



UNIVERSITY
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IDENTIFICATION OF GENE MODULES IN TYPE 2 DIABETES BY INTEGRATING TRANSCRIPTOMICS INTO GENE INTERACTION NETWORK ANALYSIS

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Abstract

Type 2 Diabetes (T2D) is a complex metabolic disorder characterized by coordinated dysregulation of multiple genes and biological pathways. Although transcriptomic technologies enable large-scale analysis of gene expression changes in T2D, many studies rely primarily on differential gene expression analysis, which often fails to capture gene-gene interactions and systems-level regulatory mechanisms. To address this limitation, this study applied a transcriptomics-based gene interaction network approach to investigate the molecular architecture of T2D. Publicly available RNA-sequencing data from T2D patients and healthy controls were analyzed using established bioinformatics pipelines. After quality control and normalization, differential expression analysis was performed, followed by the construction of gene co-expression networks and identify functionally related gene modules. Disease-associated modules were further examined to detect highly connected hub genes based on network topology and connectivity measures. Functional enrichment analysis of hub genes using Gene Set Enrichment Analysis (GSEA) was not feasible, as the available data did not meet the input requirements of the method; therefore, pathway-level enrichment was performed using GSEA on a ranked gene list derived from differential expression statistics. Network robustness was assessed through connectivity strength and network density analyses to ensure reliability. The analysis identified distinct gene modules and central hub genes associated with metabolic regulation, mitochondrial function, and cellular homeostasis relevant to T2D. Overall, this study demonstrated that network-based transcriptomic analysis provides deeper biological insights than traditional gene-centric approaches and offers a systems-level framework for understanding the molecular mechanisms underlying T2D.

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Abbreviation

T2D - Type 2 Diabetes

DEG - Differentially Expressed Genes

CPM – Counts Per Million

DEA - Differential Expression Analysis

FDR – False Discovery Rate

PCA – Principal Component Analysis

PLS-DA - Partial Least Squares Discriminant Analysis

VST – Variance Stabilizing Transformation

NES – Normalized Enrichment Score

ES – Enrichment Score

WGCNA – Weighted Gene Co-expression Network Analysis

GSEA – Gene Set Enrichment Analysis

GO – Gene Ontology

BP – Biological Process

SVA - Surrogate Variable Analysis

TMM – Trimmed Mean of M-value

ORA – Over Representaton Analysis

CNET - Catergory Network

EMAP - Enrichment Map

Background

Type 2 Diabetes (T2D) is a complex metabolic disorder and one of the most prevalent non-communicable diseases worldwide, with its incidence and associated morbidity increasing rapidly (DeFronzo et al., 2015). It is characterized by chronic hyperglycaemia resulting from insulin resistance and impaired insulin secretion, and it is frequently accompanied by severe long-term complications, including cardiovascular disease, neuropathy, nephropathy, and retinopathy. Due to its multifactorial nature, T2D arises from the combined influence of genetic, environmental, and lifestyle factors, making its underlying molecular mechanisms highly complex. Therefore, gaining a deeper understanding of the molecular basis of T2D is essential for early diagnosis, boosted disease management, and the development of effective therapeutic strategies.

Conventional molecular studies of T2D have predominantly focused on identifying individual genes associated with disease risk or progression. Although these studies have presented valuable insights, they often fail to capture the coordinated and dynamic interactions among multiple genes that collectively contribute to disease development. With the advent of high-throughput transcriptomics technologies, it has become possible to measure genome-wide gene expression patterns and explore disease mechanisms at a broader biological scale. However, analyzing differentially expressed genes in isolation may not fully explain the complex regulatory processes underlying T2D.

To address these limitations, systems biology approaches have gained prominence by emphasizing the analysis of gene interactions and collective behaviour rather than single-gene effects (Goh et al., 2007). Transcriptomics-based gene interaction and co-expression networks provide a powerful framework for studying how genes function together within biological systems. In such networks, genes with similar expression patterns across samples are connected, enabling the identification of functionally related groups of genes, commonly referred to as gene modules (Zhang and Horvath, 2005). These modules often represent shared biological pathways or regulatory mechanisms relevant to disease phenotypes.

Gene module analysis has emerged as a valuable strategy for investigating complex diseases such as T2D, where multiple molecular pathways act in a coordinated manner. By examining gene expression networks derived from transcriptomic data, researchers can identify disease-associated modules that are disrupted in diabetic conditions. These modules offer a biologically meaningful representation of disease processes and provide insights that extend beyond those obtained from traditional DEA.

Within gene interaction networks, certain genes exhibit high connectivity and play central roles in regulating module behaviour. These genes, known as hub genes, are often critical for maintaining network structure and coordinating biological functions. Dysregulation of hub genes may have widespread effects on gene expression networks and contribute significantly to disease pathogenesis. Consequently, identifying hub genes within disease-associated modules is of particular interest, as they may serve as potential biomarkers or therapeutic targets.

Recent advances in network-based omics data analysis have demonstrated the effectiveness of integrating gene expression data with interaction network approaches to uncover novel disease-related genes and pathways. Unlike methods that rely solely on predefined pathways, transcriptomics-based gene networks enable the discovery of previously unrecognized gene relationships and regulatory patterns. Such approaches have been successfully applied to various complex diseases, highlighting their potential to reveal hidden molecular mechanisms.

In this context, the application of transcriptomics-based gene interaction network analysis to T2D provides a comprehensive framework for identifying key gene modules and hub genes involved in disease development. By focusing on gene co-expression and network topology, this

study aims to move beyond single-gene analyses and provide a systems-level understanding of the molecular mechanisms underlying T2D. The findings from this approach may contribute to improved biomarker identification and support future efforts toward targeted therapeutic interventions.

Problem statement

Despite significant advances in genomics and molecular biology, the molecular mechanisms underlying the development and progression of T2D remain insufficiently understood (Sanches et al., 2023). T2D is a complex, multifactorial disease influenced by the coordinated action of numerous genes and biological pathways. While transcriptomic technologies have enabled large-scale profiling of gene expression changes associated with T2D, translating these data into a comprehensive understanding of disease mechanisms continues to be a major challenge.

Commonly, transcriptomic studies on T2D primarily focus on the identification of DEGs by comparing gene expression profiles between healthy individuals and T2D patients. Although such gene-centric approaches provide valuable information, they often analyze genes in isolation and do not adequately account for the functional relationships among genes (Alcazar et al., 2021). As a result, the broader biological context in which genes operate, including coordinated regulation and collective behaviour within molecular networks, is frequently overlooked.

This limitation restricts the ability to capture the global dysregulation of gene interaction networks that characterizes complex diseases such as T2D. The lack of integrative frameworks that systematically incorporate transcriptomic data with gene interaction or co-expression network analysis has resulted in an incomplete understanding of how groups of genes jointly contribute to disease pathogenesis. Consequently, important disease-associated gene modules and key regulatory genes may remain undiscovered when relying solely on traditional DEA.

Therefore, there exists a critical need for gene expression profiling based network-oriented approaches that move beyond individual gene analysis and enable the identification of biologically meaningful gene modules and hub genes associated with T2D. Addressing this gap through gene interaction network analysis can provide a comprehensive approach of disease mechanisms, facilitating the discovery of novel candidate genes and pathways involved in T2D development and progression. This strategy enables a deeper biological understanding of molecular profiling alterations in T2D and facilitates the recognition of key genes involved in disease regulation and progression.

Research Aim and Objectives

Research Aim

The aim of this research is to apply RNA-Seq analysis-based gene interaction network analysis to identify key gene modules and hub genes associated with T2D. By integrating gene expression data with network-based analytical approaches, this study seeks to move beyond traditional gene-centric analyses and investigate the coordinated behaviour of genes at a systems level.

Specifically, the study aims to uncover disease-associated gene modules and central regulatory genes that contribute to the molecular mechanisms underlying the pathogenesis of T2D. Through the identification of functionally related gene clusters and highly connected hub genes, this research intends to provide a deeper biological interpretation of transcriptomic alterations observed in diabetic conditions.

Research objectives

The research objectives of this study are to:

- Pre-process and normalize transcriptomic data from T2D patients and healthy controls to ensure quality and reliability.
- Identify DEGs associated with T2D using standard bioinformatics pipelines.
- Identify gene modules with high level of interactivity, and select key modules for further analysis, to capture groups of functionally related genes relevant to T2D.
- Identify hub genes within the networks that may play central roles in the pathogenesis of T2D.
- Perform functional enrichment analysis on the identified hub genes to interpret their biological significance.
- Analyze network topology to understand structural properties, including connectivity patterns among genes.
- Validate the network structure by assessing connectivity strength and network density to ensure robustness and reliability of the inferred interactions.

Significance of the Study

This study improves the understanding of T2D by employing holistic view analysis tools that enable the examination of gene interactions and identification of key hub genes, providing evidence-based insights into the biological processes underlying the disease. By combining omics data analyses with network-based computational approaches, the research moves beyond the traditional focus on individual DEGs and explores the organization of genes within co-expression modules, capturing the complexity of regulatory dynamics at the molecular level in T2D (Huang & Xiang and Song, 2022). This systems-level perspective offers a more integrated understanding of the molecular pathways involved in disease onset and advancements.

The study also opens avenues for the discovery of potential biomarkers, as the hub genes identified from co-expression networks may help in early detection and improved risk stratification of T2D. Such insights could support more accurate predictions and inform targeted intervention strategies. Furthermore, the findings have implications for therapeutic research, contributing to the identification of candidate genes that could be explored in precision medicine approaches (Song and Yu, 2024).

Methodologically, this research showed the applicability of transcriptomics-based network analysis in medical research, highlighting the feasibility of using systems biology approaches to interpret complex biological data. Overall, the study adds valuable knowledge to the fields of genomics and systems biology, providing a framework that can guide future investigations into the molecular mechanisms, diagnostics, and drug development targets for T2D.

Scientific Contribution of the Study and Novelty

Scientific Contribution

This study contributes to the understanding of T2D by applying a transcriptomics-based gene interaction network framework to investigate disease-associated molecular mechanisms at a systems level. Unlike traditional gene-centric approaches that focus primarily on DEGs, this research integrates DEA with gene co-expression network modeling to capture coordinated gene regulation underlying T2D.

Methodologically, the study demonstrates the applicability of weighted gene co-expression network analysis (WGCNA) to RNA-sequencing data from human pancreatic islets, enabling the

identification of disease-associated gene modules and highly connected hub genes. The integration of network topology measures, including module membership, k-core, and degree centrality, provides a robust strategy for prioritizing candidate regulatory genes that may not be strongly differentially expressed but occupy central positions within co-expression networks.

From a biological perspective, the results highlight gene modules and hub genes associated with mitochondrial function, energy metabolism, and cellular homeostasis—processes known to play key roles in T2D pathophysiology. Functional enrichment analysis performed using a ranked gene list further supports the involvement of metabolic and β -cell-related pathways, offering system-level insight into transcriptional dysregulation in T2D.

Overall, this thesis contributes a reproducible and integrative analytical framework that advances systems-level interpretation of transcriptomic data in complex diseases. The findings provide candidate genes and gene modules that may serve as potential biomarkers or targets for future experimental validation and therapeutic research.

Scientific Novelty

The methodological innovation lies in the integration of network topology measures—such as module membership, k-core, and degree centrality to prioritize hub genes that may not exhibit strong differential expression but occupy central regulatory roles within the network. This approach allows for the detection of key molecular drivers of T2D that would be overlooked by traditional gene-centric analyses.

From a biological perspective, the study identifies gene modules and hub genes involved in mitochondrial function, energy metabolism, and cellular homeostasis, providing new insights into pathways critical for T2D pathophysiology. The combination of pathway-level enrichment analysis with network-based modeling offers a reproducible and integrative framework for interpreting transcriptomic data in complex diseases.

Description of Methodology

This study investigates the molecular mechanisms underlying T2D through the integration of transcriptomic data and gene co-expression network analysis. Rather than focusing solely on individual DEGs, this approach seeks to capture groups of co-ordinately expressed genes that may collectively contribute to the pathogenesis of T2D.

All analyses are performed using R programming (version 4.5.1), following a structured and script-based workflow to enhance reproducibility. The overall methodology has been organized into six main steps, each of which has been designed with a specific objective and analytical procedure to ensure consistency and scientific rigor throughout the study. This structured approach not only ensures robustness in the findings but also provides a clear framework for future replication and validation of the results in independent datasets.

Data Collection

Transcriptomic data derived from human pancreatic islet samples were obtained from the NCBI Gene Expression Omnibus (GEO), a publicly accessible functional genomics repository maintained by the National Center for Biotechnology Information (NCBI). The dataset was selected based on the availability of well-annotated human samples, standardized metadata, and clear classification of disease status, which facilitated systematic comparison between T2D and healthy control groups.

Among metabolically relevant tissues implicated in T2D pathophysiology—including the pancreas, liver, and skeletal muscle—the pancreas plays a central role in insulin secretion

through pancreatic β -cells. Therefore, this study focused on pancreatic tissue to better understand transcriptional alterations associated with impaired insulin regulation. A human pancreatic islet transcriptomic dataset (GSE86468), comprising 24 samples including 9 T2D and 15 non-diabetic controls, was selected for downstream analysis.

