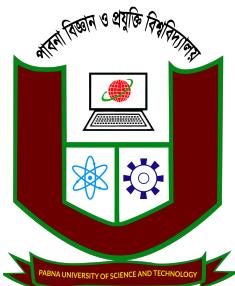


Genetic and Molecular Perspective on Endocrine and Cardiovascular Complications in Beta-Thalassemia Patients



Department of Computer Science and Engineering
Pabna University of Science and Technology, Pabna-6600

Course Title: Thesis
Course Code: CSE 4100 and CSE 4200

A thesis has been submitted to the Department of Computer Science and Engineering for the partial fulfillment of the requirement of B.Sc in Engineering in Computer Science and Engineering

Submitted By:

The Examinee of B.Sc Engineering Final Examination-2022
Mst. Shaima Ashlam Chaity
Roll Number: 200121
Registration Number: 1011879
Session: 2019-20

Supervised By:

S. M. Hasan Sazzad Iqbal

Associate Professor

Department of Computer Science and Engineering
Pabna University of Science and Technology

August, 2025

DECLARATION

I, **Shaima Aslam Chaity**, hereby declare that the work presented in this thesis is entitled "**Genetic and Molecular Perspective on Endocrine and Cardiovascular Complications in Beta-Thalassemia Patients**", is the outcome of my own research and effort carried out under the supervision of **S. M. Hasan Sazzad Iqbal**, Department of Computer Science and Engineering, Pabna University of Science and Technology (PUST), Pabna.

I also declare that this work is the best of my knowledge and does not contain any material that previously published or written by another person, nor it has been submitted in whole or in part, for the award of any degree or diploma at any other university or institution. All materials or content taken from other sources has been appropriately referenced in this thesis

This thesis reflects my own findings, opinions and conclusions and do not necessarily represent the views of Pabna University of Science and Technology or any other institution.

Signature of the Examinee

CERTIFICATION

I am happy to certify that Mst. Shaima Ashlam Chaity, Roll Number: 200121, Registration Number: 1011879, Session: 2019-2020, has completed a thesis work that enabled "**Genetic and Molecular Perspective on Endocrine and Cardiovascular Complications in Beta-Thalassemia Patients**" under my supervision to fulfill the requirements of the thesis course. This thesis was completed for a year at the Department of Computer Science and Engineering, Pabna University of Science and Technology, Pabna-6600, Bangladesh.

According to my knowledge, this thesis paper has not been submitted elsewhere or replicated by another thesis paper before being submitted to the department.

S. M. Hasan Sazzad Iqbal

Associate Professor,

Department of Computer Science and Engineering
Pabna University of Science and Technology, Pabna,
Bangladesh

ACKNOWLEDGEMENT

My deepest gratitude to the Allah for granting me the strength and guidance to complete this thesis successfully.

I am sincerely thankful to my supervisor, **S. M. Hasan Sazzad Iqbal**, Associate Professor in Department of Computer Science and Engineering at Pabna University of Science and Technology (PUST), for his continuous support, insightful advice and valuable feedback throughout the completion of this research. His insightful suggestions, encouragement, and guidance have been instrumental in shaping this work from its beginning to completion.

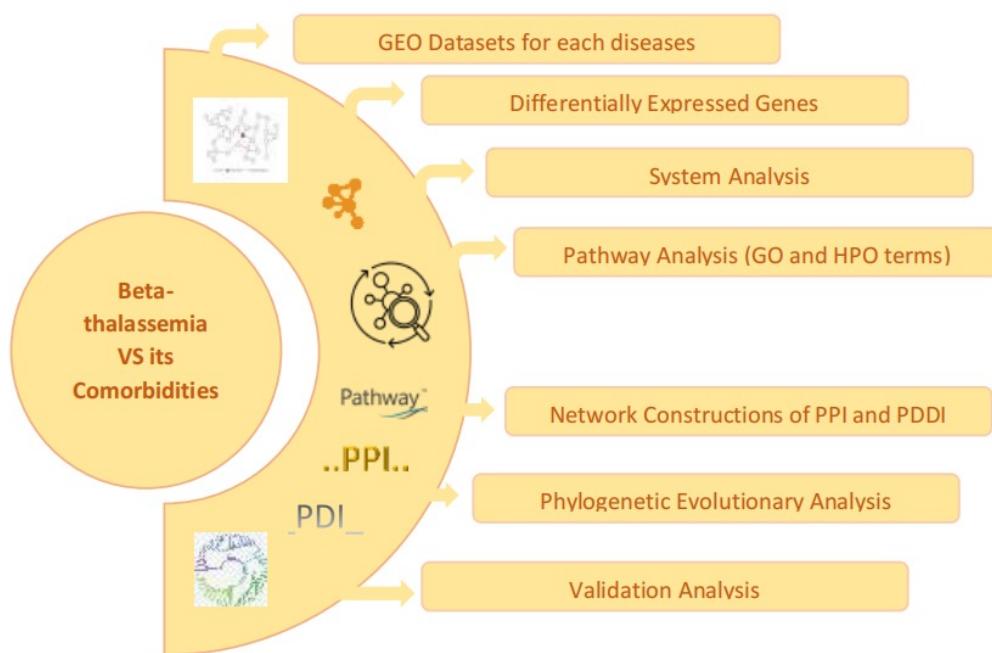
I would also like to extend my appreciation to all the respected teachers of the Department of Computer Science and Engineering, PUST, whose teachings and academic guidance have laid the foundation for my research knowledge and skills. My heartfelt thanks go to my family, especially my parents as well as my friends and well-wishers for their support, motivation, and cooperation during this work.

I express my genuine thanks to everyone who contributed by any means to the successful ending of this thesis.

August, 2025

Author

ABSTRACT



Beta-thalassemia (BT), a genetic blood anomaly brought on by abnormalities in the HBB gene that occurs when beta-globin chain production is reduced or absent in blood. It is a major worldwide health issue because of its complicated clinical management and lifetime reliance on blood transfusions. Some recent evidence highlights that beta-thalassemia is associated with several comorbidities, including endocrine diseases involving polycystic ovarian syndrome (PCOS), hypothyroidism, hypogonadism and type 2 diabetes (T2D) and cardiovascular diseases involving arrhythmogenic cardiomyopathy (ACM) and arrhythmia. These comorbidities may share common molecular mechanisms with beta-thalassemia that potentially exacerbate disease severity and treatment complications. In this study, a computational approach was applied to evaluate the genetic relationships between beta-thalassemia and its associated comorbidities using microarray and mRNA datasets that are publicly available in NCBI. Genetic profiling was constructed to identify the common matching genes and built disease-gene networks (DGNs) of matching genes. It also

visualized a heatmap to present patterns of gene expressions. We also explored multiple bioinformatics analysis including pathways, gene ontology, protein-protein interaction (PPI) and protein-drug interaction (PDI) that strongly indicate their correlation. A validation network was created to verify our selected comorbidity and then phylogenetic analysis was performed for all diseases to determine their evolutionary relationships. This study found that beta-thalassemia shares 13, 165, 13, 14, 11 and 44 significantly expressed genes with hypothyroidism, hypogonadism, PCOS, T2D, ACM and arrhythmia respectively. The outcomes of this study may help in integrative medical approaches and enhance a significant understanding of genetic and molecular structure of comorbidities in beta-thalassemia by providing valuable insights.

Keywords: Beta-thalassemia, comorbidities, genetic profiling, endocrine diseases, cardiac diseases, arrhythmogenic cardiomyopathy, pathway analysis, ontology, phylogenetic analysis.

TABLE OF CONTENTS

	Page
Abbreviations and Acronyms	viii
List of Tables	ix
List of Figures	x
1 Introduction	1
1.1 General Introduction	2
1.2 General Background	3
1.3 Motivation	4
1.4 Problem Statement	4
1.5 Thesis Objectives	5
1.6 Thesis Contribution	6
1.7 Significance	7
1.8 Thesis Layout	8
1.9 Conclusion	8
2 Literature Review	9
2.1 Review of Literature	10
2.2 Brief Summary of Related Work	12
2.3 Research Gap	15
2.4 Conclusion	16
3 Methodology	17
3.1 Introduction	18
3.2 Overview of Analytical Approaches	18
3.3 Dataset Information	19
3.4 Methodology	22
3.4.1 Selection of mRNA-seq and Microarray Datasets	22
3.4.2 Differential Gene Expression Analysis	23
3.4.3 Gene Diseases Network Analysis	25

3.4.4	Pathway and Functional Association Analysis	26
3.4.5	Gene Ontology Analysis	27
3.4.6	Up and Down-regulated Identification	28
3.4.7	Common Dysregulated Genes	28
3.4.8	Network Construction of PPI	28
3.4.9	Protein-Drug Interactions Network Analysis	29
3.4.10	Phylogenetic Analysis	30
3.4.11	Validation Analysis	31
3.4.12	Working Algorithm	31
3.4.13	Algorithm	31
3.4.14	Experimental Setup	33
3.4.15	Conclusion	33
4	Result Analysis and Discussion	34
4.1	Introduction	35
4.2	Genetic Profiling Analysis	35
4.3	Heatmap Visualization	40
4.4	Pathway and Functional Association Analysis	41
4.5	Gene Ontology (GO) Analysis	53
4.6	Protein-Protein Interactions Analysis	60
4.7	Protein-Drug Interactions Analysis	63
4.8	Results of Phylogenetic Analysis	68
4.9	Discussion	69
5	Conclusion and Future Work	71
5.1	Discussion	72
5.2	Future Work	72
5.3	Conclusion	73
REFERENCES		74

ABBREVIATIONS AND ACRONYMS

The following abbreviations and acronyms are used in this thesis:

BT	Beta-Thalassemia
GEO	Gene Expression Omnibus
DEG	Differentially Expressed Gene
GO	Gene Ontology
HPO	Human Phenotype Ontology
PPI	Protein-Protein Interaction
PDI	Protein-Drug Interaction
ACM	Arrhythmogenic Cardiomyopathy
T2D	Type 2 Diabetes
PCOS	Polycystic Ovary Syndrome
NCBI	National Center for Biotechnology Information
MEGA	Molecular Evolutionary Genetics Analysis
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
Cytoscape	Cytoscape Network Visualization Tool
Enrichr	Enrichr Gene Enrichment Analysis Tool
dbGaP	Database of Genotypes and Phenotypes
OMIM	Online Mendelian Inheritance in Man
ROS	Reactive Oxygen Species

LIST OF TABLES

TABLE	Page
2.1 Table 2-1 Brief Summary of Related Work	12
2.2 Comparison of Proposed Work with Related Studies	15
3.1 Representation of Datasets Information	20
4.1 Up-regulated and Down-regulated genes information	36
4.2 Up-regulated and down-regulated common DEGs of six comorbidities	36
4.3 Up-regulated and Down-regulated genes information for common DEGs with Beta-thalassemia	37
4.4 Most significant gene that are common for multiple diseases for BT as well as it multiple comorbidities	38
4.5 Significant pathways of Hypothyroidism of shared DEGs	42
4.6 Significant pathways of Hypogonadism of shared DEGs	44
4.7 Significant pathways of PCOS of shared DEGs	46
4.8 Significant pathways of T2D of shared DEGs	48
4.9 Significant pathways of Arrhythmogenic Cardiomyopathy (ACM) of shared DEGs	50
4.10 Significant pathways of Arrhythmia of shared DEGs	51
4.11 Gene Ontological significant pathways of Hypothyroidism of shared DEGs	54
4.12 Gene Ontological significant pathways of Hypogonadism of shared DEGs	55
4.13 Gene Ontological significant pathways of PCOS of shared DEGs.	56
4.14 Gene Ontological significant pathways of T2D of shared DEGs.	57
4.15 Gene Ontological significant pathways of ACM of shared DEGs.	58
4.16 Gene Ontological significant pathways of Arrhythmia of shared DEGs.	59
4.17 Network Stats information of PPI analysis	60
4.18 Identify Hub protein information from PPIs analysis	61
4.19 Information of drug interactions of PDI Subnetwork1	64
4.20 Information of drug interactions of PDI Subnetwork2.	65

LIST OF FIGURES

FIGURE	Page
3.1 Working flow diagram of this investigation	19
3.2 Workflow for Differential Gene Expression Analysis of Beta-Thalassemia and Comorbidities.	23
3.3 Workflow for Gene Ontology (GO) analysis using Enrichr to identify significant functional terms associated with DEGs.	27
4.1 Loss Curve (DGNs network for up-regulation of common DEGs. Orange-colored nodes are genes, pink-colored nodes are comorbidities and the center represents the beta-thalassemia.)	39
4.2 DGNs network for down-regulation of common DEGs. Orange-colored nodes are genes, pink-colored nodes are comorbidities and the center represents the beta-thalassemia. Genes that are linked to several diseases are represented by the red color.	40
4.3 Heatmap visualization of gene expression.	41
4.4 Graphical depiction of pathways of Hypothyroidism	43
4.5 Graphical depiction of pathways of Hypogonadism	45
4.6 Graphical depiction of pathways of PCOS.	47
4.7 Graphical depiction of pathways of T2D.	49
4.8 Graphical depiction of pathways of ACM.	51
4.9 Graphical depiction of pathways of Arrhythmia.	53
4.10 Clusters information's of PPI networks of hub proteins of beta-thalassemia and comorbidities.	61
4.11 PPI networks of hub proteins of beta-thalassemia and comorbidities. . .	62
4.12 Detailed cluster analysis of PPI networks for hub proteins of beta-thalassemia and comorbidities.	62
4.13 Network Topology of Subnetwork1.	65
4.14 Network Topology of Subnetwork2.	66
4.15 PDI networks of drug proteins of beta-thalassemia and comorbidities. .	67
4.16 Phylogenetic tree to illustrate associative evolutionary relationship between beta-thalassemia and its comorbidities.	68

4.17 Graphical representation of validation network of beta-thalassemia. Octagon shapes indicate the shared genes, circle shapes with brown color indicate our selected diseases and circle shapes with yellow-orange color represent our selected comorbidities.	69
---	----

C H A P T E R 1

INTRODUCTION

This chapter presents an overview of our research thesis, organized into seven sub-chapters for clarity. In section 1.1, we introduce the topic, focusing on the interplay between diabetes and cancers (breast, ovarian, cervical, and gastric). Section 1.2 outlines the motivation, emphasizing the need to understand shared biological pathways. Section 1.3 defines the problem statement, addressing the increased cancer risk in diabetic patients. Section 1.4 specifies the objectives, including identifying common differentially expressed genes (DEGs) and analyzing molecular interactions. Section 1.5 highlights the research outcomes and their potential impact on early diagnosis and treatment. Section 1.6 details the thesis structure, and section 1.7 provides a discussion on the significance and relevance of our study.

1.1 General Introduction

Beta-thalassemia is a complex hematological disorder inherited from parents to offspring. It is brought by mutations in the HBB gene and caused by the body's inability to produce enough beta-globin protein in red blood cells, which results in chronic anemia, iron overload and several medical complications. Beta-thalassemia patients require lifetime medication and often need regular blood transfusions to manage chronic anemia [1].

Some compelling research evidence shows it contributes remarkably to the development of cardiovascular diseases like ACM and arrhythmia, as well as endocrine diseases like T2D, PCOS, hypothyroidism and hypogonadism. Through gene expression analysis, this study aims to explore the link between beta-thalassemia and its associated comorbidities. Many studies show that iron overload from blood transfusions in beta-thalassemia interrupts the function of endocrine glands and reduces hormone synthesis and secretion [1]. Also, chronic anemia and iron overload from regular blood transfusion cause cardiac abnormalities [2].

To explore genetic links more deeply between beta-thalassemia and comorbidities, this study identified differentially expressed genes (DEGs) from RNA-sequence and microarray datasets. This study constructed disease-gene association networks (DGNs) by mapping the genes of up-regulated and down-regulated and then visualizing those matching genes using a heatmap [3]. Pathway analysis is conducted with graphical representation and gene ontology (GO) analysis to show molecular functions [3]. This work built the PPI and PDI networks to investigate the functional connectivity between matching genes. A phylogenetic analysis is performed to observe the interrelation of all seven diseases. Finally, this thesis validated the results using standard biomedical libraries like OMIM (Online Mendelian Inheritance in Man) and databases like dbGaP. Our proposed work has been undertaken to achieve a more comprehensive genetic understanding of beta-thalassemia with its comorbidities, which will improve the clinical approaches for its diagnosis, management and treatment.

Beta-Thalassemia

When the (β^+) or (β^0) synthesis is reduced in hemoglobin of beta globin chains, the β -thalassemia blood genetic disorder occurs in patients [4]. It manifests in three forms:

- The carrier state,
- Thalassemia intermediate (mild to severe), and
- Thalassemia major (severe, transfusion-dependent anemia).

Endocrine Disorder

An endocrine disorder is a condition resulting from abnormalities in hormone production, regulation, or action by the endocrine glands, such as the thyroid or pancreas. It includes hypothyroidism and hyperthyroidism, which can cause significant mental health changes. Diabetes mellitus, marked by hyperglycaemia due to insulin issues, is another example. These disorders often lead to systemic effects, including psychiatric symptoms, metabolic imbalances, and increased risk of comorbidities like cardiovascular diseases [5].

Cardiovascular Disorder

A cardiovascular disorder, broadly referred to as heart disease or cardiovascular disease, is any condition that negatively affects the structure or function of the heart and its associated blood vessels [6].

1.2 General Background

Beta-thalassemia is an inherited blood disorder transferred from parents in a received patterns that is characterized by absence of beta-globin production, which resulting in anemia, excessive iron accumulation and various complications affecting multiple organ systems. Over time, excessive iron deposition in endocrine glands contributes to a spectrum of complications collectively known as thalassemic endocrine disease (TED). These include hypogonadism, hypothyroidism, diabetes, adrenal dysfunction, and reduced bone mineral density [7]. It also resulting in various cardiovascular issues such as vasculopathies, cardiomyopathy, hypertension and arrhythmias. These complications significantly contribute to the risk of sudden cardiac death [8].

Although from the current existing research much is known about beta-thalassemia and its complications, the genetic and molecular links between BT and its endocrine and cardiovascular comorbidities remain unclear. Understanding of the shared genes, pathways, PPI and PDI in still limited.

This study aims to fill these gaps by analyzing gene expression data to uncover the genetic connections between BT and its related complications. This findings could improve the knowledge of disease mechanisms and help to create better diagnostic tools and therapies.

1.3 Motivation

As BT is a chronic inherited disorder that often leads to serious endocrine and cardiovascular complications. Although clinical management has improved day by day but the genetic and molecular perspectives leading these comorbidities are not still clearly understandable. The lack of knowing about internal genetic and molecular relationships limits the ability to design appropriate diagnostic ways and proper treatments.

By analyzing deeply gene expression datasets and using advanced bioinformatics methods, this work provides an opportunity to investigate the shared genetic pathways, GO analysis and gene interaction networks BT and its comorbidities. This study aims to contribute some opinions to the development of more effective diagnostic approaches and treatment strategies for BT patients, ultimately improving their quality of life and reducing the burden of associated complications.

By identifying key genes and molecular mechanisms, this study aims to contribute to the development of more effective diagnostic biomarkers, therapeutic goals and treatment strategies that obviously improve outcomes and quality of health for patients.

1.4 Problem Statement

Mutations in the HBB gene of blood causing the hereditary disorder named Beta-thalassemia (BT) because of the reduced production of hemoglobin. Hemoglobin is one type of protein found in red blood cells that contains iron and transfers oxygen to tissues across the body. Patients with BT have decreased hemoglobin levels that leads to a reduced supply of oxygen throughout the body. As a result, patients have anemia which requires regular blood transfusions for life and causes weakness, skin problems, fatigue and sometimes serious health issues. Thalassemia major and thalassemia intermediate are the two types of BT where BT major patient's dependent on regular blood transfusions [9] and BT minor patient's need not to blood transfusion. Although the blood-related symptoms of BT are well established, increasing evidence shows that the disease is linked to various serious comorbid conditions. These include endocrine diseases such as T2D, PCOS hypothyroidism and hypogonadism as well as cardiovascular complications such as ACM and arrhythmia. Such comorbidities significantly worsen patient prognosis, reduce quality of life, and increase mortality rates. These complications produced negative prognosis, life with health issues and increase the risk of death.

Despite extensive clinical research, the proper molecular and genetic mechanisms that link the BT to these comorbidities remain poorly understood. Many Current

studies focus on individual complications separately without exploring the potential shared genetic pathways or overlapping disease mechanisms. These approaches limit the identification of common genes and molecular perspectives that limit utilization of early diagnosis, prediction of future risk and specific treatment. Furthermore, most available research is clinical or descriptive lacking of integrative bioinformatics approaches. Where this work can analyze large-scale genomic data to uncover underlying relationships between BT and its associated disorders.

Facing several key challenges because of the absence of such integrative approaches. Firstly, it is difficult to develop diagnostic tools that can detect comorbidities at an early stage without identifying the shared genes and molecular relationships. Secondly, the lack of molecular insights affects the development of targeted therapies that address simultaneously both BT and its comorbidities rather than treating them as separated. Finally, the opportunities for drug recycling or the development of therapeutic approaches will be missing without mapping DGNs, PDI and PPI networks.

Addressing these gaps requires a systematic and computational approach that can integrate gene expression datasets from multiple diseases to identify overlapping genes, pathways, and protein interactions. In this study, publicly available microarray and mRNA datasets from NCBI are analyzed to perform genetic profiling of BT with its major comorbidities. The analysis involves constructing DGNs, heatmap visualization of gene patterns, pathway and ontology, PPI and PDI mapping as well as phylogenetic analysis to investigate the evolutionary connections between the diseases.

This research aims to overcome these knowledge gaps, enabling a integrative medical strategies that can improve the ways of diagnosis, treatment design and ultimately enhance the outcomes of patient situation by revealing the shared genetic relationships between BT and its comorbidities.

