UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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REDES DE INTERAÇÃO PROTEÍNA-PROTEÍNA PARA AVALIAR A ASSOCIAÇÃO ENTRE MICROBIOTA E AÇÃO NEUROINFLAMATÓRIA NA DEPRESSÃO

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TRABALHO DE CONCLUSÃO DE CURSO

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Trabalho de Conclusão de curso apresentado como requisito parcial para obtenção do título Bacharel em Ciências Biológicas na Universidade Federal do Rio Grande do Sul. Este trabalho de conclusão será apresentado na forma de artigo científico de acordo com as normas para submissão da Revista Brain, Behavior, and Immunity.

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INTRODUÇÃO GERAL

A depressão é uma das maiores comorbidades do século XXI, acometendo cerca de 30 a 40% da população mundial. Com estimativas de prevalência ao longo da vida de 6,2% entre adolescentes e até 19% entre adultos, o Transtorno Depressivo Maior (TDM) é uma das condições de saúde mental mais comuns, dispendiosas e incapacitantes em todo o mundo (1). Também é altamente recorrente, com quase três quartos das pessoas com TDM experimentando um segundo episódio em algum momento de suas vidas. A depressão contribui substancialmente para o excesso de mortalidade, diretamente por suicídio ou indiretamente. por meio de condições crônicas comórbidas, aumentando o risco de mortalidade em 60% e 80%. (1). Outro aspecto importante nessa condição é sua heterogeneidade. Conforme o Manual Diagnostico e Estatístico de Transtornos Mentais (*Diagnostic and Statistical Manual of Mental Disorders*, DSM-5), nove critérios são considerados para a realização do diagnóstico desta patologia, sendo necessário, no mínimo, dois sintomas para confirmar o episódio depressivo, como por exemplo, agitação e retardo psicomotor. Apenas humor deprimido é classificado como critério simples. Além disso, há possibilidade de duas pessoas com diagnóstico de TDM apresentarem sintomas significativamente distintos (2).

Presentes estudos e análises indicam uma forte relação entre mecanismos neuro-inflamatórios e a depressão, tendo como um fator relevante alterações na microbiota e nas respostas sucessivas ao eixo cérebro-intestino(3). Com base nesses estudos, muitos esforços têm sido feitos para entender a correlação entre os genes e o ambiente relacionados a esta doença. Evidências recentes relacionam a microbiota intestinal ao cérebro humano, dado sua influência sobre a regulação do sistema nervoso central (SNC). Achados demonstram a existência de redes de comunicação bidirecionais entre a microbiota intestinal e o SNC, e essa comunicação cruzada foi correlacionada com alterações no TDM. Sabe-se que uma diminuição em bactérias envolvidas no processo de digestão de ácidos graxos de cadeia curta promovem uma disfunção na barreira intestinal gerando um processo inflamatório que pode aumentar o risco de desenvolvimento de depressão como uma resposta inflamatória (3,4).

De modo a compreender essa complexidade, a biologia de sistemas é uma

ferramenta de análise robusta, que avalia as propriedades e interações entre componentes como genes e proteínas desde o nível molecular ao nível de organismo (5). Essa abordagem auxilia na compreensão de como o comportamento fenotípico do sistema emerge dos componentes e interações que o constituem. Deste modo, analisando as interações entre proteínas, poderemos delimitar o grau de importância destas interações e como poderiam estar associados a doenças psiquiátricas, como a depressão.Nesse contexto, um estudo analisou 255 genes associados à depressão e demonstrou sua relação com processos endócrinos, neurológicos e imunológicos (6).

Considerando o nível de centralidade das proteínas na rede, o presente estudo se deteve em analisar *node degree* (conectividade) o qual representa o número de interações de uma determinada proteína. Além disso, foi avaliado o *betweenness* (intermediação), medida que indica o quanto uma proteína pertence ao centro da rede sendo obtida pela contagem de caminhos mínimos que passam por um determinado vértice (7). A partir desses parâmetros, é possível categorizar as proteínas em hubs (proteínas com um grande número de interações) e proteínas do tipo *bottleneck* caracterizadas por unirem agrupamentos de nós. A terceira categoria, proteínas *hubs-bottleneck,* agrega proteínas com características de ambas as categorias anteriores, portanto aparecem agrupadas a outras proteínas e também intermediam conexões entre diferentes grupos de proteínas (5).

Portanto, considerando os avanços tecnológicos e as promissoras técnicas de análise de dados pela bioinformática, a criação de redes de interação proteína-proteína (PPI) são promissores meios para estudar a patofisiologia da TDM e indicar possíveis novos alvos moleculares para tratamento. O presente estudo objetiva compreender, com base na análise de biologia de sistemas, as relações estabelecidas entre o eixo cérebro-intestino e as possíveis vias inflamatórias associadas à depressão.