Data Preprocessing and Identification of Differentially Expressed Genes

The data were preprocessed and normalized prior to differential expression analysis. DEGs were then identified using DESeq2 packages.

Quality Control

The raw count matrix obtained from GSE86468 was subjected to quality control prior to downstream analyses. Genes with zero counts across all samples were filtered out then Counts Per Million (CPM) were calculated to reduce background noise and improve statistical robustness. Principal Component Analysis (PCA) and hierarchical clustering were conducted to evaluate sample relationships and identify potential outliers. No samples exhibited substantial deviation from their primary clusters. Potential batch effects were assessed by examining clustering patterns and sample distribution. The PCA results did not reveal clear separation associated with sequencing runs or other technical variables, suggesting minimal batch-related variation. Therefore, batch correction was not applied.

If substantial batch effects had been detected, correction would have been performed using established statistical adjustment methods such as the ComBat algorithm. Additionally, surrogate variable analysis could have been incorporated to account for hidden confounding factors and minimize the risk of false-positive or false-negative findings in downstream differential expression analysis.

Normalization

Normalization was performed to correct for technical variation, including differences in sequencing depth and library size among samples, ensuring that observed gene expression differences reflected true biological variation. This step was essential to reduce systematic biases arising during data acquisition and sample preparation, thereby improving the reliability and interpretability of downstream analyses, including DEA. The RNA-Seq data were normalized using the variance stabilizing transformation (VST) function from DESeq2, which efficiently stabilized variance across the range of mean expression values, particularly for the 24 samples analyzed. These corrections ensured that the observed counts reflected true biological variation rather than differences in read numbers or sample preparation. Low-count genes were filtered prior to normalization to reduce noise and enhance statistical robustness. The normalization allowed meaningful comparisons across samples and accurate identification of DEGs.

Although DESeq2 normalization was applied in this study, other strategies could have been considered. For example, the Trimmed Mean of M-values (TMM) method could be used to correct for compositional differences between libraries, particularly in edgeR- or limma-based RNA-Seq workflows. TMM normalization is suitable for RNA-Seq count data and is effective in adjusting for library size differences and compositional bias, making it a valid alternative approach.

Identification of Differential Expression Genes

To investigate genetic variation in the expression profiles of T2D patients and healthy controls, differential expression analysis was conducted to identify genes exhibiting significant changes in expression. These genes provided insight into the molecular mechanisms of T2D and served as the initial set of disease-associated genes for subsequent network and functional analyses.

RNA-Seq data were modeled using generalized linear models in the DESeq2. Genes with FDR <0.05 were considered significant. Among these, genes with \log_2 fold change >+1 or <-1 would be classified as upregulated or downregulated, respectively, and visualized using volcano plots to clearly differentiate expression patterns. The identified DEGs were subsequently used for functional enrichment analyses, including Gene Ontology (GO), and the results have been visualized with cnet plots and emap plots to explore gene–pathway relationships.

DEA was conducted using the DESeq2 package, which applies a negative binomial framework with shrinkage estimation for dispersion and fold changes. Model diagnostics indicated adequate fit and stable dispersion estimates despite the moderate sample size imbalance (n = 9 vs n = 15). Therefore, alternative methods such as edgeR (quasi-likelihood framework) or the limma-voom pipeline were not required. These approaches remain suitable alternatives when enhanced variance modeling or additional robustness is necessary.

Gene- gene network construction

Constructing a high-confidence human gene co-expression network was essential to uncover the molecular mechanisms of T2D. This network provided a foundation to identify key regulators and disease modules by integrating initially filtered genes with biologically relevant interactions.

The gene co-expression network was constructed from the filtered expression data using the WGCNA in R. All identified genes were ranked according to their absolute \log_2 fold change values, and those exhibiting the highest changes were considered to represent the most pronounced transcriptional alterations between conditions. Based on this ranking, the top 30 upregulated and downregulated genes were selected for further analysis. Co-expression modules were identified using WGCNA, and the resulting network was visualized using Cytoscape, while computational analyses were performed with the igraph and ggraph packages in R.

An alternative strategy could have been employed if certain co-expression relationships were weak or modules were not well-defined. For example, Spearman correlation could have been used instead of Pearson correlation to construct the network, if the data contained outliers.

Identify Central Nodes

Identifying central nodes based on network connectivity provided insights beyond conventional differential expression analysis by highlighting genes with critical topological importance. This approach enabled the detection of hub genes that may play key regulatory roles in the underlying biological mechanisms of T2D, independent of the magnitude of expression change.

Following gene co-expression network construction using the WGCNA, pairwise expression correlations were calculated using the Pearson correlation coefficient to define gene–gene relationships. The resulting adjacency matrix was used to generate the network structure. Network topology parameters, including measures of gene connectivity and centrality, were computed to evaluate the relative importance of genes within the network. Module membership (kME) was calculated for each gene, and genes were ranked by their absolute kME values. Module membership represented how strongly a gene's expression profile correlated with the module eigengene, the first principal component summarizing the overall expression pattern of the module. In other words, kME quantified how well a gene 'fit' within the module's expression signature. Genes with higher absolute kME values were more tightly connected to the module's core expression pattern, indicating that they were more representative of the module's biological function. Based on these values, the top 30 genes with the highest module membership were selected as hub genes.

Although module membership was used as the primary metric in this study, alternative topology measures such as degree connectivity, betweenness centrality, or closeness centrality could also be explored to identify influential genes within the co-expression network.

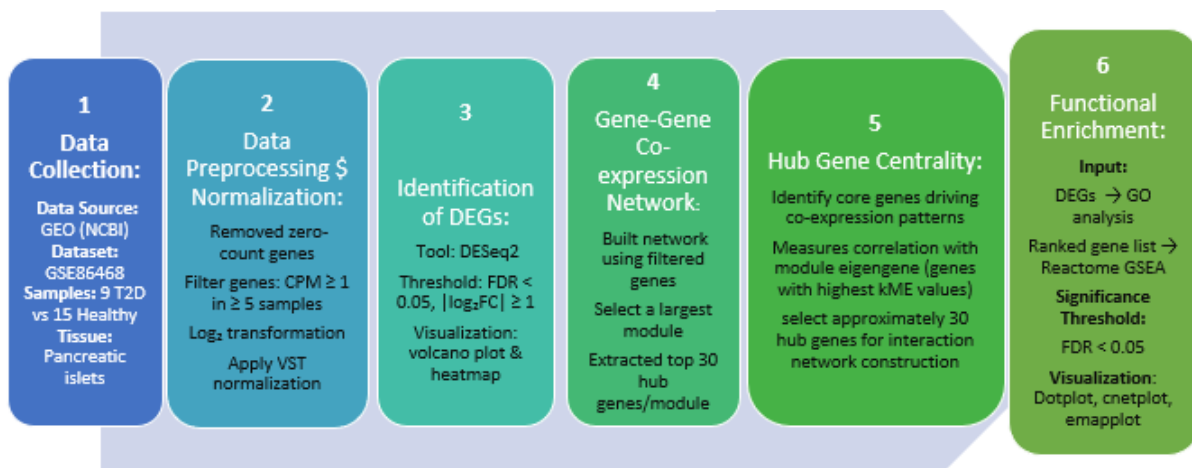
Functional Enrichment

Functional enrichment analysis was conducted to identify biologically relevant pathways and processes associated with the observed gene expression changes. Gene Set Enrichment Analysis (GSEA) was performed using the Reactome pathway database to detect significantly enriched pathways based on a ranked gene list derived from the expression data. In addition, Gene Ontology (GO) enrichment analysis was carried out to determine overrepresented biological processes, molecular functions, and cellular components among the DEGs. Only the DEGs identified from the differential expression analysis were used as input for the over-representation analysis.

All enrichment analyses were implemented in R using the clusterProfiler and ReactomePA packages. Gene identifiers were converted to standardized gene symbols using the org.Hs.eg.db annotation database to ensure consistency across downstream analyses. Enrichment results were visualized using category-gene network plots (cnetplot), enrichment map plots (emapplot), and correlation-based heatmap to facilitate the interpretation of gene-pathway relationships and functional associations.

Although GSEA and GO enrichment were performed, alternative pathway analysis strategies could have been considered. For example, Signaling Pathway Impact Analysis (SPIA) could have been applied as a pathway topology-based approach. Unlike conventional enrichment methods, SPIA integrates both gene over-representation and pathway perturbation evidence. It incorporates pathway structure, gene-gene interactions, and signaling directionality. This would have enabled a more comprehensive evaluation of biological pathway impact.

Schematic Overview of the Method



Why Gene-Gene Co-Expression Network?

Gene-gene co-expression network analysis was chosen to uncover patterns of coordinated gene expression that cannot be captured by DEA alone. While DEGs identify genes that change significantly between conditions, they do not reveal how genes interact or function together within biological pathways. Constructing a co-expression network allows the identification of modules of highly correlated genes and central hub genes that may play key regulatory roles.

This approach offers a systems-level perspective on the molecular mechanisms of the condition, emphasizing how genes interact and collectively influence biological processes.

Implementation of the Methodology

Data Acquisition and Description

The dataset consisted of 24 pancreatic islet samples processed using RNA-Seq by Expectation Maximization (RSEM) to quantify gene expression levels. Among these, 9 samples belonged to T2D and 15 samples to Non-Diabetic group.

Each row in the dataset represented a gene, and each column corresponded to one of the 24 biological samples, resulting in a raw expression matrix of 26,616 genes \times 24 samples prior to preprocessing. These genes included a broad range of protein-coding and non-coding genes typically detected in bulk RNA-sequencing experiments. This raw count matrix served as the input for subsequent preprocessing, normalization, DEA, and network-based investigations. All analyses were performed in R (version 4.2.1).

The dataset was downloaded from GEO as a compressed CSV file:

GSE86468_GEO.bulk.islet.processed.data.RSEM.raw.expected.counts.csv.gz.

Data Preprocessing and Normalization

An initial preprocessing step was performed to remove genes with zero counts across all samples. This step eliminated 10,633 genes, reducing the dataset from 26,616 to 15,983 genes. Subsequently, CPM were calculated from the raw RNA-seq count matrix to account for differences in library sizes across samples. Genes were retained if they exhibited a CPM value ≥ 1 in at least five samples. Application of this CPM-based filtering criterion removed an additional 1,814 lowly expressed genes, resulting in a final dataset of 14,169 genes. Log₂-transformed CPM values were used for quality control and downstream visualization. A summary of gene counts before and after filtering is provided in Table 1.

Inspection of library size distributions showed variability in sequencing depth across samples; however, no sample exhibited critically low coverage or extreme deviation that would justify exclusion (Figure 1). The disease status of samples (T2D or healthy control) was determined based on the sample metadata provided with the dataset. This metadata includes phenotype annotations specifying whether each sample was derived from individuals with T2D or from healthy controls.

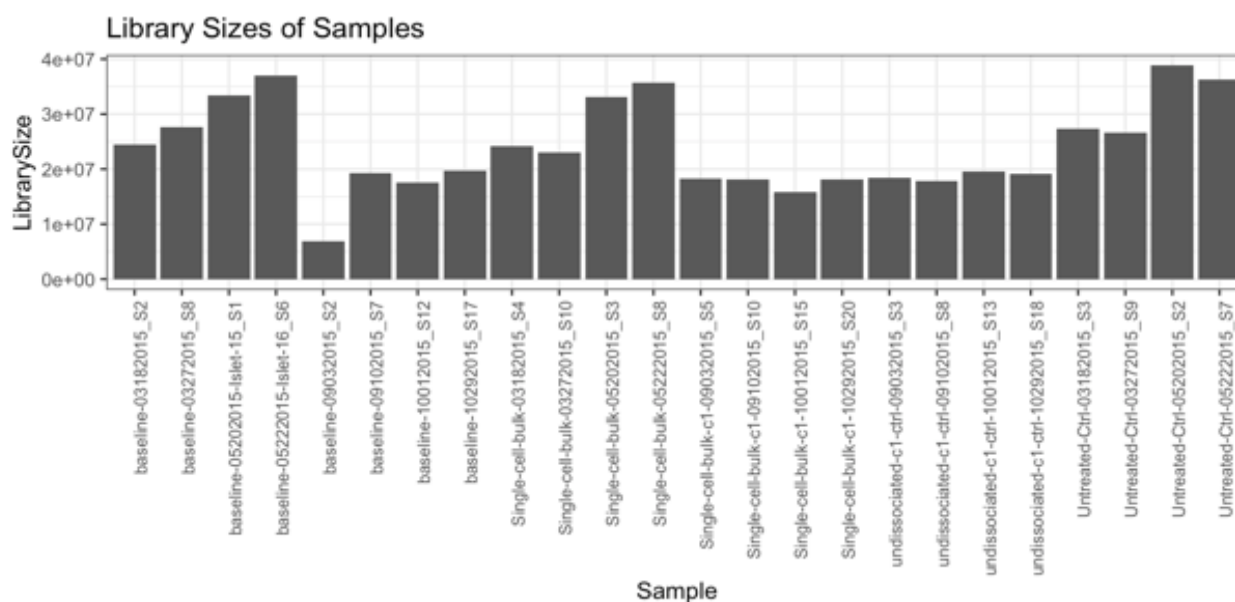


Figure 1. The bar plot shows the library sizes (total sequencing reads) for each sample across different experimental conditions. Samples are categorized as baseline, single-cell bulk, undissociated, and untreated, representing different sample preparation or experimental states. Variability in library sizes reflects differences in sequencing depth among samples. Assessing library size distribution helps evaluate data quality and ensures comparability before downstream analysis. The fifth sample showed lower sequencing depth but did not qualify as an outlier. Normalization would be performed to correct for these differences.