1.5 Thesis Objectives

The primary objectives of this research is to investigate the genetic relationships between BT and its selected endocrine and cardiovascular comorbidities. This work aims to identifying the mechanisms of shared molecular that may help in integrative diagnostic and therapeutic approaches.

The specific objectives are:

- 1) Identify the common differentially expressed genes (DEGs) between BT and its associated comorbidities including hypothyroidism, hypogonadism, PCOS,

T2D, ACM and arrhythmia by using microarray and mRNA datasets from publicly available NCBI tools.

- 2) Construct and visualize disease-gene networks (DGNs) to show the genetic overlaps between BT and each comorbidities.
- 3) Visualize heatmap analysis to reveal the gene expression patterns across BT and the selected comorbidities.
- 4) To explore the biological processes, molecular functions and cellular components of the shared genes pathway and gene ontology enrichment analysis is conducted.
- 5) Build protein-protein interaction (PPI) networks to identify the key hub proteins from molecular interaction.
- 6) Map protein-drug interactions (PDI) to highlight potential therapeutic treatments and opportunities for drug recreations.
- 7) Create a validation network to confirm that the selected comorbidities are valid for the beta-thalassemia based on genetic perspectives.
- 8) To determine the evolutionary relationships among BT and its comorbidities phylogenetic analysis is performed based on the shared genes.

This study aims to bridge the gap between genetic profiling and molecular interactions that helps in clinical application, supporting early detection of health risk, personalized treatment and improved patient outcomes affected by BT and its related comorbidities.

1.6 Thesis Contribution

The proposed computational framework provides an integrated bioinformatics approach to exploring the genetic connections of beta-thalassemia and its associated comorbidities. By analyzing microarray and mRNA datasets from National Center for Biotechnology Information NCBI, this study identifies shared genes, molecular pathways and uncovers potential therapeutic targets.

The main contributions of this research are:

- 1) A total of 15,008 differentially expressed raw genes were analyzed from NCBI platforms that identifying significantly expressed genes associated with beta-thalassemia and its comorbidities.

- 2) A total of six comorbidities have been selected to validate the connections with BT using gold benchmark.
- 3) This study discovered 13, 165, 13, 14, 11, and 44 common DEGs for hypothyroidism, hypogonadism, PCOS, T2D, ACM and arrhythmia respectively that highlighting the potential molecular links between beta-thalassemia and these conditions.
- 4) Built and analyzed DGN networks to visualize genetic interconnections and identify key hub genes that playing a central role in disease progression.
- 5) Revealed relevant signaling pathways and Gene Ontology terms strongly associated with the shared DEGs.
- 6) Conducted PPI and PDI analyses to identify the central proteins and the potential drug molecules that may target both beta-thalassemia and its comorbidities.
- 7) Developed a validation network to validate the selection of comorbidities and then performed phylogenetic analysis to determine the evolutionary relationships among the diseases.
- 8) Provides an established view of how genetic, molecular and evolutionary factors interplay in beta-thalassemia and its associated complications to providing the foundation of more targeted diagnostics and personalized treatment strategies.

1.7 Significance

This study has the substantial importance in advancing the understanding of beta-thalassemia and its associated comorbidities through an integrative genetic and bioinformatics approach. By identifying key genetic mutations, enriched pathways, functional ontologies and regulatory mRNAs, this study aims to pave the way for identifying potential biomarkers that could improve early diagnosis and help predict disease outcomes. Exploring the networks of protein-protein and protein-drug interaction will help in the identification of novel therapeutic targets and support the opportunities of drug repurposing.

Furthermore, phylogenetic analysis of disease-associated mutations will provide evolutionary insights into their origin and prevalence that enhancing the global understanding of epidemiological. Overall, the findings of this study are expected to support more personalized treatment strategies, guide future molecular research and increase the awareness about the complex health risks faced by beta-thalassemia patients.

1.8 Thesis Layout

The rest of the thesis is organized as follows.

CHAPTER 1: This chapter introduces the background of beta-thalassemia, its clinical significance and the motivation for this study. It also outlines the problem statement, objectives, scope, goal and expected contributions of the research.

CHAPTER 2: This chapter presents a comprehensive literature review, summarizing previous studies from reputable journals and conferences. It highlights the current understanding of shared molecular mechanisms and identifies the research gaps.

CHAPTER 3: This chapter describes the methodology and datasets used in the study. It details the sources of microarray and mRNA data obtained from NCBI, the pre-processing steps, and the computational approaches applied such as the Benjamini Hochberg for identifying shared genes. Another method is used for diseasesome network construction, pathway and ontology analysis, protein-protein and protein-drug interaction mapping and phylogenetic analysis.

CHAPTER 4: The most significant chapter that presents the results of the analyses, including the identification of common differentially expressed genes between beta-thalassemia and each comorbidity, visualization of gene expression patterns and functional enrichment findings. It also reports the construction of interaction networks, key hub proteins, drug association analysis, and evolutionary relationships.

CHAPTER 5: This chapter concludes the thesis by summarizing the major findings and their implications for clinical research and treatment strategies. It also outlines the limitations of the study and proposes directions for future work in exploring genetic and molecular connections between beta-thalassemia and other diseases.

1.9 Conclusion

This introductory chapter describes the main research questions, goals, objectives and the motivation of driving this study. The subsequent chapters will present a detailed review of existing literature, describe the methodology for data collection, data analysis, and discuss the results in relation to the current knowledge. The next chapters will go over each of them in depth.

C H A P T E R 2

LITERATURE REVIEW

This chapter highlights the literature-related work carried out in recent years, along with a discussion of the methodologies adopted in those studies and their identified limitations. Many related works have been conducted using a variety of bioinformatics algorithms, computational tools, and gene expression datasets to explore the genetic and molecular links between diseases, but not for beta-thalassemia and its comorbidities, which is highlighted in this work through a literature review. The main goal of this chapter is to review and discuss the latest literature, methodologies, and findings in this domain, to identify key areas where further research can be undertaken to bridge existing gaps.

2.1 Review of Literature

Some literature that triggered this study is discussed here:

In [3] they explored the genetic links between Type 2 diabetes (T2D) and its comorbidities, including kidney failure, liver cancer, myocardial infarction, endometrial cancer, embolic stroke, xanthoma, and xerostomia. They used multiple Gene Expression Omnibus (GEO) microarray datasets for each disease, constructed gene-disease networks (GDNs), performed pathway analysis on KEGG, conducted GO analysis, and built PPI networks. They identified several shared differentially expressed genes (DEGs) between T2D and each comorbidity, suggesting significant molecular associations. However, the study did not include PDI analysis, phylogenetic analysis, or validation analysis, which could have provided stronger clinical insights.

In [10] evidence suggests that COVID-19 may increase the risk of developing neurodegenerative diseases (NDGDs) like stroke, Alzheimer's disease, epilepsy, Parkinson's disease, and multiple sclerosis. GEO microarray datasets for COVID-19 and these NDGDs were analyzed to uncover shared molecular patterns. The study observed that COVID-19 shared 19, 26, 20, 19, and 22 DEGs with epilepsy, stroke, multiple sclerosis, Alzheimer's disease, and Parkinson's disease, respectively. They mapped disease-gene relationships, explored dysregulated pathways, and built PPI and PDI networks, validating their results.

The study in [11] also investigates the genetic and pathogenetic similarities between 2019-nCoV (COVID-19) and other coronaviruses, particularly SARS-CoV. They identified hundreds of dysregulated genes using genome alignment, DNA-DNA hybridization, and gene expression comparisons. They constructed an infectome-diseasome network of up- and down-regulated genes, PPI networks, protein-chemical interactions (PCI), and analyzed pathways and gene ontologies. This work aims to understand shared mechanisms between COVID-19 and related viruses for drug repurposing. In contrast, our work focuses on uncovering shared molecular mechanisms between beta-thalassemia and its comorbidities.

[12] This study use GEO2R tool to determine DEGs and then perform pathway and GO analysis for beta-thalassemia. And also construct PPI network.

The study in [14] explored the molecular relationships between COVID-19 and its comorbidities, including lung cancer, hypertension, myocardial infarction, and diabetes mellitus. They identified 93 upregulated and 15 downregulated genes in COVID-19,

with overlaps of 28 shared genes with diabetes mellitus, 17 with lung cancer, 6 with myocardial infarction, and 7 with hypertension. They performed signaling pathway analysis, GO analysis, PPI and hub protein analysis, PDI analysis, and constructed networks of dysregulated genes. However, this study did not validate their work or perform phylogenetic analysis.

The study in [15] investigates the genetic connections between gastric cancer and its common comorbidities, including kidney disease, diabetes, stroke, and liver cancer. Using mRNA-seq and microarray datasets, they identified matching shared genes, constructed gene-disease networks, analyzed pathways, ontologies, and protein interactions, and validated their work with benchmark databases. This computational work highlights significant genetic associations between gastric cancer and these comorbid conditions. However, it did not analyze PDI interactions or phylogenetic analysis.

In [18] the focus was on identifying influential genes (IFGs) in glioblastoma using the Cancer Genome Atlas (TCGA) dataset to understand its genetic links with various comorbidities. They identified 26 dysregulated IFGs from over 16,261 genes through statistical analysis, conducting further analyses including protein-protein and protein-drug interactions, comorbidity networks, and phylogenetic analysis. However, they did not analyze signaling pathways, GO, or validation networks, limiting their work compared to ours.

The work in [22] identified matching genes among welding fume (WF) and respiratory system diseases (RSDs) by developing a quantitative framework. Using microarray data for WF and RSDs (e.g., asthma, lung cancer, chronic bronchitis, pulmonary edema), they focused on identifying common genes, their networks, pathway analysis, GO analysis, and PPI analysis, validating their results.

The study in [10] analyzes gene expression to reveal genetic links between Parkinson's disease (PD) and other neurodegenerative diseases (Alzheimer's, ALS, Huntington's, and multiple sclerosis). They identified shared dysregulated genes, pathways, GO analysis, phylogenetic analysis, and protein interactions, validating their findings to highlight PD's potential role in the progression of these disorders.

To analyze molecular phylogenetic relationships, we follow the approaches in research evidence [23] and network-based strategies from the system biology paper [25], to study associative relationships through nucleotide sequences. Our analysis primarily depend on traditional tree construction.

In [24] the paper showed bidirectional connections between T2D and breast cancer using GSE 29231, GSE70905, and GSE50586 for diabetes, malignant breast tissue, and both biopsies, respectively. They identified 94 common DEGs, constructed a PPI network, performed limited pathway and ontology analysis, and identified hub proteins and survival construction.

In [26] differentially expressed genes (DEGs) were identified for colorectal cancer (CRC) and eight related comorbidities. Protein interaction analysis uncovered four sub-networks and eight key hub genes as potential therapeutic targets, predicting clinical outcomes and highlighting genes linked to CRC progression and patient survival. The study reviews machine learning and network-based methods for discovering genetic risk factors for CRC.

The study in [29] applied bioinformatics and systems biology methods to identify risk factors for cardiovascular disease (CVD) progression. They found 32, 17, 53, 70, and 89 common DEGs between CVD and its associated risk factors, identifying potential biomarkers through PPI analysis, pathway analysis, and ontology analysis, validated using benchmark databases.

2.2 Brief Summary of Related Work

The existing related works, their methods, datasets, techniques, and limitations are highlighted in Table 2-1 below. The proposed work aims to overcome these limitations after preprocessing microarray and mRNA-seq datasets.

Table 2.1: Table 2-1 Brief Summary of Related Work

Authors Name	Datasets	Methods	Limitations of Work
Malik, S.E., Kanwal, S., Javed, J., Hidayat, W., Ghaffar, T. and Aamir, A.H., 2023 [1]	135 Beta-Thalassemia Major (BTM) patients	Statistical analysis and laboratory methods	No genetic and molecular relationships

Table 2.1: Table 2-1 Brief Summary of Related Work (Continued)

Authors Name	Datasets	Methods	Limitations of Work
Akiki, N., Hodroj, M.H., Bou-Fakhredin, R., Matli, K., Taher, A.T., 2023 [2]	Secondary data	Summarizing patterns, mechanisms, diagnostic methods, preventive measures, and treatments reported in the literature	No relationships shown among disease and its comorbidities
Podder, N.K., Rana, H.K., Azam, M.S., Rana, M.S., Akhtar, M.R., Rahman, M.R., Rahman, M.H. and Moni, M.A., 2020 [3]	GEO microarray datasets	Statistical methods, quantitative model, z-transform, and multi-layered topologies	Fails to calculate large-scale datasets; No drug protein identification; No phylogenetic analysis
Podder, N.K., Shill, P.C., Rana, H.K., Omit, S.B.S., Al Shahriar, M.M.H. and Azam, M.S., 2021 [10]	mRNA and microarray datasets	Statistical methods and algorithm, and Benjamini-Hochberg algorithm	No phylogenetic analysis; Fails to validate their selected comorbidities
Khare, Soumya, et al. [12]	RNA sequencing GEO datasets	Complex detection algorithm, ClueGo and CluePedia methods	-Fails to create a relationships of between BT with its any comorbidities.
Datta, R., Podder, N.K., Rana, H.K., Islam, M.K.B. and Moni, M.A., 2020 [15]	Microarray and mRNA-seq datasets	Statistical methods (t-test), z-transformation, multilayer topology, and neighborhood benchmark method	No drug protein identification; No phylogenetic analysis

Table 2.1: Table 2-1 Brief Summary of Related Work (Continued)

Authors Name	Datasets	Methods	Limitations of Work
Podder, N.K. and Shill, P.C., 2022 [18]	TCGA datasets	Statistical and bioinformatics model	No significant identification of signaling pathway and GO analysis; Fails to validate their work
Rana, M.S., Podder, N.K., Rana, H.K., Hasan, M.I., Azam, M.S., Rahim, M.A., Iqbal, S.H.S. and Saha, S., 2023 [24]	mRNA and microarray datasets	Benjamini-Hochberg algorithm, neighborhood-based benchmarks, and multilayer topology	Fails to identify protein-drug intersections
Durrani, I.A., Bhatti, A. and John, P., 2023 [26]	Microarray and mRNA-seq datasets	Integrated in silico analyses approach	No phylogenetic analysis; Fails to identify drug protein
Talihati, Z., Abudurousuli, K., Hailati, S., Han, M., Nuer, M., Khan, N., Maihemuti, N., Simayi, J., Zhang, W. and Zhou, W., 2025 [29]	TCGA database, genomic database, transcriptomic, and GEO datasets	Limma package in R, STRING database, molecular docking, etc.	Fails to analyze PDI, phylogenetic, and validation analysis
Barua, J.D., Omit, S.B.S., Rana, H.K., Podder, N.K., Chowdhury, U.N. and Rahman, M.H., 2022 [34]	GEO microarray datasets	Z-transformation, neighborhood-benchmark, and multilayered topology	Fails to identify drug protein; No phylogenetic analysis

Table 2.1: Table 2-1 Brief Summary of Related Work (Continued)

Authors Name	Datasets	Methods	Limitations of Work
Rahman, M.H., et al., 2023 [35]	GEO datasets	Design matrix model, fit-linear, and Bayesian model	Fails to identify hub and drug proteins; Fails to validate the work; Fails to calculate large-scale data

2.3 Research Gap

Our proposed work offers a more comprehensive level of analysis compared to the referenced studies, as it integrates all analytical approaches previously applied separately in the existing literature. This study comparatively investigates all analyses to explore the deep genetic and molecular correlation of beta-thalassemia with associated endocrine and cardiac diseases. This is shown in Table 2-2.

Table 2.2: Comparison of Proposed Work with Related Studies

Related Work	Datasets	Diseases	DGN, Pathways, GO, PPI, Validation Network	PDI, Phylogenetic Analysis
[3]	Microarray datasets	T2D vs. comorbidities; Gastric cancer vs. comorbidities; Welding fumes vs. respiratory system	YES	NO
[10], [14]	mRNA and microarray datasets	COVID-19 vs. comorbidities	YES but Validation: NO	NO but PDI: YES
[15], [22]	Microarray datasets	T2D vs. comorbidities; Gastric cancer vs. comorbidities; Welding fumes vs. respiratory system	YES	NO
[18]	TCGA datasets	Glioblastoma vs. comorbidities	NO but DGN: YES; PPI: YES	YES

Table 2.2: Comparison of Proposed Work with Related Studies (Continued)

Related Work	Datasets	Diseases	DGN, Pathways, GO, PPI, Validation Network	PDI, Phylogenetic Analysis
[24]	mRNA and microarray datasets	Parkinson's vs. neurodegenerative	YES	NO but Phylogenetic: YES
Proposed Work	mRNA and microarray datasets	Beta-thalassemia vs. endocrine and cardiac diseases	YES	YES

Previous studies on any main diseases and its comorbidities have performed individual analyses such as gene expression profiling, pathway analysis, gene ontology, protein-protein interaction or drug interaction were applied separately and not in an integrated manner. No existing work has combined all of these analytical approaches together to provide a comprehensive genetic, molecular and evolutionary understanding of beta-thalassemia with its associated endocrine and cardiac complications. This gap limits the discovery of common biomarkers, therapeutic targets, and evolutionary insights. Where our work have performed these analyses in combined manner to give a clear and straight visions of BT and its comorbidities connections.

2.4 Conclusion

This chapter has explored previous research relevant to beta-thalassemia and those research that relevant to system biological approaches. While earlier studies have provided valuable insights into gene expression with its clinical outcomes in a separated manner. But this literature review highlights the absence of an integrated framework that connects genetic profiling with pathway enrichment, ontology, interaction networks and evolutionary perspectives. Recognizing these gaps has guided the direction of the present study, which aims to bring these analyses together to achieve a more clear understanding of beta-thalassemia and its related comorbidities.

C H A P T E R 3

METHODOLOGY

In this chapter, the methodology for analyzing beta-thalassemia and its comorbidities has been detailed. For discussion convenience, there are a total of 4 sub-chapters under Chapter 3. In section 3.1, we discussed the collection of genetic datasets; in section 3.2, we described the differential gene expression analysis; in section 3.3, we outlined the pathway and network analysis; and in section 3.4, we explained the validation and visualization techniques.

3.1 Introduction

This chapter outlines the proposed model and methodology for analyzing the genetic profiling between BT and its comorbidities. The study integrates bioinformatics and system biological techniques to process gene expression data of BT and its complications such as PCOS, hypothyroidism, hypogonadism, diabetes, cardiomyopathy and arrhythmia.

The methodology involves data preprocessing, genetic profiling, pathway enrichment, functional ontology, protein-protein and protein-drug interaction networks, phylogenetic analysis and validation analysis. These approaches aim to identify key biomarkers, therapeutic targets and evolutionary insights between the genes of BT and its comorbidities. The details of these analytical approaches are presented in the following sections.

3.2 Overview of Analytical Approaches

This chapter shows a standard analytical procedure to gain the genetic link between beta-thalassemia and its comorbidities by analyzing the microarray and mRNA sequence datasets. We have used Gene Expression Omnibus (GEO) datasets for each disease where each dataset has two groups: normal tissue (healthy or control samples) and malignant tissue (affected samples) [13]. Comparing these normal and malignant tissues by using Benjamini-Hochberg method to make sure that the significant genes are reliable and control the false discovery rate by adjusting p-value. A Limma package of R language to identify differentially expressed genes (DEGs) between BT and PCOS, T2D, hypothyroidism, hypogonadism, ACM, arrhythmia respectively. After applying the Benjamini-Hochberg correction to control the false discovery rate, this study applies statistical thresholds to differentiate up-regulated and down-regulated genes. And then by finding common DEGs, disease-gene network was constructed to visualize their co-relations. This study performed ontological analysis, pathway analysis, PPI, PDI, phylogenetic analysis and their respective networks. After that it performed validation analysis using gold benchmark datasets including dbGaP and OMIM.

A working flowchart representing this quantitative method in Figure 3-1.

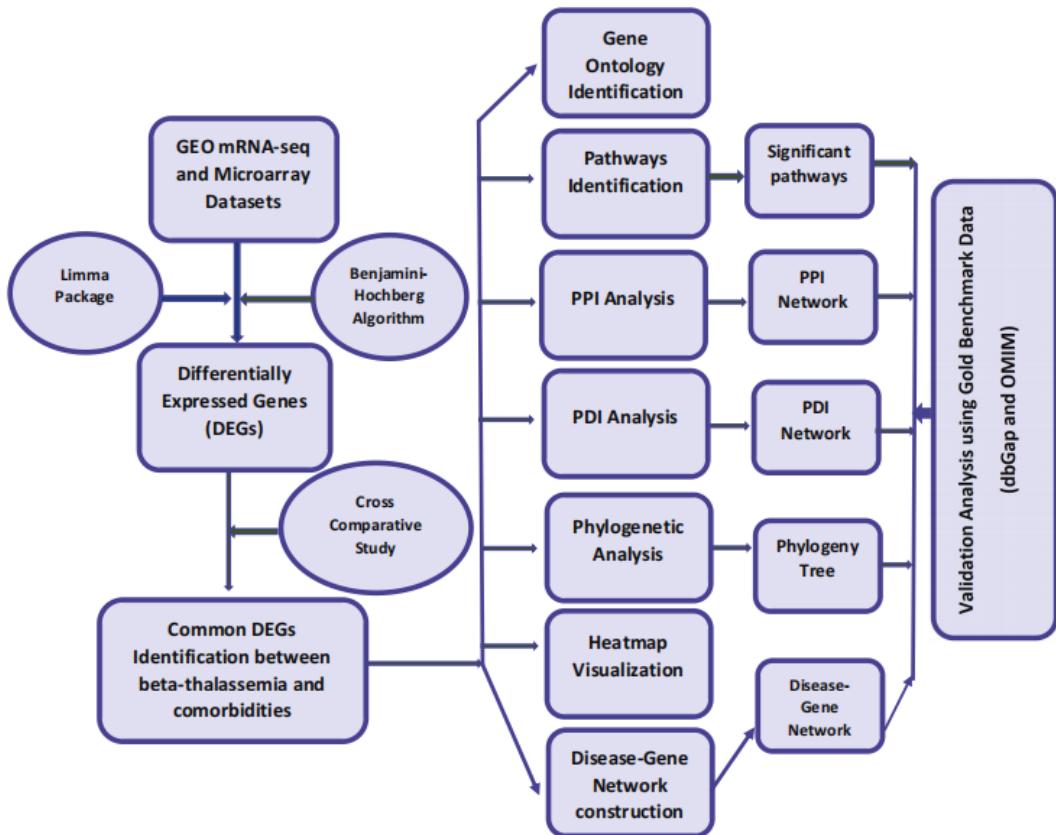


Figure 3.1: Working flow diagram of this investigation

3.3 Dataset Information

This study investigated mRNA-seq datasets for BT, PCOS, hypothyroidism, ACM, and arrhythmia, and microarray datasets for T2D and hypogonadism, available in Gene Expression Omnibus (GEO), which is maintained by National Center for Biotechnology Information (NCBI) [13], to identify the genetic link between beta-thalassemia and its comorbidities. Each and every datasets has two group normal tissue and malignant or affected tissue. This work compares these normal and malignant samples to indicate which genes are expressed differentially. Those differences can then point out the possible genetic links between BT and its comorbidities.