Similarities in genetic structure in major depression and microbiome

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Abstract

INTRODUCTION: Major depressive disorder (MDD) has a heritability of 37% and is one of the most common, costly and disabling mental health conditions worldwide with lifetime prevalence estimates of 19% among adults. There is evidence of MDD alterations correlated with bidirectional communication between CNS and microbiota. Considered as the “second brain”, the microbiota plays a key role in regulating the central nervous system (CNS) and immune system. Analysis of the network of proteins encoded by genes associated with MDD and the microbiota can help to elucidate the molecular systems involved. **METHODS:** To establish the relationships between intestinal microbiota and MDD, protein-protein interaction networks (PPIN) were constructed from human genetic variants identified in the literature. The R Studio v 4.0.2 interface, the STRING v11 database and Cytoscape v 3.7.2. were used for the generation and visualization of networks. The protein from each gene was connected to 10 other proteins at three successive levels. An intersection network was generated between PPIN built from microbiota-associated variants and PPIN from MDD variants. **RESULTS:** One of the main biological processes was the exocytosis of proteins from

the lumen of azurophile granules (code: HSA:6798751, p-corrected: 2.20E-18). The proteins that act on this phenomenon interact with proteins encoded by the DENND1A and DENND1B genes, which are associated with MDD. **DISCUSSION**: Exocytosis of proteins from the lumen of azurophilic neutrophil granules are involved in the first immune response when the permeability of the intestinal barrier is altered, which can be caused by dysbiosis. Additionally, alterations in proteins encoded by variants of DENND1A and DENND1B genes could deregulate neutrophil degranulation, altering the organism's inflammatory response. **CONCLUSION:** Understanding the interaction between the gut-brain axis and MDD are important steps to elucidate the inflammatory pathway present in depression and the immune dysregulation associated with this pathology

Key-words : Major Depressive Disorder, Gut-Brain-Axis, System Biology

1.Introduction

Depression is one of the most frequent comorbidities in this century, affecting around from 30% to 40% of the worldwide population (1). The lifetime prevalence is 6.2% in adolescents and up to 19% in adults, which shows that major depressive disorder (MDD) is one of the most common disorders, expensive and disabling(2). Additionally, it is highly recurrent, in which three quarters MDD subjects have a second episode at any moment in their lives. Furthermore, MDD considerably contributes to an excess of mortality, directly through suicides or indirectly through comorbid and chronic conditions, increasing the mortality risk in 60% to 80% (2). Studies have found strong association between neuroinflammation and depression, being microbiota one important factor in triggering them, changes in gut microbiota and in the brain-gut axis (3).

Much effort has been made to understand how genes and the environment work together to shape lifelong risk of depression (2). One way can be the gut microbiota, the “second human brain", due to its role in the regulation of the central nervous system (CNS). Recent findings provide strong evidence for the existence of bidirectional communication networks between the gut microbiota and the CNS, and this cross communication has been correlated with changes in MDD (4). The latest findings related to MDD and the microbiome demonstrate that a decrease in bacteria involved in the digestion process of short-chain fatty acids promotes a dysfunction in the intestinal barrier, generating an inflammatory process that can increase the risk of developing depression (4–6).

Inflammation is an important pathological feature of depression (7). A subpopulation of depressed patients present immune dysregulation and chronic inflammation. The main hypothesis of the cause of these alterations is the involvement of cytokines. It is postulated that in depression, pro-inflammatory cytokines, including IL-6 and TNF-α, increase in quantity while anti-inflammatory cytokines decrease (8)..

MDD has 35–50% adult heritability (9) and advances in '–omics' approaches have created opportunities for the identification of molecular signatures in various fields, such as psychiatry. Genomics is the science that allows the large-scale identification of all genes in the organism, an assessment that is essential for understanding the biological processes associated with health and disease, especially for complex diseases such as depression (10).

In order to understand this complexity, systems biology can be an interesting analysis tool, which consists of a field of study that assesses the properties and interactions between many components, such as genes and proteins at various scales, from the subcellular level to the organism level (11). This approach focuses on developing an understanding of how the phenotypic behavior of the system as a whole emerges from its constituent components and interactions (11,12). Thus, analyzing the interactions between proteins, we will be able to delimit the degree of importance of these interactions.

Therefore, taking into account technological advances and promising data analysis and bioinformatics techniques, the creation of protein-protein networks (PPIN) are promising ways to elucidate unknowns about MDD. The present study aims to

understand, based on systems biology, the relationships established between the brain-intestinal axis and its possible inflammatory pathways associated with depression.

2. Methods

2.1 Prospection of protein from literature

SNPs (Single nucleotide polymorphisms) associated with depression were extracted from Howard et. al (2019) (13), a meta-analysis of the three largest cohorts of depression (total n = 807,553, with 246,363 cases and 561,190 controls). The study identified 102 independently segregated genetic variants associated with depression at 101 loci and demonstrated a consistency of effect directionality in a large replication sample (total n = 1,306,354, with 414,055 cases and 892,299 controls) and in the three contributing studies, allowing greater confidence in the conclusions.

