Title: Human Gene Expression Profile Analysis of Insomnia and Pre-insomnia Disorders: A Cellular Study

Running Title: Human Gene Expression Profile Analysis of Insomnia

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Abstract

Background: Sleep is an essential process for restoring brain function and is recognized as a fundamental aspect of physical and mental health. The aim of this study is to assess the molecular mechanisms of insomnia disorder and to identify the key dysregulated genes associated with it.
Methods: To study molecular mechanisms of insomnia, GSE208668 was selected from the Gene Expression Omnibus (GEO) database. Total RNA from peripheral blood mononuclear cells (PBMCs) of 17 individuals with insomnia disorder was analyzed and compared to 25 controls using the GEO2R program. The gene expression profiles were assessed using box plot, Uniform Manifold Approximation and Projection (UMAP) plot, expression density diagram, and Venn diagram. The significant differentially expressed genes (DEGs) were evaluated through a directed protein-protein interaction (PPI) network using the CluePedia plugin of Cytoscape software, considering co-expression interactions. The central nodes were identified as the most influential and regulated genes.
Results: Pre-evaluation analysis revealed that insomnia exhibits heterogeneity and can be divided into two groups. The gene expression profiles of the first group were similar to those of the insomnia group, while the second group of controls was distinguished from the insomnia group by genes such as TP53, CCND1, IL1B, SOX1, and NOTCH1, which were identified as key actor genes. Additionally, IL10, IL6, TP53, PTGS2, ESR1, PTEN, JUN, CREB1, CDKN1ACDKN2A, CXCR4, and GATA3 were identified as important regulatory genes.
Conclusion: It can be concluded that many individuals may be potentially involved in insomnia disorder as pre-insomnia. The findings demonstrate that pre-insomnia and insomnia share very similar molecular mechanisms. The critical genes TP53, CCND1, IL1B, SOX1, and NOTCH1, along with pathways related to apoptosis, inflammation, immunological response, and changes in sleep quality, are emphasized as particularly relevant to insomnia disorder.
Keywords: Insomnia, Gene expression, Network analysis, Pre-insomnia, Human

Introduction

Sleep is a fundamental process for restoring brain function and is recognized as a basic dimension of physical and mental health (1). On the other hand, sleep disturbances, such as insomnia, are associated with an increased risk of dementia (2), a higher likelihood of dying from cardiovascular diseases (3), and an elevated risk of mental disorders (4). The association between insomnia and depression has been acknowledged, with key indicators including high levels of negative emotion and low levels of positive emotion dysregulation (5). Franzen et al. revealed that that 90 percent of individuals with major depression report disturbances in normal sleep (6). However, recent meta analytic data concluded there is no consistent evidence for an intervention effect between sleep disturbances and improvement in depressive symptoms (7). Circadian systems and sleep are key modulators of immune system function, and experimental sleep deprivation leads to peaks in the expression of IL6 and TNF from night-time to day time (8). Sleep loss may activate nuclear factor-Kappa B, a key regulatory pathway in the inflammatory response, increasing levels of IL6 and TNF (9). Interventional studies have revealed an association between insomnia and inflammation (10). Evidence suggests that inflammation caused by insomnia may be related to hypothalamic-pituitary-adrenal axis activation and glucocorticoid resistance (11). Genome wide association studies of insomnia have identified MEIS1 as having a strong association signal, suggesting that MEIS1 may play a role in insomnia and restless leg syndrome (12). However, the inflammation associated with insomnia and depression has not been thoroughly analyzed and remains largely hypothetical (13).

Today, with the assistance of bioinformatics knowledge and powerful data analysis software, gene network analysis and the interpretation of gene interactions are possible (12). Since bioinformatics tools are suitable for detecting the molecular mechanisms of diseases (14), the aim of this study is to assess the molecular mechanisms of insomnia and identify the related dysregulated key genes. The findings may be important for managing individuals with insomnia or related disorders.

Methods

Data collection: To study the molecular mechanisms of insomnia, relevant data were obtained from the GEO database (GSE208668) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=gse208668). Total RNA obtained from peripheral blood mononuclear cells (PBMCs) of 17 people with insomnia disorder, compared to 25 controls, was retrieved for analysis. The data are linked to the published document of Piber et al, titled; Sleep disturbance and activation of cellular and transcriptional mechanisms of inflammation in older adults (15). There are evidences, a patient cells are a suitable source to study molecular mechanism of the studied disorder or disease (16, 17).

Pre-evaluation of data: Data were evaluated using the GEO2R program to find possible comparison between samples through box plot, Uniform Manifold Approximation and Projection (UMAP) plot, expression density diagram, and Venn diagram. The gene expression profiles that did not match statistically were normalized using the "force normalization" option of the GEO2R program. UMAP plot analysis indicated; normalization had not unfavorable consequence. Significant differentially expressed genes (DEGs) were selected based on adjusted p-value < 0.05 and a fold change > 2. Data were cleaned, and uncharacterized individuals were excluded from further analysis.

PPI network analysis: The selected significant DEGs were included in a directed protein-protein interaction (PPI) network using the CluePedia plugin of Cytoscape software, considering co-expression interactions. The main connected components of the network that were eligible for network topology analysis were assessed using the "Network Analyzer" application of Cytoscape software in "direct" mode. The main connected components were laid out based on outdegree and indegree centrality parameters to identify the critical actor and controlled genes, respectively. The central nodes and their centrality parameters were visualized to highlight the crucial genes.

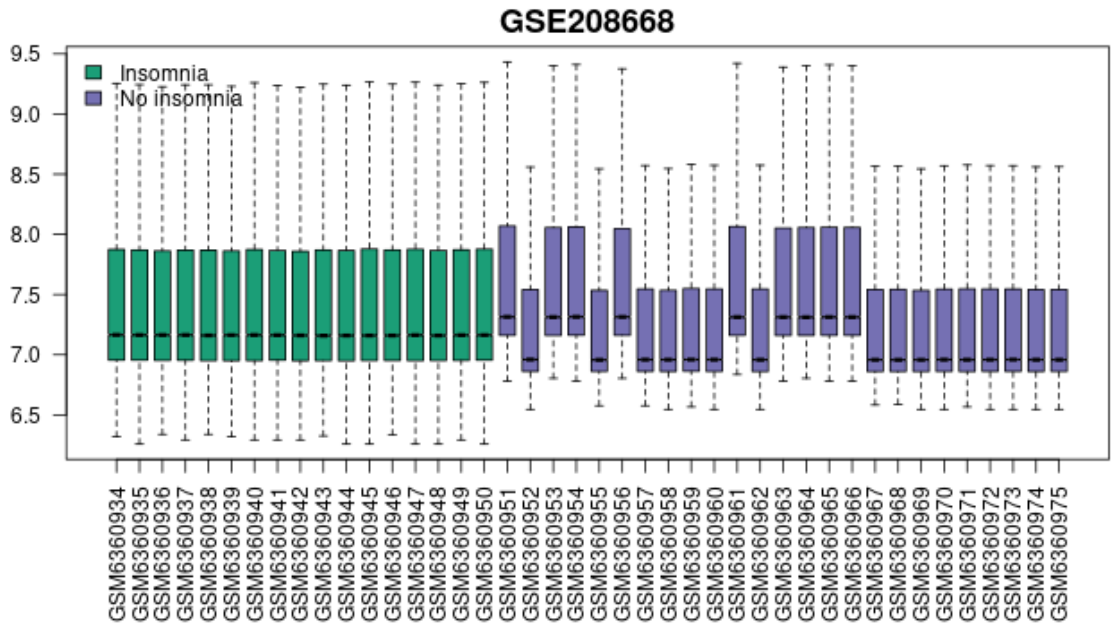
Statistical analysis: The significant DEGs were selected based on (adjusted p-value) < 0.05 and (fold change) > 2.