For BT, GSE117221 GEO mRNA-seq dataset are selected which has total 49 samples. 17 samples are normal tissue that means healthy patient and 32 samples are malignant tissue where (15 samples for thalassemia intermediate and 17 samples for thalassemia major) both type of patients are either female or male.

For T2D, GSE25724 expression profiling by array genes are selected with 13 samples where 7 samples for type 2 diabetes patients and 6 samples for non-diabetic patients. GSE216609 mRNA-seq datasets are selected for PCOS with total 7 samples where 4 sample for control and 3 samples for polycystic ovary syndrome.

With 8 samples GSE176153 GEO mRNA genes are selected for hypothyroidism where 4 samples for healthy patients and 4 samples for malignant patients for both male and female. For ACM RNA-seq datasets are selected from NCBI. Its GEO accession is GSE233780 with 12 samples where 6 samples for ACM and other 6 for healthy control.

GSE26966 GEO microarray datasets are used for hypogonadism with 23 samples where 9 samples for normal pituitary and 14 samples for gonadotrope tumor.

For arrhythmia GSE175944 mRNA-seq datasets are collected which introduced the most common arrhythmia is atrial fibrillation with 6 samples where 3 samples for control patient and 3 samples for case samples.

Table 3-1 provides a detailed representation of datasets including GEO accession id, Gender (no. of male and no. of female), samples (divided into case and control groups), associated diseases and source name that indicates the tissue type, cell type or experimental conditions.

Table 3.1: Representation of Datasets Information

S. No.	Disease Name	GEO Accession Id.	Gender (Male + Female)	Sample (Case + Control)	Disease status (case, control)	Source Name
1	Beta-thalassemia	GSE-117221	49 total, (23 + 26)	49 samples, (32 + 17)	Thalassemia intermediate (TI) and Thalassemia major (TM), and Healthy patient	Healthy-ErPCs, TI-ErPCs, TM-ErPCs
2	T2D	GSE-25724	13 total, (07 + 06)	13 samples, (06 + 07)	Non-diabetic, and Type 2 diabetes	Human islets, non-diabetic and Human islets, diabetic
3	PCOS	GSE-216609	07 total, (07 female)	07 samples, (03 + 04)	Control, and PCOS	Cumulus granule cells
4	Hypothyroidism	GSE-176153	08 total, (02 + 06)	08 samples, (04 + 04)	Control, and Hypothyroidism	Whole blood

Table 3.1: Representation of Datasets Information (Continued)

S. No.	Disease Name	GEO Accession Id.	Gender (Male + Female)	Sample (Case + Control)	Disease status (case, control)	Source Name
5	ACM	GSE-233780	12 total, (10 + 02)	12 samples, (06 + 06)	ACM, and Healthy control	Cardiac mesenchymal stromal cells
6	Hypogonadism	GSE-26966	23 total, (12 + 11)	23 samples, (09 + 14)	Normal pituitary (NP), and Gonadotrope tumor (GT)	NP at autopsy within 2–18 hr. of death, and GT at time of transsphenoidal surgery
7	Arrhythmia	GSE-175944	Not mentioned	06 samples, (03 + 03)	Control, and PITX2 knock-out	Cell line of human induced pluripotent stem cells (hiPSC)

3.4 Methodology

This section provides a detailed overview of the methodology applied in this study. Different analytical approaches have been applied to investigate the genetic profiling of beta-thalassemia and its associated comorbidities. The process includes data collection, preprocessing, identification of important functional genes, enrichment analysis, and network-based evaluations such as protein-protein and protein-drug interactions. Additionally, evolutionary relationships has been incorporated to strengthen the findings. By combining these diverse methods, the study aims to ensure a comprehensive and reliable analysis framework that captures both the molecular and clinical perspectives of beta-thalassemia.

3.4.1 Selection of mRNA-seq and Microarray Datasets

For BdSL classification, this study used pre-trained convolutional neural networks, ResNet-50 for feature extraction and evaluating two classifiers SVM (Support Vector Machine) and CNN (Convolutional Neural Network) for the final classification job. For this study, a reliable and relevant data was selected for investigating the genetic association between BT and its comorbidities. Two types of datasets were utilized that are mRNA-seq datasets and microarray datasets which were selected from the Gene Expression Omnibus (GEO), a public functional genomics data repository maintained by the National Center for Biotechnology Information (NCBI) [10].

- mRNA-seq datasets (gene expression that is profiling by high throughput sequencing) were selected for BT, PCOS, hypothyroidism, ACM and arrhythmia. These datasets provide a comprehensive view of transcriptional expression.
- Microarray datasets (gene expression that is profiling by array) were selected for T2D and hypogonadism from GEO. Microarray datasets remain valuable for differential expression analysis for experimental designs.

3.4.2 Differential Gene Expression Analysis

Differential gene expression analysis is a base of bioinformatics for understanding the molecular and genetic inter-relations of complex diseases like beta-thalassemia and its comorbidities. In this study, we performed gene expression analysis on microarray and mRNA-seq datasets to identify differentially expressed genes (DEGs) between case disease and control samples for BT, PCOS, hypothyroidism, hypogonadism, T2D, ACM and arrhythmia. The analysis was conducted using the GEO2R tool on NCBI Gene Expression Omnibus (GEO) website and the Limma package in R which are widely used for strong statistical frameworks.

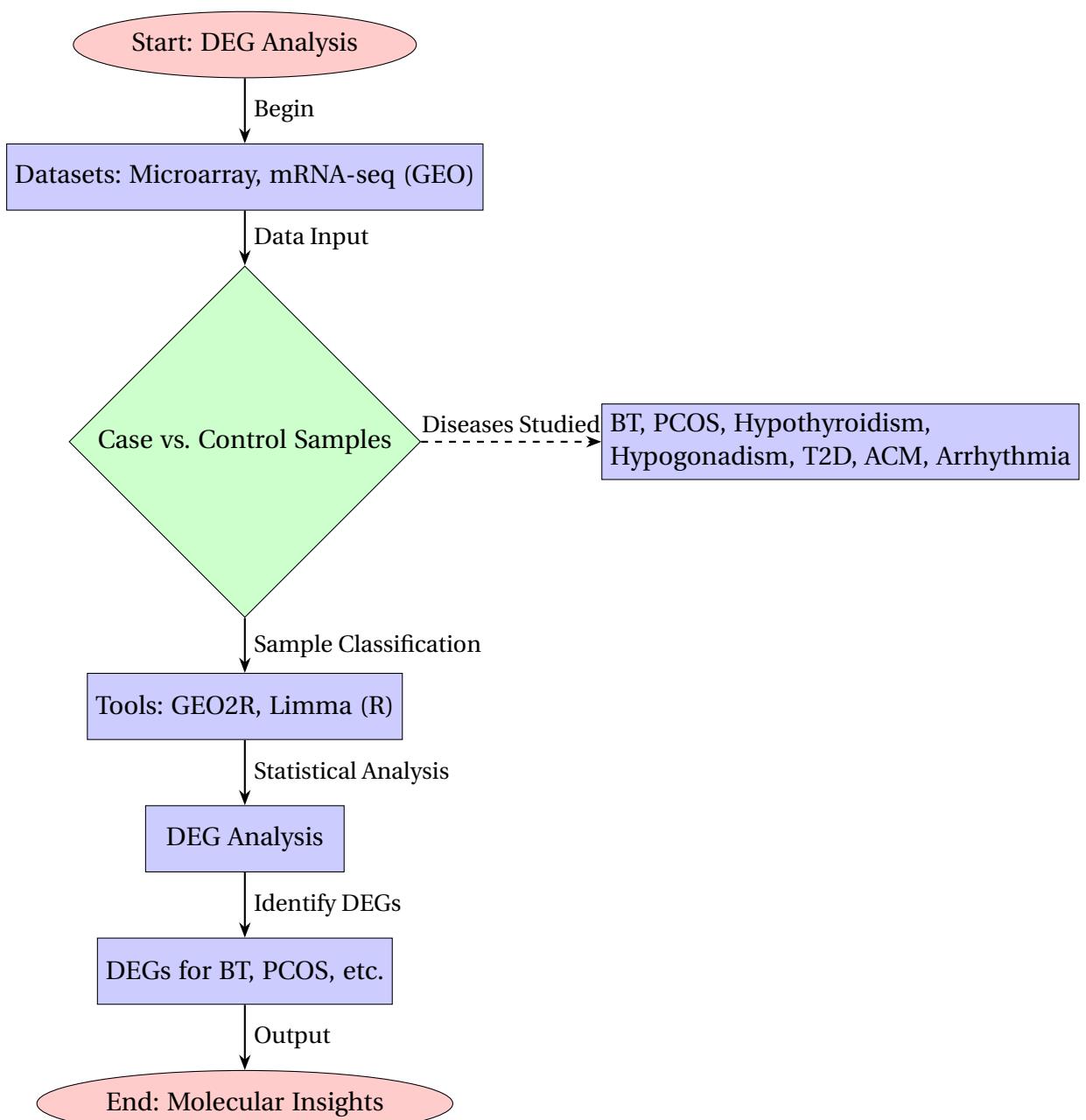


Figure 3.2: Workflow for Differential Gene Expression Analysis of Beta-Thalassemia and Comorbidities.

Data Pre-processing and Normalization

To reduce experimental differences and keep the data consistent, gene expression values in each dataset were normalized using the Z-score method. This approach standardizes the expression values to make them comparable across different samples [3]. The Z-score transformation for a gene expression value is calculated as:

$$z_{ij} = \frac{x_{ij} - \mu_i}{\sigma_i} \quad (3.1)$$

where:

- z_{ij} : Normalized gene expression value for gene i in sample j ,
- x_{ij} : The value of gene expression i in sample j ,
- μ_i : Mean value of gene expression i in all samples,
- σ_i : The standard deviation of gene expression values i .

The normalization method was applied to both microarray (for T2D and hypogonadism) and mRNA-seq (for BT, hypothyroidism, PCOS, ACM, and arrhythmia) datasets to ensure reliable identification of differentially expressed genes (DEGs).

Statistical Analysis for DEG Identification

We identified the differentially expressed genes (DEGs) by applying the Benjamini-Hochberg (BH) correction method, which minimizes false positives by controlling the false discovery rate (FDR) [10]. The BH formula is:

$$\text{BH adjusted } p\text{-value} = \frac{i}{m}Q \quad (3.2)$$

where:

- i : Rank of individual p -value,
- m : Total number of tests,
- Q : False discovery rate threshold.

The adjusted p -value threshold of BH was set to ≤ 0.05 to ensure statistical significance. These genes were classified based on the \log_2 fold change ($\log FC$) values by applying the following conditions:

- To identify up-regulated genes: $|\log FC| > 1$,

- To identify down-regulated genes: $|\log FC| < -1$.

These threshold values were used to identify significant DEGs between case and control groups.

3.4.3 Gene Diseases Network Analysis

This study constructed Disease-Gene Networks (DGNs) using a neighborhood-based benchmarking and topological approach to investigate the genetic associations between beta-thalassemia and its comorbidities. These networks of up- and down-regulated genes show the relationships between diseases and differentially expressed genes (DEGs), providing a visual framework to identify shared genetic signatures. Each node represents either a disease or a gene, where diseases are source nodes and their associated genes are target nodes in the DGN network. This disease-gene connection forms a bipartite graph. A connection exists if at least one significant DEG is shared between a disease and beta-thalassemia. Let D represent the set of diseases and Z represent the set of dysregulated genes identified from the gene expression analysis, where a gene $z \in Z$ is connected to a disease $d \in D$ if z is significantly dysregulated in the dataset for d . If associated diseases d_1 and d_2 have sets of significant dysregulated genes Z_1 and Z_2 , respectively, then the number of shared genes is defined as:

$$|Z_1 \cap Z_2| \quad (3.3)$$

By using the Jaccard Coefficient, this study measured the similarity between disease pairs, which evaluates the overlap relative to the total unique genes. The edge weight or similarity score between diseases d_1 and d_2 is calculated as [16]:

$$X(d_1, d_2) = \frac{|Z_1 \cap Z_2|}{|Z_1 \cup Z_2|} \quad (3.4)$$

Here, $|Z_1 \cap Z_2|$ represents the set of common DEGs, and $|Z_1 \cup Z_2|$ represents the union of DEGs for the two diseases. The Jaccard Coefficient $X(d_1, d_2)$ ranges from 0 to 1, where a higher value indicates greater similarity in the genetic profiles of the diseases [20].

Two separate networks were constructed for up-regulated and down-regulated DEGs to show the distinct regulatory patterns. The DGNs were visualized and analyzed using the Cytoscape platform. Cytoscape enabled the mapping of disease-gene associations by optimizing the layouts to highlight connectivity patterns, such as hub genes across multiple comorbidities.

3.4.4 Pathway and Functional Association Analysis

Pathway analysis involves the systematic identification of enriched biological pathways from sets of differentially expressed genes (DEGs) that are shared between beta-thalassemia and its comorbidities. This study identifies significant pathways using Enrichr, a public tool developed by the Ma'ayan Laboratory for Computational Systems Biology for gene set enrichment analysis (GSEA). Enrichr works by comparing input gene lists with collections of curated biological databases. The method applies statistical tests to measure significance, ensuring reliable and meaningful insights into the underlying molecular mechanisms.

A pathway is a sequence of molecular interactions that results in a specific change or product in a cell. It is a standard method for understanding the connections between complex diseases [14]. We investigated dysregulated gene pathways across four databases using Enrichr: Reactome, KEGG, WikiPathways, and BioCarta.

1. Preparation of Input Gene Sets

Enrichr compares the input gene list against a reference set that includes all known annotated genes in the human genome (approximately 20,000–30,000), depending on each library. Using this background, it ensures that the analysis is fair and considers the full range of genes that could potentially be expressed [17].

2. Selection of Pathway Libraries

Enrichr compares the input gene list against multiple pathway-focused databases. This study selects four databases based on their relevance to biological processes [19]:

- **KEGG** (Kyoto Encyclopedia of Genes and Genomes) provides correlated sets of genes involved in metabolic, signaling, and disease pathways [20].
- **Reactome** is an open-source database of manually curated biological pathways, focusing on reactions and interactions.
- **WikiPathways** is a community-curated resource with pathways linked to drug interactions and biological processes.
- **BioCarta** focuses on gene interaction models in signaling and metabolic pathways.

Using Enrichr, we analyzed the common DEGs identified from the cross-comparative analysis of beta-thalassemia and its comorbidities. Pathways were considered significant if their adjusted *p*-value was ≤ 0.05 , ensuring robust statistical reliability. The analysis identified key pathways for each comorbidity, such as GPCR ligand binding

for PCOS, interferon gamma signaling for hypogonadism, and zinc homeostasis for arrhythmia, among others.

3.4.5 Gene Ontology Analysis

Gene Ontology (GO) analysis is a structured framework for describing genes by their molecular functions, biological processes, and cellular components, enabling a deeper understanding of their roles in disease mechanisms [15]. In this study, we conducted GO analysis to explore the functional associations of common differentially expressed genes (DEGs) shared between beta-thalassemia and its comorbidities. The analysis was performed using Enrichr, a web-based enrichment tool developed by the Ma'ayan Laboratory, which queries gene sets against standardized ontology databases to identify significant functional terms [31].

GO Analysis Workflow The common DEGs identified through cross-comparative analysis of GEO datasets were used as input for Enrichr. We focused on two key ontology databases:

- **GO Biological Process (GO Term)** categorizes genes based on their involvement in coordinated biological processes, such as signaling pathways or metabolic activities.
- **Human Phenotype Ontology (HP Term)** links genes to phenotypic abnormalities observed in diseases, facilitating the identification of clinical manifestations associated with DEGs.

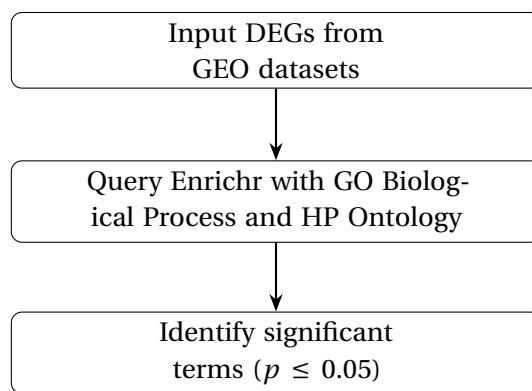


Figure 3.3: Workflow for Gene Ontology (GO) analysis using Enrichr to identify significant functional terms associated with DEGs.

3.4.6 Up and Down-regulated Identification

Downregulation is the process in which a cell reduces the expression level of a gene compared to a reference in response to an external variable. Upregulation refers to an increase in expression level compared to a reference. Here, the expression level indicates the abundance of biological components, such as RNA or protein [32]. These changes are typically determined by comparing two groups: a reference or control sample, often healthy tissue, and a test sample, such as diseased or mutant tissue. The expression values of the test sample are compared against those of the reference sample to generate expression ratios, which is standard practice in gene expression analysis. These ratios are typically transformed into a logarithmic scale for easier interpretation. A positive log value indicates that the gene expression is higher in the test sample compared to the reference, indicating upregulation. A negative log value signifies reduced expression in the test sample relative to the reference, indicating downregulation. Thus, by comparing the expression profiles of two samples, it is possible to determine whether a gene is up- or down-regulated under specific conditions.

3.4.7 Common Dysregulated Genes

This study identified common differentially expressed genes (DEGs) shared between beta-thalassemia (BT) and its comorbidities by using statistical analysis to explore genetic relationships among these diseases. We retrieved GEO datasets from NCBI for the diseases under investigation. To reject the null hypothesis, a statistical p -value of ≤ 0.05 was used. For up-regulated gene identification, a threshold of $|\log FC| \geq 1$ was applied, and for down-regulated gene identification, a threshold of $|\log FC| \leq -1$ was used [11]. By performing an intersection set operation using the R `limma` package, we identified common genes between BT and its comorbidities [26]. Using these datasets and methods, we obtained results that demonstrate the connections between BT and the selected diseases. Other detailed information is discussed in the next chapter.

3.4.8 Network Construction of PPI

Protein-Protein Interaction (PPI) analysis is a primary goal of systems biology, predicting protein functions and drug targets through molecular interactions [15]. These interactions are significant for driving cellular processes and mapping connections between beta-thalassemia (BT) and its comorbidities [29]. We constructed PPI networks to explore how proteins encoded by shared differentially expressed genes (DEGs) interact.

We used the STRING database, inputting common DEGs and setting a high confidence score of 0.9 for reliable interactions. The NetworkAnalyst platform, utilizing the Markov Clustering algorithm, facilitated clustering of proteins based on connectivity. The resulting networks were visualized in Cytoscape, with proteins represented as nodes and interactions as edges [18].

Hub proteins were selected based on topological parameters, specifically a degree greater than 10. The distance between a pair of proteins (i, j) is defined as follows:

$$d(i, j) = 1 - \frac{|N_i \cap N_j|}{|N_i \cup N_j|} \quad (3.5)$$

where N_i and N_j are the neighbor sets of proteins i and j , respectively.

3.4.9 Protein-Drug Interactions Network Analysis

The primary objective of this investigation is to identify potential therapeutic drug particles [18]. The Protein-Drug Interaction (PDI) network was constructed using NetworkAnalyst and prepared with the DrugBank database, which is designed for common genes of beta-thalassemia and its comorbidities. To explore potential therapeutic options for beta-thalassemia and its comorbidities, we constructed PDI networks. These networks map how proteins encoded by the shared differentially expressed genes (DEGs) interact with drug molecules, which is used to identify possible treatments [33]. Using systems biology-based strategies for studying protein-drug interactions, the goal of this work was to identify potential drug targets capable of addressing the molecular mechanisms that connect these diseases.

Building the PDI Networks

Firstly, we identified the common DEGs between beta-thalassemia and each comorbidity. These genes were input into the NetworkAnalyst platform, which integrates the DrugBank database to map interactions between proteins and known drug compounds [18]. DrugBank provides a comprehensive repository of drugs and their protein targets, including approved, experimental, and investigational compounds, which is used to ensure a broad scope of potential interactions.

Analytical Approach

Following the systems biology framework outlined by Colinge et al. (2012) [4], we analyzed the PDI network by:

- Topological Analysis is used to identify hub proteins with multiple drug interactions by using metrics like degree (the number of connections) to prioritize potential therapeutic targets.
- Functional Mapping is used for cross-referenced protein functions to ensure drugs targeted biologically relevant proteins, such as those involved in immune response or signaling pathways linked to beta-thalassemia's comorbidities.
- Drug Prioritization were ranked based on the number of protein targets and their relevance to disease pathways, ensuring focus on compounds with potential clinical applicability.