As GWAS has a great complexity to analyze, it seeks to correlate genetic and phenotypic factors to elucidate possible heritability biases in depression. Thus, the biggest challenge is probably the identification of genetic variants that are causally involved in MDD. These challenges are not unique to depression, but apply to varying degrees to highly polygenic conditions in general. (36)

Microbiota-associated SNPs were extracted from the systematic review by Jun Wang et al. (2016) who studied genome-wide association (GWAS) using two northern German sections with 1812 individuals. A set of dense genomic markers comprising a

total of 6,344,846 genotyped SNPs and extensive metadata were included in the analyses, which allowed them to study the influence of the host genotype, along with dietary and other environmental factors, on the variability among individuals in the intestinal microbiome. Inter-individual variability was measured by β diversity indices, which represent general differences between microbial communities in the population and are influenced by variation between multiple taxa (14), in total, 343 SNPs were included in the analyses, of which 63 are associated with microbiota and 278 are associated with depression.

2.2 Systems Biology Analysis

Protein-protein interaction networks are composed of nodes (proteins) and lines that connect them represent types of interaction or association between proteins (physical interaction, co-expression, experimental).

Interactions between proteins were obtained from the STRING v11 database (15) Only interactions with a combined score greater than or equal to 0.7 were maintained and only experimental, database and co-expression evidence was considered. For the generation of these networks, the R Studio v 4.0.2 interface was used (16,17). The protein of each gene in the list of SNPs extracted from the articles was mapped using the R package BioMart (18).(Figure 1)

2.2.1 Protein-protein interaction network

A protein-protein interaction network was created based on the SNPs of the depression meta-analysis and another network based on the SNPs identified by the systematicreview of microbiota, so the expansion of the networks were conducted independently. For this purpose, protein-protein interaction data were obtained from the STRING database, then imported into R 3.6. Only interactions with a combined score equal to or greater than 0.7 (high confidence interactions) were maintained. Given the proteins of the SNPs, the 10 neighboring proteins of up to three degrees were selected for each one, since closely related proteins tend to participate in similar biological processes. Protein-protein interaction networks were visualized in the Cytoscape v 3.7.2 software (19), in which the networks were intersected in order to identify connections and proteins in common between them.(Figure 1)

2.2.2 Functional enrichment

The ClueGO plugin(20)of the Cytoscape v 3.7.2 software was used to obtain non-redundant biological terms for large clusters of genes. The tool integrates terms from Gene Ontology (GO), as well as KEGG pathways and BioCarta, and establishes a functionally organized list of GO terms/pathways. Analyze one or compare two lists of genes and comprehensively visualize functionally grouped terms (21). The following parameters were used in ClueGO: two-sided hypergeometric (statistical test for the enrichment), Bonferroni step down correction, and kappa score of 0.4.(Figure S1)

2.2.3 Centrality measures

To assess the degree of importance of proteins in networks, two measures of centrality were used. Node degree (connectivity) corresponds to the number of interactions of a given protein. Betweenness (intermediation) is how much a protein belongs to the center of the network and is obtained by counting the shortest paths that pass through a certain vertex. A node with high centrality has a great influence on the transfer of information across the network, under the assumption that transfers follow the shortest paths (22). In other words, the node acts as a bridge between different parts of the network.(Figure 2)

From the centrality measures, the following protein categories were created: hub, bottleneck and hub-bottleneck. Hub nodes tend to form clusters. In protein-protein interaction networks (PPI), hubs represent proteins with a large number of interactions (in this study, 99th percentile of node degree). Nodes in the bottleneck category (99th percentile of betweenness) correspond to nodes that bridge two or more clusters of nodes (11). Hub-bottlenecks are distinguished by being nodes that, in addition to grouping interactions, bridge several groups of them (Figure 3).

3.Results

Biomart extracted 278 Depression SNPs and 63 Microbiota SNPs and 30 SNPs were mapped for depression and 12 SNPs for microbiota. Based on these SNPs a network with 1217 proteins and 2143 proteins for microbiota and depression, respectively. Analyzing the centrality of the network, some proteins stand out with a large number of connections forming hubs, others that form bottlenecks and proteins

that act as both hubs and bottlenecks (HB) (Figure 3). Thus, the Rab GDP dissociation inhibitor beta (GDI2) protein stands out with the highest node degree. In addition to the GDI2 protein, we can highlight as HB the Neutrophil elastase (ELANE), Arginase 1 (ARG1), Alpha-galactosidase A (GLA), KxDL motif-containing protein 1 (KXD1), Myeloperoxidase (MPO), Kelch-like protein 12 (KLHL12), 26S proteasome regulatory subunit 7(PSMC2), 26S proteasome regulatory subunit 8 (PSMC5) 26S proteasome non-ATPase regulatory subunit 1 (PSMD1) protein. Note the presence of proteins PSMC2, PSMC5 and PSMD1 in more than one process as shown in table 1.

Among the identified processes, those involved in the action of the proteasome in the immune reaction and exocytosis were the most significant.

4. Discussion

In the present study, we gathered data on SNPs associated with depression and changes in gut microbiota. Hence, we sought to identify common biological processes and potential molecular targets for treatment and dysfunctional molecular pathways.