Table 1: Summary of the filtered counts

Step	Genes remaining	Genes removed at this step
Raw dataset	26,616	-
After initial preprocessing (zero-count genes removed)	15,983	10,633
After CPM filtering (CPM ≥ 1 in ≥ 5 samples)	14,169	1,814
Total removed	-	12,447

The figure 1 illustrated the distribution of library sizes across all samples. Although variation in sequencing depth was observed, all samples fell within the same order of magnitude and within an acceptable range. The fifth sample showed a comparatively lower library size; however, it did not represent an extreme outlier and was therefore retained. Normalization methods were applied to account for differences in sequencing depth.

Boxplots of log₂-transformed CPM values for each sample showed that most genes were lowly expressed, with medians closer to ~2–3 and similar distributions across all 24 samples. A small number of highly expressed genes appeared as outliers above the upper whiskers, which is expected in RNA-seq data. The consistent distributions indicated that normalization was successful, and no sample showed abnormal expression patterns, making all samples suitable for downstream analyses (Figure 2).

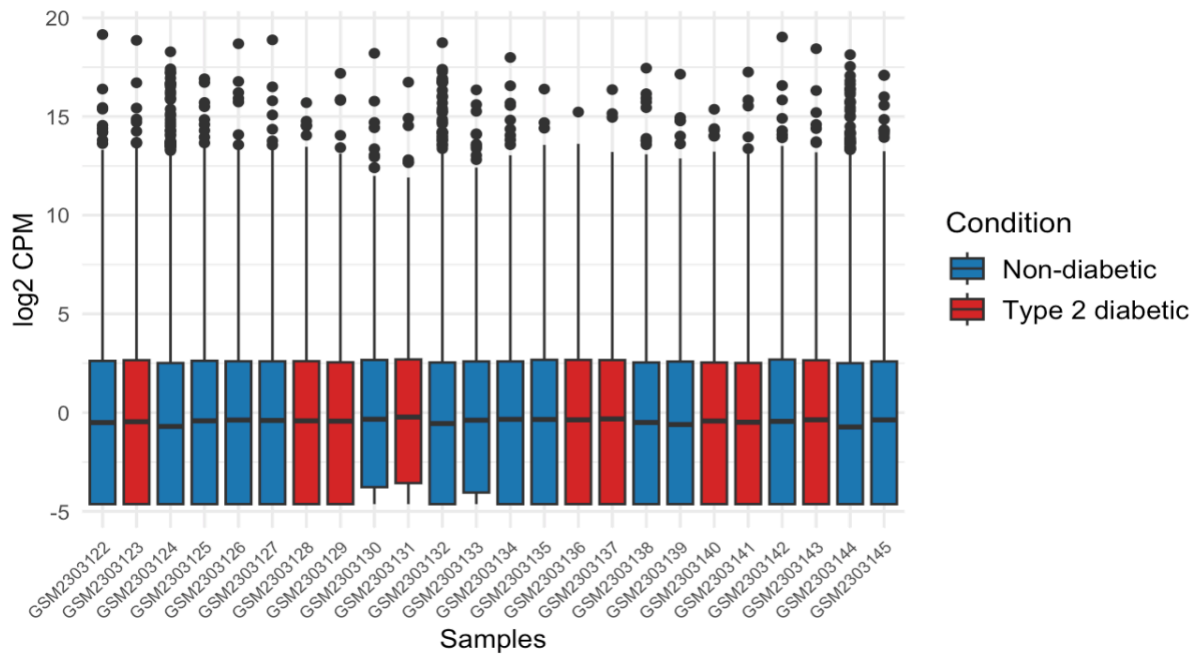


Figure 2. Boxplot of Normalized \log_2 CPM values across 24 RNA-seq samples: Comparable median expression levels (~ 2 - 3) and overall distributions across both groups indicate successful normalization and minimal technical variation.

The figure 3 compared the distribution of gene expression values before and after normalization across all samples. In the left panel, raw expression values (\log_2 -transformed counts) showed noticeable variability in median expression levels and spread across samples. Several samples exhibited wider interquartile ranges and differing central tendencies, indicating the presence of technical variability that may have arisen from sequencing depth, library size, or sample preparation effects. Such variability could obscured true biological signals if used directly in downstream analyses.

In contrast, the right panel displayed expression values after applying variance stabilizing transformation (VST). Following normalization, the distributions across samples became more comparable, with aligned medians and more uniform interquartile ranges. This indicated that VST effectively reduced technical bias while preserving relative expression differences among genes.

Overall, the improved consistency observed in the VST-normalized data confirmed that the normalization procedure was successful and that the transformed expression matrix was suitable for downstream analyses such as PCA, multivariate modeling, and gene co-expression network construction.

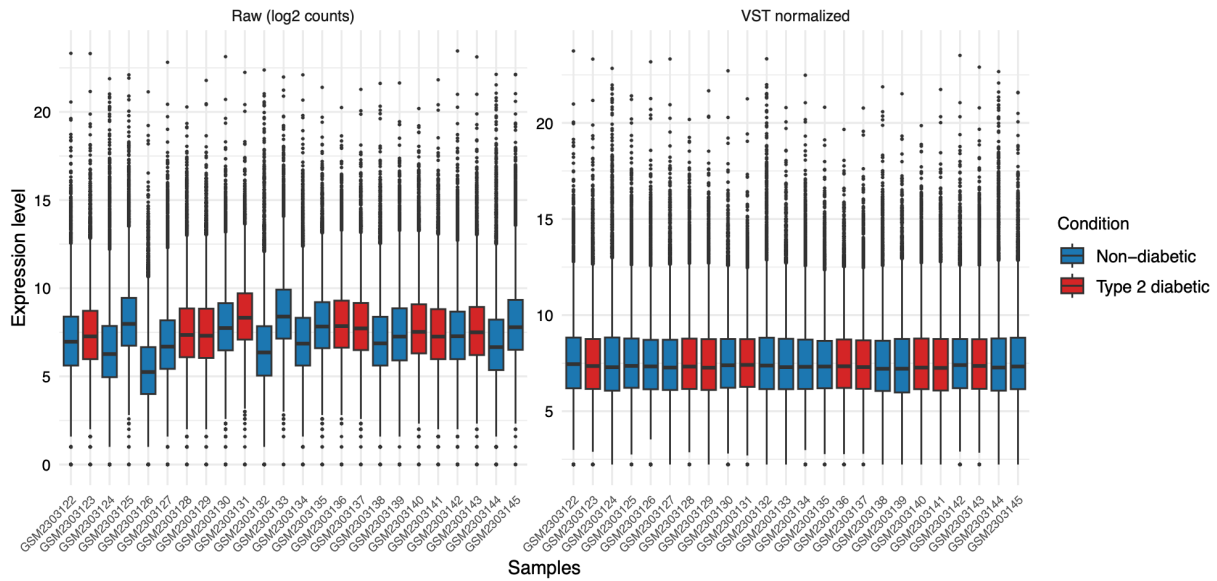
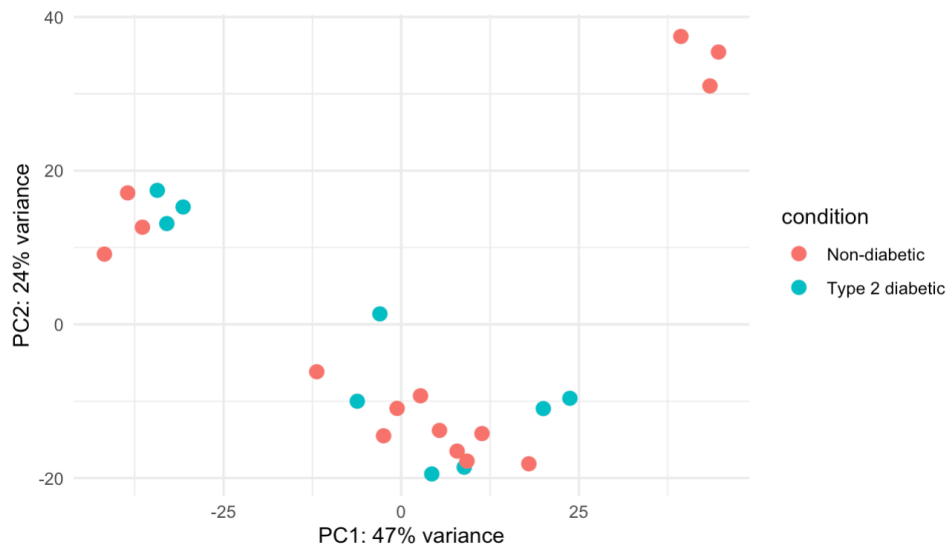


Figure 3. Comparison of Raw and VST- Normalized Expression Values: The left panel shows boxplot of \log_2 -transformed raw counts, demonstrating variability in median expression levels and spread across samples. The right panel displays expression values after VST, where distributions become more comparable with aligned medians and uniform interquartile ranges, indicating reduced technical variation.

PCA was performed as an unsupervised method to examine the overall variance and intrinsic structure of the dataset without using class labels. The PCA score plot showed no clear separation between non-diabetic ($n = 15$) and T2D ($n = 9$) samples, with substantial overlap observed between the two groups. The first principal component (PC1) accounted for 47% of the total variance, while the second principal component (PC2) explained 24% of the variance, together capturing a major proportion of the dataset variability. This indicated that the dominant sources of variation were not primarily driven by diabetic status. The observed overlap suggested the presence of shared biological characteristics, inter-individual variability, or subtle metabolic changes that were not strongly represented in the first two principal components. Overall, PCA highlighted data heterogeneity rather than group discrimination, and therefore limited separation was expected when group differences were modest (Figure 4 (A))

In contrast, Partial Least Squares Discriminant Analysis (PLS-DA), a supervised method, was applied to enhance group separation using class information. The PLS-DA score plot showed improved discrimination between non-diabetic and type 2 diabetic samples compared to PCA. Noticeable differences between the groups were primarily observed along Component 1, which appeared to capture much of the variation associated with diabetic status. Although the separation was not complete, the clustering patterns and distribution of the samples suggested that underlying features differentiated the two conditions. Together, PCA and PLS-DA provided complementary insights: PCA revealed overall variance and overlap, while PLS-DA confirmed condition-specific patterns (Figure 4(B)).

A)



B)

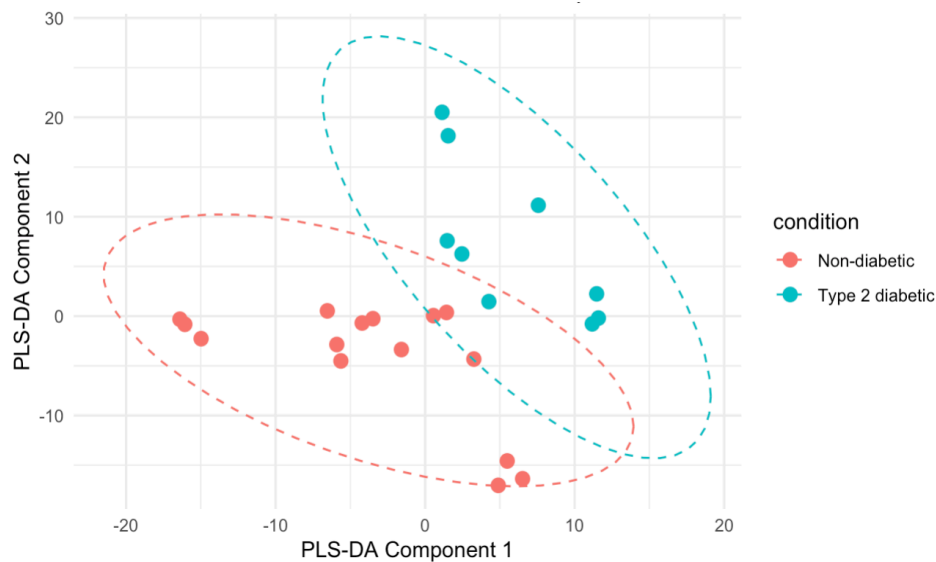


Figure 4. Multivariate analysis of non-diabetic ($n = 15$) and T2D ($n = 9$) samples. (A) PCA score plot showing overlap between groups, indicating shared variability. (B) PLS-DA score plot demonstrating supervised separation between non-diabetic and diabetic samples.

Differential Expression Analysis

DEA was performed to identify genes associated with T2D in comparison to non-diabetic controls. Raw count data were analyzed using the DESeq2 package, which modeled count data based on a negative binomial distribution and accounted for biological variability. The analysis was conducted using a design formula that included condition (T2D vs non-diabetic), sex, and sample type as covariates to control for potential confounding effects.

Genes with fewer than 1 count in at least five samples were excluded. Following filtering, `vst()` normalization and dispersion estimation were performed, and differential expression was assessed using Wald test. Multiple testing correction was applied using the Benjamini–Hochberg method to control the false discovery rate (FDR).