The PDI network helps us see which drugs might influence the proteins driving beta-thalassemia and its comorbidities.

3.4.10 Phylogenetic Analysis

Phylogenetic analysis enhances the understanding of the evolutionary relationships among genes, proteins, and diseases [18]. We constructed a phylogenetic tree for beta-thalassemia and its comorbidities to illustrate the associative relationships among them by using Molecular Evolutionary Genetics Analysis (MEGA) tools and FASTA sequences of nucleotide datasets from NCBI [18]. The tree shows a strong evolutionary relationship between beta-thalassemia and hypogonadism as they belong to a common species. We retrieved nucleotide sequences in FASTA format from the NCBI database for representative genes associated with beta-thalassemia and its comorbidities by selecting sequences based on their relevance to shared differentially expressed genes (DEGs). To ensure comprehensive coverage, we included multiple sequences per disease and prioritized those linked to key pathways like iron metabolism or endocrine signaling. Following standard molecular phylogenetic protocols [23]:

- Sequence Alignment: Sequences were aligned using ClustalX to identify homologous regions, accounting for insertions, deletions, and substitutions. This step ensures accurate comparison for evolutionary inferences.
- Tree Construction: We used the Molecular Evolutionary Genetics Analysis (MEGA) software to generate phylogenetic trees. The Neighbor-Joining (NJ) method was employed, with the Kimura 2-parameter model for distance calculation to handle nucleotide substitutions [36].

3.4.11 Validation Analysis

To ensure the reliability of our findings on the genetic links between beta-thalassemia and its comorbidities, we conducted a validation analysis using gold-standard biomedical databases. This step confirms that the DEGs and their disease associations are consistent [22]. We used two benchmark databases, including dbGaP and OMIM from Enrichr tools, on the up-regulated and down-regulated genes of beta-thalassemia to validate our findings [22]:

- dbGaP (Database of Genotypes and Phenotypes): This NCBI-hosted database contains genotype-phenotype relationships from large-scale genomic studies. We queried dbGaP with our shared DEGs to identify diseases with overlapping genetic profiles.
- OMIM (Online Mendelian Inheritance in Man): This comprehensive catalog of human genes and genetic disorders was used to verify whether our DEGs are linked to beta-thalassemia or its comorbidities in Mendelian or complex disease contexts.

3.4.12 Working Algorithm

A systematic methodology outlines the entire operational process as a set of structured steps. The following guidelines offer a detailed summary of all procedures carried out in our research [38].

3.4.13 Algorithm

Input: Microarray and mRNA-Seq GEO datasets.

Output: Differentially Expressed Genes (DEGs), Common DEGs, Disease-Gene Networks (DGNs), Signaling Pathways, Ontological Pathways, Protein-Protein Interactions (PPIs), Protein-Drug Interactions (PDIs), Phylogenetic Tree, and Validation Network.

1. Dataset Selection:

- Selecting relevant Gene Expression Omnibus (GEO) datasets from the NCBI using disease-specific criteria.

2. Differential Gene Expression Analysis:

- For every dataset $i = 1, 2, 3, \dots, N$
 - a) Load the datasets.

- b) Normalize datasets using Z-score transformation to ensure comparability.
- c) Create a case vs. control design matrix.
- d) Apply the R Limma package and GEO2R to compute DEGs using the Benjamini-Hochberg method.
- e) Filter DEGs based on adjusted p -value ≤ 0.05 .
- f) Modify $|\log FC| \geq 1$ for Upregulation and $|\log FC| \leq -1$ for Downregulation.
- g) Identify significant DEGs.

3. Cross-Comparative Analysis:

- Compare DEG gene sets between beta-thalassemia and each comorbidity including T2D, PCOS, ACM, hypothyroidism, hypogonadism, and arrhythmia to identify common up- and down-regulated genes.
- Use the Jaccard Coefficient to quantify similarity between gene sets.

4. Some Analysis for Common DEGs:

- Disease-Gene Networks (DGNs) construction.
- Enrichment analysis for significant signaling pathways.
- Enrichment analysis for Ontological pathways.
- PPI network construction.
- PDI network construction.
- Evolutionary phylogenetic Analysis.
- Plot these pathways in tabular form.

5. Validation Analysis:

- Build a validation network in Cytoscape.

6. Results:

- List of common DEGs.
- DGNs for up- and down-regulated genes.
- Heatmap Visualization.
- Enriched signaling and ontological pathways.
- PPI networks and hub proteins.
- PDI networks with potential drug targets.

- Phylogenetic tree showing disease relationships.
- Validated disease-gene associations.

3.4.14 Experimental Setup

To conduct the genetic and molecular analysis of beta-thalassemia and its comorbidities, we used the following computational setup and tools:

- **Hardware:**

- Device: Desktop PC
- Processor: Intel Core i5-7200U CPU @ 2.50GHz (2.71 GHz)
- RAM: 4.00 GB (3.26 GB usable)
- System: 64-bit Windows 10 Pro, Version 21H2, OS Build 19044.2006

- **Software and Tools:**

- R Studio: For differential gene expression analysis using the Limma package.
- Cytoscape: For constructing and visualizing disease-gene and validation networks.
- MEGA: For phylogenetic tree construction.
- STRING: For protein-protein interaction (PPI) network analysis.
- Enrichr: For pathway and gene ontology enrichment analysis.
- Network Analyst: For PPI and protein-drug interaction (PDI) network construction with DrugBank integration.

3.4.15 Conclusion

This chapter outlined the methodology for investigating the genetic and molecular links between beta-thalassemia and its comorbidities. Using GEO datasets, we identified DEGs, constructed DGNs, performed pathway and GO enrichment, built PPI and PDI networks, conducted phylogenetic analysis, and validated findings with dbGaP and OMIM. The systematic approach, supported by tools like R, Cytoscape, Enrichr, STRING, NetworkAnalyst, and MEGA, ensures robust and reproducible results, setting the stage for detailed findings in Chapter 4.

C H A P T E R 4

RESULT ANALYSIS AND DISCUSSION

In this chapter, the result analysis and discussion have been clarified. For discussion convenience, there are a total of 10 sub-chapters under chapter 4, which we introduced. In section 4.1, we discussed experimental tools. From section 4.2 to 4.9, we discussed results of each analyses with tables and figures including genetic profiling analysis, heatmap visualization, pathway analysis, GO analysis, PPI analysis, PDI analysis, phylogenetic analysis and validation analysis. In section 4.10, we discussed the discussion part.

4.1 Introduction

This chapter represents the results of our computational approach to investigate the genetic and molecular relationships between beta-thalassemia and its comorbidities. By applying bioinformatics techniques, we analyzed microarray and mRNA-seq datasets from GEO to identify the DEGs. Based on these DEGs, we then constructed DGNs network, performed pathway and gene ontology pathway, build PPI and PDI networks, conducted phylogenetic analysis and at last validate our findings. The analyses were executed by using some tools such as R Limma package, Cytoscape, Enrichr [39], STRING [39], Network Analyst [40] and MEGA tool. Each section below of this chapter provides a detailed evaluation of all results. The chapter concludes with a summary of the findings and their implications for understanding beta-thalassemia's comorbidities.

4.2 Genetic Profiling Analysis

We identified common dysregulated genes among beta-thalassemia (BT) and its comorbidities, including T2D, PCOS, hypothyroidism, hypogonadism, ACM, and arrhythmia, by analyzing the GEO microarray and mRNA datasets from NCBI. Each dataset was analyzed using the R Limma package and GEO2R by applying a statistical threshold of adjusted p -value ≤ 0.05 to reject the null hypothesis. Genes were classified as up-regulated with $|\log FC| \geq 1$ and down-regulated with $|\log FC| \leq -1$, indicating significant expression changes between case and control groups [4].

We have 15,008 differentially expressed raw genes, from which we achieved 123 up-regulated genes and 991 down-regulated genes for beta-thalassemia. Similarly, these dysregulated genes were obtained for T2D, PCOS, hypogonadism, hypothyroidism, ACM, and arrhythmia, and applied to cross-comparative studies between beta-thalassemia and these comorbidities to identify common genes. After that, we obtained common genes, such as:

- Hypothyroidism: 13 DEGs (1 up-regulated, 12 down-regulated).
- Hypogonadism: 165 DEGs (4 up-regulated, 161 down-regulated).
- PCOS: 13 DEGs (1 up-regulated, 12 down-regulated).
- T2D: 14 DEGs (all down-regulated).
- ACM: 11 DEGs (1 up-regulated, 10 down-regulated).
- Arrhythmia: 44 DEGs (14 up-regulated, 30 down-regulated).

Tabular representation of DEGs is given below in Table 4-1 and common DEGs in Tables 4-2 and 4-3.

TABLE 4.1. Up-regulated and Down-regulated genes information

Serial No.	Diseases Name	Up-regulated gene	Down-regulated gene	Total
1	Beta-thalassemia	123	991	1114
2	PCOS	139	533	672
3	Hypothyroidism	208	78	286
4	Hypogonadism	2479	3213	5692
5	T2D	123	1844	1967
6	ACM	47	101	148
7	Arrhythmia	1173	799	1972

Table 4.2: Up-regulated and down-regulated common DEGs of six comorbidities

	Hypogona-dism	Arrhyth-mia	T2D	PCOS	Hypothyro-i-dism	ACM
Up-regulat-ed	GLUD2; EPS8; PCDH9; XPO7	MT1G; MT2A; PTGES; MT1E; MT1X; MRC2; VCAN; AXL; TMEM158; PLPP4; PCDH1; GAL3ST4; PKP1; FN1	CRHBP	LOC10050-7634	MT2A	

Down-regulated	ASS1; NUPR1; SOD2; RPS6KA2; TMEM176B; C1QB; NNMT; PHGDH; PTGDS; SH3PXD2B; GPNMB; TRIB3; MGAT1; NINJ1; SPON2; TPM2; POR; MGLL; MIB2; ADAT3 etc.	IL3RA; PTGDS; AKR1B1; RPP25; SPON2; TNNT1; SLC1A3; RAB20; FXYD6; STXBP6; ACSF2; SYP; SOCS1; MAOA; STAT4 etc.	KCNMA1; AKR1B1; CLU; GPX3; MRC1; FUCA1; CD14; ABCG1; RRAGD; NFIL3; CEBPD; ARID5B; RIN2	SH3D21; LINGO3; TFAP2E; HRH2; SLCO5A1; OSCAR; HLA-J; NCF1; MLPH; LOC11226-8292; RRAD; ZFYVE28	TYMP; CDC42EP2; LRP3; ATF5; RAB20; SECTM1; C4orf48; JDP2; GPR162; HLA-B; DTX4; LY6E	MIR3648-2; MIR3648-1; RNA45SN4; RNA45SN2; RNA45SN3; KIF21B; DUSP4; RRAGD; CD24; CD96
----------------	--	--	--	---	---	--

TABLE 4.3. Up-regulated and Down-regulated genes information for common DEGs with Beta-thalassemia

Serial No.	Diseases Name	Up-regulated Genes	Down-regulated gene	Total
1	Hypogonadism	4	161	165
2	Arrhythmia	14	30	44
3	T2D	0	14	14
4	PCOS	1	12	13
5	Hypothyroidism	1	12	13
7	ACM	1	10	11

Two distinct disease-gene networks (DGNs) were constructed using Cytoscape to visualize these relationships for matching up-regulated genes in Figure 4-1 and down-regulated genes in Figure 4-2, where disease nodes are source nodes and common gene nodes are target nodes [5]. In these networks, Orange-colored nodes are genes, pink-colored nodes are comorbidities and the center represents the main Beta-Thalassemia disease. Connections between diseases and genes are weighted using the Jaccard Coefficient. These DGNs illustrate the interconnections of beta-thalassemia

with its comorbidities that showing how shared genes may drive common molecular mechanisms. The networks highlight that the strong genetic overlap is particularly with hypogonadism has 165 shared DEGs which suggesting a deep molecular relationship. Here arrhythmia (44 DEGs) also shows significant connectivity.

Some common genes that have connection with beta-thalassemia as well as multiple comorbidities. These genes are indicated as red color in the network of down regulated DGNs network that has significant molecular connection. These genes are listed below in Table 4-3 with their connected diseases. For example, gene 'DUSP4' and 'CD24' belongs to BT, ACM and as well as hypogonadism. That make our investigation more strong for the genetic connection between BT and its comorbidities.

TABLE 4.4. Most significant gene that are common for multiple diseases for BT as well as it multiple comorbidities

Gene Name	Connection with multiple diseases
DUSP4; CD24	BT, ACM and hypogonadism
DTX4	BT, hypothyroidism and hypogonadism
RRAGD	BT, ACM and diabetes
RAB20	BT, hypothyroidism, hypogonadism and arrhythmia
ARID5B; RIN2; NFIL3; MAFB	BT, hypogonadism and diabetes
AKR1B1	BT, arrhythmia and diabetes
CEBD; CD14; MRC1	BT, hypogonadism and diabetes
CXCR4; PHACTR1; LIN7A; PTGDS; FXYD6; SOCS1; SPON2; PTGER4; SLC1A3; CHI3L1	BT, arrhythmia and hypogonadism
ZFYVE26; RRAD; SLCO5A1; MLPH	BT, PCOS and hypogonadism

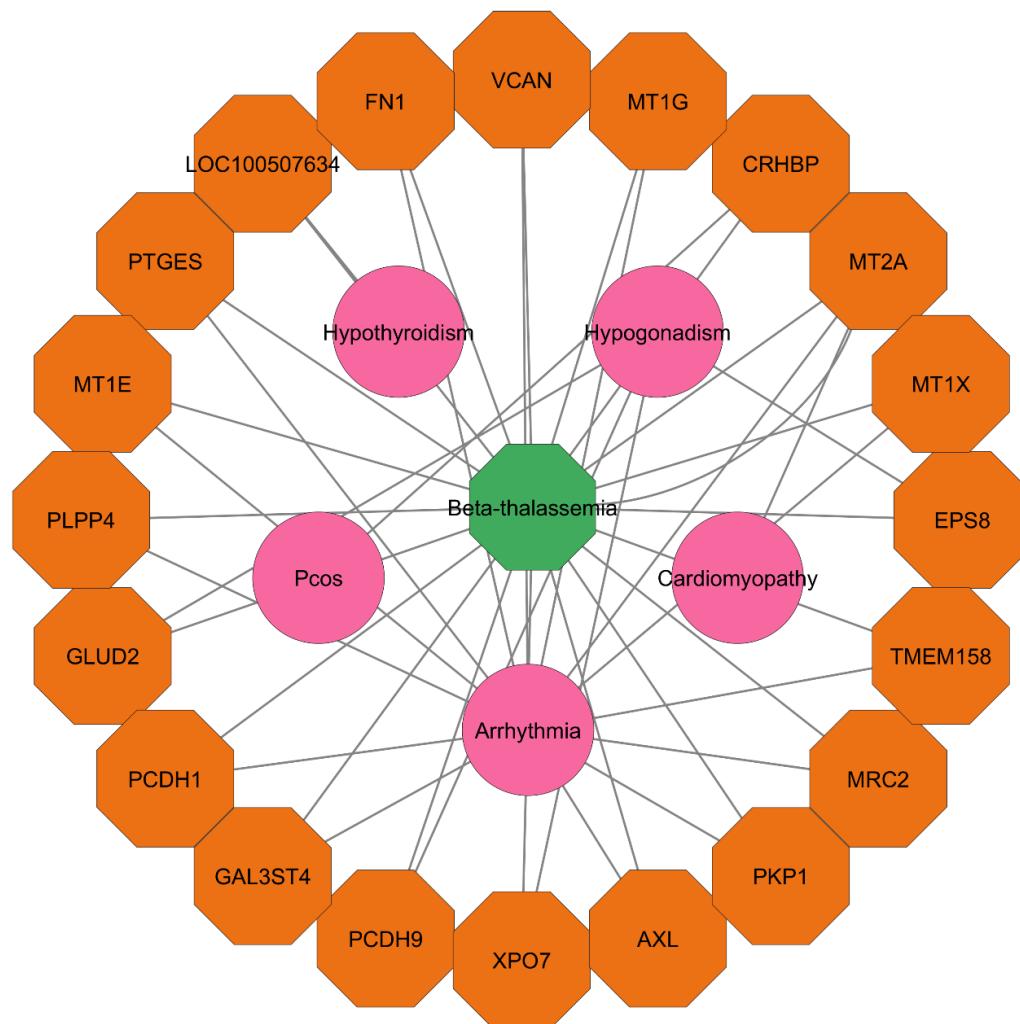


Figure 4.1: Loss Curve (DGNs network for up-regulation of common DEGs. Orange-colored nodes are genes, pink-colored nodes are comorbidities and the center represents the beta-thalassemia.)

From Table 4-4, we can explore that genes those most significantly common for multiple diseases have must connection with 'hypogonadism'. For ever genes in that table have a common connected disease that is'hypogonadism'. Thus this study can claim that hypogonadism has the evolutionary connection with beta-thalassemia that can affect beta-thalassemia patient.

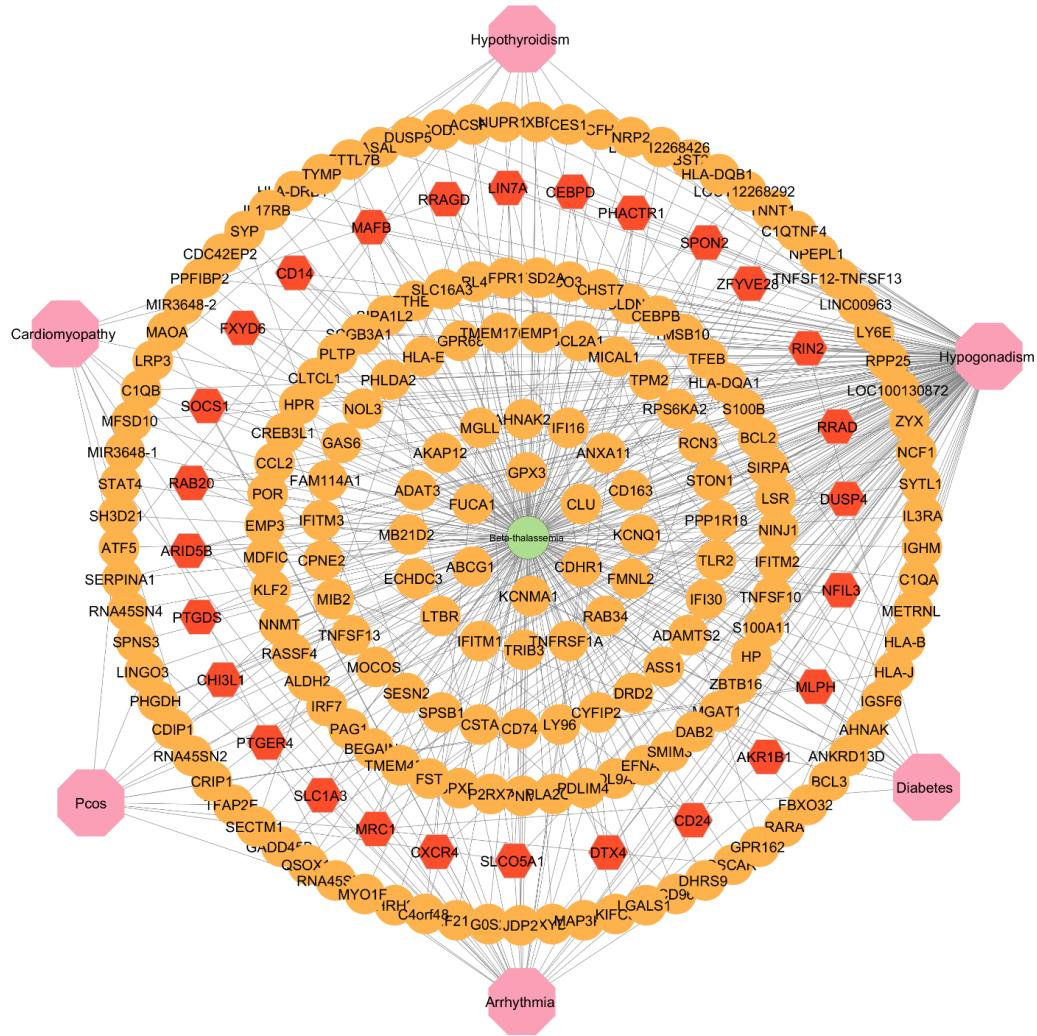


Figure 4.2: DGNs network for down-regulation of common DEGs. Orange-colored nodes are genes, pink-colored nodes are comorbidities and the center represents the beta-thalassemia. Genes that are linked to several diseases are represented by the red color.

4.3 Heatmap Visualization

This study visualizes a heat-map to represent the expression levels of differentially expressed genes among beta-thalassemia and its comorbidities. To visualize the expression level, we generate a heatmap using R's pheatmap package. Columns represent individual diseases like arrhythmia, PCOS, hypothyroidism, cardiomyopathy, hypogonadism, and T2D, and rows represent specific genes [6]. In this visualization, Red color visualizes down-regulated genes with lower expression, blue color visualizes up-regulated genes with higher expression, white or neutral colors represent no changes in expression.