Here, we identified ten proteins: GDI2, ELANE, ARG1, GLA, KXD1, MPO, KLHL12, PSMC2, PSMC5 e PSMD1. To understand the high-level functions and utilities of the uncovered proteins, gene ontology analysis revealed exocytosis of azurophil granule lumen proteins and processes related to degradation by proteasome. (Figure 4). The relevance of each protein and potential interaction mechanisms in the context of gut microbiota and MDD are described below.

4.1 Most significant biological processes

We found that Exocytosis of azurophil granule lumen proteins was the most significant process in the intersection network, suggesting that this process may be associated with both depression and changes in the microbiota. The azurophile or primary granules were originally defined by their high myeloperoxidase (MPO) contentand their affinity for the basic dye blue A (23) Azurophil granules are generally described as spherical. Like lysosomes, they contain CD63 on their membrane. (24), but are considered specialized secretory granules (25). Azurophil granules undergo limited exocytosis in response to stimulation (26,27) , its main role is believed to be to kill and degrade microbes involved in the phagolysosome. (28). MPO reacts with H2O2 formed by NADPH oxidase, increasing its toxicity by the formation of hypochlorous acid and other chlorination products, tyrosine radicals and reactive nitrogen intermediates that attack the surface of microbes (29). Thus, the pathways of action of the azurophilic granules are associated with the first immune response. Proteins such as GDI2, ELANE, ARG1, GLA, MPO and PSMD1 participate in this biological process and play an important role in the network.

4.1.1 GDI2

The action of GDI2 is to inhibit the dissociation of GDP by regulating the GDP-

GTP exchange reaction of members of the rab family, small GTP-binding proteins of the ras superfamily, which are involved in vesicular trafficking of molecules between cell organelles. GDIs decrease the rate of dissociation of GDP from rab proteins and release GDP from membrane-bound rabs. GDI2 is ubiquitously expressed. GDI2 gene contains many repetitive elements, indicating that it may be subject to inversion/deletion rearrangements. Alternative splicing results in multiple transcriptional variants that encode distinct isoforms. There is evidence that it acts in the neutrophil degranulation process, aiding in the cellular phagocytosis process. Studies show that Rab GDP dissociation inhibitors (GDI) are evolutionarily conserved proteins that play an essential role in the recycling of Rab GTPases necessary for vesicular transport (secretory pathway). GDI2 has a paralog called GDI1, in which mutations were found in two affected families with X-linked non-specific mental retardation. One of the mutations caused a non-conservative substitution (L92P) that reduced the binding and recycling of RAB3A, the second was a null mutation. Thus, mutations in GDI1 can lead to functional and developmental changes in the neuron and, consequently, severe impairment of learning abilities. Furthermore, studies demonstrate a significant importance in some neurological pathologies (such as Alzheimer's)(30). Thus, given the similarity of GDI1 and GDI2 and the studies mentioned above, mutations in GDI1 could cause neurological damage. Therefore, further studies are needed to better elucidate the role of GDI1 in depression and changes in the gut microbiota.

4.1.2 DENND1A

We found that the protein encoded by the DENN Domain Containing 1 gene (DENND1A) is directly connected to GDI2, an important protein for the network. Furthermore, DENN1A is a SNP associated with major depression, which was prospected from the meta-analysis. It is a guanine nucleotide exchange factor (GEF) that regulates clathrin-mediated endocytosis through the activation of RAB35. It promotes the exchange of GDP to GTP, converting RAB35 bound to inactive GDP to its active GTP-bound form. It regulates clathrin-mediated endocytosis of synaptic vesicles and mediates the egress of early endosomes. DENN domains are found in a widevariety of proteins of seemingly unrelated functions, including 5 and 13 related to myotubularin, DENN / MADD / Rab3GEP, Rab6 interaction protein 1 and tumorigenicity suppressor , many of which have been linked to Human diseases Interestingly, in addition to DENND1A being associated with depression, studies suggest that variants of this gene are associated with polycystic ovary syndrome (PCOS), a more common endocrine disorder in women affecting 6% to 10% of women of childbearing age. Depression and stress are high risk factors among patients with PCOS, along with impaired metabolic and reproductive characteristics. (31), being changes in the microbiota correlated with the metabolic health of women with PCOS (32). Future studies could better assess the interaction of DENND1A variants with GDI1, which could lead to changes in signaling pathways associated with synaptic vesicle endocytosis and possible alteration in the interaction of the immune system with the microbiota.

4.1.3 ELANE

We found that the ELANE protein is important in the intersection network (hub-botteneck). The ELANE protein has a strong relationship with the action of neutrophils, being associated with neutropenia, a reduction in these cells, which provides a higher incidence of bacterial infections. The ELANE gene encodes a neutrophil elastase (NE), a serine protease highly expressed in the promyelocyte stage of granulocyte differentiation (33). Like ARG1 (hub-bottleneck), ELANE is associated with diseases of the immune system, in which there is an overactive immune response of the body against substances and tissues, resulting from an abnormal functioning of the immune system that results in the production of antibodies or targeted T cells against host tissues.

