleftharpoons$ 2 acetyl-CoA	Tryptophan metabolism
HMR_3889	2 H ₂ O + ATP + 5-oxoproline $\rightarrow$ ADP + glutamate + Pi	Glutathione metabolism
HMR_3899	AKG + alanine $\rightleftharpoons$ glutamate + pyruvate	Alanine, aspartate and glutamate metabolism
HMR_3912	cysteine + AKG $\rightleftharpoons$ glutamate + mercaptopyruvate	Cysteine and methionine metabolism
HMR_3933	formylglutathione + H ⁺ + NADH $\rightleftharpoons$ hydroxymethylglutathione + NAD ⁺	Glutathione metabolism
HMR_4038	FAICAR $\rightleftharpoons$ IMP + H ₂ O	Purine metabolism
HMR_4097	ATP + CoA + acetate $\rightarrow$ AMP + PPi + acetyl-CoA	Glycolysis / Gluconeogenesis
HMR_4099	ATP + CoA + acetate $\rightarrow$ AMP + PPi + acetyl-CoA	Glycolysis / Gluconeogenesis
HMR_4101	GTP + OAA $\rightarrow$ GDP + CO ₂ + PEP	Pyruvate metabolism
HMR_4103	GTP + OAA $\rightarrow$ GDP + CO ₂ + PEP	Pyruvate metabolism
HMR_4108	acetyl adenylate + CoA $\rightarrow$ AMP + acetyl-CoA	Glycolysis / Gluconeogenesis
HMR_4109	AKG + alanine $\rightleftharpoons$ glutamate + pyruvate	Alanine, aspartate and glutamate metabolism
HMR_4133	ATP + acetate $\rightarrow$ acetyl adenylate + PPi	Glycolysis / Gluconeogenesis
HMR_4280	H ⁺ + pyruvate + NADH $\rightleftharpoons$ L-lactate + NAD ⁺	Pyruvate metabolism
HMR_4281	pyruvate + NADH + H ⁺ $\rightleftharpoons$ L-lactate + NAD ⁺	Glycolysis / Gluconeogenesis
HMR_4283	H ₂ O + NADP ⁺ + acetaldehyde $\rightarrow$ H ⁺ + NADPH + acetate	Glycolysis / Gluconeogenesis
HMR_4285	succinate semialdehyde + H ₂ O + NAD ⁺ $\rightarrow$ H ⁺ + NADH + succinate	Alanine, aspartate and glutamate metabolism
HMR_4287	succinate semialdehyde + H ₂ O + NADP ⁺ $\rightarrow$ H ⁺ + NADPH + succinate	Alanine, aspartate and glutamate metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_4302	H ₂ O + cysteine -> H ₂ S + pyruvate + NH ₃	Cysteine and methionine metabolism
HMR_4324	ATP + cysteine + glutamate -> ADP + gamma-glutamyl-cysteine + Pi	Glutathione metabolism
HMR_4326	ATP + glycine + gamma-glutamyl-cysteine -> ADP + GSH + Pi	Glutathione metabolism
HMR_4328	H ₂ O + cys-gly -> cysteine + glycine	Glutathione metabolism
HMR_4330	3-oxopropanoate + glutamate <=> beta-alanine + AKG	Beta-Alanine metabolism
HMR_4340	formate + ATP + THF -> 10-formyl-THF + ADP + Pi	Folate metabolism
HMR_4345	5,6-Dihydrouracil + NADP ⁺ <=> H ⁺ + NADPH + uracil	Pyrimidine metabolism
HMR_4355	DHAP + erythrose-4-phosphate -> sedoheptulose-1,7-bisphosphate	Glycolysis / Gluconeogenesis
HMR_4375	DHAP + GAP <=> fructose-1,6-bisphosphate	Glycolysis / Gluconeogenesis
HMR_4377	H ₂ O + fructose-1,6-bisphosphate -> Pi + fructose-6-phosphate	Glycolysis / Gluconeogenesis
HMR_4388	H ⁺ + NADH + pyruvate <=> NAD ⁺ + L-lactate	Glycolysis / Gluconeogenesis
HMR_4406	H ₂ O + glutamine + PRPP -> 5-phosphoribosylamine + glutamate + PPi	Purine metabolism
HMR_4454	citrate <=> isocitrate	Tricarboxylic acid cycle, glyoxylate/dicarboxylate metabolism
HMR_4485	thymine + 2-deoxy-D-ribose-1-phosphate <=> thymidine + Pi	Pyrimidine metabolism