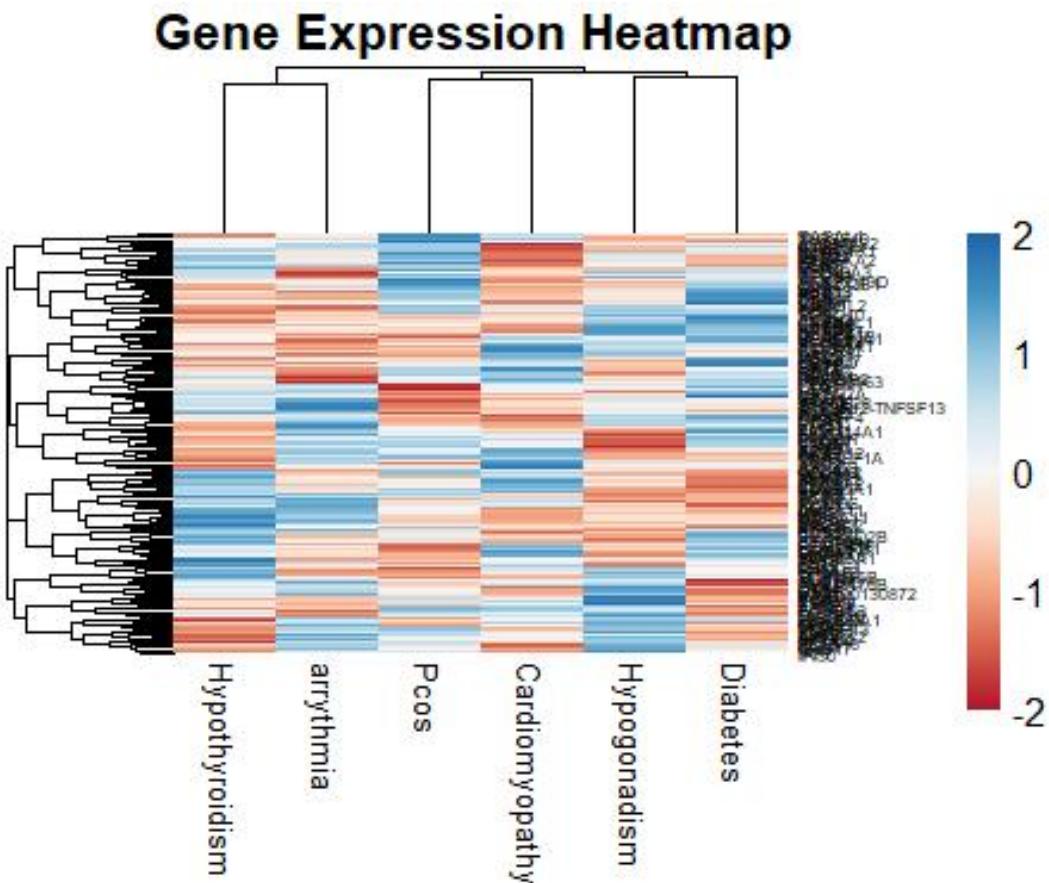


Figure 4.3: Heatmap visualization of gene expression.

The heatmap highlights clear differences in gene activity. For example, in hypogonadism, almost all genes are switched off (161 out of 165). And in arrhythmia, most are also turned down (30 out of 44). This pattern helps to underlying the causes of possibly related to iron overload or long-term anemia in beta-thalassemia. At the same time, 14 genes of arrhythmia are switched on, which could point to the activation of certain biological pathways. Overall, this visualization shows how gene expression varies across conditions and sets the stage for deeper pathway and interaction studies [6].

4.4 Pathway and Functional Association Analysis

A Pathway is a sequence of molecular interactions that results in a specific change and product in a cell. It is a standard method for understanding the connection between complex diseases [6]. We investigated dysregulated gene pathways across four databases using Enrichr, such as Reactome, KEGG, WikiPathways and BioCarta. Significant pathways with adjusted p-value ≤ 0.05 were selected through statistical analysis. From the analysis, we identified 11, 10, 13, 14, 13 and 13 significant

pathways for hypothyroidism, hypogonadism, PCOS, T2D, ACM, and arrhythmia respectively. Significant pathways for each disease were identified and summarized in Table 4-5 to Table 4-10 and in Figure 4-4 to Figure 4-9 [7]:

TABLE 4.5. Significant pathways of Hypothyroidism of shared DEGs

Significant Pathways	Gene Symbols	Adjusted P-Value	Most relevant disease for this pathway
Response of EIF2AK1 (HRI) to Heme Deficiency	ATF5	0.071135712	Beta-thalassemia
Regulation of Innate Immune Responses to Cytosolic DNA	DTX4	0.071135712	Particularly T2D, arrhythmia, ACM, and potentially thalassemia
Mitochondrial Unfolded Protein Response (UPRmt)	ATF5	0.071135712	highly relevant to ACM, arrhythmia and diabetes
Nucleotide Salvage	TYMP	0.074257875	ACM and arrhythmia
Fluoropyrimidine Ac-tivity WP1601	TYMP	0.069079732	T2D, ACM and arrhythmia
Type II Interferon Signaling WP619	HLA-B	0.069079732	Most directly related to ACM and arrhythmia
Notch Signaling WP268	DTX4	0.069079732	ACM and arrhythmia
Proteasome Degradation WP183	HLA-B	0.069079732	Particularly diabetes, cardiomyopathy, and arrhythmia
Allograft rejection	HLA-B	0.110160639	Cardiomyopathy
Graft-versus-host disease	HLA-B	0.110160639	diabetes
Autoimmune thyroid disease	HLA-B	0.110160639	Diabetes and hypothyroidism

- Reactome Database for Hypothyroidism
 - The significant pathway “Response of EIF2AK1 (HRI) to Heme Deficiency” is specifically relevant to beta-thalassemia. It is a hallmark of BT, and the gene *ATF5* is mainly responsible for thyroid problems.
 - “Regulation of Innate Immune Responses to Cytosolic DNA” is particularly relevant to T2D, arrhythmia, ACM, and potentially to thalassemia. The gene *DTX4* is responsible for beta-thalassemia.
 - The significant pathway “Mitochondrial Unfolded Protein Response (UPRmt)” is highly relevant to ACM, arrhythmia, and diabetes, where the gene *ATF5* may cause complications of BT.

- The “Nucleotide Salvage” pathway term significantly supports our findings with strong relevance to ACM and arrhythmia, where the responsible gene is *TYMP*.
- WikiPathways Database for Hypothyroidism
 - For the pathway “Type II Interferon Signaling WP619,” the most directly related diseases are ACM and arrhythmia.
 - For the pathway “Proteasome Degradation WP183,” the particularly relevant diseases are diabetes, cardiomyopathy, and arrhythmia. For both pathways, the gene *HLA-B* is responsible.
- KEGG Database for Hypothyroidism
 - “Allograft rejection,” “Graft-versus-host disease,” and “Autoimmune thyroid disease” pathways involve the gene *HLA-B*. These immune-related pathways are linked to cardiomyopathy, diabetes, and hypothyroidism.

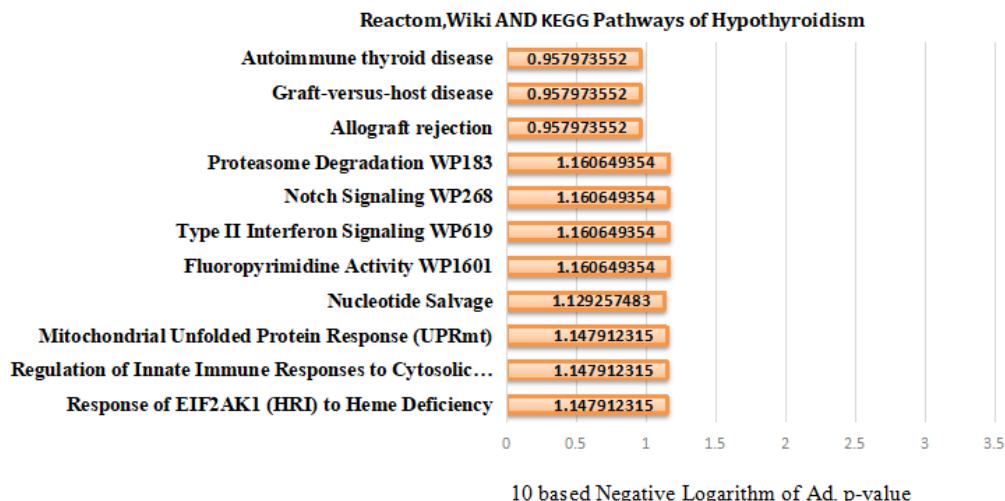


Figure 4.4: Graphical depiction of pathways of Hypothyroidism

Table 4.6: Significant pathways of Hypogonadism of shared DEGs

Significant Pathways	Gene Symbols	Adjusted P-Value	Most relevant disease for this pathway
Innate Immune System	CYFIP2; C1QB; C1QA; SERPINA1; CFH; HP; FPR1; LY96; DTX4; SOCS1; IFI16; RPS6KA2; SIRPA; QSOX1; CD14; S100A11; DUSP4; S100B; HLA-E; BST2; P2RX7; BCL2; IRF7; CHI3L1; TLR2	0.001030984	Diabetes
Interferon Gamma Signaling	DSOCS1; IRF7; IFI30; HLA-DQA1; HLA-DRB1; HLA-E; HLA-DQB1	0.001785204	Cardiomyopathy
Interferon Signaling	IFITM3; BST2; IFITM1; IFITM2; SOCS1; IRF7; IFI30; HLA-DQA1; HLA-DRB1; HLA-E; HLA-DQB1	0.001798407	Cardiomyopathy
Network Map Of SARS CoV 2 Signaling WP5115	IFITM3; BST2; IFITM1; CD163; CEBPB; CFH; HP; TNFSF10; CCL2; CD14; HLA-DRB1	0.00125113	T2D
Ebola Virus Infection In Host WP4217	BST2; CLTCL1; IRF7; GAS6; HLA-DQA1; HLA-DRB1; HLA-E; HLA-DQB1	0.00125113	Arrhythmia
Macrophage Markers WP4146	CD74; CD163; CD14	0.002831209	Cardiomyopathy
Tuberculosis	CD74; CEBPB; MRC1; BCL2; CD14; HLA-DQA1; HLA-DRB1; TNFRSF1A; HLA-DQB1; TLR2	0.00027045	T2D
Toxoplasmosis	SOCS1; BCL2; LY96; HLA-DQA1; HLA-DRB1; TNFRSF1A; HLA-DQB1; TLR2	0.000282742	Diabetes and cardiomyopathy

Lipid and atherosclerosis	TNFSF10; BCL2; IRF7; MIB2; LY96; CCL2; CD14; SOD2; TNFRSF1A; TLR2	0.0005613633	Arrhythmia
NF-kappa B signaling pathway	GADD45B; BCL2A1; BCL2; LY96; CD14; LTBR; TNFRSF1A	0.000845249	Diabetes

Total ten significant pathways with adjusted p-value ≤ 0.05 were identified due to their relevance to hypogonadism pathophysiology. "Innate Immune System", "Interferon Gamma Signaling" and "Interferon Signaling" most significant pathways were selected from Reactome databases. "Network Map of SARS CoV 2 Signaling WP5115", "Ebola Virus Infection In Host WP4217" and "Macrophage Markers WP4146" were selected from WikiPathways databases. And the other four in the table 4-6 were selected from KEEG pathways. These significant pathways shows e relevance with our comorbidities that make a strong evidence for our findings.

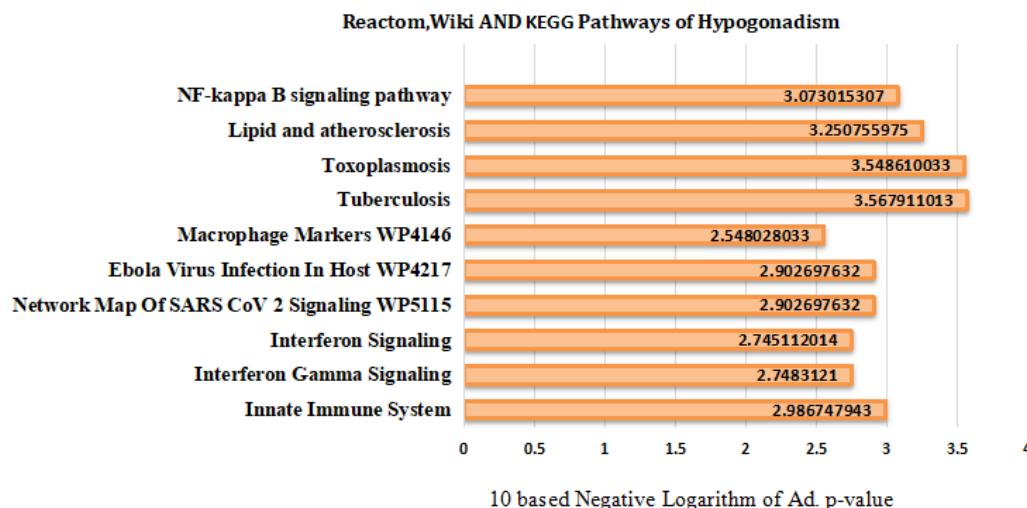


Figure 4.5: Graphical depiction of pathways of Hypogonadism

Table 4.7: Significant pathways of PCOS of shared DEGs

Significant Pathways	Gene Symbols	Adjusted P-Value	Most relevant disease for this pathway
Cross-presentation of Particulate Exogenous Antigens (Phagosomes)	NCF1	0.119204664	Diabetes
RHO GTPases Activate NADPH Oxidases	NCF1	0.12696123	Diabetes
Detoxification of Reactive Oxygen Species	NCF1	0.12696123	Diabetes
GPCR Ligand Binding	CRHBP;HRH2	0.14928425	Hypogonadism, hypothyroidism, diabetes, cardiomyopathy, and arrhythmia
Signaling by Receptor Tyrosine Kinases	NCF1;RRAD	0.151294003	Arrhythmia
AGE RAGE Pathway WP2324	NCF1	0.094169866	T2D and cardiovascular
Urotensin II Mediated Signaling WP5158	NCF1	0.094169866	Diabetes and cardiomyopathy
Osteoclast differentiation	NCF1;OSCAR	0.041730274	Most relevant to diabetes and potentially related to thalassemia
Gastric acid secretion	HRH2	0.16687814	Particularly Diabetes, Hypothyroidism, and potentially Thalassemia
Leishmaniasis	NCF1	0.16687814	Arrhythmia and cardiomyopathy and potential indirect links to diabetes and thalassemia

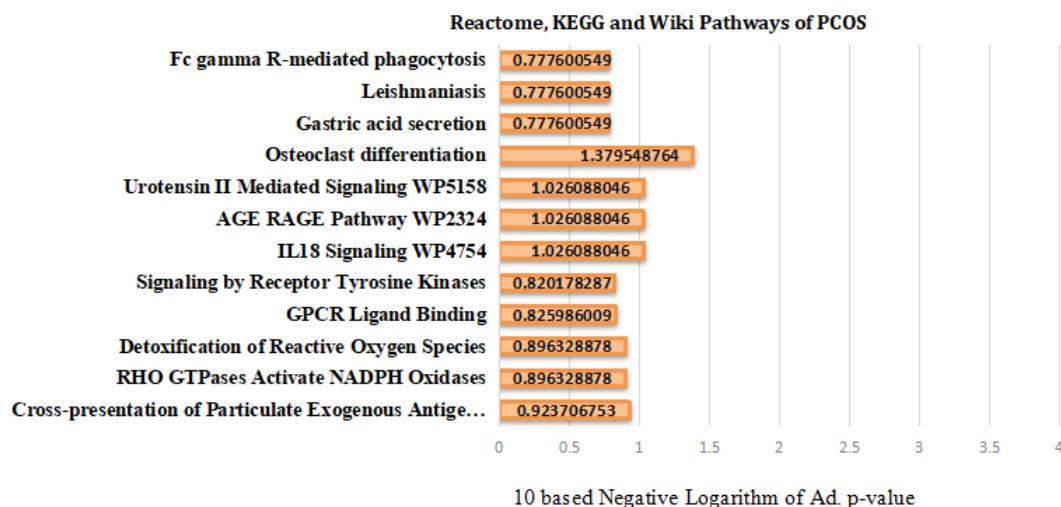


Figure 4.6: Graphical depiction of pathways of PCOS.

Pathway enrichment analysis for PCOS was performed by querying KEGG, Reactome, WikiPathways, and BioCarta databases. Below, we critically analyze these pathways to explain their biological significance and interconnections.

- The GPCR ligand binding pathway, which includes *CRHBP* (corticotropin-releasing hormone binding protein) and *HRH2* (histamine receptor H2), plays an important role in several conditions such as hypogonadism, hypothyroidism, type 2 diabetes, cardiomyopathy, and arrhythmia. GPCR signaling helps control hormonal activity and inflammatory responses, which are directly tied to the hormonal disturbances seen in PCOS. In beta-thalassemia, iron overload interferes with GPCR-mediated hormonal pathways, which may intensify hormonal disturbances such as the rise in androgen levels often observed in PCOS.
- Osteoclast Differentiation involving *NCF1* (neutrophil cytosolic factor 1) and OSCAR (osteoclast-associated receptor) is most relevant to T2D and potentially beta-thalassemia. It governs bone remodeling, which is disrupted in metabolic and hematologic disorders. Iron overload in beta-thalassemia may impair bone metabolism through oxidative stress, linking to PCOS's metabolic complications.
- “RHO GTPases Activate NADPH Oxidases,” “Detoxification of Reactive Oxygen Species,” “AGE-RAGE Pathway,” “IL18 Signaling,” “Urotensin II Mediated Signaling,” and “Leishmaniasis” are significant pathways primarily driven by *NCF1*. These are linked to T2D, cardiomyopathy, arrhythmia, and indirectly to beta-thalassemia. They involve oxidative stress (*NCF1* activates NADPH oxidases), inflammation (IL18 signaling), and metabolic dysfunction (AGE-

RAGE pathway). The role of *NCF1* in oxidative stress pathways aligns with beta-thalassemia's iron-induced reactive oxygen species (ROS) production.

Table 4.8: Significant pathways of T2D of shared DEGs

Significant Pathways	Gene Symbols	Adjusted P-Value	Most Relevant Disease for this Pathway
Fructose Metabolism	AKR1B1	0.086097405	Diabetes
Terminal Pathway of Complement	CLU	0.086097405	Diabetes
Ca ²⁺ Activated K ⁺ Channels	KCNMA1	0.086097405	Diabetes, arrhythmias and ACM
HDL Remodeling	ABCG1	0.086097405	Cardiomyopathy
FTO Obesity Variant Mechanism WP3407	ARID5B	0.067455203	Diabetes and PCOS
Macrophage Markers WP4146	CD14	0.067455203	Diabetes
Circadian Rhythm Genes WP3594	NFIL3; KCNMA1	0.067455203	Arrhythmia
Tuberculosis	MRC1; CD14	0.095231698	Diabetes
Lipid and Atherosclerosis	CD14; ABCG1	0.095231698	Diabetes and arrhythmias
Galactose Metabolism	AKR1B1	0.099417263	Hypogonadism, diabetes and cardiomyopathy
Toll-Like Receptor Pathway (Homo sapiens, h_tollPathway)	CD14	0.026965134	Diabetes
Inactivation of Gsk3 by AKT causes accumulation of β-catenin in Alveolar Macrophages (Homo sapiens, h_gsk3Pathway)	CD14	0.026965134	Diabetes

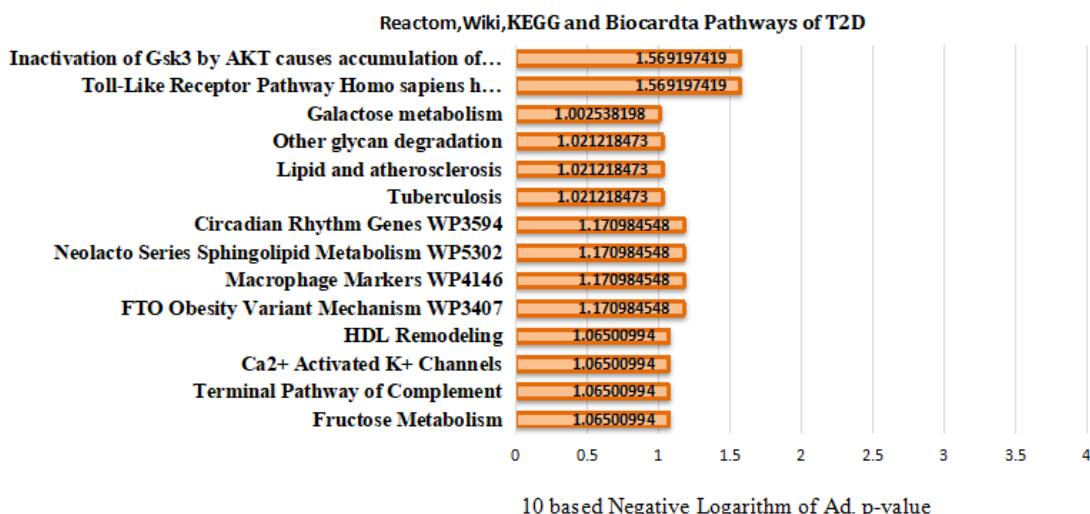


Figure 4.7: Graphical depiction of pathways of T2D.

- Fructose Metabolism and Galactose Metabolism pathways, both driven by *AKR1B1* (aldo-keto reductase family 1 member B1), are highly relevant to T2D. In addition, Galactose Metabolism is also relevant to hypogonadism and cardiomyopathy. These pathways regulate sugar metabolism, which is critical in the insulin resistance profile of T2D. In beta-thalassemia, iron overload may exacerbate oxidative stress and upregulate *AKR1B1* to handle sugar metabolites.
- Macrophage Markers and Toll-Like Receptor Pathway involve *CD14*. Iron accumulation in beta-thalassemia likely triggers *CD14*-mediated inflammation through TLR signaling.
- Tuberculosis and Lipid and Atherosclerosis pathways involve *MRC1* (mannose receptor C-type 1), *CD14*, and *ABCG1* (ATP-binding cassette subfamily G member 1). These pathways govern immune responses and lipid metabolism. Iron overload in beta-thalassemia may disrupt lipid homeostasis (*ABCG1*) and immune regulation (*MRC1*, *CD14*), thereby promoting atherosclerosis and inflammation in T2D.