Through protein-protein network analysis, as shown in Figure 4, we show that exocytosis of proteins from the lumen of azurophile granules (code: HSA:6798751, p-corrected: 2.20E-18) was the main identified biological process. The proteins that act on this phenomenon interact with proteins encoded by the DENND1A and DENND1B genes, which have variants associated with depression and act on TCR signaling. Therefore, they regulate TH2 cells (34). Analyzing the centrality of the network, some proteins with a large number of connections forming Hubs stand out, others that formed bottlenecks and still proteins that act as both hubs and bottlenecks (HB) (Figure 3). Thus, the GDI2 protein stands out, with a higher betweenness and degree.

4.1.4 GLA

The GLA gene is associated with the process of exocytosis of proteins in the

azurophilic granule lumen. Among its related pathways are glycolipid metabolism and the innate immune system. This being related to Fabry disease in which there is an accumulation of globotriaosylceramide in many affected tissues, including the peripheral and central nervous system, heart, kidneys and skin. (35). Within this aspect it would be possible to relate to a dysregulation of apolipoproteins (https://www.genecards.org/cgi-bin/carddisp.pl?gene=GLA).PSMD1

The PSMD1 gene, in addition to being associated with the process of exocytosis of proteins from the lumen of the azurophile granule, is linked to the proteasomal cleavage of the substrate (code: R-HSA:983150, p-corrected: 1.05E-14) which acts on the complex of 26S proteasome into the 20S catalytic core particle that houses the proteolytically active sites and the regulatory 19S particle responsible for substrate interaction. This subunit is involved in antigen processing to generate class I binding peptides. Together the replacement of PSMB6 by PSMB9 increases the immunoproteasome's ability to cleave template peptides after hydrophobic and basic residues.

This process generates a large number (perhaps hundreds) of different peptides, depending on the length and sequence of the substrate protein. Only a small fraction of these peptides (almost 10%) form the exact length to be presented by class I MHC; most (approximately 70%) are too short to call. The remaining proteasome products (10-20%) are N-terminally extended precursors that require additional cleavage by cytosolic aminopeptidases for presentation by class I MHC molecules.

Proteosomes are distributed by eukaryotic cells in high concentration and cleave peptides in an ATP/ubiquitin dependent process in a non-lysosomal pathway. This gene

encodes a member of the triple-A family of ATPases that is a component of the 19S regulatory subunit and plays a role in the assembly of the 26S proteasome. Thus, the proteasome has an ATP-dependent proteolytic activity. This subunit is involved inantigen processing to generate class I binding peptides. Substitution of PSMB6 for PSMB9 increases the ability of the immunoproteasome to cleave template peptides after hydrophobic and basic residues.

The encoded protein interacts with gankyrin, a hepatic oncoprotein, and may also play a role in Parkinson's disease through interactions with symphilin-1. Alternately spliced transcriptional variants that encode multiple isoforms for this gene have been observed. [provided by RefSeq, Jul 2012].

4.2 Limitations

This study has to be interpreted in the light of some limitations. The first is that we used a systematic review to prospect SNPs associated with the microbiota and it was restricted to the population in Germany maintaining a sparsely broad beta microbiota diversity. However, this may have resulted in a smaller collection of SNPs compared to the depression data. It could use the beta diversity data to improve the microbiota network. Although we have validated genes by building a genome-wide network using the GWAS dataset, further validation of specific new genes using more samples would be needed.

5. Conclusion

According to our results, the biological process that stands out is the inflammatory. Furthermore, it was possible to understand that the interaction between the intestine-brain axis and depression are important steps to elucidate the proteins and pathways involved in the immune dysregulation associated with this pathology. Therefore, an action of neutrophils linked to an inflammatory condition resulting from the response of the innate immune system.