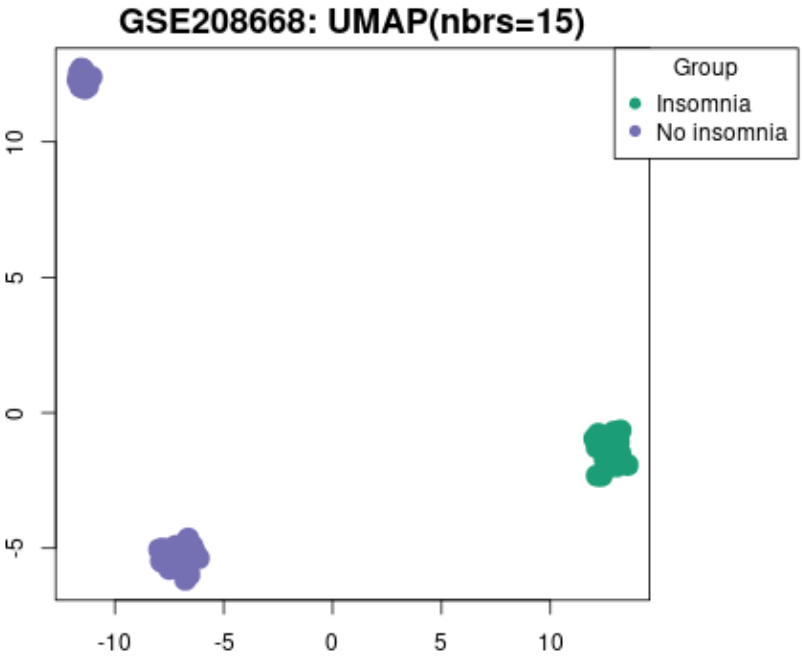
Results

The visualization of Insomnia – No insomnia (I-NI) samples, using box plots, UMAP plots, and expression density diagram, is presented in Figure 1. As depicted in Figure 1, No insomnia samples are not uniform and do not match the Insomnia individuals. The UMAP plot and density diagram correspond to the two sets of samples in No insomnia group. Based on the box plot of Figure 1, the gene expression profiles of the No insomnia group were divided into two groups including No insomnia 1(NI1) and No insomnia 2 (NI2). NI1 includes: GSM6360952, GSM6360955, GSM6360957-60, GSM6360962, and GSM6360967-75, while, GSM6360951, GSM6360953-4, GSM6360956, GSM63609561, and GSM6360963-6 are grouped as NI2. The Box plot, UMAP scheme, expression density diagram and Ven diagram of the I-NI1-NI2 analysis indicate that the two groups of No insomnia gene expression profiles are completely separated from the Insomnia group (see Figure 2). The samples were normalized and compared, and the results of the I-NI1-NI2 reanalysis are shown in Figure 3. As depicted in Figure 3, the three groups are discriminated via gene expression profiles (see UMAP plot). Since the intensity diagrams of samples have a similar pattern the gene expression profiles of the studied groups are comparable. UMAP plot demonstrated exactly, two distinct groups of No insomnia samples.

To identify the differentiation among NI1, NI2, and Insomnia groups, the gene expression profiles of groups were compared with each other. The results of this analysis are depicted in Figure 4. As shown in Figure 4, the three groups are completely separated from each other based on the significant DEGs. As depicted in Figures 2-4 (the Venn diagrams), many DEGs are dysregulated significantly. The central part of the main connected component of the NI1-NI2 PPI network is shown in Figure 5. According to Figure 5, the genes CCND1, STAT3, SOX9, NOTCH1, IL1B, HMOX1, EGR1, SPI1, YY1, SP1, PTGS2, and PTEN are identified as the main actors that differentiate the two No insomnia groups. These genes are arranged in descending order based on their outdegree values, with CCND1 and PTEN being the strongest and weakest actors, respectively. IL6, PTGS2, PTEN, ESR1, JUN, CREB1, CDKN1A, and CDKN2A were highlighted as the critical controlled DEGs, with IL6 and CDKN2A identified as the most and least influential controlled DEGs, respectively. The genes are laid out based on out-degree and in-value values via color and the related amounts of the central parameters.

As presented in Figure 6, there are no crucial DEGs that separate the Insomnia samples from those in the NI1 group. The central part of the main connected component of the PPI network from the I-NI2 analysis, which is laid out based on outdegree and indegree values (via color and amounts of central parameters) is shown in Figure 7. TP53, CCND1, IL1B, SOX1, and NOTCH1 are identified as the principal actors in the analyzed network, with TP53 being the most influential actor. IL10, IL6, TP53, PTGS2, ESR1, PTEN, JUN, CREB1, CDKN1ACDKN2A, CXCR4, and GATA3 are the key controlled DEGs.