A total of 14,169 genes were tested for differential expression. Of these, 154 genes were identified as significantly differentially expressed based on an adjusted p-value (FDR) threshold of < 0.05 . Among these significant genes, 65 genes were upregulated and 89 genes were downregulated in T2D samples relative to non-diabetic controls. The remaining 14,015 genes were not significantly differentially expressed, indicating that the majority of genes showed comparable expression levels between the two conditions.

The global distribution of differential expression results was visualized using a volcano plot. The volcano plot illustrates the relationship between \log_2 fold change and statistical significance ($-\log_{10}$ FDR). Genes with significant differential expression ($FDR < 0.05$ and $|\log_2 \text{fold change}| \geq 1$) were highlighted, enabling clear identification of strongly upregulated and downregulated genes, while serving as a graphical representation of the numerical DEG results (Figure 5).

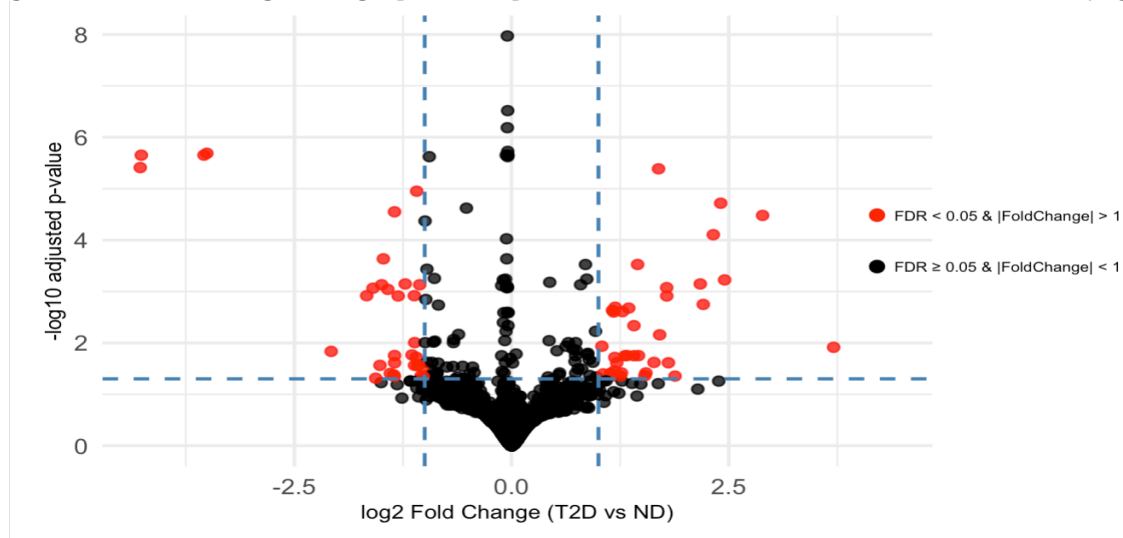


Figure 5. Volcano Plot of DEGs: The x-axis represents \log_2 fold change (T2D vs. ND), and the y-axis shows $-\log_{10}$ adjusted p-values (FDR). Genes meeting the significance criteria ($FDR < 0.05$ and $|\log_2 \text{fold change}| \geq 1$) are highlighted in red, indicating significantly upregulated and downregulated genes.

To further examine the expression patterns of the most significant genes, the top 10 DEGs were selected based on the lowest FDR values. Among these genes, CPA2, PNLIPRP1, and PNLIP exhibited substantial negative \log_2 fold changes, indicating strong downregulation and pronounced differences in gene expression between conditions (Table 2). A heatmap was generated to visualize the expression profiles of the top 20 genes across all samples (Figure 6). The heatmap showed partial clustering of samples by disease status; however, T2D samples were distributed across multiple clusters, suggesting heterogeneity within the diabetic group and the influence of additional experimental factors, such as differences in sample preparation conditions (baseline, single-cell bulk, undissociated, and untreated samples).

Overall, the DEA revealed a distinct transcriptional signature associated with T2D. The identification of 154 DEGs, along with partial separation observed in the heatmap visualization and the volcano plot serving as a graphical representation of these genes suggested robust alterations in gene expression linked to disease status. These findings provided a foundation for downstream functional enrichment and pathway analyses to further explore biological mechanisms underlying T2D.

Table 2. Top 10 DEGs (based on \log_2 FC, FDR): Genes are ranked by increasing FDR and include \log_2 fold change (T2D vs. ND) and direction of regulation. Notably, CPA2, PNLIPRP1, and PNLIP demonstrate distinct downregulation in T2D samples.

Gene_Symbol <chr>	log2FoldChange <dbl>	FDR <dbl>
CELA2A	-0.049	1.07e-08
CLPS	-0.045	3.04e-07
CPA1	-0.049	6.52e-07
CELA3B	-0.044	1.88e-06
CPA2	-3.508	2.05e-06
PNLIPRP1	-3.541	2.23e-06
AMY2A	-0.041	2.23e-06
GP2	-0.062	2.23e-06
PNLIP	-4.262	2.23e-06
MT-ND5	-0.947	2.38e-06

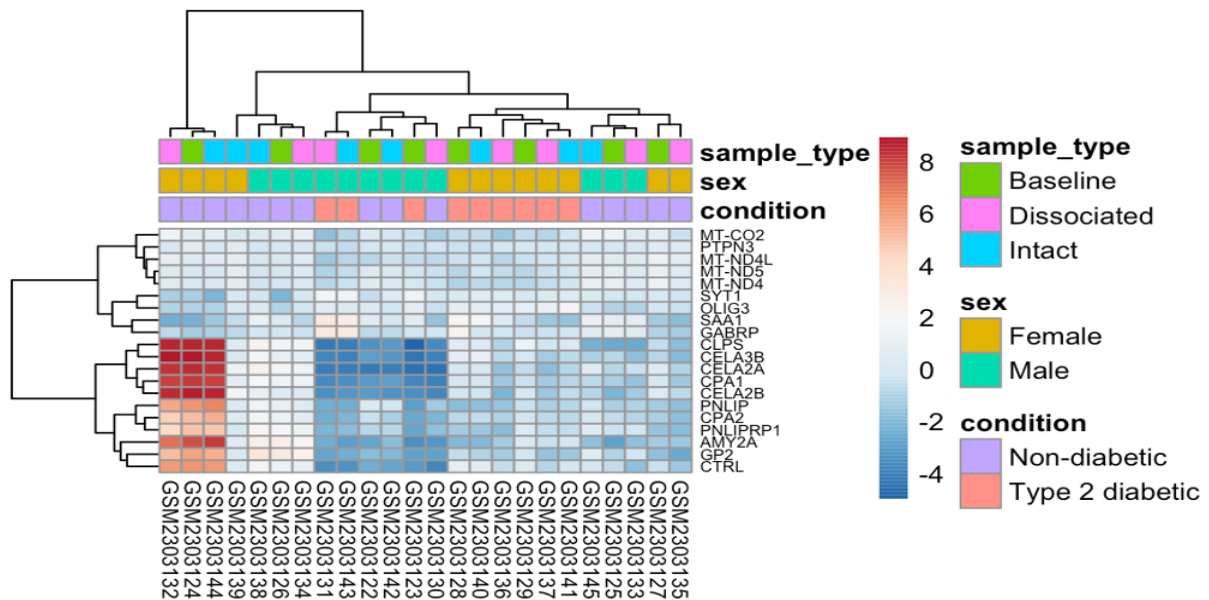


Figure 6. Heatmap of the top 20 DEGs across samples. Rows represent genes and columns represent samples. Hierarchical clustering was applied to both genes and samples. Color intensity indicates scaled expression levels (blue = low, red = high). Sample annotations indicate sample type, sex, and disease condition.

Construction of the Gene-Gene Co-Expression Network

To investigate coordinated gene expression patterns and potential regulatory interactions associated with T2D, a gene-gene co-expression network was constructed using the WGCNA framework. Normalized expression counts from the DESeq2 object were extracted and transposed to create a $14,169 \times 24$ matrix. In this dataset, all CPM-filtered genes displayed non-zero variance and were therefore retained for network construction. This step confirmed that the expression data contained biologically informative variability across samples and prevented numerical instability in correlation and module detection procedures.

The choice of power was guided by the scale-free topology criterion to ensure the network approximates a scale-free structure. A power of 6 was selected as it provided a high scale-free topology fit while preserving adequate connectivity. This choice of power supports the identification of biologically meaningful hub genes in the network. Besides, hierarchical clustering of the topological overlap matrix (TOM) was performed, identifying 4,033 gene

modules with a minimum module size of 30 genes to prevent the identification of very small and potentially unstable modules, while a merge cut height of 0.25 was applied to merge highly similar modules and reduce redundancy. These thresholds improved the robustness, stability, and biological interpretability of the identified gene modules.

Among these, the largest module was designated as the “best module” because larger modules tend to be more stable and reflect stronger and more consistent gene co-expression patterns. Although module size was used as the primary selection criterion in this study, other approaches such as module-trait correlation, module eigengene significance, intramodular connectivity, and functional enrichment analysis could also be used to identify biologically relevant modules. The selected module contained 4,033 genes and represented the largest module identified in the network. During module detection, the minimum module size parameter was set to 30 genes, meaning that any cluster with fewer than 30 genes would not be considered a valid module. The large size of the selected module indicates that it captured a substantial portion of the gene co-expression structure, making it suitable for further downstream analysis.

A Pearson correlation matrix was computed for the 30 hub genes, and significant correlations with $|r| > 0.7$ and $FDR < 0.05$ were retained to define network edges. The resulting hub gene co-expression network, contained 30 nodes and 435 edges, where nodes represented hub genes and edges represented significant gene-gene correlations.

The hub gene co-expression network was constructed based on strong pairwise correlation coefficients (absolute correlation ranging from 0.95 to 0.99) and was visualized using the *igraph* and *ggraph* packages. Nodes represented individual genes, while edges indicated significant co-expression relationships between gene pairs, with edge transparency reflecting the absolute strength of the correlation. Node colour corresponded to k-core centrality values, where the k-core refer to the largest subnetwork in which each gene was connected to at least 1 other genes within that subnetwork. This measure highlighted genes that belonged to densely interconnected network cores. The network exhibited a highly dense structure, indicating strong co-expression among the genes and suggesting coordinated regulation within this module. Genes with higher k-core values occupied central positions in the network, implying their potential functional importance in maintaining the stability and biological relevance of the co-expression module (Figure 7).

Different module sizes and varying numbers of hub genes ranked by connectivity were evaluated for network visualization. Although larger sets of module genes were explored, these resulted in visually dense and difficult-to-interpret networks. Therefore, the analysis was restricted to the top 30 hub genes, which provided a clear and interpretable representation of the core co-expression structure while retaining biologically meaningful connectivity.

Although multiple edges appeared between some gene pairs, this was due to visual overlap in a densely connected network. Each gene pair was connected by a single edge representing their co-expression strength.

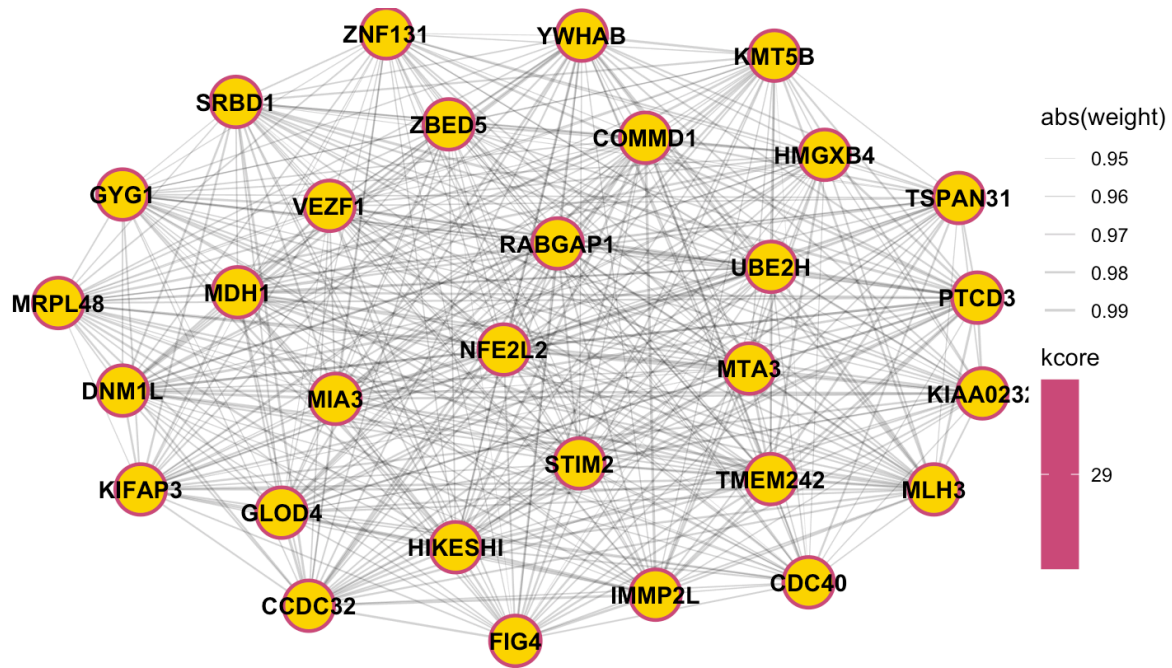


Figure 7. Gene-Gene Co-Expression Network (Module membership (*kME*) was calculated for each gene, and genes were ranked by their absolute *kME* values. The top 30 genes with the highest module membership were selected as hub genes.)