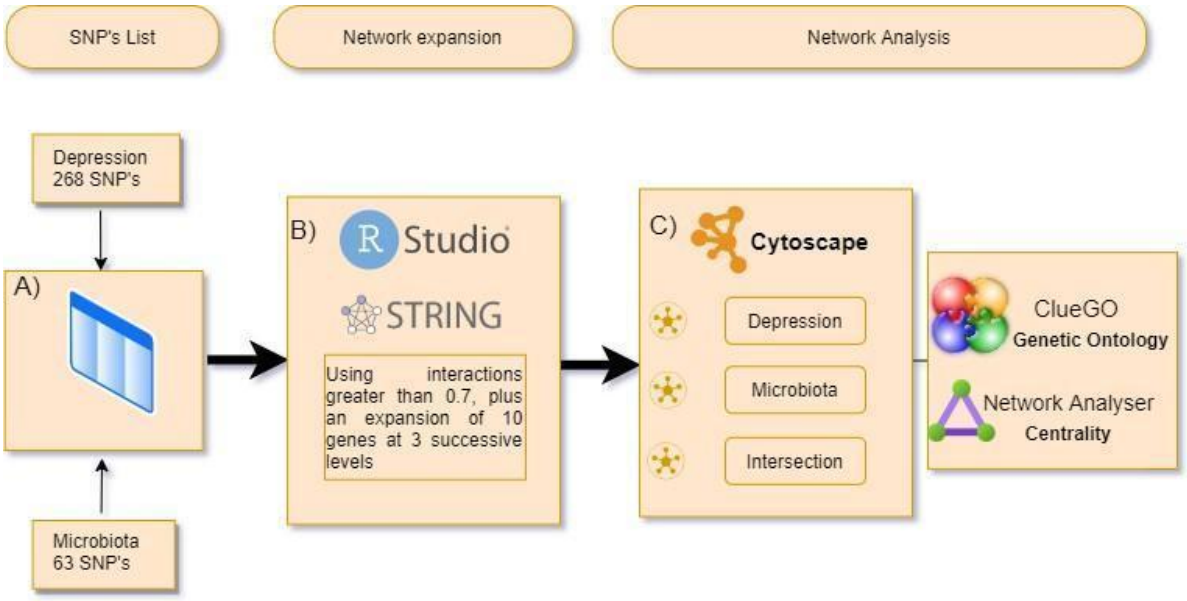

Figure 1: Outline of materials and methods. A) SNPs were prospected from a meta-analysis on genetic variants associated with depression and from a systematic review on variants associated with gut microbiota diversity. B) The SNPs were used to expand the networks using the R Studio v 4.0.2 interface and the STRING v11 database. C) The networks were imported into the Cytoscape software, where they were visualized and manipulated. In this software, two independent networks were evaluated and an intersection network was created between the protein-protein interaction networks of depression and microbiota. The ClueGO plugin was used for gene ontology and Network analyzer for centrality analysis.

Table 1: Main biological processes of the intersection network. The 10 most relevant biological processes in the network , highlighting Exocytosis of azurophil granule lumen proteins

GOID	GOTerm	Nr. Genes	% Associated Genes	Term PValue	Term PValue Corrected with Bonferroni step down	Associated Genes Found
R-HSA:6798751	**Exocytosis of azurophil granule lumen proteins**	**21**	**23.59**	**1.58E-20**	**2.20E-18**	**[AGA, ANXA2, ARG1, ARHGAP45, ARSA, C3, CCT8, ELANE, GCA, GDI2, GLA, GM2A, MPO, PA2G4, PRKCD, PSMD1, PTGES2, PYCARD, RNASET2, TUBB, VCP]**
R-HSA:180573	Degradation of ubiquitinated CD4	16	30.77	6.29E-18	8.68E-16	[BTRC, PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]
R-HSA:1504193	Ubiquitinated DVL is degraded by the proteasome	16	29.09	1.74E-17	2.39E-15	[KLHL12, PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]
R-HSA:174203	**SCF-mediated degradation of Emi1**	**16**	**29.09**	**1.74E-17**	**2.39E-15**	**[BTRC, PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]**
R-HSA:1236935	Proteasomal cleavage of substrate	15	30.61	7.75E-17	1.05E-14	[PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]
R-HSA:983150	Proteasomal cleavage of substrate	15	30.61	7.75E-17	1.05E-14	[PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]
R-HSA:211715	Proteasome mediated degradation of PAK-2p34	15	30	1.09E-16	1.48E-14	[PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]

R-HSA:264458	Proteasome mediated degradation of COP1	15	30	1.09E-16	1.48E-14	[PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]
R-HSA:5387392	processing defective Hh variants are degraded by the proteasome	15	30	1.09E-16	1.48E-14	[PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]
R-HSA:5687112	MAPK6 is degraded by the 26S proteasome	15	30	1.09E-16	1.48E-14	[PSMA5, PSMB11, PSMB2, PSMB4, PSMB9, PSMC1, PSMC2, PSMC4, PSMC5, PSMD1, PSMD11, PSME2, PSMF1, UBB, UBC]