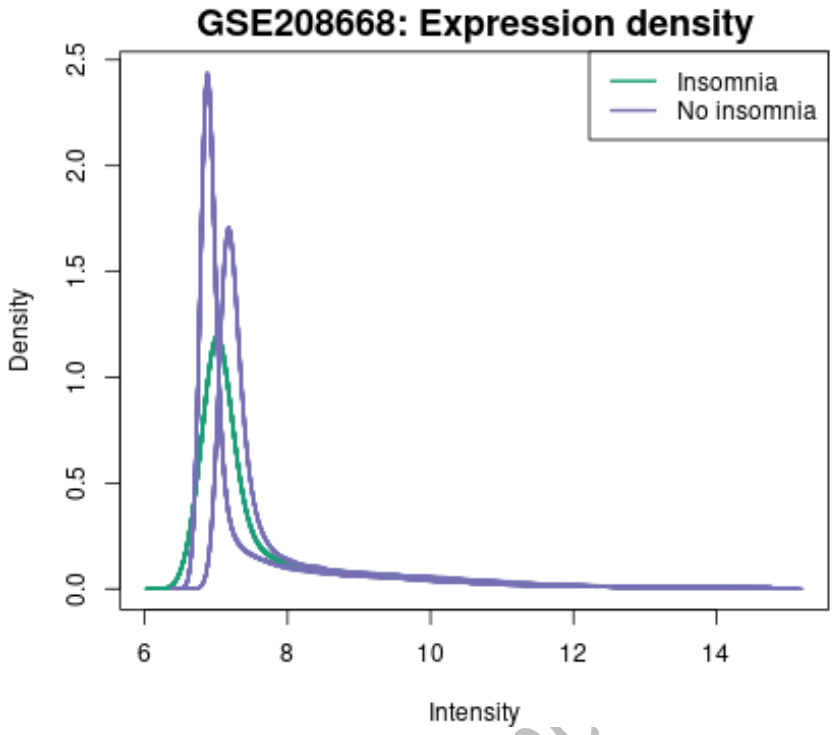
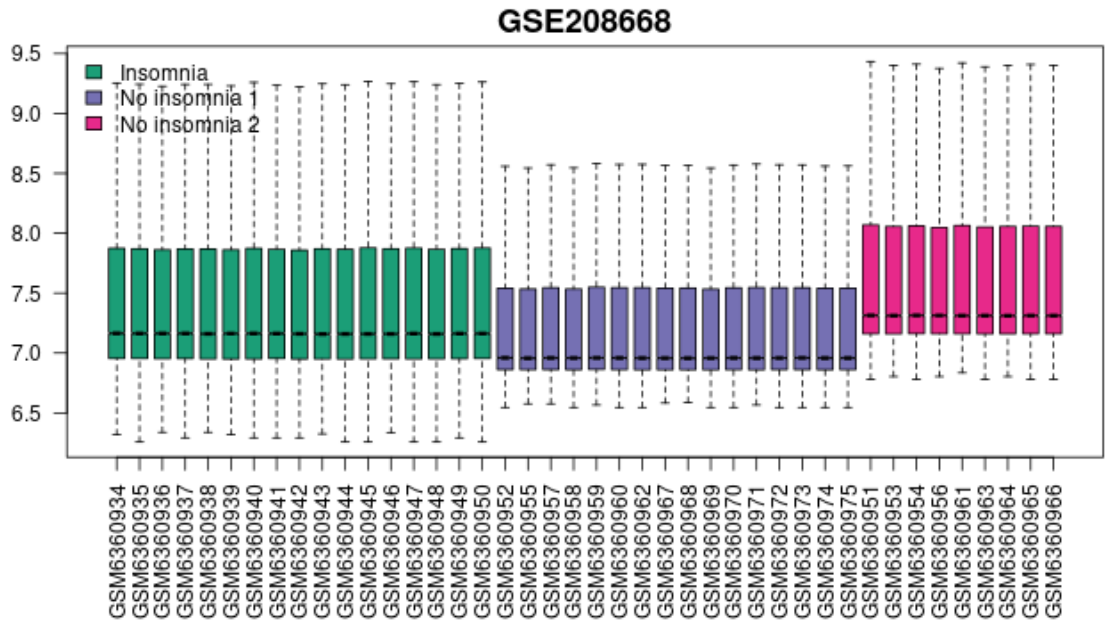
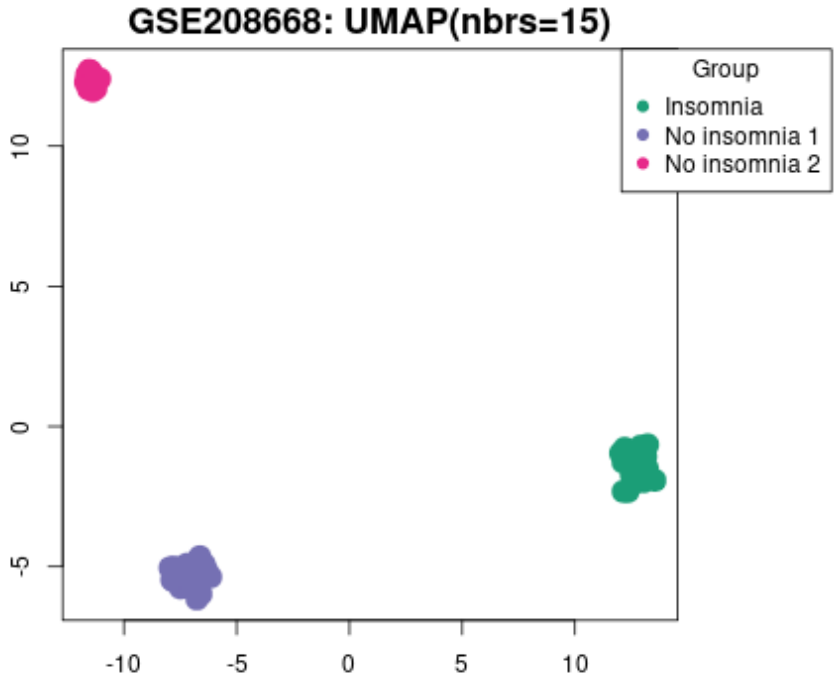
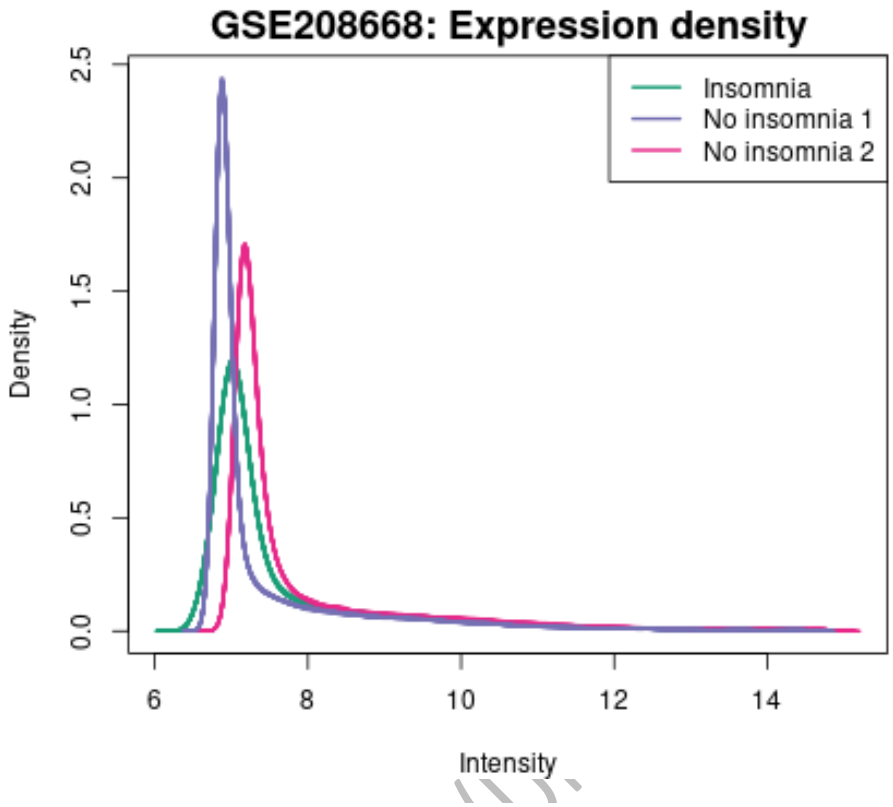


Figure 1. Box plot, UMAP plot, and density diagram of I–NI gene expression profile analysis. "NBRS 15" in UMAP plot refers to the number of neighbors that a certain individual is compared with them.







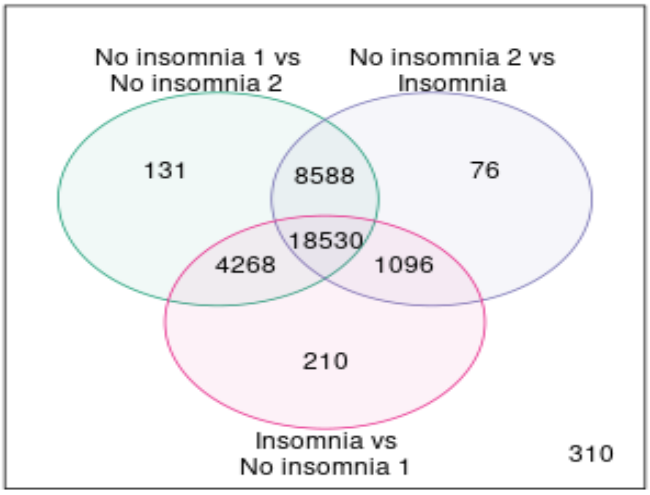
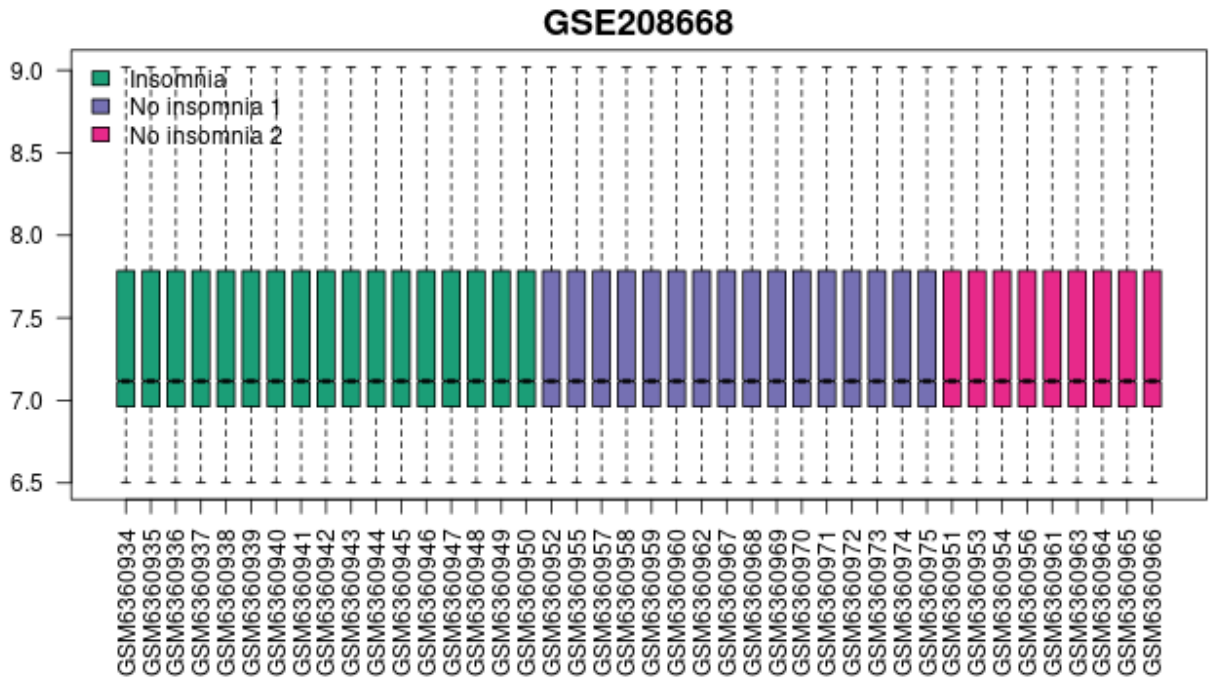