Table 4.9: Significant pathways of Arrhythmogenic Cardiomyopathy (ACM) of shared DEGs

for readability

Significant Pathways	Gene Symbols	Adjusted P-Value	Most Relevant Disease for this Pathway
Metallothioneins Bind Metals	<i>MT2A</i>	0.12931125	Diabetes and cardiomyopathy
ERKs Are Inactivated	<i>DUSP4</i>	0.12931125	Cardiomyopathy
Response to Metal Ions	<i>MT2A</i>	0.12931125	Diabetes
ERK MAPK Targets	<i>DUSP4</i>	0.12931125	Diabetes
mTORC1-mediated Signalling	<i>RRAGD</i>	0.12931125	Diabetes
Zinc Homeostasis (WP3529)	<i>MT2A</i>	0.065424906	Diabetes and hypogonadism
Target Of Rapamycin Signaling (WP1471)	<i>RRAGD</i>	0.065424906	Diabetes and cardiomyopathy
Copper Homeostasis (WP3286)	<i>MT2A</i>	0.068235169	Cardiomyopathy and arrhythmia
Endoderm Differentiation (WP2853)	<i>DUSP4</i>	0.082642753	Diabetes
Mineral Absorption	<i>MT2A</i>	0.122298814	T2D
Hematopoietic Cell Lineage	<i>CD24</i>	0.122298814	Beta-thalassemia
Autophagy	<i>RRAGD</i>	0.122298814	ACM
Regulation of MAP Kinase Pathways Through Dual Specificity Phosphatases (Homo sapiens, h_dspPathway)	<i>DUSP4</i>	0.004940043	Diabetes and cardiomyopathy

- **Metallothioneins Bind Metals, Response to Metal Ions, Copper Homeostasis, and Mineral Absorption** are driven by *MT2A* (metallothionein 2A). *MT2A* regulates metal ion homeostasis, particularly zinc and copper, thereby protecting against oxidative stress. Iron overload in beta-thalassemia likely induces oxidative stress, upregulating *MT2A* to mitigate metal toxicity.
- **ERKs Are Inactivated, ERK MAPK Targets, and Regulation of MAP Kinase Pathways** involve *DUSP4*, which regulates cell proliferation and stress responses. Iron-induced oxidative stress in beta-thalassemia may suppress *DUSP4*, leading to dysregulated MAPK signaling.
- **Target of Rapamycin Signaling and Autophagy** are driven by *RRAGD*. These pathways regulate mTORC1 signaling and autophagy, which are critical for

cellular homeostasis and stress responses. In beta-thalassemia, iron overload may impair autophagy via *RRAGD* dysregulation, leading to protein aggregation and cardiac damage in ACM.

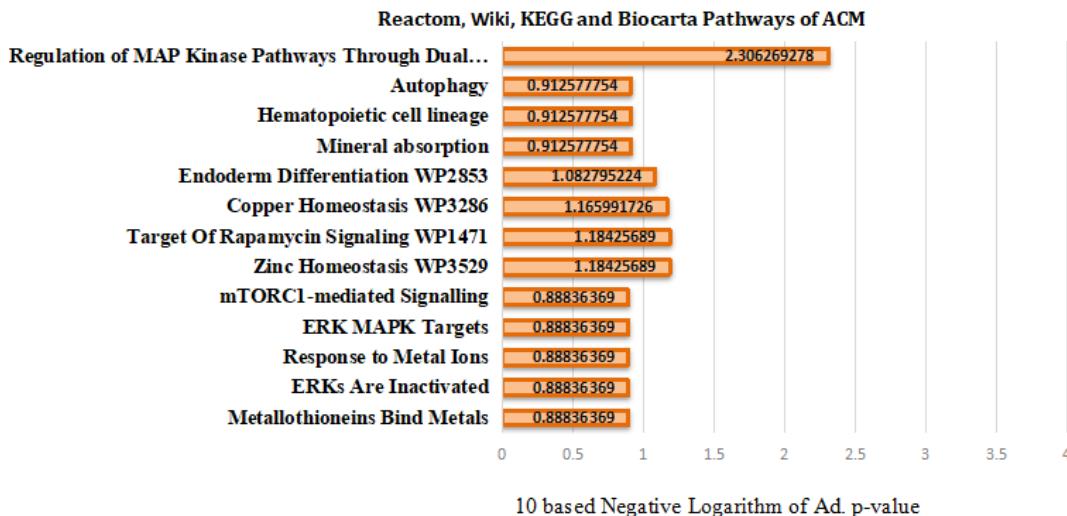


Figure 4.8: Graphical depiction of pathways of ACM.

Table 4.10: Significant pathways of Arrhythmia of shared DEGs

Significant Pathways	Gene Symbols	Adjusted P-Value	Most Relevant Disease for this Pathway
Metallothioneins Bind Metals	<i>MT2A; MT1G; MT1X; MT1E</i>	1.362354723	T2D and ACM
Response to Metal Ions	<i>MT2A; MT1G; MT1X; MT1E</i>	0.0000020563	Diabetes
Neurotransmitter Release Cycle	<i>MAOA; SLC1A3; LIN7A</i>	0.013131521	Diabetes
Synthesis of Prostaglandins (PG) and Thromboxanes (TX)	<i>PTGDS; PTGES</i>	0.02499481	Arrhythmia
Zinc Homeostasis (WP3529)	<i>MT2A; MT1G; MT1X; MT1E</i>	0.0001265177	Diabetes and hypogonadism
Prostaglandin Synthesis and Regulation (WP98)	<i>PTGER4; AKR1B1; PTGDS; PTGES</i>	0.0001577216	Diabetes

Copper Homeostasis (WP3286)	<i>MT2A; MT1G; MT1X; MT1E</i>	0.000174674	Arrhythmia and cardiomyopathy
Mineral Absorption	<i>MT2A; MT1G; MT1X; MT1E</i>	0.000644711	Diabetes
JAK-STAT Signaling Pathway	<i>SOCS1; IL3RA; STAT4</i>	0.113203704	ACM
Pathways in Cancer	<i>PTGER4; IL3RA; STAT4; FN1; CXCR4</i>	0.113203704	Hypogonadism, diabetes, arrhythmia, cardiomyopathy
Eicosanoid Metabolism (Homo sapiens, h_eicosanoidPathway)	<i>PTGER4; PTGES</i>	0.018593144	Diabetes
Pertussis Toxin-Insensitive CCR5 Signaling in Macrophage (Homo sapiens, h_Ccr5Pathway)	<i>CXCR4</i>	0.083487378	Diabetes
CXCR4 Signaling Pathway (Homo sapiens, h_cxcr4Pathway)	<i>CXCR4</i>	0.083487378	Diabetes

- **Synthesis of Prostaglandins (PG) and Thromboxanes (TX) and Prostaglandin Synthesis and Regulation** involve *PTGDS*, *PTGES*, *PTGER4*, and *AKR1B1*, and are strongly linked to arrhythmia and diabetes. These pathways regulate prostaglandin synthesis, which influences inflammation and vascular function critical to cardiac rhythm. Iron overload in beta-thalassemia may enhance prostaglandin-mediated inflammation, contributing to the cardiac stress seen in arrhythmia.
- **Copper Homeostasis** is driven by metallothionein genes, including *MT2A*, *MT1G*, *MT1X*, and *MT1E*. This pathway regulates copper ion balance and protects against oxidative stress. Iron accumulation in beta-thalassemia likely induces oxidative stress, upregulating metallothioneins to mitigate metal toxicity, which is critical for protecting cardiac tissue in arrhythmia.

- **Pathways in Cancer** involve *PTGER4*, *IL3RA*, *STAT4*, *FN1*, and *CXCR4*. These govern cell proliferation and signaling pathways often dysregulated in chronic diseases. In beta-thalassemia, iron-induced oxidative stress may dysregulate *FN1* and *CXCR4*, contributing to cardiac remodeling in arrhythmia and overlapping with metabolic (diabetes) and endocrine (hypogonadism) dysfunction.
- **JAK-STAT Signaling Pathway** is linked to arrhythmia and ACM. This pathway involves *SOCS1*, *IL3RA*, and *STAT4*, which regulate cytokine signaling and immune responses. Chronic inflammation from beta-thalassemia's iron overload may activate JAK-STAT signaling, contributing to cardiac inflammation in arrhythmia.

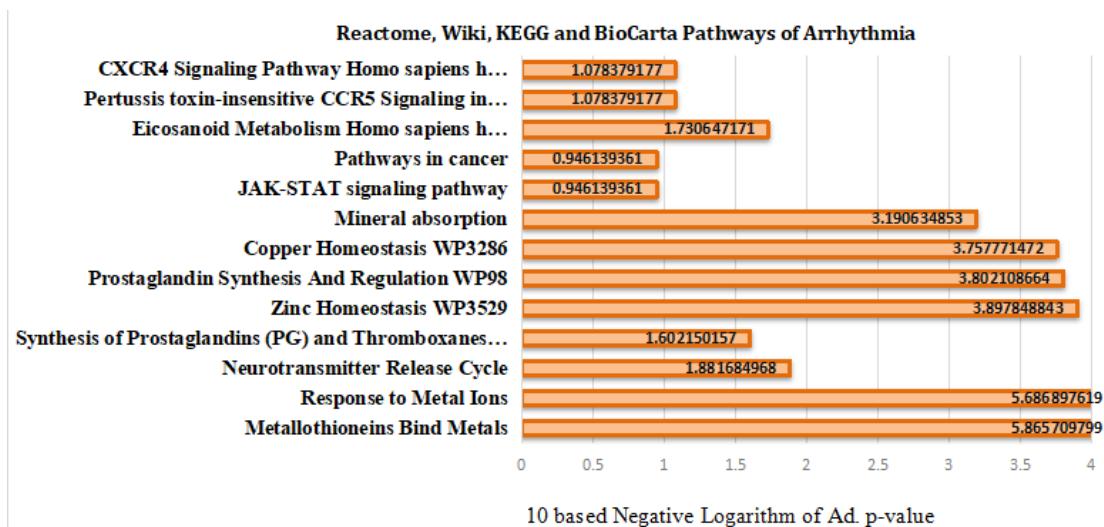


Figure 4.9: Graphical depiction of pathways of Arrhythmia.

4.5 Gene Ontology (GO) Analysis

Gene Ontology (GO) analysis provides a structured framework to characterize the molecular functions, biological processes, and cellular components of genes which offering insights into their roles in disease mechanisms. We performed GO analysis with Enrichr by querying the GO Biological Process and Human Phenotype Ontology (HPO) databases. This analysis describes detailed in Table 4-11 to Table 4-16 to explain the molecular and phenotypic connections linking these diseases.

Table 4.11: Gene Ontological significant pathways of Hypothyroidism of shared DEGs

Term	Name	Adjusted P-Value	Genes	Most Relevant Disease for this Pathway
GO:0002664	Regulation of T Cell Tolerance Induction	0.060741016	<i>HLA-B</i>	Diabetes
GO:0007219	Notch Signaling Pathway	0.085112911	<i>DTX4</i>	Cardiomyopathy
HP:0001824	Weight loss	0.044703647	<i>HLA-B; TYMP</i>	T2D
HP:0002027	Abdominal pain	0.044703647	<i>HLA-B; TYMP</i>	T2D and cardiomyopathy
HP:0004395	Malnutrition	0.044703647	<i>TYMP</i>	Diabetes and hypothyroidism
HP:0100653	Optic neuritis	0.044703647	<i>HLA-B</i>	Diabetes and arrhythmias

1. GO Terms:

1. The term **GO:0002664** is driven by *HLA-B*, which regulates immune tolerance and is particularly relevant to diabetes. In beta-thalassemia (BT), iron overload may disrupt *HLA-B*-mediated immune regulation, contributing to autoimmune thyroid dysfunction, a hallmark of hypothyroidism.
2. The Notch Signaling Pathway (**GO:0007219**) involves *DTX4*. This pathway governs cell differentiation and is linked to cardiomyopathy. Its relevance to hypothyroidism suggests that iron-induced stress in BT may affect thyroid cell differentiation, potentially exacerbating hormonal imbalances.

2. HPO Terms:

1. The phenotype **Weight Loss (HP:0001824)** is associated with T2D. This reflects metabolic dysregulation, which may also occur in hypothyroidism due to thyroid hormone imbalances. Iron overload in BT could exacerbate metabolic stress, contributing to weight loss.
2. **Abdominal Pain (HP:0002027)** is linked to T2D and cardiomyopathy. This phenotype may indicate systemic inflammation or gastrointestinal complications in hypothyroidism, potentially driven by BT's iron toxicity.
3. **Malnutrition (HP:0004395)** is relevant to diabetes and hypothyroidism. This term suggests nutrient absorption issues and may be linked to systemic effects of iron overload on metabolism in BT patients.

4. **Optic Neuritis (HP:0100653)**, involving the gene *HLA-B* (adjusted *p*-value = 0.045), is associated with diabetes and arrhythmia. This inflammatory phenotype may reflect immune dysregulation in hypothyroidism, possibly amplified by chronic inflammation in BT.

Table 4.12: Gene Ontological significant pathways of Hypogonadism of shared DEGs.

Term	Name	Adjusted p-value	Genes	Most relevant disease for this pathway
GO:0050727	Regulation of Inflammatory Response	0.004340401	<i>PTGER4; LGALS1; IFI16; NINJ1; SIRPA; METRNL; PLA2G7; MGLL; TNFRSF1A; TLR2</i>	Diabetes
GO:0032753	Positive Regulation of Interleukin-4 Production	0.013597802	<i>CEBPB; RARA; HLA-E</i>	Diabetes
GO:0032675	Regulation of Interleukin-6 Production	0.013795602	<i>C1QTNF4; CD74; GPNMB; SIRPA; GAS6; TLR2</i>	Diabetes
GO:0097193	Intrinsic Apoptotic Signaling Pathway	0.014095553	<i>CEPB; BCL2A1; IFI16; BCL2; TRIB3; CD24</i>	Diabetes
HP:0000099	Glomerulonephritis	0.042357015	<i>C1QB; C1QA; CFH</i>	Diabetes
HP:0100539	Periorbital edema	0.099130412	<i>ADAMTS2; RIN2; TNFRSF1A</i>	Hypothyroidism
HP:0002725	Systemic lupus erythematosus	0.099691906	<i>C1QB; C1QA</i>	T2D
HP:0001287	Meningitis	0.156489906	<i>IGHM; BCL2; HLA-DRB1</i>	T2D

Using Enrichr, we queried the GO and HPO databases to identify four significant GO terms and four HPO terms.

1. GO Terms:

1. **Regulation of Inflammatory Response:** Involving genes such as *PTGER4*, *LGALS1*, *SIRPA*, and *TLR2*. This pathway underscores inflammation modulation in hypogonadism. Iron overload in beta-thalassemia (BT) likely triggers

TLR2-mediated inflammatory responses, contributing to gonadal dysfunction through chronic inflammation.

2. **Positive Regulation of Interleukin-4 Production:** Driven by *CEBPP*, *RARA*, and *HLA-E*. This pathway regulates IL-4, an anti-inflammatory cytokine. Dysregulation suggests an altered immune balance in hypogonadism, potentially exacerbated by BT's oxidative stress affecting hormonal regulation.
3. **Regulation of Interleukin-6 Production:** Involving *CD74*, *GPNMB*, and *TLR2*. This pathway indicates IL-6-driven inflammation. Iron-induced oxidative stress in BT may amplify IL-6, disrupting gonadal function in hypogonadism.

2. HPO Terms:

1. **Glomerulonephritis:** Linked to diabetes but relevant to hypogonadism, involving *C1QB*, *C1QA*, and *CFH*. This phenotype suggests inflammatory damage, potentially driven by BT's systemic inflammation affecting gonadal tissues.
2. **Systemic Lupus Erythematosus:** Involving *C1QB* and *C1QA*, this term indicates autoimmune-like mechanisms that may contribute to hypogonadism's immune dysregulation in the context of BT.
3. **Meningitis:** Linked to *IGHM*, *BCL2*, and *HLA-DRB1*. This phenotype suggests broader inflammatory effects.

Table 4.13: Gene Ontological significant pathways of PCOS of shared DEGs.

Term	Name	Adjusted p-value	Genes	Most relevant disease for this pathway
GO:0030316	Osteoclast Differentiation	0.046477967	OSCAR	Diabetes
HP:0005406	Recurrent bacterial skin infections	0.04671673	<i>NCF1</i>	Diabetes
HP:0100721	Mediastinal lymphadenopathy	0.04671673	<i>NCF1</i>	Diabetes and ACM
HP:0006510	Chronic obstructive pulmonary disease	0.04671673	<i>NCF1</i>	Diabetes
HP:0000230	Gingivitis	0.04671673	<i>NCF1</i>	Diabetes

In GO pathway analysis of PCOS, it involves 1 GO terms and 4 HP terms. We can determine from table 4-13 that all HP term is involving the *NCF1* gene. The GO term Osteoclast Differentiation highlights bone metabolism dysregulation that linking

PCOS to BT's iron overload through OSCAR-mediated oxidative stress with overlaps in diabetes's metabolic profile. All HPO terms is driven by NCF1 that emphasize inflammatory and immune dysregulation which suggesting that iron-induced oxidative stress in BT amplifies NCF1-mediated inflammation and contributing to PCOS's clinical phenotypes like infections and systemic inflammation.

Table 4.14: Gene Ontological significant pathways of T2D of shared DEGs.

Term	Name	Adjusted p-value	Genes	Most relevant disease for this pathway
GO:0043691	Reverse Cholesterol Transport	0.00445197	<i>CLU; ABCG1</i>	Diabetes
GO:1902003	Regulation of Amyloid-Beta Formation	0.007020347	<i>CLU; ABCG1</i>	T2D
GO:0140467	Integrated Stress Response Signaling	0.007020347	<i>MAFB; CEBPD</i>	T2D
GO:0030301	Cholesterol Transport	0.0144153	<i>CLU; ABCG1</i>	Diabetes
HP:0009120	Aplasia/Hypoplasia involving the sinuses	0.0455770	<i>FUCA1</i>	Diabetes
HP:0000970	Anhidrosis	0.045577	<i>FUCA1</i>	Diabetes
HP:0003541	Urinary glycosaminoglycan excretion	0.04557702	<i>FUCA1</i>	Diabetes
HP:0000815	Hypergonadotropic hypogonadism	0.0532044	<i>RIN2</i>	Hypogonadism and PCOS

Four significant GO terms and four HPO terms with adjusted p-value ≤ 0.05 were identified for T2D. The GO terms, including "Reverse Cholesterol Transport" and "Regulation of Amyloid-Beta Formation," are driven by *CLU* and *ABCG1* genes, highlighting lipid metabolism and stress response. These terms link T2D to beta-thalassemia's (BT) iron-induced metabolic dysregulation, and "Integrated Stress Response Signaling" driven by *MAFB* and *CEBPD* genes suggests cellular stress.

HPO terms like "Aplasia/Hypoplasia Involving the Sinuses," "Anhidrosis," and "Urinary Glycosaminoglycan Excretion" involving *FUCA1* indicate metabolic and inflammatory phenotypes. "Hypergonadotropic Hypogonadism" connects to hypogonadism and PCOS, reflecting shared endocrine disruptions. These findings underscore iron overload's role in T2D's metabolic and inflammatory pathology, suggesting potential therapeutic targets such as lipid-modulating agents.

Table 4.15: Gene Ontological significant pathways of ACM of shared DEGs.

Term	Name	Adjusted p-value	Genes	Most relevant disease for this pathway
GO:0072311	Glomerular Epithelial Cell Differentiation	0.037250334	<i>CD24</i>	Diabetes
GO:0072112	Podocyte Differentiation	0.037250334	<i>CD24</i>	T2D
GO:0030856	Regulation of Epithelial Cell Differentiation	0.039402171	<i>CD24</i>	Cardiomyopathy and arrhythmia
GO:0043627	Response to Estrogen	0.048014194	<i>CD24</i>	Diabetes
HP:0000243	Trigonocephaly	0.064644186	<i>CD96</i>	Hypothyroidism
HP:0000057	Clitoromegaly	0.064644186	<i>CD96</i>	PCOS
HP:0010318	Aplasia/Hypoplasia of the abdominal wall musculature	0.074611621	<i>CD96</i>	Diabetes

Four significant GO terms and three HPO terms were identified for ACM. The GO terms, including “Glomerular Epithelial Cell Differentiation,” “Podocyte Differentiation,” and “Regulation of Epithelial Cell Differentiation,” are driven by *CD24*, highlighting epithelial cell regulation relevant to cardiomyopathy and arrhythmia. These terms suggest iron overload in beta-thalassemia (BT) disrupts *CD24*-mediated cellular differentiation, contributing to cardiac tissue remodeling. HPO terms like “Trigonocephaly,” “Clitoromegaly,” and “Aplasia/Hypoplasia of the abdominal wall musculature” are driven by *CD96*, reflecting developmental and endocrine phenotypes connecting to hypothyroidism, PCOS, and diabetes, and may indicate systemic effects of iron toxicity. These findings suggest therapeutic targets such as anti-inflammatory or hormonal modulators.

Table 4.16: Gene Ontological significant pathways of Arrhythmia of shared DEGs.