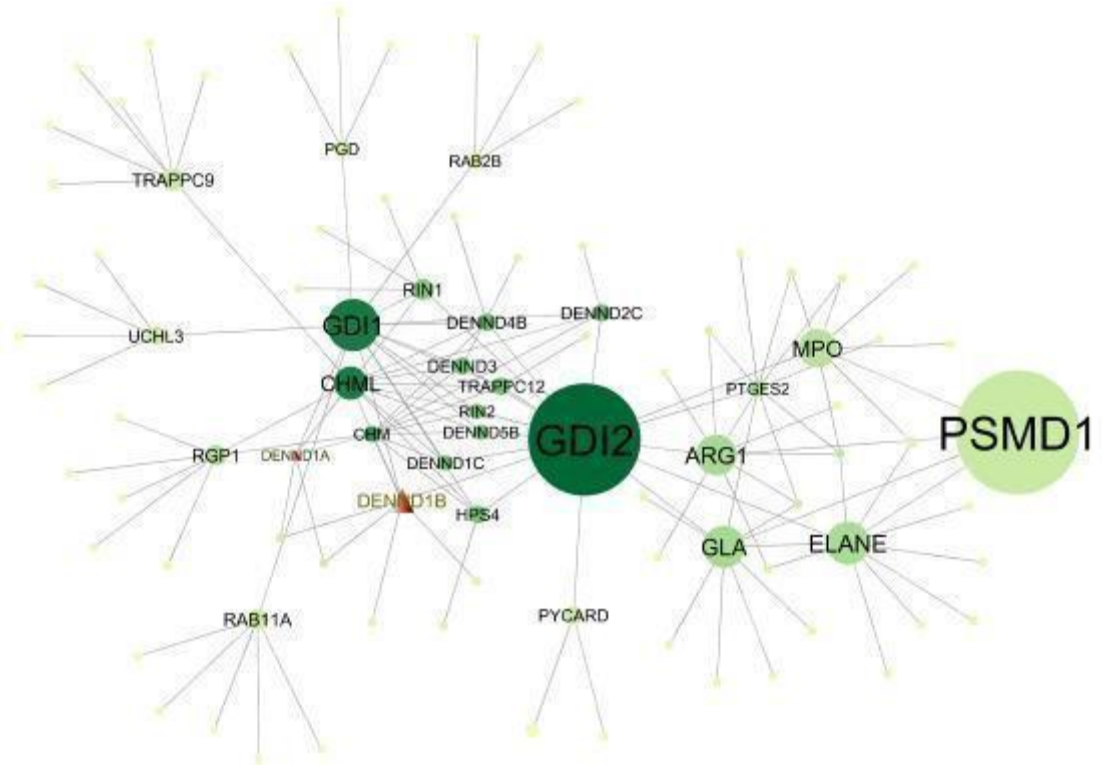

Figure 2: Network (highlight for SNPs and bottlenecks). The larger the node, the greater is its betweenness. The lines connecting two nodes represent an interaction between two proteins. Dark green circles indicates that the node has high connectivity and the SNPs are represented by triangles.

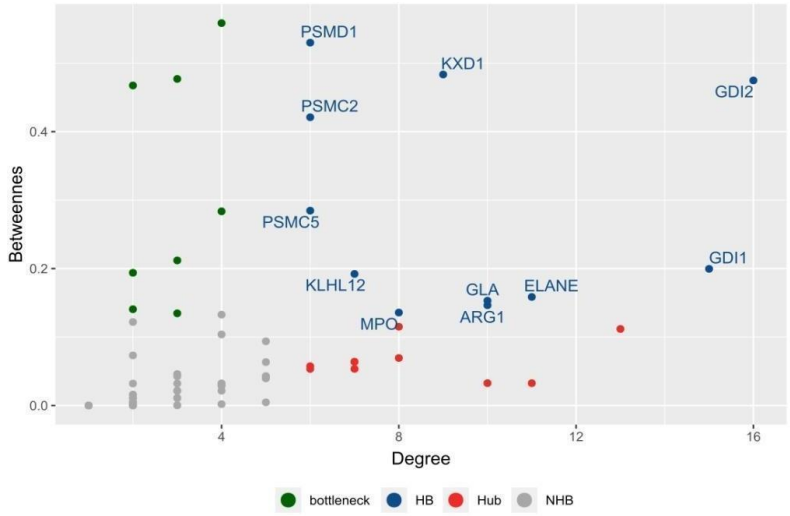

Figure 3: Centrality of intersecting network proteins. Proteins that presented degree of knot (degree, number of proteins that bind to it) and betweenness (importance for the network as a whole) above the 99th percentile were considered as hub-bottleneck proteins.

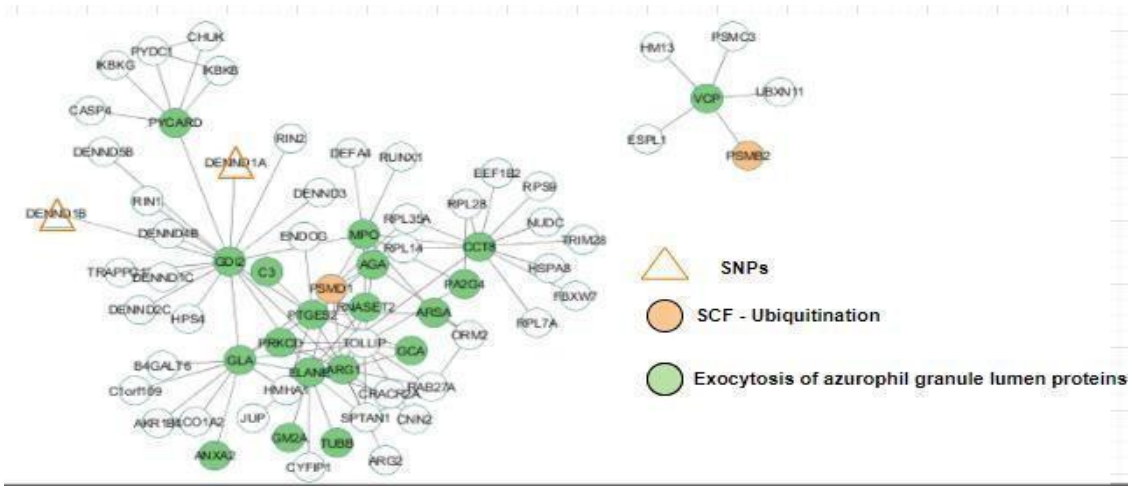

Figure 4: Protein-protein interaction network highlighting one of the main biological processes obtained by the analysis of gene ontology (exocytosis of proteins from the lumen, represented in green) and the variants prospected in the literature (triangles).