HMR_4486	ITP + glucose -> IDP + glucose-6-phosphate	Purine metabolism
HMR_4487	glucose + dATP -> glucose-6-phosphate + dADP	Purine metabolism
HMR_4488	ITP + fructose -> IDP + fructose-6-phosphate	Purine metabolism
HMR_4489	dATP + fructose -> dADP + fructose-6-phosphate	Purine metabolism
HMR_4503	10-formyl-THF + H ⁺ <=> 5,10-methenyl-THF + H ₂ O	Folate metabolism
HMR_4505	10-formyl-THF + H ⁺ <=> 5,10-methenyl-THF + H ₂ O	Folate metabolism
HMR_4574	inosine + Pi <=> ribose-1-phosphate + hypoxanthine	Purine metabolism
HMR_4586	oxalosuccinate + H ⁺ + NADPH -> isocitrate + NADP ⁺	Tricarboxylic acid cycle and glyoxylate/dicarboxylate metabolism
HMR_4588	oxalosuccinate + H ⁺ + NADH <=> isocitrate + NAD ⁺	Tricarboxylic acid cycle and glyoxylate/dicarboxylate metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_4603	2-deoxy-D-ribose-1-phosphate + guanine $\rightleftharpoons$ deoxyguanosine + Pi	Purine metabolism
HMR_4651	guanosine + Pi $\rightleftharpoons$ ribose-1-phosphate + guanine	Purine metabolism
HMR_4693	4-aminobutyrate + AKG $\rightleftharpoons$ succinate semialdehyde + glutamate	Alanine, aspartate and glutamate metabolism
HMR_4695	2-deoxy-D-ribose-1-phosphate + adenine $\rightleftharpoons$ deoxyadenosine + Pi	Purine metabolism
HMR_4699	betaine + homocysteine $\rightarrow$ dimethylglycine + methionine	Glycine, serine and threonine metabolism
HMR_4708	gamma-glutamyl-cysteine $\rightarrow$ cysteine + 5-oxoproline	Glutathione metabolism
HMR_4714	pantetheine + ATP $\rightarrow$ phosphopantetheine + ADP	Pantothenate and CoA biosynthesis
HMR_4739	2-aminoadipate 6-semialdehyde + H ₂ O + NAD ⁺ $\rightarrow$ L-2-aminoadipate + H ⁺ + NADH	Lysine metabolism
HMR_4808	N-formyl-GAR + H ₂ O + ATP + glutamine $\rightarrow$ 5-phosphoribosylformylglycinamide + ADP + glutamate + Pi	Purine metabolism
HMR_4814	AICAR + 10-formyl-THF $\rightleftharpoons$ FAICAR + THF	Purine metabolism
HMR_4841	ribose + H ⁺ + NADH $\rightleftharpoons$ ribitol + NAD ⁺	Pentose phosphate pathway
HMR_4843	GDP + GTP $\rightleftharpoons$ GTP + GDP	Transport reactions
HMR_4858	galactose $\rightleftharpoons$ galactose	Transport reactions
HMR_6246	prostaglandin E2 $\rightarrow$ prostaglandin E2	Transport reactions
HMR_6328	ADP + ATP $\rightleftharpoons$ ATP + ADP	Transport reactions
HMR_6409	lipoamide + H ⁺ + NADH $\rightleftharpoons$ dihydrolipoamide + NAD ⁺	Glycine, serine and threonine metabolism
HMR_6537	H ⁺ + NADPH + glucuronate $\rightleftharpoons$ L-gulonate + NADP ⁺	Pentose and glucuronate interconversions
HMR_6608	hydroxide $\rightleftharpoons$ hydroxyl radical	ROS detoxification
HMR_6647	H ₂ O + NAD ⁺ + retinal $\rightarrow$ H ⁺ + NADH + retinoate	Retinol metabolism
HMR_6652	H ₂ O + NAD ⁺ + 9-cis-retinal $\rightleftharpoons$ 9-cis-retinoate + H ⁺ + NADH	Retinol metabolism
HMR_6674	13-cis-retinal + H ₂ O + NAD ⁺ $\rightleftharpoons$ H ⁺ + NADH + retinoate	Retinol metabolism