Term	Name	Adjusted p-value	Genes	Most relevant disease for this pathway
GO:0042093	T-helper Cell Differentiation	0.024608573	<i>PTGER4; STAT4</i>	Diabetes
GO:0006692	Prostanoid Metabolic Process	0.024608573	<i>PTGDS; PTGES</i>	T2D
GO:0007399	Nervous System Development	0.067160207	<i>VCAN; AXL; FN1; CXCR4; PCDH1</i>	PCOS
HP:0100820	Glomerulopathy	0.137761059	<i>STAT4; FN1</i>	Diabetes
HP:0003323	Progressive muscle weakness	0.137761059	<i>TNNT1</i>	Hypothyroidism
HP:0100614	Myositis	0.137761059	<i>STAT4</i>	Arrhythmia

Three significant GO terms and three HPO terms were identified for arrhythmia. The GO terms, including “T-helper Cell Differentiation” and “Prostanoid Metabolic Process,” are driven by *PTGER4*, *STAT4*, *PTGDS*, and *PTGES*, highlighting immune and inflammatory processes. Iron overload in BT drives *STAT4*-mediated immune dysregulation and *PTGDS/PTGES*-related prostaglandin synthesis. “Nervous System Development,” involving *VCAN*, *AXL*, *FN1*, *CXCR4*, and *PCDH1*, indicates neural influences relevant to PCOS and potentially links to autonomic nervous system effects on cardiac rhythm. HPO terms like “Glomerulopathy” and “Myositis” reflect inflammatory phenotypes directly relevant to arrhythmia, while “Progressive Muscle Weakness” connects to hypothyroidism, suggesting muscle-related cardiac impacts.

The GO and HPO analyses, conducted using Enrichr between BT and its comorbidities, reveal shared molecular and phenotypic mechanisms driven by iron overload. GO terms underscore immune and metabolic pathways, while HPO terms reflect clinical phenotypes linked to endocrine, inflammatory, and cardiac complications. These findings indicate that iron-induced oxidative stress and inflammation are central to comorbidity development in beta-thalassemia. The overlap across diseases suggests shared therapeutic targets, such as anti-inflammatory or iron-chelating agents, to mitigate these conditions. This integrative analysis provides a molecular framework for understanding beta-thalassemia’s systemic impact, guiding future research and clinical strategies.

4.6 Protein-Protein Interactions Analysis

Protein-Protein Interaction (PPI) analysis is a cornerstone of systems biology, enabling the prediction of protein functions and potential drug targets through molecular interactions. Using the STRING database with a high confidence score (900), we analyzed PPIs of the shared differentially expressed genes (DEGs) between BT and its comorbidities. We constructed PPI networks using the Network Analyst platform as visualized in Figure 4-10. The identified hub proteins play critical roles in immune regulation, inflammation, and cellular stress that linking BT iron overload to endocrine and cardiac comorbidities. The high connectivity of CD74 and BCL2 suggests their central role in inflammatory and apoptotic pathways. These findings highlight potential therapeutic targets, such as anti-inflammatory or immune-modulating agents, for managing comorbidities in beta-thalassemia. Network Stats of our findings in Table 4-17. We identified mainly 86 seeds from where top 15 hub proteins are given in Table 4-18.

TABLE 4.17. Network Stats information of PPI analysis

Description	Value
Number of nodes:	219
Number of edges:	462
Average node degree:	4.22
Avg. local clustering coefficient:	0.344

Degree: The degree of a gene or node in a PPI network is the number of direct interactions through edges with other genes/proteins. It measures how connected a gene is within the network. A high degree indicates that a gene is a hub that interacting with many other proteins and suggesting it plays a central role in biological processes.

Betweenness: It centrality measures how often a gene or node lies on the shortest paths between other pairs of nodes in the network. It quantifies a gene's role as a "bridge" or mediator in facilitating interactions between other proteins. A high betweenness centrality indicates that a gene is crucial for connecting different parts of the network, controlling the flow of information or signals, and is often pivotal in coordinating biological functions.

TABLE 4.18. Identify Hub protein information from PPIs analysis

Serial No.	Hub Protein	Degree	Betweeness
1	CD74	132	139810.9
2	BCL2	61	122437.4
3	FN1	56	126043.7
4	TNFRSF1A	41	41685.53
5	CXCR4	41	76314.66
6	TLR2	34	46163.96
7	CEBPB	29	53645.13
8	HLA-DRB1	25	21809.87
9	IRF7	25	20416.19
10	SOCS1	24	27432.88
11	HLA-B	23	13507.96
12	RARA	22	21166.3
13	CLTCL1	22	18312
14	HLA-E	20	20407.77
15	CYFIP2	20	15689.23

color	cluster Id	gene count	description
●	Cluster 1	11	+ Acute phase
●	Cluster 2	10	+ Lipopolysaccharide binding
●	Cluster 3	9	Basic region leucin zipper
●	Cluster 4	8	+ Negative regulation of viral life cycle
●	Cluster 5	7	Plasma lipoprotein particle
●	Cluster 6	6	MHC class II protein complex binding
●	Cluster 7	5	+ Negative regulation of natural killer cell mediated immunity
●	Cluster 8	5	BCL (B-Cell lymphoma)
●	Cluster 9	5	+ TNFR1-induced proapoptotic signaling
●	Cluster 10	4	CCL2, CHI3L1, EFNA1, SOCS1
●	Cluster 11	4	+ Detoxification of copper ion
●	Cluster 12	4	+ Metabolism of serotonin

Figure 4.10: Clusters information's of PPI networks of hub proteins of beta-thalassemia and comorbidities.

The PPI network is shown below in Figure 4.11:

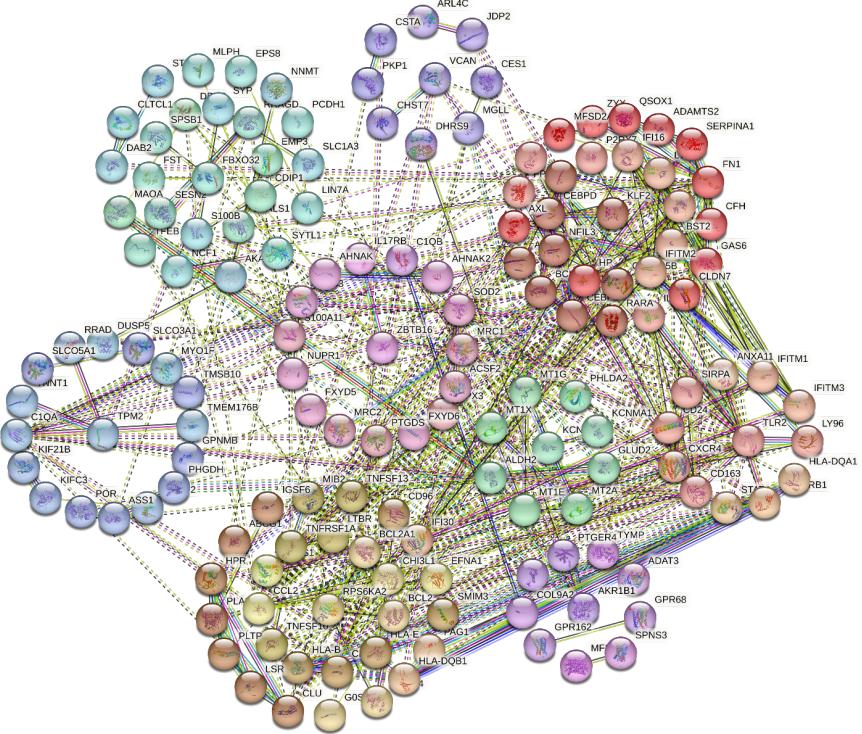


Figure 4.11: PPI networks of hub proteins of beta-thalassemia and comorbidities.

To further elucidate the structural organization of the PPI network, we performed a cluster analysis to identify densely connected regions of hub proteins, providing deeper insights into their functional roles. The results are visualized in the following figure.

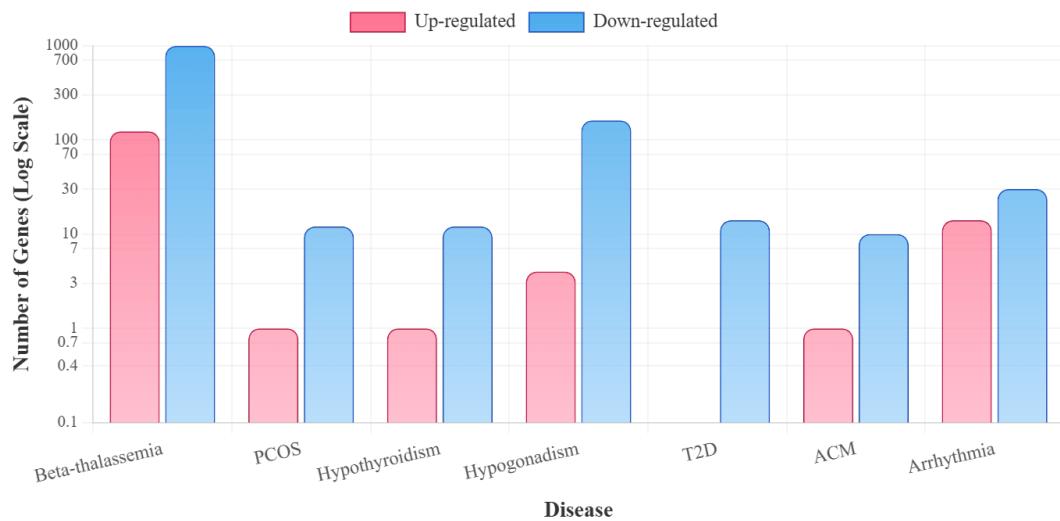


Figure 4.12: Detailed cluster analysis of PPI networks for hub proteins of beta-thalassemia and comorbidities.

The cluster analysis highlights distinct modules within the PPI network, with CD74 and BCL2 forming central clusters linked to inflammation and apoptosis. These

modules underscore the critical roles of these hub proteins in mediating iron overload effects across comorbidities, supporting their potential as therapeutic targets.

- **CD74** is a central hub in the PPI network. *CD74* manages MHC class II antigen processing, guiding peptide-free complexes to endosomal/lysosomal systems. In beta-thalassemia, it drives immune dysregulation worsened by iron overload, fueling inflammation in hypogonadism, T2D, and arrhythmia. Its link to MIF amplifies systemic inflammation across these comorbidities.
- **BCL2** is a key hub in the PPI network that blocks apoptosis by regulating mitochondrial permeability and caspase activity. In beta-thalassemia, it counters iron-driven cell death in cardiac and endocrine (hypogonadism) tissues.
- **FN1** is involved in cell adhesion, motility, and extracellular matrix (ECM) remodeling. In BT, iron overload disrupts ECM integrity, impacting cardiac and endocrine tissues. *FN1*'s role in osteoblast compaction and collagen deposition connects to bone metabolism issues observed in PCOS.
- **CXCR4** is a hub protein that mediates cell migration and inflammation via CXCL12/SDF-1 signaling. Its role in nervous system development (Table 4-16) and inflammation links to arrhythmia and PCOS, where iron-induced oxidative stress amplifies inflammatory responses.
- **TLR2** (Toll-like receptor 2) mediates innate immune responses to bacterial lipoproteins, driving NF-kappa-B activation and inflammation. In BT, *TLR2* contributes to chronic inflammation, exacerbating comorbidities like hypogonadism, T2D, and arrhythmia.
- **SOCS1** is a hub protein that regulates cytokine signaling via JAK/STAT pathways, inhibiting IL-6 and interferon-gamma signaling. Its role in immune modulation is critical for BT's inflammatory environment, impacting T2D and ACM.
- **HLA-B** (HLA class I histocompatibility antigen) regulates immune tolerance. Its high connectivity in the PPI network suggests it contributes to autoimmune aspects of hypothyroidism and T2D in BT.
- **NCF1** drives oxidative stress via NADPH oxidase, exacerbating inflammation in beta-thalassemia and contributing to PCOS's inflammatory phenotypes.

4.7 Protein-Drug Interactions Analysis

Protein-Drug Interaction (PDI) analysis was conducted to identify potential therapeutic drug targets for beta-thalassemia and its comorbidities by leveraging the Network

Analyst platform and the DrugBank database. Two PDI networks were constructed based on shared differentially expressed genes (DEGs) identified by uploading 231 genes in Network Analyst.

The first PDI network comprises 20 hub genes, represented as circles, including *C1QA* and *C1QB*, with squares indicating their interacting drug molecules. These hubs, part of the complement system, suggest potential anti-inflammatory drug targets to mitigate iron-induced immune dysregulation in comorbidities like T2D and arrhythmia.

The second PDI network includes 15 hub genes, highlighting *PLA2G7* and *CES1*, alongside their interacting drug molecules. *PLA2G7* (platelet-activating factor acetyl-hydrolase) and *CES1* (liver carboxylesterase 1) are linked to lipid metabolism and xenobiotic detoxification, respectively, pointing to therapeutic strategies for endocrine and cardiac complications.

These PDI networks are visualized in Figure 4-12 to reveal key molecular targets for drug development, emphasizing anti-inflammatory and metabolic modulators to address iron overload's systemic effects in beta-thalassemia comorbidities.

TABLE 4.19. Information of drug interactions of PDI Subnetwork1

Serial No.	Drug Name	Degree	Betweeness
1	<i>C1QB</i>	18	76.5
2	<i>C1QA</i>	18	76.5
3	Cetuximab	2	0.05555556
4	Etanercept	2	0.05555556
5	Adalimumab	2	0.05555556
6	Abciximab	2	0.05555556
7	Gemtuzumab ozogamicin	2	0.05555556
8	Trastuzumab	2	0.05555556
9	Rituximab	2	0.05555556
10	Basiliximab	2	0.05555556

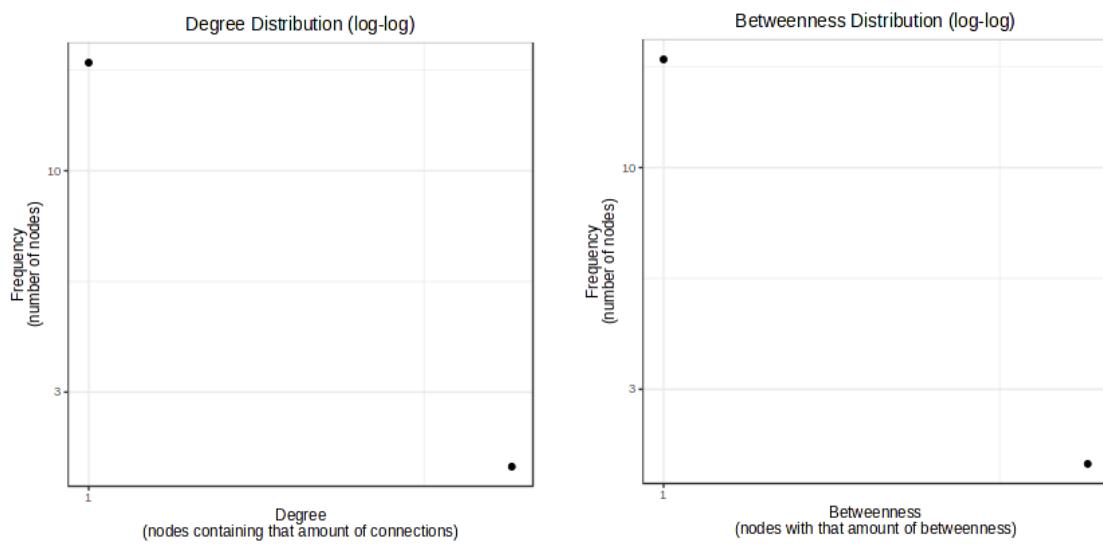


Figure 4.13: Network Topology of Subnetwork1.

Table 4.20: Information of drug interactions of PDI Subnetwork2.

Serial No.	Drug Name	Degree	Betweenness
1	CES1	12	88
2	PLA2G7	2	13
3	(1R)-1,2,2-TRIMETHYLPROPYL (R)-METHYLPHOSPHINATE	2	24
4	Oseltamivir	1	0
5	L-Carnitine	1	0
6	Probucol	1	0
7	Hydroxy-Phenyl-Acetic Acid 8-Methyl-8-Aza-Bicyclo[3.2.1]Oct-3-Yl Ester	1	0
8	Cholic Acid	1	0
9	4-Piperidino-Piperidine	1	0
10	N-acetyl-alpha-neuraminic acid	1	0

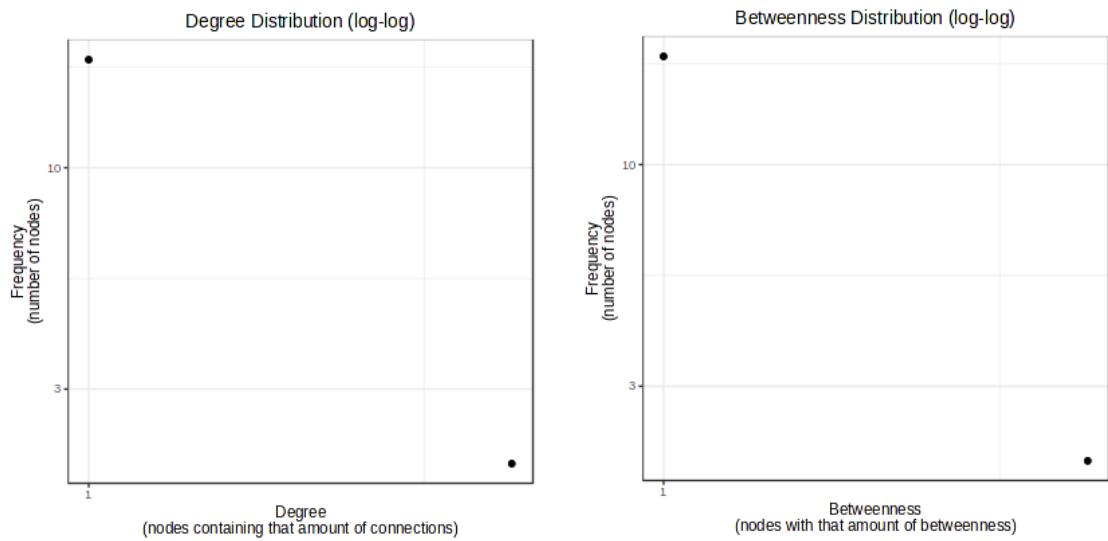


Figure 4.14: Network Topology of Subnetwork2.

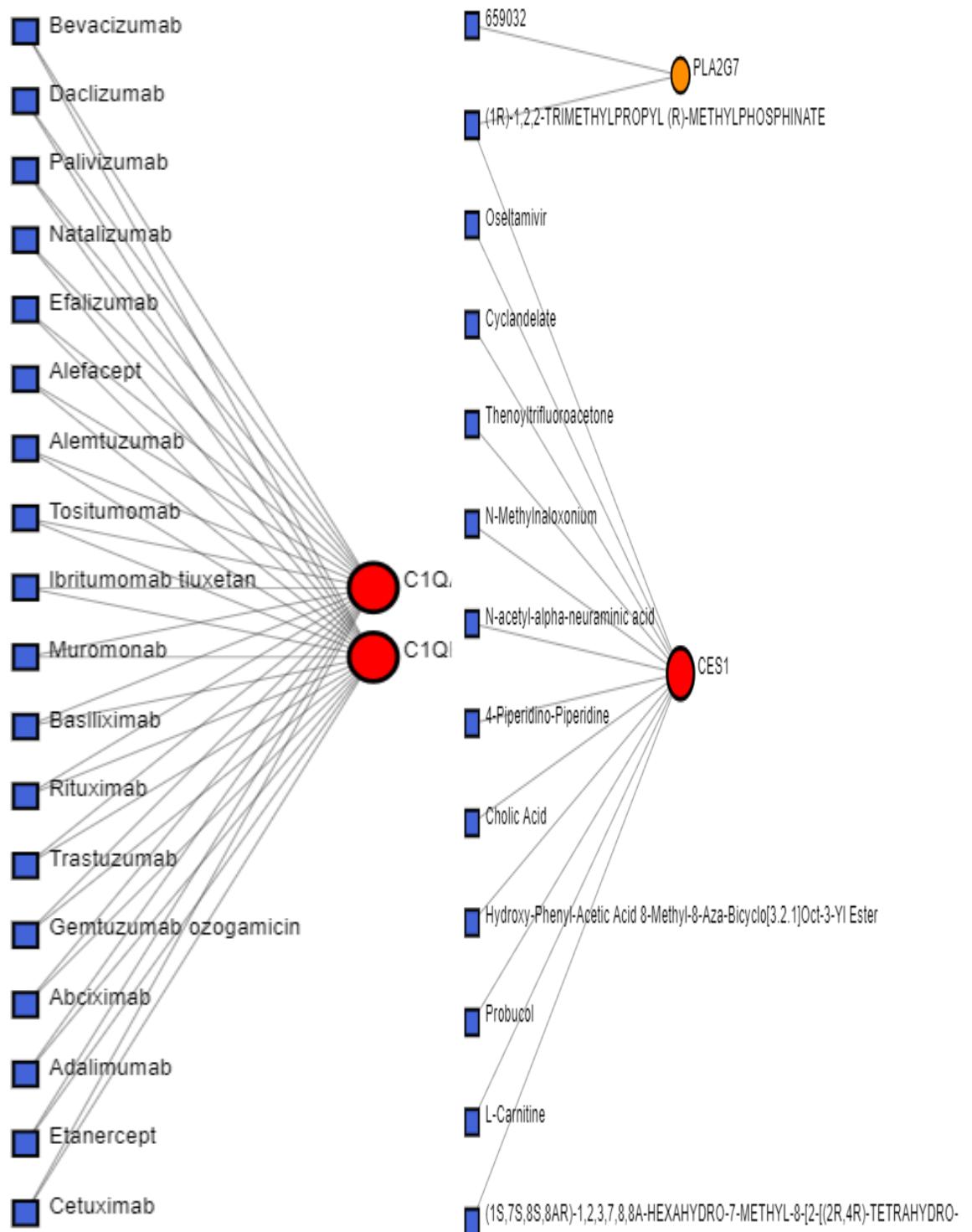


Figure 4.15: PDI networks of drug proteins of beta-thalassemia and comorbidities.