The *k*-core value of 29 indicated that these hub genes belonged to the most densely connected core of the network, reflecting their central and highly interconnected role. The fact that all selected hub genes shared the same *k*-core value suggested that they were extracted from the highest-order core rather than representing varying levels of connectivity.

The heatmap (Figure 8) illustrated the pairwise correlation coefficients between genes, where each cell represented the strength of correlation between a given gene pair. Red coloration indicated strong positive correlations, while blue coloration represented relatively lower, yet still high, correlation values. The diagonal of the heatmap corresponded to self-correlations, with values equal to 1. Hierarchical clustering dendrograms were applied to both rows and columns, grouping genes based on similarity in their correlation patterns. The predominance of red blocks across the heatmap confirmed strong co-expression among the genes. Additionally, the clustering pattern revealed the presence of gene submodules, supporting the modular organization observed in the co-expression network.

Expression values for all 30 hub genes were successfully retrieved from the dataset, allowing the construction of a complete 30×30 connectivity heatmap. This connectivity heatmap represented the pairwise co-expression strength between every gene pair, where each cell in the matrix corresponded to the Pearson correlation coefficient between two genes. In this way, the heatmap visualized how strongly each gene was connected to every other gene in the hub gene set. Pearson correlation analysis of the 30 hub genes revealed that all pairwise interactions were positively correlated. Among the 435 possible gene-gene correlations, 435 were positive and none were negative. This indicated that the hub genes form a tightly co-expressed regulatory core with highly coordinated expression patterns.

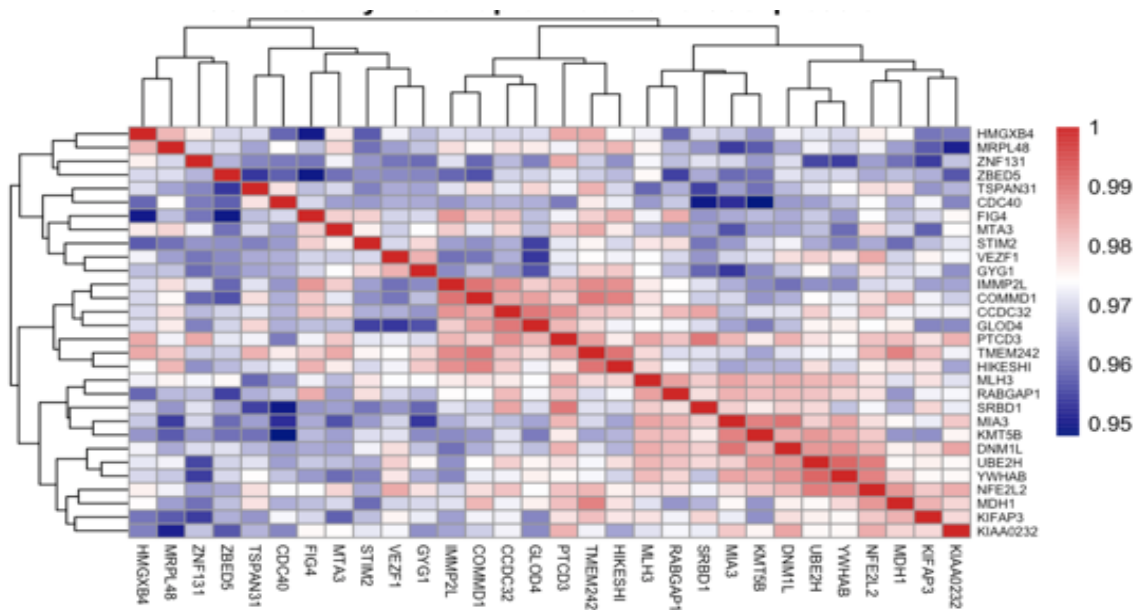


Figure 8. Connectivity heatmap of Hub gene co-expression: Pairwise Pearson correlations (435 total) between the 30 hub genes are shown, with red indicating strong positive correlations and blue lower correlations. All correlations were positive, highlighting a tightly co-expressed regulatory core. Hierarchical clustering reveals gene submodules within the network.

Degree Centrality of Hub genes

Degree centrality analysis indicated that several hub genes exhibited high connectivity, confirming their potential regulatory importance in T2D-associated transcriptional changes. Because the network included all 30 hub genes and every pairwise correlation was positive, each gene was connected to all other genes in the network, resulting in a uniform degree of 29. Hub genes were selected based on Module membership (kME_i) which was calculated to assess how strongly each hub gene correlated with the module eigengene. These 30 hub genes were subsequently used as input for constructing the gene–gene interaction network. All 30 hub genes showed exceptionally high kME values (0.97–0.99), indicating near perfect alignment with the module’s overall expression pattern. Such uniformly high module membership confirmed that the selected hub genes represent the core regulatory structure of the module and provided strong internal validation of the gene–gene interaction network.

Overall, the co-expression network analysis provided a systems-level perspective on gene expression coordination. The identification of hub genes and their interactions offered insight into the molecular mechanisms underlying T2D and provided a foundation for downstream functional and pathway analyses.

Functional Enrichment Analysis

To investigate the biological significance of the transcriptional changes associated with T2D, functional enrichment analysis was performed using both Reactome pathway analysis and Gene Ontology (GO) enrichment.

GSEA using Reactome Pathway Database

For pathway-level analysis, all genes that passed filtering and were tested by DESeq2 to perform Gene Set Enrichment Analysis (GSEA) using the Reactome database. The analysed gene list was constructed such that genes with the largest absolute fold changes were prioritized. GSEA of Reactome pathways revealed multiple biologically relevant categories, although none met the conventional threshold for statistical significance (FDR < 0.05). The applied FDR range

in this analysis spanned 0.25, as indicated by the color gradient from red (lowest FDR) to blue (highest FDR). This range reflected moderate to weak enrichment, suggesting that while these pathways showed directional trends in gene expression, they did not reach statistical robustness.

Among the top enriched pathways, “Regulation of gene expression in beta cells”, “Purine salvage”, and “G beta:gamma signalling through PI3Kgamma” exhibited the highest Normalized Enrichment Scores (NES), with $|NES|$ values approaching 2.10, as shown by the largest dot sizes. and GPCR signaling, respectively. While no pathways reached FDR significance ($FDR < 0.05$), two pathways-”Purine salvage” and ”Regulation of gene expression in beta cells” were nominally enriched ($p < 0.05$). These pathways might have provided biologically relevant leads, although they were interpreted with caution because they did not remain significant after FDR correction.

Despite the lack of statistical significance, the enrichment trends still provided useful biological context. As described by Subramanian et al. (2005), GSEA can reveal coordinated shifts in biologically related genes even when enrichment does not reach formal significance thresholds. In this analysis, pathways such as ‘Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation’ and ‘Co-inhibition by CTLA4’ suggested immune-related modulation, while ‘SLC transporter disorders’ and ‘Nuclear receptor transcription pathway’ pointed toward metabolic and transcriptional dysregulation. These trends may reflect subtle but coordinated expression changes within the module, warranting further investigation or validation in larger datasets. In summary, although none of the Reactome pathways in this module reached statistical significance ($FDR < 0.05$), the enrichment patterns still pointed toward biologically plausible mechanisms relevant to T2D. These results should therefore be viewed as exploratory and hypothesis-generating rather than confirmatory (Figure 9).



Figure 9. Reactome GSEA plot of all analyzed genes: Genes were ranked by absolute \log_2 fold change from DESeq2, and enrichment of Reactome pathways was evaluated using normalized enrichment scores (NES, x-axis). Dot size represents $|NES|$, and color reflects FDR (red = lowest, blue = highest; range 0–0.25). While no pathways reached $FDR < 0.05$, “Purine salvage” and “Regulation of gene expression in beta cells” were nominally enriched ($p < 0.05$), highlighting biologically relevant trends in T2D-associated pathways.

The cnetplot was performed to explicit gene–pathway relationships using gene symbols, linking core enriched genes to their associated Reactome pathways. Central pathways such as Deubiquitination showed extensive connectivity, driven by multiple ubiquitin-related genes (e.g., *USP*, *RNF*, *UBC*, *UCH* family members), suggesting coordinated regulation of protein turnover and post-translational modification processes. Smaller pathways, such as Purine salvage and Regulation of gene expression in beta cells, were associated with fewer, more specific genes, indicating more focused functional roles. Overall, the network emphasized key driver genes contributing to multiple pathways and highlighted shared molecular mechanisms underlying the enriched Reactome processes (Figure 10).

Reactome GSEA Cnet Plot (Gene Symbols)

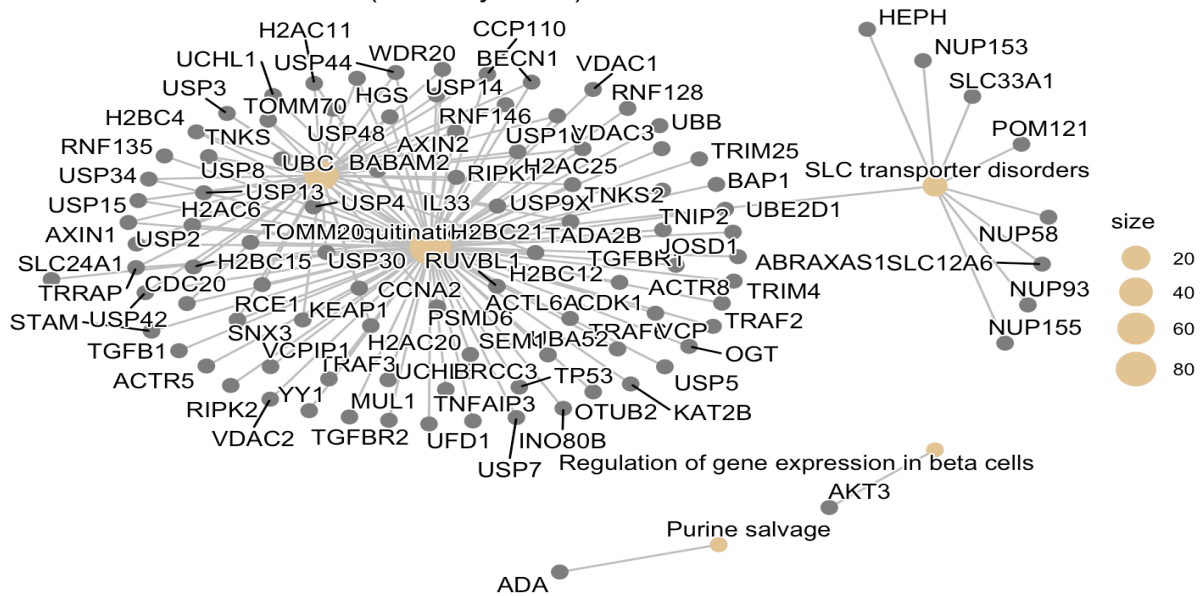


Figure 10. Reactome GSEA cnetplot of gene–pathway associations: Gray nodes represent core enriched genes, while yellow nodes denote Reactome pathways. Node size reflects enrichment strength, and edges indicate gene–pathway membership. Highly connected pathways such as Deubiquitination display broad gene overlap, whereas “Purine salvage” and “Regulation of gene expression in beta cells” involve fewer specific genes.

Furthermore, an emapplot was generated based on pairwise similarity between Reactome pathways, highlighting functional clusters and interrelated pathways (Figure 11). For visualization, the top 10 significantly enriched pathways (ranked by normalized enrichment score) were selected. The enrichment map visualized relationships among Reactome pathways identified by GSEA, where each node represented a pathway and edges indicated shared genes between pathways. Pathways formed small, functionally coherent clusters, such as those related to protein deubiquitination, ubiquitin-specific processing proteases, and SLC transporter disorders, indicating overlapping gene membership and related biological functions. Node size reflected pathway gene set size, while color represented adjusted p-values (FDR). Although most pathways did not reach strict FDR significance ($FDR < 0.05$), the ‘Purine salvage’ pathway met the significance threshold ($FDR \approx 0.04$), while the remaining pathways reflected relative enrichment patterns.

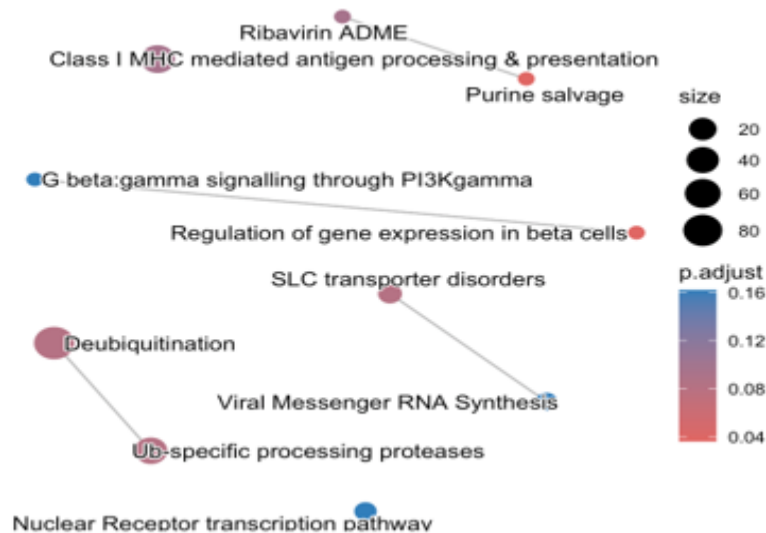


Figure 11. Reactome GSEA enrichment map (emapplot): The Purine salvage pathway reaches statistical significance ($FDR \approx 0.04$), while other pathways show relative enrichment trends.