Figure 2. Box plot, UMAP scheme, expression density diagram and Ven diagram of I-NI1-NI2 groups analysis. "NBRS 15" in UMAP plot refers to the number of neighbors that a certain individual is compared with them.


GSE208668
Insomnia
No insomnia 1
No insomnia 2
9.0
8.5
8.0
7.5
7.0
6.5
GSM6360934
GSM6360935
GSM6360936
GSM6360937
GSM6360938
GSM6360939
GSM6360940
GSM6360941
GSM6360942
GSM6360943
GSM6360944
GSM6360945
GSM6360946
GSM6360947
GSM6360948
GSM6360949
GSM6360950
GSM6360952
GSM6360955
GSM6360957
GSM6360958
GSM6360959
GSM6360960
GSM6360962
GSM6360967
GSM6360968
GSM6360969
GSM6360970
GSM6360971
GSM6360972
GSM6360973
GSM6360974
GSM6360975
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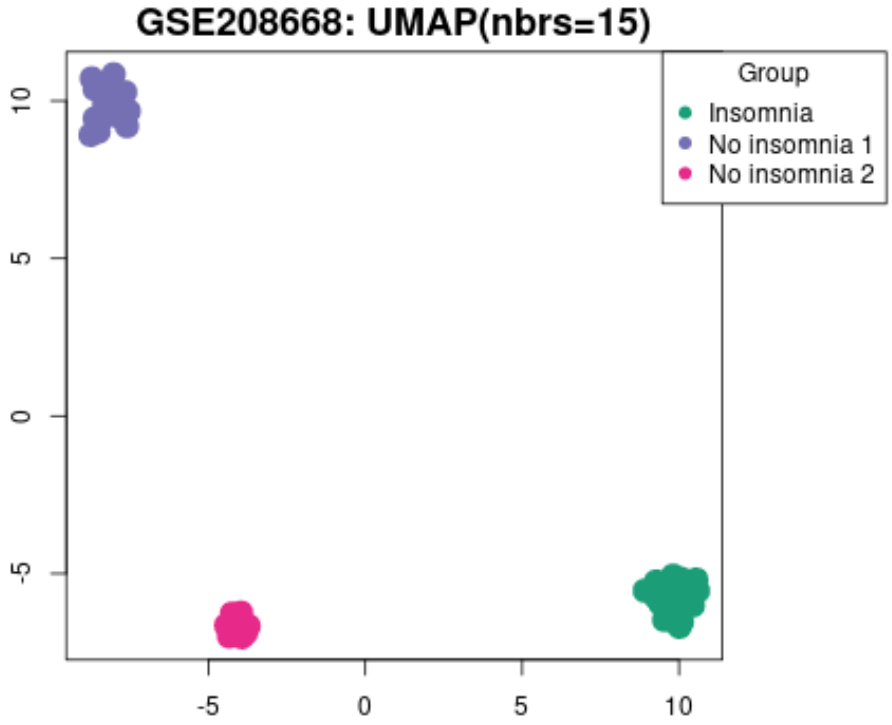
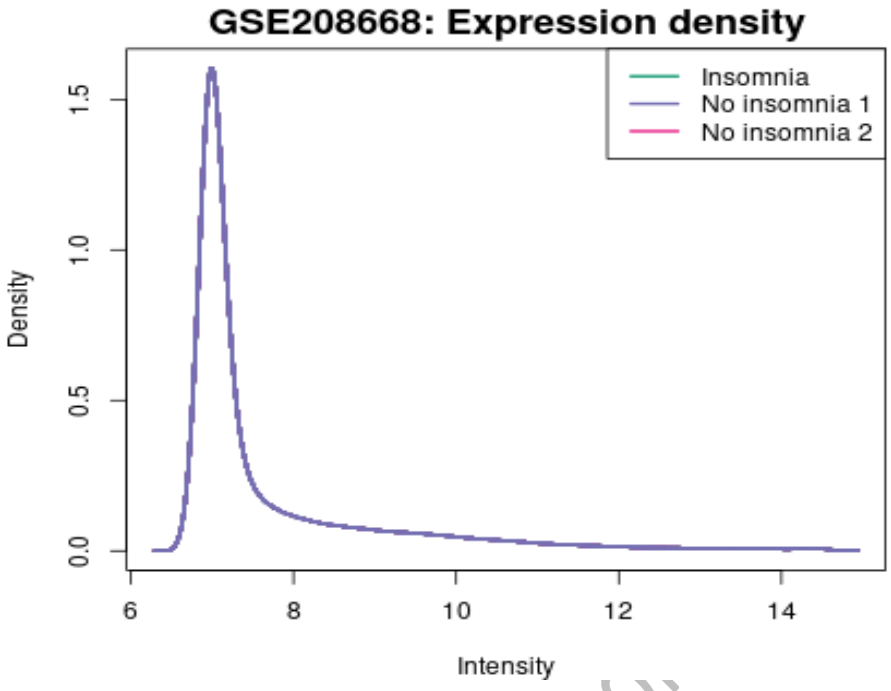
GSM6360961
GSM6360963
GSM6360964
GSM6360965
GSM6360966


GSE208668: UMAP(nbrs=15)
Group
Insomnia
No insomnia 1
No insomnia 2
10
5
0
-5
-5
0
5
10