4.8 Results of Phylogenetic Analysis

Phylogenetic analysis enhances the understanding of the evolutionary relationships among genes, proteins and diseases. We constructed a phylogenetic tree for beta-thalassemia and its comorbidities to illustrate the associative relationships among them by using Molecular Evolutionary Genetics Analysis (MEGA) tools and FASTA sequences of nucleotide datasets from NCBI. The phylogenetic tree shows the relationship between beta-thalassemia and the comorbidities including PCOS, T2D, hypogonadism, hypothyroidism, ACM and arrhythmia in Figure 4.16. The tree shows a strong evolutionary relationship between beta-thalassemia and hypogonadism as they belong to a common species.

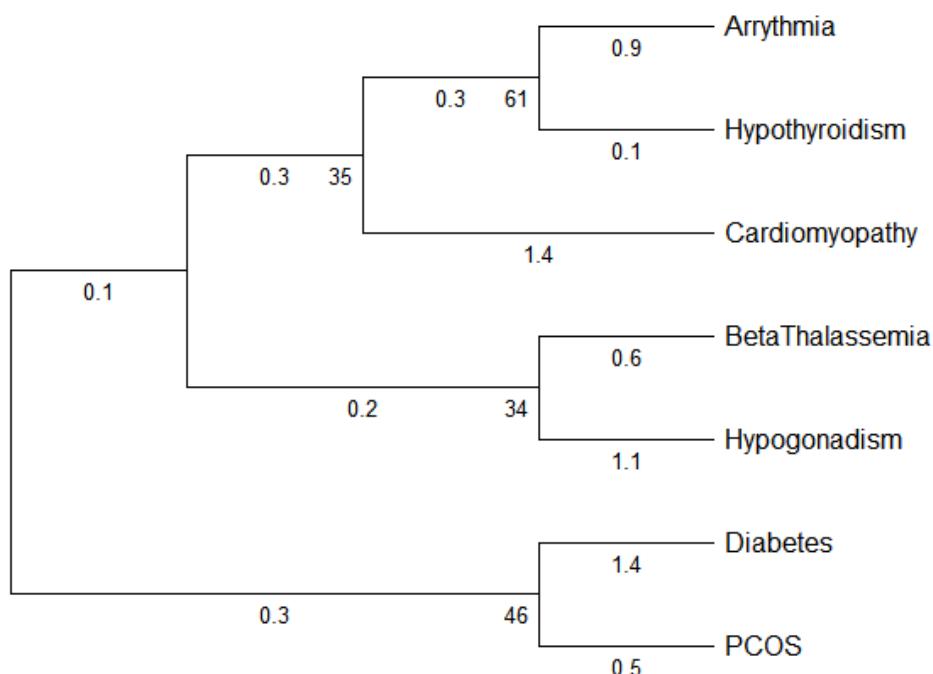


Figure 4.16: Phylogenetic tree to illustrate associative evolutionary relationship between beta-thalassemia and its comorbidities.

To validate our findings, we used two benchmark databases including dbGap and OMIM from Enrichr tools on the up-regulated and down-regulated genes of beta-thalassemia . The analysis identified several drugs and diseases from which we identified twelve diseases whose genes are similar to the genes of beta-thalassemia. Among those identified diseases, our six selected comorbidities were present that support the validity of our study. The other six diseases may be the potential comorbidities of beta-thalassemia, T2D, hypothyroidism, hypogonadism, PCOS, arrhythmogenic cardiomyopathy and arrhythmia. To illustrate these associations, we constructed a

drug-disease validation network using Cytoscape, as shown in Figure 4.17 .

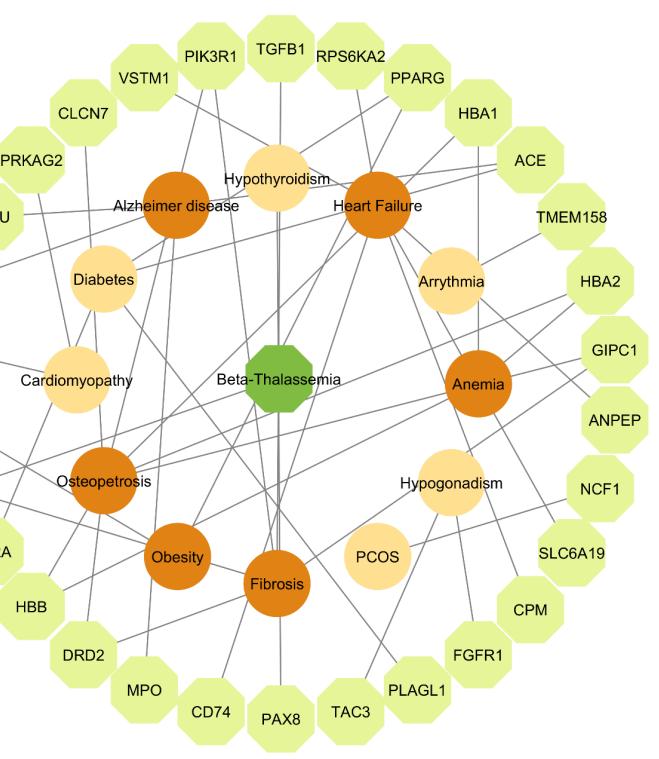


Figure 4.17: Graphical representation of validation network of beta-thalassemia. Octagon shapes indicate the shared genes, circle shapes with brown color indicate our selected diseases and circle shapes with yellow-orange color represent our selected comorbidities.

4.9 Discussion

This chapter presents the comprehensive outcomes of the proposed investigation into beta-thalassemia and its comorbidities. We explored dysregulated genes, disease gene networks, signaling pathways, Gene Ontology (GO) and Human Phenotype Ontology (HPO) terms, Protein-Protein Interaction (PPI) networks, and Protein-Drug Interaction (PDI) networks in detail. Key findings reveal that iron overload drives inflammation, apoptosis, and metabolic dysregulation, linking beta-thalassemia to its comorbidities through hub genes and therapeutic targets such as in the PDI analysis.

The simulation environment effectively mapped the regulatory networks, identifying critical pathways which underpin the pathophysiological overlap across comorbidities. The phylogenetic tree illustrates associative patterns, suggesting stronger molecular ties between BT and hypogonadism. These results provide a solid foundation for future therapeutic strategies targeting iron overload and its systemic effects

in beta-thalassemia.

C H A P T E R 5

CONCLUSION AND FUTURE WORK

In this chapter, the conclusion and future work have been clarified. For discussion convenience, there are a total of 2 sub-chapters under Chapter 5, which we introduced. In Section 5.1, we discussed the conclusion part; in Section 5.2, we discussed our thesis future work.

5.1 Discussion

This study has conducted a thorough genetic profiling analysis to unravel the molecular connections between beta-thalassemia and its comorbidities including cardiac complications such as arrhythmogenic cardiomyopathy (ACM), arrhythmia and endocrine complications such as polycystic ovary syndrome (PCOS), type 2 diabetes (T2D), hypothyroidism, and hypogonadism by using Gene Expression Omnibus (GEO) datasets from NCBI. By identifying differentially expressed genes (DEGs), we uncovered shared dysregulated genes across these conditions, and then visualized these DEGs through heatmap representation which highlight common molecular mechanisms driven by iron overload. Our analysis of signaling pathways, Gene Ontology with GO terms and Human Phenotype Ontology (HPO) terms, alongside Protein-Protein Interaction (PPI) and Protein-Drug Interaction (PDI) networks, reveals a robust genetic and molecular linkage between beta-thalassemia and its comorbidities. The PPI networks was constructed using the STRING database, identified hub genes like CD74, BCL2 and FN1 as central players in inflammation, apoptosis, and extracellular matrix remodeling. While PDI networks identify potential drug targets such as C1QA and PLA2G7 for anti-inflammatory and metabolic therapies. Phylogenetic analysis based on FASTA sequences from NCBI offers evolutionary insights into the associative relationships and suggesting a stronger ties between T2D-arrhythmia, hypothyroidism-PCOS and the main strong connections between BT-hypogonadism. These findings are validated against gold-standard databases like dbGaP and OMIM, reinforcing the genetic association and providing a solid foundation for understanding the interplay of these conditions. The identified pathways underscore the role of iron-induced oxidative stress and inflammation in comorbidity progression. The PDI results open avenues for developing targeted treatments, potentially reducing the burden of cardiac and endocrine complications.

Overall, this investigation deepens the molecular understanding of beta-thalassemia's systemic effects, offering valuable insights for designing diagnostic tools and therapeutic strategies. By targeting shared molecular pathways and leveraging the identified drug interactions, this work supports precision medicine approaches and advances systems biology, paving the way for improved management and treatment of beta-thalassemia and its associated conditions.

5.2 Future Work

This study has focused on the molecular relationship between beta-thalassemia and its comorbidities. However, the impact of iron overload and related dysregu-

lated pathways may extend to additional conditions beyond those explored here. Future research can broaden the scope to include other potential comorbidities, such as liver fibrosis or osteoporosis or its potential cancer diseases with survival analysis, which are also influenced by iron metabolism. To deepen our insights, more advanced methodologies, including cutting-edge bioinformatics tools, can be employed to pinpoint critical disease markers. Given the global significance of conditions linked to iron overload, like beta-thalassemia, further investigation into its diverse manifestations is essential. Analyzing larger and more diverse datasets this study will integrating genetic and clinical information by using Artificial Intelligence and Machine Learning techniques which will enhance our ability to predict disease outcomes and tailor personalized treatment strategies.

5.3 Conclusion

Beta-thalassemia, a prevalent genetic disorder, significantly influences the development of its comorbidities such as which is very necessitating greater awareness of these interconnected health challenges. Through the analysis of common differentially expressed genes (DEGs), signaling pathways, Gene Ontology (GO) and Human Phenotype Ontology (HPO) terms, Protein-Protein Interaction (PPI) networks, and Protein-Drug Interaction (PDI) networks, this study has established a robust genetic and molecular relationship between beta-thalassemia and its comorbidities. We identified sufficient dysregulated genes, such as CD74, BCL2, and FN1, which underscore a strong association and heightened risk factor across these conditions that supported by PPI hubs and PDI targets like C1QA and PLA2G7. Our pathway analyses, including T-helper Cell Differentiation and Reverse Cholesterol Transport elucidated critical biological processes, cellular functions, and molecular mechanisms, offering vital insights into disease progression and potential therapeutic targets driven by iron overload.

Validation through gold-standard databases like dbGaP and OMIM, combined with phylogenetic insights reinforces the genetic linkage. Understanding these biological connections enables the identification of individuals at higher risk for comorbidities, facilitating early detection, timely intervention, and increased awareness, ultimately improving management and treatment strategies for beta-thalassemia and its associated conditions.

REFERENCES

- [1] Malik, S.E., Kanwal, S., Javed, J., Hidayat, W., Ghaffar, T., Aamir, A.H.: Endocrine disorders in Beta-Thalassemia major patients at a Tertiary Care Hospital. *Pakistan Journal of Medical Sciences* **39**(3), 726 (2023).
- [2] Akiki, N., Hodroj, M.H., Bou-Fakhredin, R., Matli, K., Taher, A.T.: Cardiovascular complications in β -thalassemia: getting to the heart of it. *Thalassemia Reports* **13**(1), 38-50 (2023).
- [3] Podder, N.K., Rana, H.K., Azam, M.S., Rana, M.S., Akhtar, M.R., Rahman, M.R., Rahman, M.H., Moni, M.A.: A system biological approach to investigate the genetic profiling and comorbidities of type 2 diabetes. *Gene Reports* **21**, 100830 (2020).
- [4] Cao, A., Galanello, R.: Beta-thalassemia. *Genetics in Medicine* **12**(2), 61-76 (2010).
- [5] Harrison, N.A., Kopelman, M.D., David, A.S., Fleminger, S.: Endocrine diseases and metabolic disorders. In: *Lishman's Organic Psychiatry: A Textbook of Neuropsychiatry*, 4th ed., pp. 617-688. Wiley-Blackwell, Chichester (2009).
- [6] Wuche, C.: The cardiovascular system and associated disorders. *British Journal of Nursing* **31**(17), 886-892 (2022).
- [7] Carsote, M., Vasiliu, C., Trandafir, A.I., Albu, S.E., Dumitrascu, M.C., Popa, A., Mehedintu, C., Petca, R.C., Petca, A., Sandru, F.: New entity, $\tilde{\alpha}$ thalassemic endocrine disease: major beta-thalassemia and endocrine involvement. *Diagnostics* **12**(8), 1921 (2022).
- [8] Akiki, N., Hodroj, M.H., Bou-Fakhredin, R., Matli, K., Taher, A.T.: Cardiovascular complications in β -thalassemia: getting to the heart of it. *Thalassemia Reports* **13**(1), 38-50 (2023).
- [9] Ali, S., Mumtaz, S., Shakir, H.A., Khan, M., Tahir, H.M., Mumtaz, S., Mughal, T.A., Hassan, A., Kazmi, S.A.R., Sadia, Irfan, M.: Current status of beta-thalassemia and its treatment strategies. *Molecular Genetics & Genomic Medicine* **9**(12), e1788 (2021).

- [10] Podder, N.K., Shill, P.C., Rana, H.K., Omit, S.B.S., Al Shahriar, M.M.H., Azam, M.S.: Genetic effects of COVID-19 on the development of neurodegenerative diseases. In: *2021 5th International Conference on Electrical Information and Communication Technology (EICT)*, pp. 1-6. IEEE, Piscataway (2021).
- [11] Nain, Z., Rana, H.K., Lio, P., Islam, S.M.S., Summers, M.A., Moni, M.A.: Pathogenetic profiling of COVID-19 and SARS-like viruses. *Briefings in Bioinformatics* **22**(2), 1175-1196 (2021).
- [12] Khare, S., Chatterjee, T., Gupta, S., Patel, A.: Molecular pathways and gene networks in fetal hemoglobin as a novel protein target for beta-thalassemia. *MGM Journal of Medical Sciences* **10**(3), 485-492 (2023).
- [13] Sarkar, M.S., Mia, M.M., Al Amin, M., Hossain, M.S., Islam, M.Z.: Bioinformatics and network biology approach to identifying type 2 diabetes genes and pathways that influence the progression of breast cancer. *Helioyon* **9**(5) (2023).
- [14] Nain, Z., Barman, S.K., Sheam, M.M., Syed, S.B., Samad, A., Quinn, J.M., Karim, M.M., Himel, M.K., Roy, R.K., Moni, M.A., Biswas, S.K.: Transcriptomic studies revealed pathophysiological impact of COVID-19 to predominant health conditions. *Briefings in Bioinformatics* **22**(6), bbab197 (2021).
- [15] Datta, R., Podder, N.K., Rana, H.K., Islam, M.K.B., Moni, M.A.: Bioinformatics approach to analyze gene expression profile and comorbidities of gastric cancer. In: *2020 23rd International Conference on Computer and Information Technology (ICCIT)*, pp. 1-6. IEEE, Piscataway (2020).
- [16] Deshpande, R., VanderSluis, B., Myers, C.L.: Comparison of profile similarity measures for genetic interaction networks. *PloS One* **8**(7), e68664 (2013).
- [17] Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G.: Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Research* **44**(W1), W90-W97 (2016).
- [18] Podder, N.K., Shill, P.C.: Statistical and bioinformatics model to identify the influential genes and comorbidities of glioblastoma. In: *International Conference on Machine Intelligence and Emerging Technologies*, pp. 763-774. Springer, Cham (2022).
- [19] Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G.V., Clark, N.R., Ma'ayan, A.: Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC Bioinformatics* **14**(1), 128 (2013).

- [20] Wheelock, C.E., Wheelock, A.M., Kawashima, S., Diez, D., Kanehisa, M., van Erk, M., Kleemann, R., Haeggstrom, J.Z., Goto, S.: Systems biology approaches and pathway tools for investigating cardiovascular disease. *Molecular BioSystems* **5**(6), 588-602 (2009).
- [21] Croft, D., O'Kelly, G., Wu, G., et al.: Reactome: a database of reactions, pathways and biological processes. *Nucleic Acids Research* **39**(Database), D691-D697 (2011).
- [22] Rana, H.K., Akhtar, M.R., Islam, M.B., Ahmed, M.B., Lio, P., Quinn, J.M., Huq, F., Moni, M.A.: Genetic effects of welding fumes on the development of respiratory system diseases. *Computers in Biology and Medicine* **108**, 142-149 (2019).
- [23] Morrison, D.A.: Networks in phylogenetic analysis: new tools for population biology. *International Journal for Parasitology* **35**(5), 567-582 (2005).
- [24] Rana, M.S., Podder, N.K., Rana, H.K., Hasan, M.I., Azam, M.S., Rahim, M.A., Iqbal, S.H.S., Saha, S.: Identification of genomic associations between Parkinson's and neurodegenerative diseases using bioinformatics models. In: *2023 International Conference on Electrical, Computer and Communication Engineering (ECCE)*, pp. 1-6. IEEE, Piscataway (2023).
- [25] Slavov, S.N., Haddad, S.K., Silva-Pinto, A.C., Amarilla, A.A., Alfonso, H.L., Aquino, V.H., Covas, D.T.: Molecular and phylogenetic analyses of human Parvovirus B19 isolated from Brazilian patients with sickle cell disease and β -thalassemia major and healthy blood donors. *Journal of Medical Virology* **84**(10), 1652-1665 (2012).
- [26] Durrani, I.A., Bhatti, A., John, P.: Integrated bioinformatics analyses identifying potential biomarkers for type 2 diabetes mellitus and breast cancer: In SIK1-ness and health. *PLoS One* **18**(8), e0289839 (2023).
- [27] Slenter, D.N., Kutmon, M., Hanspers, K., et al.: WikiPathways: a multifaceted pathway database bridging metabolomics to other omics research. *Nucleic Acids Research* **46**(D1), D661-D667 (2018).
- [28] Barrett, T., Wilhite, S.E., Ledoux, P., et al.: NCBI GEO: archive for functional genomics data sets, update. *Nucleic Acids Research* **41**(D1), D991-D995 (2012).
- [29] Talihati, Z., Abudurousuli, K., Hailati, S., Han, M., Nuer, M., Khan, N., ... Zhou, W.: Screening of hub genes and therapeutic drugs in cervical cancer using integrated bioinformatics analysis. *Journal of Cancer* **16**(1), 92 (2025).

- [30] Caso, V.M., Manzo, V., Pecchillo Cimmino, T., et al.: Regulation of inflammation and oxidative stress by formyl peptide receptors in cardiovascular disease progression. *Lifestyles* **11**(3), 243 (2021).
- [31] Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A.: Gene ontology: tool for the unification of biology. *Nature Genetics* **25**(1), 25-29 (2000).
- [32] Podder, N.K., Shill, P.C.: Statistical and bioinformatics model to identify the influential genes and comorbidities of glioblastoma. In: *International Conference on Machine Intelligence and Emerging Technologies*, pp. 763-774. Springer, Cham (2022).
- [33] Wishart, D.S., Feunang, Y.D., Guo, A.C., Lo, E.J., Marcu, A., Grant, J.R., Sajed, T., Johnson, D., Li, C., Sayeeda, Z., Assempour, N.: DrugBank 5.0: a major update to the DrugBank database for 2018. *Nucleic Acids Research* **46**(D1), D1074-D1082 (2018).
- [34] Barua, J.D., Omit, S.B.S., Rana, H.K., Podder, N.K., Chowdhury, U.N., Rahman, M.H.: Bioinformatics and system biological approaches for the identification of genetic risk factors in the progression of cardiovascular disease. *Cardiovascular Therapeutics* **2022**(1), 9034996 (2022).
- [35] Rahman, M.H., Peng, S., Hu, X., Chen, C., Uddin, S., Quinn, J.M., Moni, M.A.: Bioinformatics methodologies to identify interactions between type 2 diabetes and neurological comorbidities. *IEEE Access* **7**, 183948-183970 (2019).
- [36] Colinge, J., Rix, U., Bennett, K.L., Superti-Furga, G.: Systems biology analysis of protein-drug interactions. *PROTEOMICS-Clinical Applications* **6**(1-2), 102-116 (2012).
- [37] Kumar, S., Tamura, K., Nei, M.: MEGA: molecular evolutionary genetics analysis software for microcomputers. *Bioinformatics* **10**(2), 189-191 (1994).
- [38] Rahman, M.H., Rana, H.K., Peng, S., Hu, X., Chen, C., Quinn, J.M., Moni, M.A.: Bioinformatics and machine learning methodologies to identify the effects of central nervous system disorders on glioblastoma progression. *Briefings in Bioinformatics* **22**(5), bbaa365 (2021).
- [39] Kuleshov, M.V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G.: Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Research* **44**(W1), W90-W97 (2016).

- [40] Zhou, G., Soufan, O., Ewald, J., Hancock, R.E., Basu, N., Xia, J.: NetworkAnalyst 3.0: a visual analytics platform for comprehensive gene expression profiling and meta-analysis. *Nucleic Acids Research* **47**(W1), W234-W241 (2019).
- [41] Moni, M.A., Lio, P.: Genetic profiling and comorbidities of zika infection. *The Journal of Infectious Diseases* **216**(6), 703-712 (2017).
- [42] Podder, N.K., Akter, F., Puza, A.K., Shill, P.C., Rana, H.K., Saha, K.K., Datta, R., Hossain, M.A.: A bioinformatics approach to identify the influences of diabetes on the progression of cancers. In: *2022 International Conference on Advancement in Electrical and Electronic Engineering (ICAEEE)*, pp. 1-6. IEEE (2022).
- [43] Yan, S., Meng, L., Guo, X., Chen, Z., Zhang, Y., Li, Y.: Identification of ITGAX and CCR1 as potential biomarkers of atherosclerosis via gene set enrichment analysis. *Journal of International Medical Research* **50**(3), 03000605211039480 (2022).
- [44] Liu, R., Chen, Y.: Comprehensive analysis of hub genes, biological mechanism and predicted drugs related to iron metabolism in β -thalassemia. (2023).