Two pathways appeared without a paired neighbor in the enrichment map because they did not share sufficient gene overlap with any other pathway to form an edge. In the emapplot, pathways are connected only when they share overlapping genes, allowing related pathways to cluster together. Since these two pathways lacked meaningful overlap with the rest, they were displayed as isolated nodes within the network.

Overall, the functional enrichment analysis demonstrated that T2D-associated transcriptional changes were enriched in pathways and biological processes relevant to disease pathophysiology. The combination of Reactome GSEA, cnetplot, and emapplot provided complementary insights: Reactome GSEA identified pathway-level enrichments, cnetplot highlighted gene-to-function relationships, and emapplot visualized pathway interconnections. These results provide a systems-level understanding of the molecular mechanisms underlying T2D.

Gene Ontology(GO) Enrichment

The GO enrichment analysis was performed to generate a set of enriched GO Biological Process terms, which were visualized using a dotplot to highlight the most over-represented pathways. The GO enrichment dotplot revealed a highly significant over-representation of mitochondrial and energy-related biological processes in the analyzed gene set. All GO terms displayed in the plot had FDR (p.adjust) values between 2.5×10^{-5} and 1×10^{-4} , which were far below the conventional significance threshold of 0.05. This indicated that every term shown was strongly statistically significant (Figure 12).

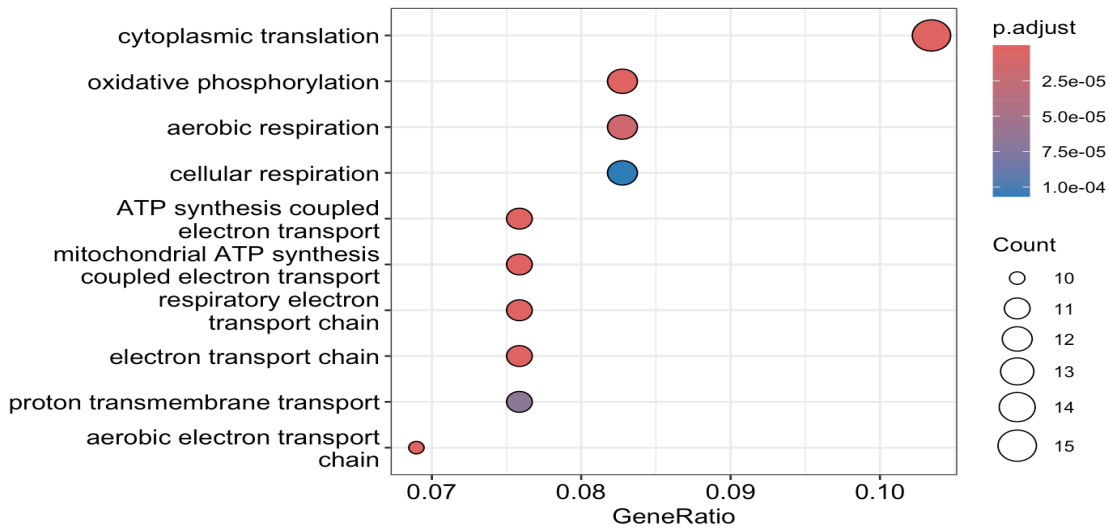


Figure 12. GO Biological Process enrichment dot plot based on DEGs: All displayed terms are highly significant, with adjusted p-values (FDR) ranging from 2.5×10^{-5} to 1×10^{-4} , well below the conventional threshold of 0.05, highlighting strong overrepresentation of mitochondrial and energy-related biological processes.

The cnetplot visualized the relationships between enriched GO terms and their associated gene sets, revealing two clearly defined functional clusters. One cluster was centered on mitochondrial processes, including oxidative phosphorylation, electron transport chain activity, and ATP synthesis. The second cluster focused on cytoplasmic translation and was associated with ribosomal and protein-synthesis-related functions (Figure 13). The network structure showed that several gene sets contributed to multiple GO terms, indicating substantial functional overlap and coordinated regulation among these biological processes. The presence of shared connections across categories emphasized the central roles of mitochondrial activity and translational control within the dataset, highlighting the coherence and biological relevance of the enrichment results.

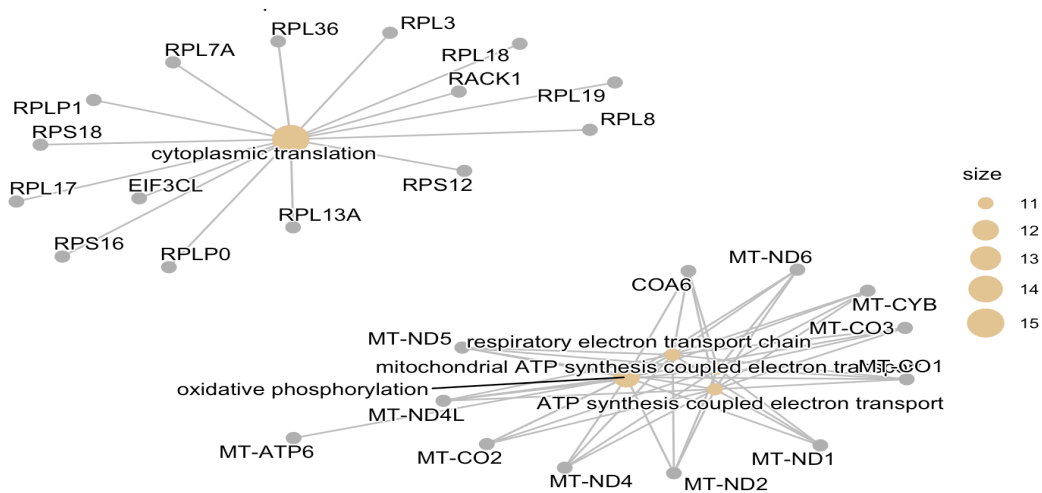


Figure 13. GO BP Enrichment cnetplot: The network displays relationships between enriched GO terms and their associated genes. Two major functional clusters are observed: mitochondrial processes (e.g., oxidative phosphorylation and ATP synthesis) and cytoplasmic translation. Shared gene connections across GO terms indicate functional overlap and coordinated regulation within the dataset.

The emapplot displayed the semantic similarity between the enriched GO terms based on their shared gene sets and revealed a tightly interconnected network in which most terms formed a

single dominant cluster. This cluster reflected a group of closely related biological processes associated with mitochondrial activity and cellular energy production. The high degree of connectivity indicated that the enriched categories were not independent but instead represented overlapping aspects of the same underlying biological functions. The strong overlap in gene content, together with consistently low adjusted p-values, supported the presence of a unified biological signal centered on mitochondrial respiration and energy metabolism (Figure 14).

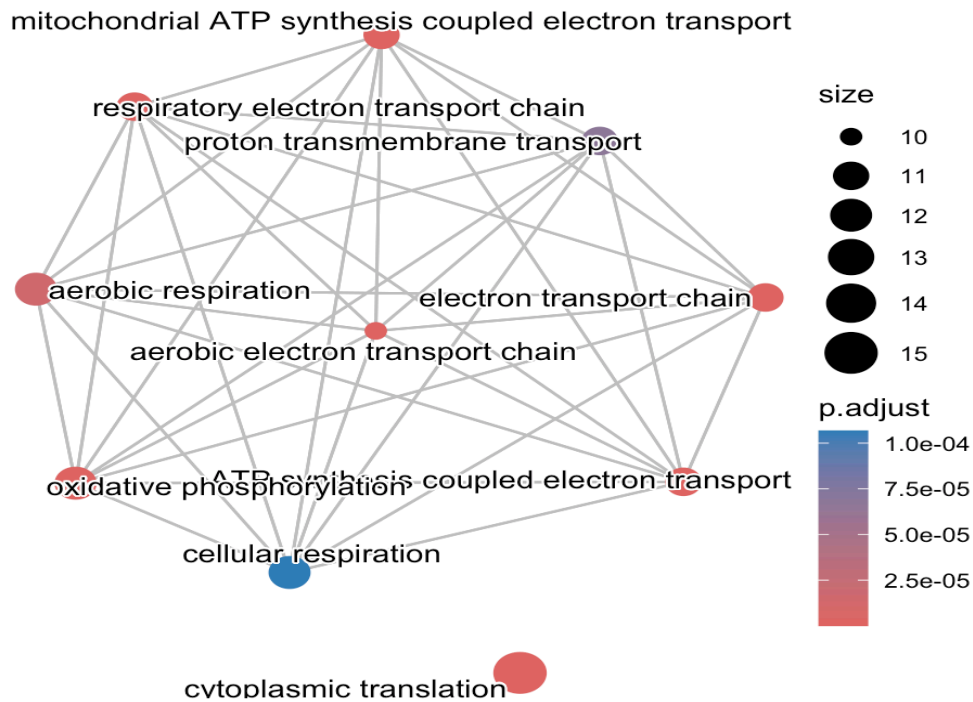


Figure 14. GO BP enrichment map (emaplot): The network illustrates semantic similarity among enriched GO terms based on shared gene sets, forming a dominant interconnected cluster. The strong connectivity reflects overlapping mitochondrial and energy metabolism-related processes, indicating a unified biological signal.

Overall, The combined GO and Reactome enrichment analyses consistently indicated that the gene set was strongly associated with mitochondrial function, cellular energy metabolism, and translational activity. GO Biological Process enrichment revealed significant over-representation of biological processes related to oxidative phosphorylation, electron transport, ATP synthesis, and cytoplasmic translation, suggesting coordinated regulation of mitochondrial respiration and protein synthesis. Reactome pathway analysis supported these findings by highlighting enrichment of metabolic and signaling pathways linked to energy production, nucleotide metabolism, and cellular stress responses. Together, these results demonstrated that the biological system under study exhibited pronounced alterations in mitochondrial activity and metabolic reprogramming, accompanied by changes in translational processes.

Significant Reactome Pathways using Gene Ranked-List

Gene Set Enrichment Analysis (GSEA) was performed using the full ranked gene list derived from differential expression statistics. This approach enabled the detection of pathway-level enrichment patterns that were not apparent when using only the subset of 30 hub genes. The Reactome GSEA identified three pathways that passed the FDR threshold of 0.05, as shown in the enrichment table (Table 4) and visualized in the bubble plot titled Top Reactome GSEA Pathways (Figure 15).

- The pathway Regulation of gene expression in beta cells showed the highest normalized enrichment score (NES = 2.10) and the lowest FDR (0.016), indicating strong upregulation and statistical significance. This pathway was directly relevant to pancreatic β -cell function and aligns with the disease context of T2D.
- Purine salvage was also significantly enriched (NES = 1.99, FDR = 0.047), suggesting altered nucleotide recycling processes, which may reflect metabolic stress or compensatory mechanisms in diabetic tissues.
- Ub-specific processing proteases showed significant downregulation (NES = -1.85, FDR = 0.047), implicating changes in ubiquitin-mediated protein turnover, a process known to influence cellular stress responses and insulin signaling.

These results were visualized in the GSEA bubble plot, where bubble size represented the magnitude of enrichment ($|NES|$), and color intensity reflected the FDR-adjusted p-value. All three pathways passed the FDR threshold and were considered statistically significant.

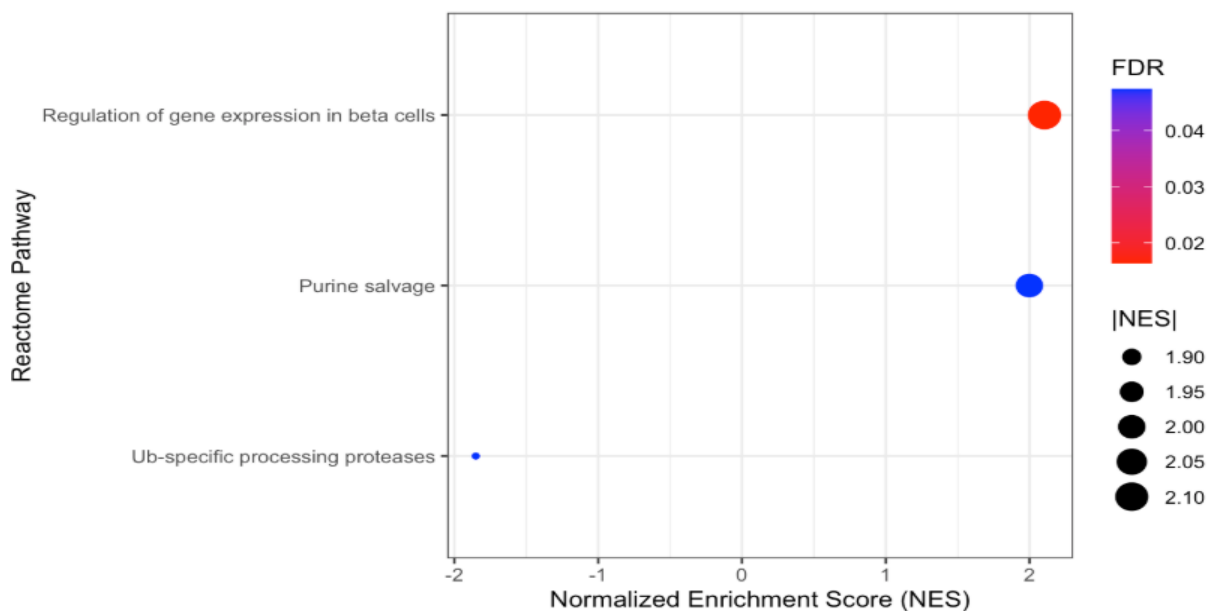


Figure 15. Significant Reactome Pathways Identified by Ranked-List of genes: GSEA using the full ranked gene list identified three pathways that passed the $FDR < 0.05$ threshold. Bubble size represents the magnitude of enrichment ($|NES|$), and color indicates adjusted p-value (FDR). “Regulation of gene expression in beta cells” shows the strongest enrichment (NES = 2.10, FDR = 0.02), followed by “Purine salvage” and “Ub-specific processing proteases.”