HMR_6675	13-cis-retinal + H ₂ O + NAD ⁺ $\rightleftharpoons$ 13-cis-retinoate + H ⁺ + NADH	Retinol metabolism
HMR_6676	13-cis-retinal + H ₂ O + NAD ⁺ $\rightleftharpoons$ 13-cis-retinoate + H ⁺ + NADH	Retinol metabolism
HMR_6784	ATP + CoA + 4-coumarate $\rightarrow$ AMP + PPi + 4-coumaroyl-CoA	Phenylalanine, tyrosine and tryptophan biosynthesis
HMR_6903	4-hydroxybenzoate + all-trans-decaprenyl-diphosphate $\rightarrow$ 3-decaprenyl-4-hydroxybenzoate + PPi	Terpenoid backbone biosynthesis

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_6904	3-decaprenyl-4-hydroxybenzoate + H ⁺ + NADPH + O ₂ -> 3,4-dihydroxy-5-all-trans-decaprenylbenzoate + H ₂ O + NADP ⁺	Terpenoid backbone biosynthesis
HMR_6907	2-methoxy-6-(all-trans-decaprenyl)phenol + 3 H ⁺ + NADPH + O ₂ -> 2-methoxy-6-all-trans-decaprenyl-2-methoxy-1,4-benzoquinol + H ₂ O + NADP ⁺	Terpenoid backbone biosynthesis
HMR_6981	4-trimethylammoniobutanal + H ₂ O + NAD ⁺ -> 2 H ⁺ + NADH + gamma-butyrobetaine	Lysine metabolism
HMR_7613	1-organyl-2-lyso-sn-glycero-3-phosphocholine + acetyl-CoA -> 2-acetyl-1-alkyl-sn-glycero-3-phosphocholine + CoA	Glycerophospholipid metabolism
HMR_7713	AMP + H ₂ O -> adenosine + Pi	Nucleotide metabolism
HMR_7716	UMP + H ₂ O -> Pi + uridine	Nucleotide metabolism
HMR_7721	CMP + H ₂ O -> Pi + cytidine	Nucleotide metabolism
HMR_7728	GMP + H ₂ O -> Pi + guanosine	Nucleotide metabolism
HMR_8066	glutamate + 3-sulfolpyruvate <=> AKG + L-cysteate	Cysteine and methionine metabolism
HMR_8088	glutamate + 2-methyl-3-oxopropanoate <=> L-3-amino-isobutanoate + AKG	Valine, leucine, and isoleucine metabolism
HMR_8144	5,10-methenyl-THF + NADH <=> 5,10-methylene-THF + NAD ⁺	Folate metabolism
HMR_8357	H ₂ O + NAD ⁺ + acetaldehyde -> H ⁺ + acetate + NADH	Glycolysis / Gluconeogenesis
HMR_8506	lactaldehyde + H ₂ O + NAD ⁺ -> L-lactate + H ⁺ + NADH	Pyruvate metabolism
HMR_8512	NAD ⁺ + D-lactate <=> H ⁺ + NADH + pyruvate	Pyruvate metabolism
HMR_8736	oxalate + 2 sulfate -> oxalate + 2 sulfate	Transport reactions
HMR_8769	GSH + alanine -> (5-L-glutamyl)-L-amino acid + cys-gly	Glutathione metabolism
HMR_8770	(5-L-glutamyl)-L-amino acid -> alanine + 5-oxoproline	Glutathione metabolism
HMR_8773	D-xylulose-1-phosphate <=> glycolaldehyde + DHAP	Tricarboxylic acid cycle and glyoxylate/dicarboxylate metabolism
HMR_8775	glycolaldehyde + H ₂ O + NAD ⁺ -> glycolate + H ⁺ + NADH	Tricarboxylic acid cycle and glyoxylate/dicarboxylate metabolism
HMR_8778	glycolaldehyde + H ₂ O + NAD ⁺ -> H ⁺ + NADH + glycolate	Tricarboxylic acid cycle and glyoxylate/dicarboxylate metabolism

Table A1.2: Continue

Reaction	Equation	Pathway
HMR_8780	$\text{H}_2\text{O} + \text{NAD}^+ + \text{glyoxalate} \rightarrow \text{oxalate} + \text{H}^+ + \text{NADH}$	Tricarboxylic acid cycle and glyoxylate/dicarboxylate metabolism
KMR_1076	$\text{GSH} \rightarrow \text{cys-gly} + 5\text{-oxoproline}$	KM pathways
pj32	$(2\text{E},5\text{Z})\text{-dodecadienoyl-CoA} \rightleftharpoons (3\text{E},5\text{Z})\text{-dodecadienoyl-CoA}$	Isolated