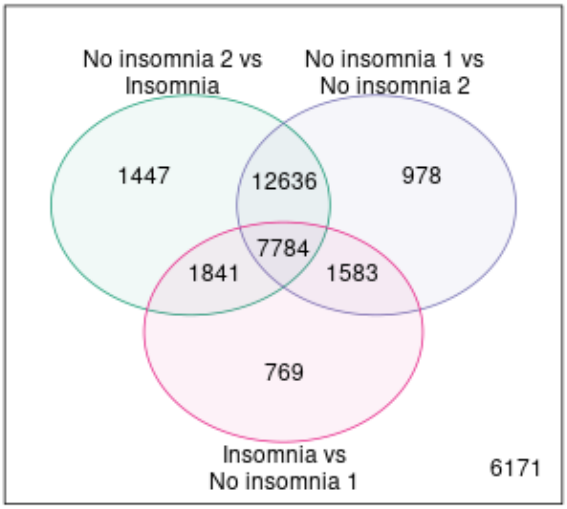
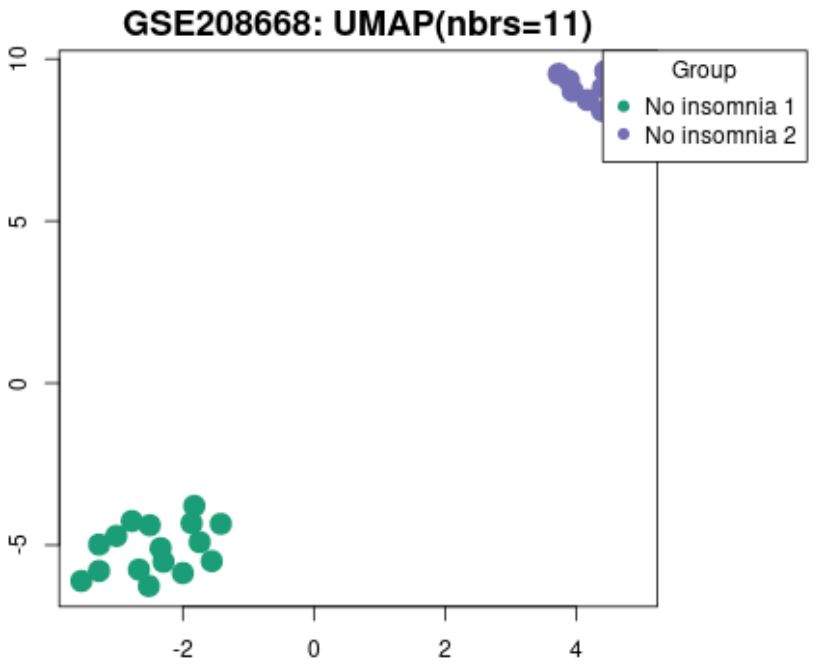
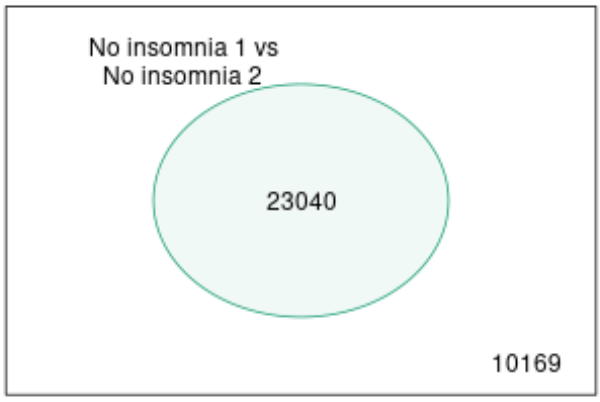
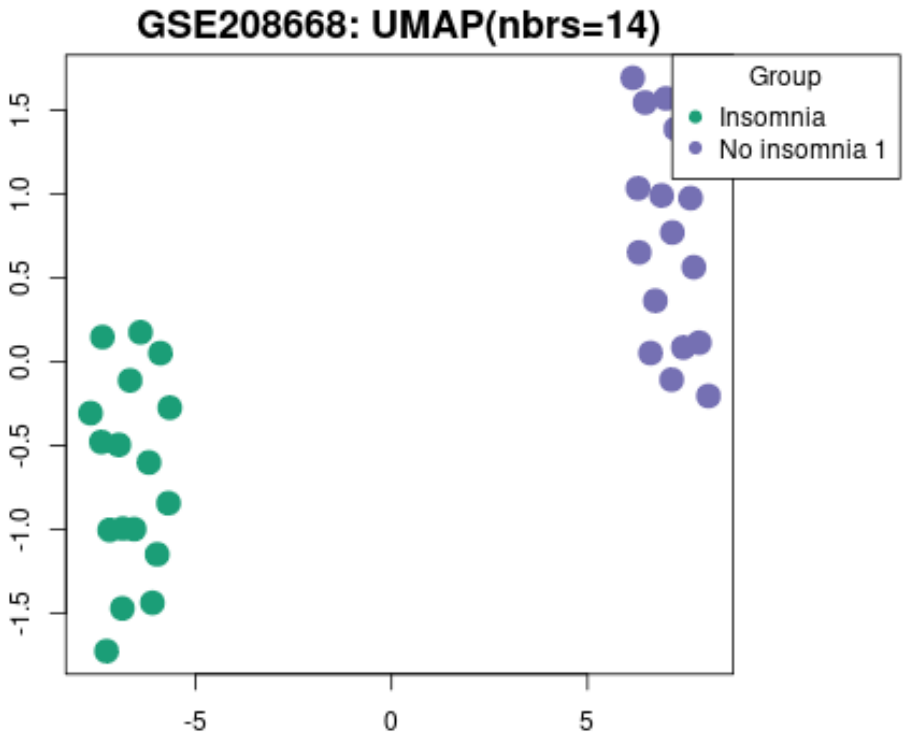


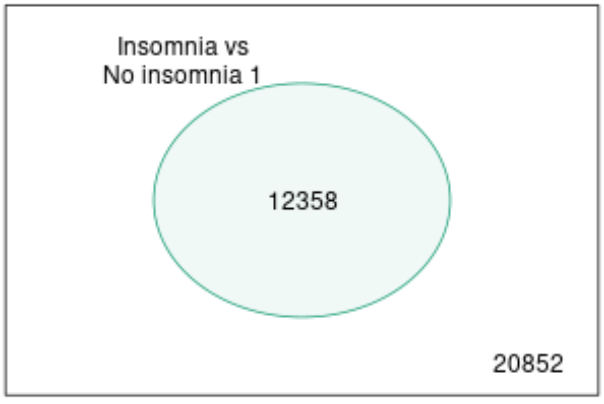
Figure 3. Box plot, UMAP scheme, expression density diagram and Ven diagram of I-NI1-NI2 groups analysis after normalization. "NBRS 15" in UMAP plot refers to the number of neighbors that a certain individual is compared with them.



GSE208668: limma, Padj<0.05




GSE208668: UMAP(nbrs=14)
Group
Insomnia
No insomnia 1
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-5
0
5