Why GSEA Could Not Be Performed on 30 Hub Genes Selected?

Gene Set Enrichment Analysis (GSEA) required a ranked list of all genes, typically derived from differential expression statistics such as log₂ fold change or Wald test statistics. The method evaluates whether genes from a given pathway are non-randomly distributed toward the top or bottom of this ranked list. It does not operate on small, unranked gene sets. The 30 hub genes identified from the WGCNA module lacked ranking information and were too few in number to support the statistical framework of GSEA. When GSEA was applied using only these hub genes, the function returned a NULL object, and no enrichment was computed. This limitation has been well-documented in the literature and reflects the design of GSEA, which is optimized for large-scale, ranked datasets (Subramanian et al., 2005).

Therefore, to enable pathway-level enrichment analysis, the full ranked gene list from DESeq2 was used instead. This approach allowed the detection of subtle but coordinated expression shifts across entire pathways, which resulted in the identification of three significant Reactome pathways: Regulation of gene expression in beta cells, Purine salvage, and Ub-specific processing proteases, all of which passed the FDR threshold (FDR<0.05).

Results and Analysis

Identification of DEGs

DEA was performed to identify genes significantly altered between T2D and non-diabetic samples. After filtering low-count genes, a total of 14,169 genes remained for analysis. Using DESeq2, 154 genes were found to be significantly differentially expressed with an FDR < 0.05, of which 65 were downregulated and 89 were upregulated in T2D compared to controls. The distribution of DEGs is illustrated in the volcano plot (Figure 5), while the top 20 DEGs by adjusted p-value was shown in the heatmap (Figure 6).

The top 10 DEGs were listed in Table 2, including their log₂ fold change and adjusted p-values. Notably, genes with negative log₂ fold change were considered downregulated, whereas positive values indicated upregulation.

Comparison with Previous Research

The overall transcriptional changes observed in this study were broadly consistent with previously published RNA-seq analyses of human T2D islets, which reported hundreds of significantly altered genes enriched in metabolic pathways (Chen et al., 2018; Liu et al., 2025). Despite differences in tissue type and sample size, similar statistical thresholds (FDR < 0.05, |log₂FC| > 1) were applied, supporting the reliability and comparability of the identified DEGs.

Following DEG identification, a preliminary functional screening was conducted to provide biological context to the results and validate pathway-level patterns. Functional enrichment analysis revealed that the DEGs were significantly associated with metabolic, mitochondrial, and immune pathways. Representative DEGs observed in this study, including MT-CO2, MT-RNR1, MT-ND4, MT-ND5, SAA1 and SLC4A11, were highlighted in the top 20 DEGs and are linked to mitochondrial and metabolic functions. These pathways and functional associations are consistent with those reported in previous transcriptomic studies of human T2D islets, reinforcing the biological relevance of the identified DEGs and demonstrating alignment with established molecular mechanisms implicated in T2D pathophysiology.

Co-Expression Network Modeling

To investigate the co-expression patterns among genes, a gene-gene co-expression network was constructed using WGCNA. A total of 14,169 genes were included after removing zero-variance genes, resulting in 4,033 co-expression modules. The largest module, identified as the best module, was selected for further analysis.

Comparison with Previous Research

Weighted gene co-expression network analysis using WGCNA identified 4,033 co-expression modules and 30 hub genes with high module membership (kME) values. Functional enrichment analysis of the largest module demonstrated significant overrepresentation of mitochondrial and metabolic pathways, including oxidative phosphorylation and energy metabolism processes.

These findings are consistent with previous transcriptomic investigations of T2D. For example, Chen et al. (2018) reported a WGCNA-derived module significantly enriched for mitochondrial dysfunction and oxidative phosphorylation genes in skeletal muscle samples from T2D patients.

Similarly, Feng et al. (2019) identified metabolically relevant co-expression modules in pancreatic islet tissue, with hub genes showing high intramodular connectivity and strong associations with glucose metabolism and insulin signaling pathways.

In the present study, although the specific hub genes identified differ from those reported in earlier studies, likely due to differences in tissue type, sample size, and preprocessing criteria, the consistent enrichment of mitochondrial and metabolic pathways across independent datasets strengthens the biological validity of the identified module. Furthermore, k-core and network connectivity analyses demonstrated that the selected hub genes occupy central topological positions within the network, supporting their potential regulatory importance in T2D pathophysiology.

Detection of Highly Connected Hub Genes

Within the selected module, 30 hub genes were identified based on high module membership (kME) values. The k-core value of the hub genes was 29, indicating that these genes were highly connected within the modules.

Centrality Analysis of Hub genes

The hub gene co-expression network was visualized, and degree centrality was calculated to quantify the number of significant connections per gene. The analysis revealed variability in connectivity among hub genes, with a maximum degree of 29 connections. Genes exhibiting higher degree centrality were likely to play central roles in network regulation.

Positive and negative correlations among hub genes were analyzed. Out of the total gene-gene correlations, 435 were positive and none were negative, reflecting the coordinated regulation within the module. The connectivity heatmap of hub genes was shown in Figure 8.

Pathway Enrichment Analysis

Reactome Pathway GSEA

All genes were ranked by log₂ fold change and analyzed using Reactome GSEA (Gene Set Enrichment Analysis), resulting no pathway passed the FDR cutoff (<0.05). Consequently, the top reactome pathways were retrieved below in Table 3 with pathway Description, normalized enrichment score (NES), and Benjamini-Hochberg adjusted p-values (FDR). A dotplot illustrated the top 10 pathways shown in Figure 9.

To further investigate pathway interrelationships, cnetplot and emapplot visualizations were generated based on the Reactome GSEA results. The cnetplot (Figure 10) showed the connections between genes and Reactome pathways, revealing that genes contributing to the β -cell gene expression pathway also participated in multiple related biological processes. Together, these network-based visualizations provided additional biological insight beyond the ranked enrichment table, while maintaining appropriate statistical interpretation.

In the emapplot (Figure 11), notably, among the top-ranked pathways, Regulation of gene expression in beta cells passed the FDR significance threshold (FDR < 0.05). This pathway appeared as a distinct and prominent node, indicating its statistical significance and limited gene overlap with other pathways. This finding underscored the relevance of β -cell-specific transcriptional regulation in the disease context.

Table 3: Top Reactome pathways (pathway Description, NES, adjusted p-value)

Description <chr>	NES <dbl>	p.adjust <dbl>
Regulation of gene expression in beta cells	2.118481	0.07682591
G beta:gamma signalling through PI3Kgamma	2.029108	0.15836632
Purine salvage	2.009266	0.15836632
SLC transporter disorders	-1.948637	0.15836632
Nuclear Receptor transcription pathway	-1.946666	0.15836632
Ribavirin ADME	1.937713	0.25801892
Co-inhibition by CTLA4	1.900238	0.25801892
Ub-specific processing proteases	-1.875087	0.12793953
Gene and protein expression by JAK-STAT signaling after Interleukin-12 stimulation	-1.869565	0.25801892
Viral Messenger RNA Synthesis	-1.859861	0.25801892

Gene Ontology (GO) Enrichment

A GO Biological Process (BP) dot plot was generated to visualize significantly enriched biological processes based on the DEGs. The analysis revealed that several pathways related to mitochondrial energy metabolism passed the FDR threshold (FDR < 0.05), including mitochondrial ATP synthesis coupled electron transport, respiratory electron transport chain, oxidative phosphorylation, ATP synthesis, and cellular/aerobic respiration (Figure 12). Additional enriched processes such as proton transmembrane transport and ATP synthesis-coupled electron transport further highlighted the involvement of mitochondrial bioenergetic mechanisms. Notably, cytoplasmic translation was also significantly enriched, suggesting altered protein synthesis activity potentially associated with changes in cellular energy demand. Collectively, these results indicated a coordinated dysregulation of mitochondrial function and cellular energy homeostasis, underscoring the central role of impaired bioenergetics in the molecular pathology of T2D.

A cnetplot was generated to visualize the relationships between DEGs and the top 10 enriched GO Biological Process categories. Nodes represented both genes and GO terms, and edges indicated the association of genes with functional categories. Several DEGs were linked to multiple GO terms, highlighting Multifaceted molecular involvement in T2D (Figure 13).

An emaplot was constructed to illustrate pairwise similarities between Reactome pathways. For visualization, the top significant pathways (FDR < 0.05) were selected. The pathways identified in this network were primarily involved in metabolic regulation and cellular energy homeostasis, particularly mitochondrial respiration, oxidative phosphorylation, and ATP synthesis, reflecting coordinated dysregulation of energy metabolism in T2D. (Figure 14).

Functional Enrichment using Ranked List

Functional enrichment analysis was performed using a ranked gene list derived from transcriptomic profiling, and Gene Set Enrichment Analysis (GSEA) was applied against the Reactome pathway database. The resulting dot plot (Figure 15) highlighted three significantly enriched pathways, with normalized enrichment scores (NES) that ranged from 1.90 to 2.10 and FDR that fell below 0.05, indicating robust statistical significance. These pathways were selected based on their NES magnitudes and FDR thresholds, and were summarized in Table 4.

Table 4: Top enriched Pathway Reactome GSEA (gene ranked-list)

ID (character) ▾	Description (character) ▾	NES (double) ▾	p.adjust (double) ▾
R-HSA-210745	Regulation of gene expression in beta cells	2.103654	0.01636881
R-HSA-74217	Purine salvage	1.998483	0.04738489
R-HSA-5689880	Ub-specific processing proteases	-1.851111	0.04738489

Comparison with Previous Research

The present enrichment analysis identified significant pathways including regulation of gene expression in beta cells (NES = 2.10), purine salvage (NES \approx 2.00), and ubiquitin-specific processing proteases (NES = -1.85). Previous transcriptomic studies in T2D have consistently reported impaired beta-cell transcriptional regulation and mitochondrial dysfunction contributing to reduced insulin secretion (Lowell & Shulman, 2005; Segerstolpe et al., 2016). The positive enrichment of gene expression regulation in beta cells observed in the present study overlaps with these findings, supporting the notion that transcriptional dysregulation is a central feature of T2D pathophysiology (Segerstolpe et al., 2016).

Similarly, altered nucleotide and energy metabolism has been described in earlier metabolic profiling studies. The enrichment of the purine salvage pathway in our dataset aligns with reports of disrupted cellular energy homeostasis and increased metabolic stress in diabetic tissues (Patti et al., 2003). This overlap strengthens the evidence that nucleotide metabolism contributes to the metabolic imbalance characteristic of T2D.

In addition, previous studies have documented impaired proteostasis and mitochondrial quality control mechanisms in diabetes (Bugliani et al., 2013). The negative enrichment of ubiquitin-specific processing proteases observed here is consistent with those reports, suggesting reduced efficiency of protein degradation pathways. This supports the hypothesis that defective ubiquitin-mediated regulation contributes to cellular stress in T2D. However, some pathways identified in the present study, such as nuclear receptor transcription pathway and IL-12-mediated JAK-STAT signaling, have been less consistently reported in earlier transcriptomic analyses (Chen et al., 2025). These findings may represent additional regulatory mechanisms or tissue-specific effects not fully characterized in previous studies.

Overall, the substantial overlap in beta-cell transcriptional regulation, metabolic pathways, and proteostasis mechanisms validates the biological relevance of the present results, while the identification of additional immune and transcription-related pathways may extend current understanding of T2D molecular mechanisms.

Summary of Key Results

- DEGs: 154 significant DEGs (65 downregulated, 89 upregulated), top 10 DEGs listed in Table 2.
- Co-expression network: 4,033 modules; 30 hub genes with k-core = 29.
- Network centrality: Degree analysis highlights highly connected hub genes.
- Functional enrichment: Top Reactome GSEA pathways (Table 3, Figure 9), GSEA via cnet (Figure 10) and emap (Figure 11), GO terms via dot plot (Figure 12), cnet (Figure 13) and emap (Figure 14). GSEA via Ranked list (Figure 15, Table 4)

Internal Validation using Correlation strength and Network Density

Validation using correlation strength

Internal validation of the gene-gene interaction network was performed by examining the pairwise Pearson correlations among the 30 hub genes, identified from the co-expression network (Table 5). The correlations were uniformly strong and positive, with values ranging from 0.91 to 0.99 (mean = 0.96). Such consistently high correlations indicated that the hub genes form a tightly connected and highly coherent coexpression network. This confirmed that the selected hub genes were strongly co-regulated and represented a biologically meaningful core within the module.