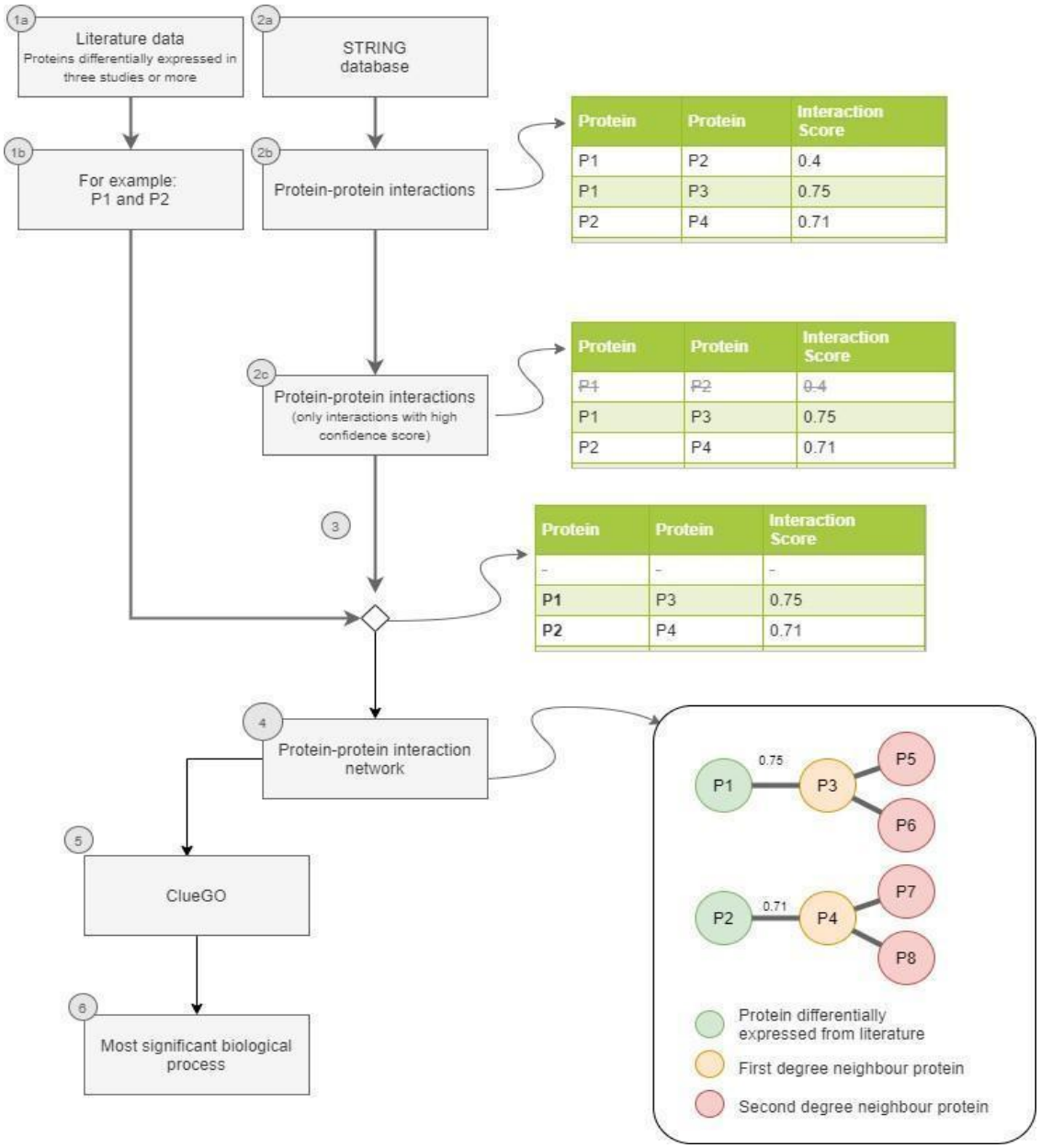

Figure 5 1a) We prospected differentially expressed proteins. 2b) For example, P1 and P2 represent two proteins differentially expressed. 3) P1 and P2 were mapped in a dataset containing protein-protein interactions. This dataset was created in three steps (2a – 2c): 2a) A dataset with protein-protein interactions was downloaded from STRING. 2b) The dataset was imported to R. 2c) After, interactions with confidence score less than 0.7 were excluded. 4) The protein-protein interaction was created based on interactions with high confidence. Neighboring proteins of up to two degrees were selected (orange and red protein in illustration in step 4. 5) Each identified protein was converted and mapped onto its corresponding gene object. 6) The final set of proteins was employed as input in ClueGO v2.5.7 (a Cytoscape v3.8 plug-in. In order to visualize the main biological process, the R package pathview version 1.24 was used

6. References

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