GSE208668: limma, Padj<0.05
Insomnia vs
No insomnia 1
12358
20852

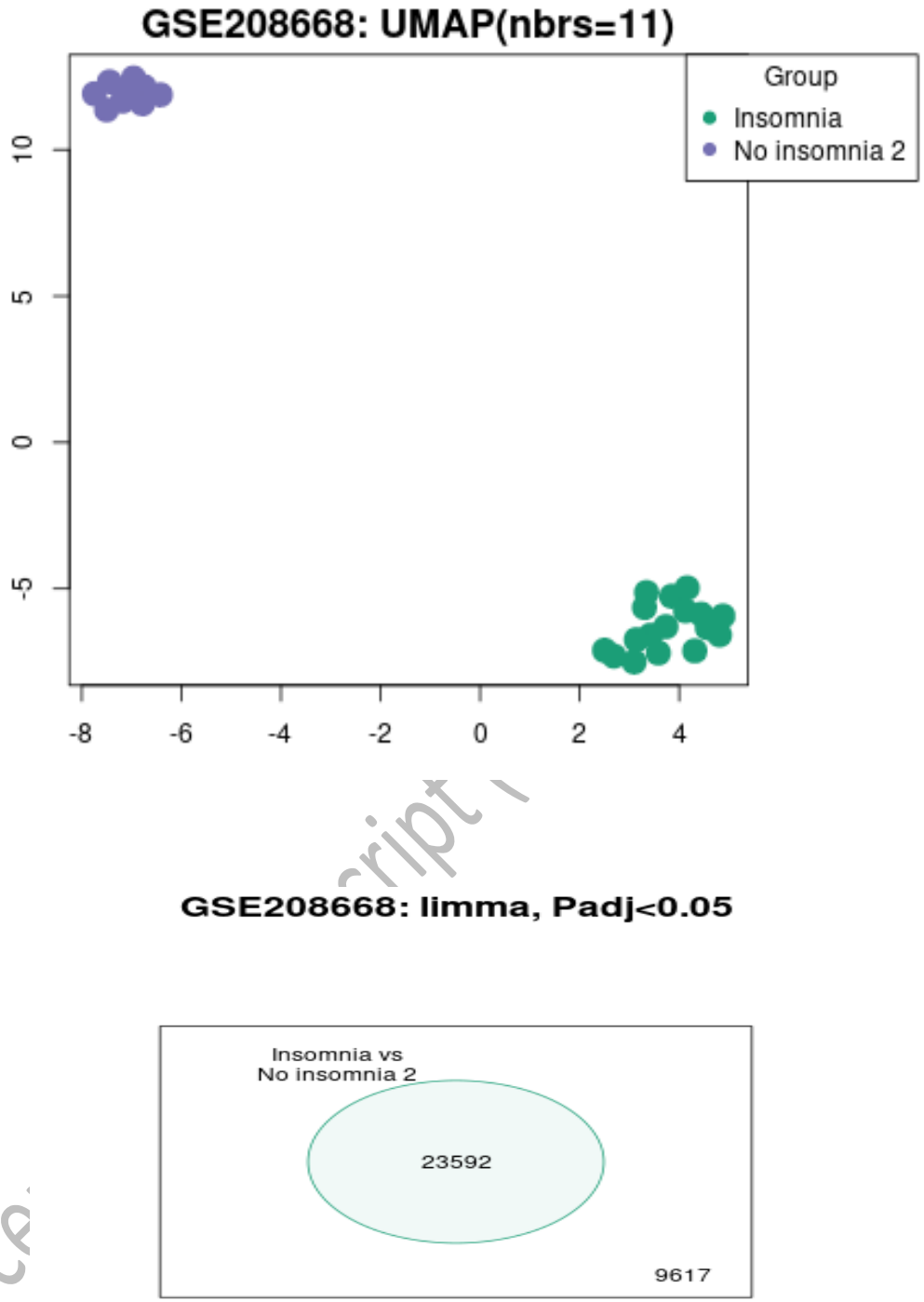
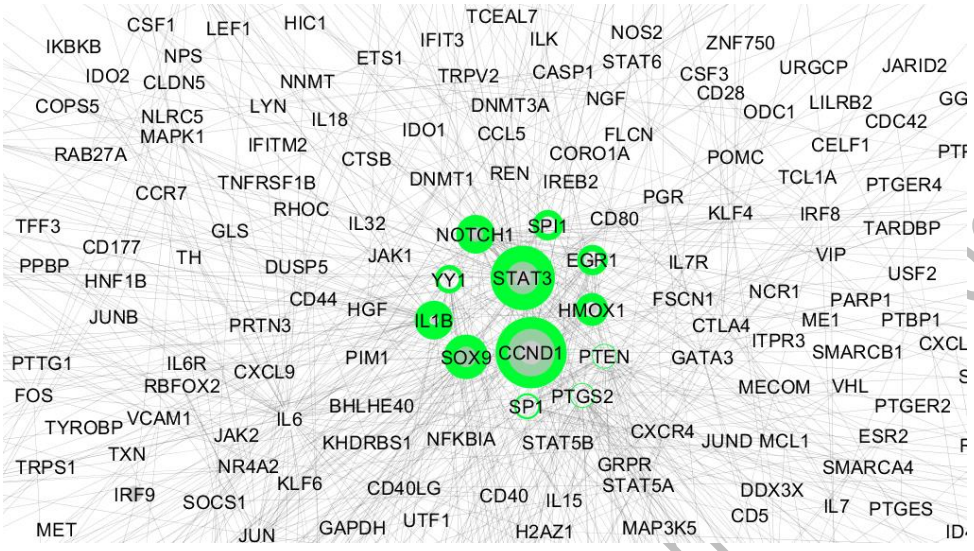
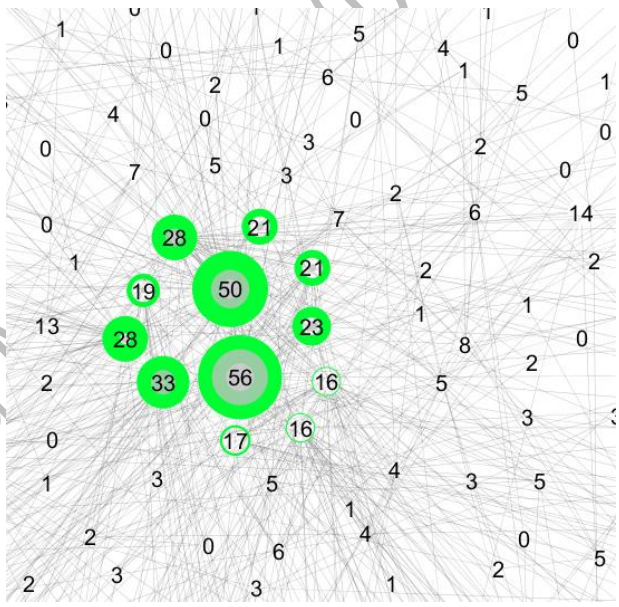


Figure 4. UMAP plot and Ven diagram of NI1-NI2, I-NI1, and I-NI2 groups analyses. "NBRS 11 and 14" in UMAP plots refer to the number of neighbors that a certain individual is compared with them.