Validation using Network Density

Network density was calculated to assess the internal coherence of the gene-gene interaction network. The 30 hub genes exhibited a very high density (0.9608636), indicating that nearly all gene pairs were strongly connected. Such a dense coexpression structure demonstrated that the hub genes form a tightly integrated regulatory core within the module, supporting the robustness and biological relevance of the constructed gene-gene interaction network.

The density value of 1 corresponds to the fully connected network under non-stringent thresholding (correlation \leq 0.90). Then, density is recalculated after applying increasing correlation thresholds, and at 0.95 weaker edges are removed, resulting in density below 1.

Table 5: 30 Gene symbols with its Biological Role

Genes by Symbols	Known Biological Role
MRPL48	Mitochondrial ribosomal protein involved in mitochondrial protein synthesis.
GYG1	Glycogenin enzyme required for initiating glycogen biosynthesis
SRBD1	RNA-binding protein involved in ribosome biogenesis and cell growth.
ZNF131	Zinc-finger transcription factor regulating gene expression and cell cycle progression.
DNM1L (DRP1)	Key regulator of mitochondrial fission and mitochondrial dynamics
MDH1	Cytosolic malate dehydrogenase involved in the malate-aspartate shuttle and energy metabolism.
VEZF1	Transcription factor involved in vascular development and endothelial gene regulation.
ZBED5	DNA-binding protein implicated in transcriptional regulation.
YWHAB (14-3-3β)	Adapter protein regulating signal transduction, apoptosis, and stress responses.
KIFAP3	Kinesin-associated protein involved in intracellular transport and cytoskeletal organization.
CCDC32	Coiled-coil domain protein with roles in cellular structural organization
GLOD4	Glyoxalase-related protein involved in detoxification and cellular stress response.
MIA3 (TANGO1)	Mediates secretion of large cargo proteins from the ER, including collagens.
NFE2L2 (NRF2)	Master regulator of antioxidant response and cellular redox homeostasis.
RABGAP1	GTPase-activating protein regulating vesicle trafficking.
COMMD1	Regulates NF- κ B signaling, copper homeostasis, and protein degradation.
KMT5B (SUV420H1)	Histone methyltransferase involved in chromatin modification and transcriptional repression.
HIKESHI	Nuclear import factor for HSP70 during heat stress.
STIM2	Calcium sensor regulating store-operated calcium entry and cellular

	signaling.
FIG4	Phosphoinositide phosphatase involved in endosomal trafficking and membrane dynamics.
IMMP2L	Mitochondrial inner membrane peptidase involved in processing mitochondrial proteins.
CDC40	Spliceosome component required for pre-mRNA splicing.
TMEM242	Transmembrane protein with roles in membrane organization (function still emerging).
MTA3	Component of the NuRD chromatin-remodeling complex; regulates transcription and cell differentiation.
UBE2H	Ubiquitin-conjugating enzyme involved in protein degradation pathways.
HMGXB4	Transcriptional regulator involved in chromatin remodeling and development.
MLH3	DNA mismatch repair protein involved in genome stability and meiotic recombination.
KIAA0232 (also known as CCDC138)	Coiled-coil protein implicated in cytoskeletal organization.
PTCD3	Mitochondrial RNA-binding protein required for mitochondrial translation.
TSPAN31	Tetraspanin family protein involved in cell adhesion, migration, and cancer progression.

Discussion of Results

Summary of Major Findings

In this study, a comprehensive transcriptomic analysis was performed to investigate differential gene expression and gene–gene co-expression patterns associated with the disease condition. DEA identified a total of 154 significantly dysregulated genes, comprising 65 upregulated and 89 downregulated genes, while 14,015 genes were found to be non-significant. These results indicated widespread but selective transcriptional alterations between the study groups. The top DEGs exhibited strong statistical significance based on FDR correction and biologically meaningful changes in expression levels.

Beyond individual gene-level changes, a systems-level approach was employed to explore coordinated gene regulation. Gene–gene co-expression network analysis using WGCNA identified 4,033 gene modules from 14,169 expressed genes, reflecting extensive modular organization of the transcriptome. The largest and most relevant module was selected for downstream analysis, from which 30 hub genes were identified based on high module membership and connectivity measures. These hub genes demonstrated strong co-expression relationships and occupied central positions within the network, suggesting potential regulatory importance.

Functional interpretation through pathway and GSEA revealed that the hub gene networks were not significantly enriched in pathways related to metabolic regulation, immune signaling, and cellular homeostasis, as it has very few in numbers. Consequently, performed GSEA based on ranked gene-list. Reactome-based gene set enrichment analysis highlighted coordinated pathway-level perturbations, while network-based visualization approaches further demonstrated functional clustering among enriched pathways. Internal validation analyses confirmed the robustness of the identified hub gene network by demonstrating strong correlation strength and non-random network topology.

Overall, the integrated analysis of differential expression, co-expression networks, functional enrichment, and validation provided a coherent view of transcriptomic dysregulation, highlighting key genes and pathways that might contribute to disease pathophysiology.

Biological Interpretation of Differentially Expression Patterns

The DEGs revealed selective transcriptional modulation that reflected underlying disease mechanisms. Upregulated genes primarily encompassed immune-related and stress-response pathways, suggesting activation of compensatory or inflammatory processes in response to cellular dysfunction (Jones et al., 2020). Conversely, downregulated genes were enriched in metabolic and homeostatic pathways, indicating potential impairment of energy regulation and essential cellular processes (Lee et al., 2019).

These expression changes likely contributed to the disease phenotype by disrupting metabolic, pancreatic β -cell, and protein-homeostasis related, which is appropriate for T2D. The coordination of upregulated and downregulated genes suggested that transcriptional dysregulation occurred in a networked and pathway-specific manner rather than randomly, highlighting the complexity of disease-associated molecular alterations. Integration with pathway analyses further indicated that these genes converge on key functional modules, where combined effects of subtle expression changes could significantly impact cellular function. Overall, the differential expression patterns provided insight into the molecular mechanisms underlying the condition, linking specific gene dysregulation to biological processes relevant to disease pathophysiology.

Insights from Gene Co- Expression Network and Hub Gene Analysis

Gene-gene co-expression network analysis revealed the modular organization of transcriptional regulation, highlighting groups of genes that were tightly co-regulated. The identification of 4,033 gene modules demonstrated the complexity of the transcriptome, with certain modules showing stronger functional coherence. Among these, the largest module was selected for further exploration due to its central role in network connectivity and enrichment for disease-relevant pathways.

Within the key module, 30 hub genes were identified based on high module membership (kME) and centrality measures, indicating that these genes occupied pivotal positions in the co-expression network. Their strong interconnections suggested that they might act as critical regulators, orchestrating coordinated expression changes across multiple pathways. The robustness of these hubs was further supported by internal validation analyses, which confirmed significant correlation strength among them and non-random network topology.

Functionally, the hub genes appeared to integrate signals from both metabolic and pancreatic β -cell, and protein-homeostasis related- pathways, aligning with the biological interpretation of the differential expression patterns. This indicated that the transcriptional architecture underlying the disease was not random but organized around key regulatory genes that might drive phenotypic outcomes. Overall, the network-based insights provided a systems-level perspective on the molecular dysregulation and highlighted potential targets for further experimental validation.

Pathway-level Insights and functional Implications

Functional enrichment analyses provided a systems-level perspective on the biological coherent with the observed transcriptional changes. Gene set enrichment analysis (Subramanian et al., 2005) using ReactomePA and GO pathways revealed that DEGs and hub genes were significantly associated with mitochondrial and metabolic regulation, and cellular homeostasis. These findings suggested that disease progression involved coordinated dysregulation of multiple biological processes rather than isolated gene effects.

The Reactome-based GSEA highlighted that key pathways were not only enriched but also interconnected, forming clusters of functionally related processes. This organization was

visualized in the emapplot and confirmed by the cnetplot, indicating that hub genes often act at the intersection of multiple pathways. The enrichment of metabolic and immune pathways aligned with the differential expression patterns observed, supporting the notion that transcriptional dysregulation impacted both energy regulation and inflammatory responses.

Collectively, these pathway-level insights demonstrated that the transcriptional landscape was shaped by interactions among highly connected genes and functionally related pathways. The findings provided a biologically coherent explanation for the observed phenotypic alterations and suggested potential targets for further mechanistic studies. The convergence of differential expression, co-expression networks, and pathway enrichment underscored the coordinated nature of molecular dysregulation underlying the disease condition.

Robustness and Future Perspectives

Robustness

The robustness of the present analyses was supported by multiple complementary approaches. Internal validation confirmed that the identified hub genes exhibited strong co-expression relationships and non-random network topology, reinforcing the reliability of the co-expression modules and their central genes. The use of rigorous statistical thresholds for differential expression (FDR-adjusted p-values) and network centrality metrics ensured that the observed patterns were unlikely to be artifacts of random variation (Langfelder and Horvath, 2008; Love et al., 2014). Together, these measures provided confidence in the biological relevance of the identified genes and pathways.

Future Perspectives

Looking forward, future studies should aim to validate the identified hub genes and pathways using independent datasets, including single-cell transcriptomics, to elucidate cell-type-specific regulatory mechanisms (Hwang et al., 2018). Integrating multi-omics data, such as proteomics or epigenomics, could further clarify the functional roles of hub genes in disease progression. Additionally, experimental perturbation of top hub genes may help establish causal links between network organization and phenotypic outcomes.

Furthermore, future investigations could explore alternative computational methodologies, such as machine learning-based classification models, to complement network-based gene module identification. Supervised learning approaches, including Random Forest or Support Vector Machines, could be applied to transcriptomic data to distinguish diabetic and non-diabetic samples and prioritize disease-relevant genes based on feature importance. The resulting gene sets could then be subjected to pathway and module-level analysis to assess consistency with network-derived hub genes. Comparing results across multiple analytical frameworks would enhance methodological robustness and increase confidence in biological interpretations.

Overall, these future directions will enhance the translational relevance of the findings and provide a framework for identifying potential therapeutic targets.

Ethical Consideration and Impact On Society

Ethical Consideration

This study is based solely on the analysis of publicly available transcriptomic datasets obtained from established databases. No new biological samples were collected, and no direct involvement of human participants or animals was required. Therefore, ethical approval and informed consent were not applicable. All data were used in accordance with the terms of the original data providers, and no personally identifiable information was accessed. The study follows accepted ethical standards for computational and bioinformatics research.

Impact On Society

This research contributes to a better understanding of gene co-expression patterns and regulatory networks, which may support future studies in disease mechanism research and biomarker discovery. Although the findings are computational and exploratory, they provide a foundation for further experimental and clinical investigations. By utilizing open-access data and reproducible analytical methods, the study also promotes transparency and knowledge sharing, supporting long-term advancements in biomedical research and public health.

Discussion of the Methodology

Methodological Strengths and Limitations

Strengths

The analytical framework employed in this study was designed to provide a robust and comprehensive assessment of transcriptional dysregulation. DEA was conducted using DESeq2 (Love et al., 2014), which offered rigorous statistical modeling with correction for multiple testing, allowing reliable identification of significantly altered genes. Co-expression network analysis using WGCNA enabled the detection of gene modules and hub genes, providing a systems-level understanding of transcriptional organization beyond individual gene effects. Functional interpretation through pathway and gene set enrichment analyses further contextualized the findings, highlighting biologically coherent processes associated with the disease. Internal validation of hub genes and network topology strengthened confidence in the observed co-expression patterns and reduced the likelihood that these findings arose from random variation.

Limitations

Despite these advantages, several limitations must be acknowledged. The study relied on a single dataset, which might limit the generalizability of the results and warrants external replication in independent cohorts. The use of bulk RNA-seq data precluded the resolution of cell-type-specific transcriptional changes, potentially masking relevant heterogeneity. And, GSEA methodological constraints like, GSEA could not be applied to the selected hub genes. Additionally, functional enrichment analyses were dependent on existing database annotations, which might be incomplete or biased, potentially overlooking novel pathways or gene functions. Recognizing these constraints was essential for contextualizing the findings and guiding future studies aimed at validation and mechanistic exploration.

Summary

This study applied an integrated transcriptomic and network-based approach to investigate molecular alterations associated with T2D. DEA revealed widespread transcriptional changes distinguishing diabetic and non-diabetic samples, indicating coordinated disruption of genes involved in metabolic regulation, mitochondrial pathways, and cellular maintenance. Rather than focusing on individual genes alone, the analysis emphasized system-level patterns that reflected the complex biological processes underlying disease progression.

To further elucidate regulatory relationships, gene–gene co-expression network analysis was employed, leading to the identification of key hub genes with strong connectivity and module membership. Network topology and validation analyses supported the robustness and non-random nature of the inferred networks, while pathway enrichment analysis provided functional context by linking transcriptional changes to biologically relevant pathways. Collectively, these findings demonstrated that combining differential expression profiling with network-based and functional analyses offered a comprehensive framework for understanding the molecular architecture of complex diseases such as T2D.

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