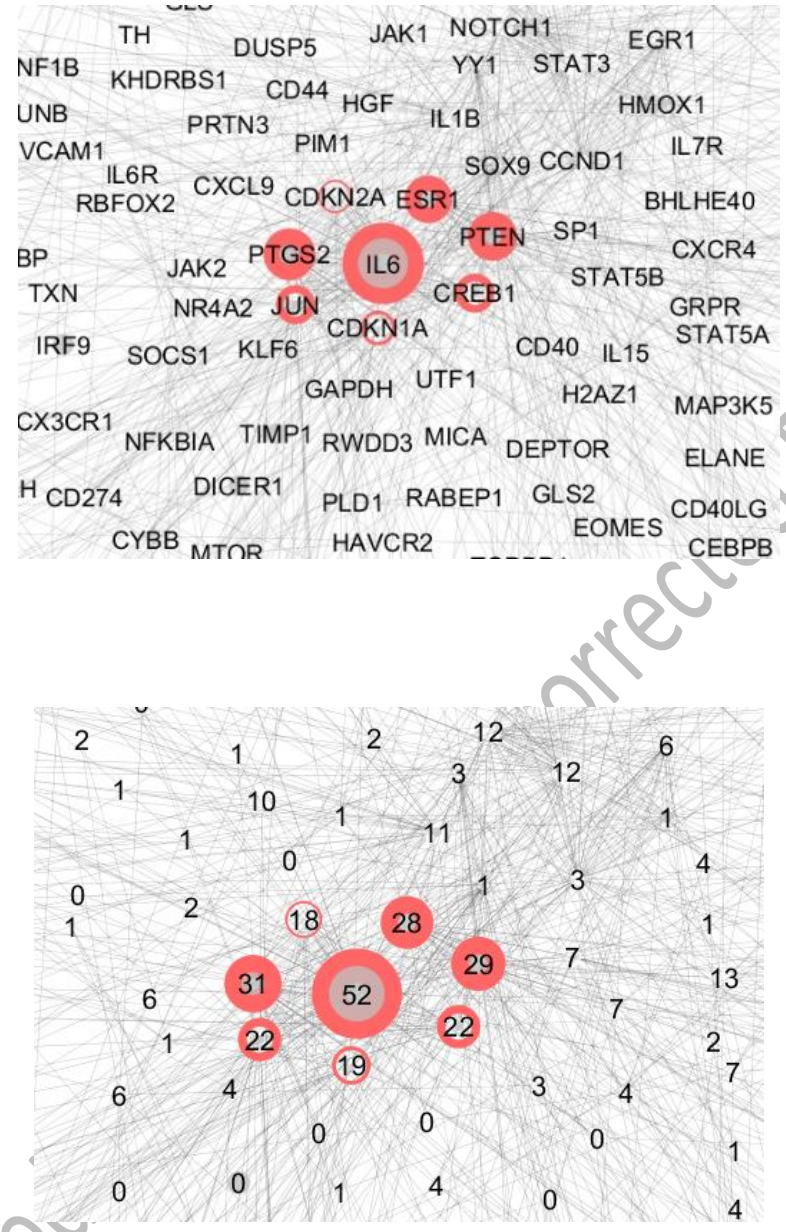


Figure 5. The central part of the main connected component of directed PPI network of NI1-NI2 gene expression analysis via co-expression action. The network was formed from 6611 recognized DEGs (include 5692 isolated nodes) and 1522 edges. The actor genes and the related outdegree values are appeared in green while, the controlled individuals and the associated indegree values are shown in red.

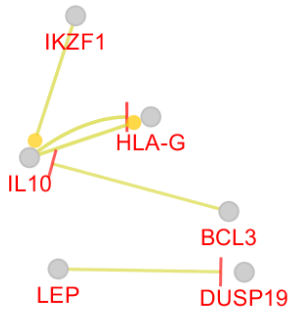
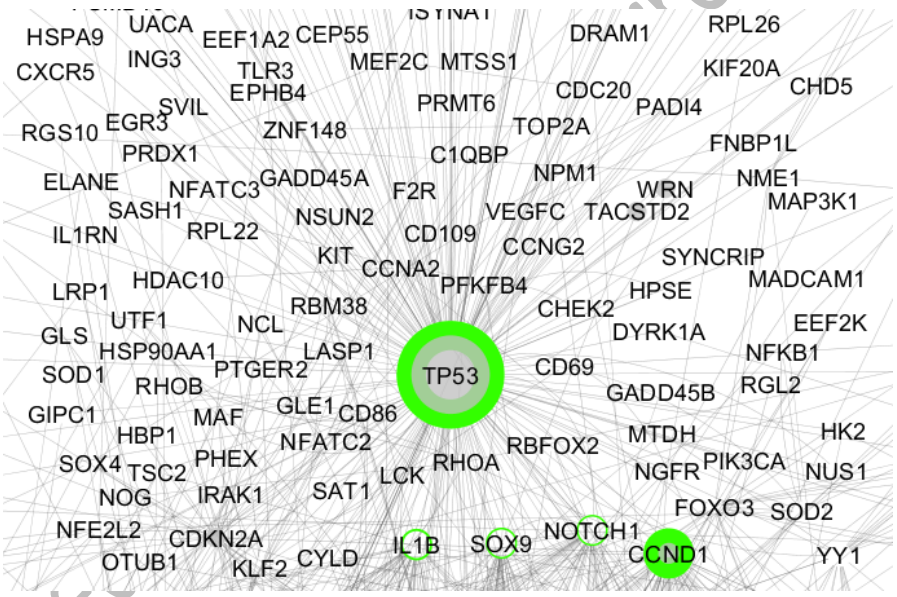
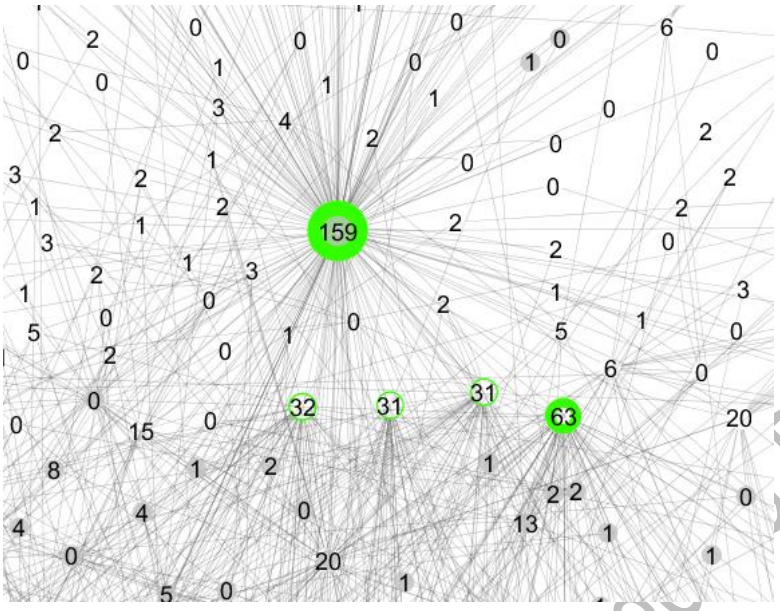
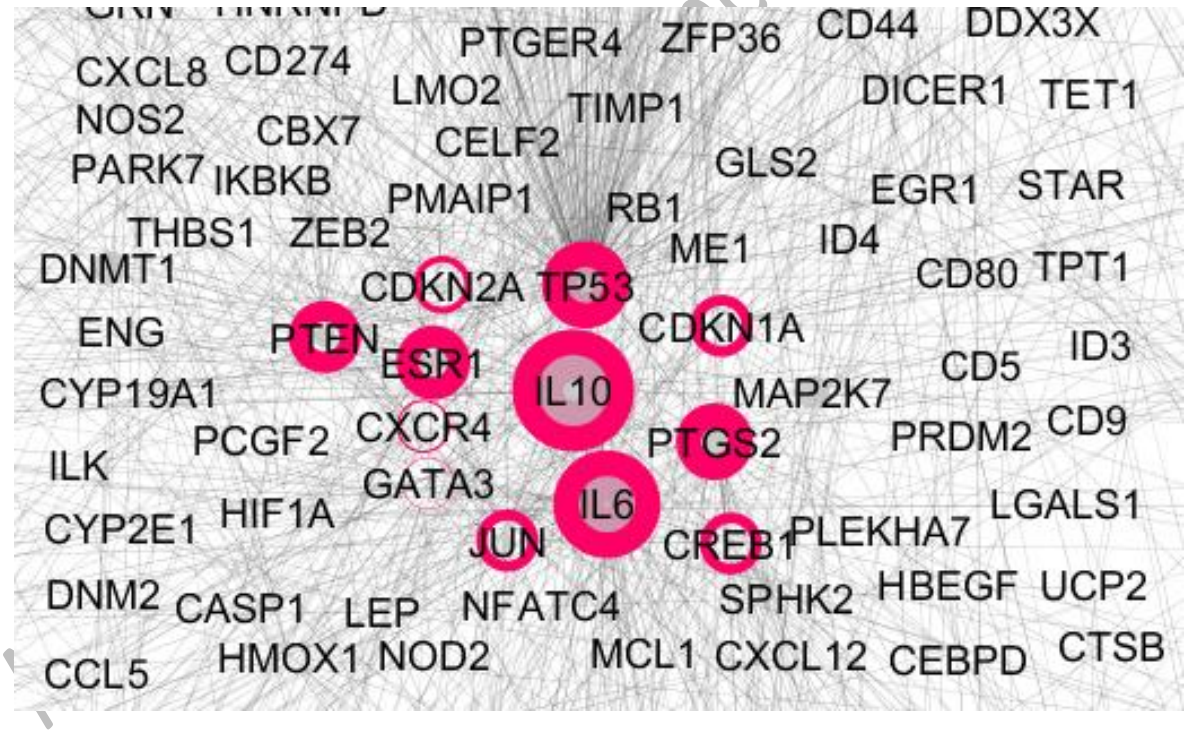


Figure 6. The main connected component of directed PPI network of I-NI1 gene expression analysis via co-expression action.







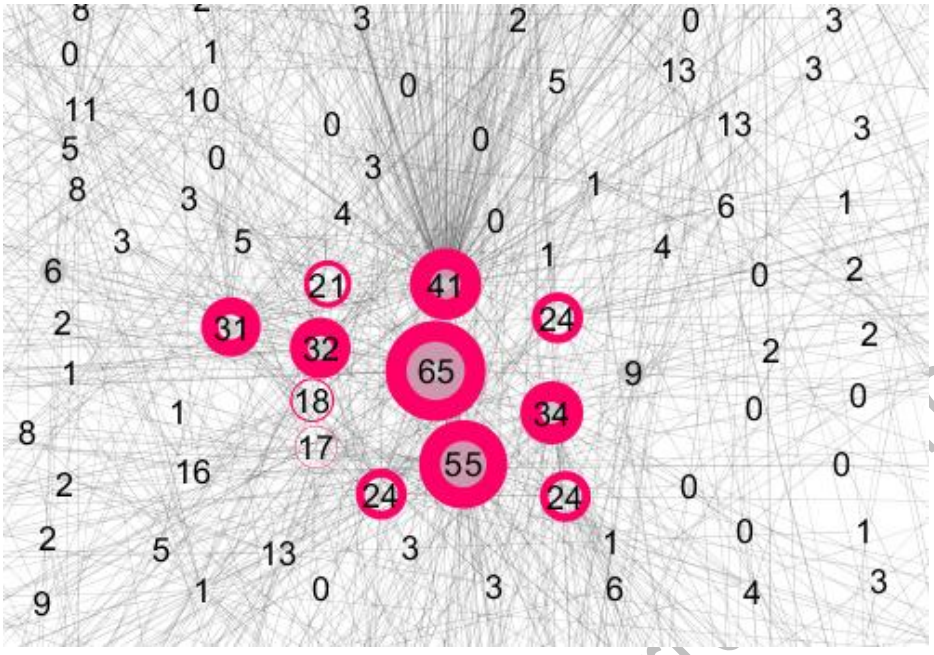


Figure 7. The central part of the main connected component of directed PPI network of I-NI2 gene expression analysis via co-expression action. The network was formed from 6958 recognized DEGs (include 5910 isolated nodes) and 1882 edges. The actor genes and the related outdegree values are appeared in green while, the controlled individuals and the associated indegree values are shown in red.

Discussion

Insomnia disorder is reported to be associated with abnormalities in brain function and structure in patients. The investigation has established an association between these abnormalities and gene expression. A study by Zhang et al. revealed the involvement of insomnia disorder-related genes in functions such as brain development, endocrine regulation, and ion transport (18). In the present study, the gene expression of patients was analyzed and compared to controls. As depicted in Figures 1-4, the analyses indicate the heterogeneity of gene expression profiles among the control samples. Although the control samples exhibited heterogeneity, it was possible to cluster them into distinct groups; NI1 and NI2. The issue of sample heterogeneity is addressed in many studies, and various methods have been suggested to resolve it (19, 20). As shown in Figure 4, the control samples are divided into distinct groups and can be compared to each other.

The genes CCND1, STAT3, SOX9, NOTCH1, IL1B, HMOX1, EGR1, SPI1, YY1, SP1, PTGS2, and PTEN serve as crucial actors, while IL6, PTGS2, PTEN, ESR1, JUN, CREB1, CDKN1A, and CDKN2A function as key controlled genes that differentiate the two compared groups of control samples (see figure 5). This indicates that the clustered control samples exhibit significant differences at least at the gene expression level, differences that cannot be overlooked in the analysis of insomnia disorder samples. Since the categorization of insomnia disorder is considered a "work in progress" (21) it seems that some control samples are classified as normal but may actually belong to the insomnia patient group. This suggests that a portion of the normal samples could be individuals potentially suffering from insomnia. The results illustrated in Figure 6 support this notion. As shown in Figure 6 there are no significant differences between the insomnia group and the NI1 group. The main differences are detected between insomnia patients and the control group of nine samples (see Figure 7). The genes TP53, CCND1, IL1B, SOX1, and NOTCH1 are identified as the main actors, while IL10, IL6, TP53, PTGS2, ESR1, PTEN, JUN, CREB1, CDKN1ACDKN2A, CXCR4, and GATA3 are recognized as the key controlled genes that distinguish insomnia disorder patients from normal controls. Liang et al.'s investigation, utilizing PPI network analysis and molecular complex detection, introduced ten hub genes related to insomnia (22). The crucial genes in our analysis, such as TP53, JUN, IL6, and CREB1 included in this set of hub genes. As depicted in Figure 7, TP53 is the primary actor in the I-NI2 analysis. Experiments indicate that ketamine has behavioral effects on exposed animals. Exposure of zebrafish larvae to ketamine resulted in difficulty initiating sleep, which was associated with the upregulation of the TP53 apoptotic gene. After a week of recovery, a shorter duration of sleep (insomnia-like behavior) was observed in the treated zebrafish larvae (23). In the present study, TP53 is upregulated two-fold in the Insomnia group compared to the NI2 group. Several cytokines and immune genes, such as IL6, IL10, and IL1B have been highlighted in mammalians for their association with sleep regulation (24). As mentioned, IL1B ranks as the third most significant actor in our analysis, while IL10 and IL6 are the first and second-ranked controlled genes, respectively. Cyclin D1 (CCND1) is an oncogene, and its role in several cancers, such as melanoma, breast cancer, and hepatocellular carcinoma has been highlighted (25-27). CCND1 appears as the second-ranked actor in insomnia. Furthermore, literature suggests a correlation between positive anti-SOX1 in the serum of individuals with fatal familial insomnia, a condition characterized by dysautonomia, motor disorder, and disturbed sleep (28). SOX1 is identified as the fourth actor in

insomnia. The last actor introduced is NOTCH1, which has been shown to be regulated by melatonin in rats (29). The significant role of melatonin in sleep quality has been both investigated and confirmed (30). PTGS2 and chemokine receptor CXCR4 are identified as critical controlled genes in insomnia. As reported in the literature both PTGS2 and CXCR4 are involved in the inflammatory process (31, 32). These findings, alongside the presence of the other mentioned interleukins, underscore the prominent role of inflammation in insomnia.

Comparison of critical genes between the NI1-NI2 and I-NI2 analyses indicates that CCND1, IL1B, NOTCH1, and members of the SOX gene family are common actors in both analyses. This suggests that 80% of the actors identified in the I-NI2 analysis overlap with those of the NI1-NI2 analysis. It can be concluded that a significant number of control samples may be involved in insomnia and could develop insomnia disorder soon. This idea is further supported by the controlled genes; 100% of the controlled genes in the NI1-NI2 analysis (IL6, PTGS2, PTEN, ESR1, JUN, CREB1, CDKN1A, and CDKN2A) are common to the individuals assessed in the I-NI2 analysis. As highlighted in previous investigations, insomnia is categorized as severe, moderate, and mild (33). Additionally, sleep duration is classified as very short, short, normal, or long (34). Based on these findings, the term "pre-insomnia" is suitable to describe the situation of the NI1 group.

Conclusion

In conclusion, the findings indicate that many people may potentially be involved in insomnia disorder. It can be concluded that pre-insomnia occurs prior to the onset of insomnia. There is a close relationship between the molecular mechanisms of pre-insomnia and insomnia. The genes TP53, CCND1, IL1B, SOX1, and NOTCH1 were highlighted as critical actors in insomnia disorder. Apoptosis, inflammation and immunological response, and changes in sleep quality (due to dysregulation of melatonin) were emphasized as prominent events in insomnia disorder.

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