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A Systems Biological Approach to the Identification of Exosomal NTRK1 as a Potent Biomarker for Prolonged type II Diabetes-Induced Alzheimer's Disease

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ABSTRACT

A prolonged diabetic state leading to Alzheimer's disease (AD), attributable to diminished insulin action in the brain, is referred to as "type 3 diabetes"(DMIII). Recent studies have confirmed that signaling extracellular vesicles (EVs) or exosomes serve as potential biomarkers for AD and is also involved in the neuropathology of brain diabetes. They provide a possible link between DMII and AD, which remains unknown due to complex pathophysiological mechanism, and a lack of an integrative approach to identify novel molecular pathways. To elucidate the biological pathways, we used a systems biological method that integrates genes, interactomes and biological pathways involved, to reveal the underlying molecular mechanisms and also candidate biomarkers. Causative genes were identified from the literature and databases for AD, diabetes and exosomes, to create protein interactomes which were examined to find 214 common molecular signatures. Analysis of the subnetwork provides functional modules representing various mechanistic molecular and cellular pathway including tyrosine kinase signaling, apoptosis, cyclin-dependent kinase signaling and the homeostatic pathway. Furthermore, the prioritization of functional modules identifies NTRK1, as a potential candidate marker. These results support the possible use of these candidate biomarkers for diagnosis of diabetic-mediated AD. Overall, our molecular annotations of exosome reveal the pathophysiology of AD, to identify circulating markers for diagnosis. More studies are required to validate NTRK1 as a biomarker, in vivo and in vitro.

Keywords
Diabetes; Alzheimer's disease; exosomes; system biology; Protein-protein interaction network;

Introduction

Currently, Alzheimer's disease (AD) has been proposed as a foremost contributor to dementia, accounting for 60 to 80% of all cases (Alzheimer's disease facts and figures; Alzheimer's Dement. 2015 [1]. The disease is clinically characterized by cognitive decline, various neuropsychiatric symptoms and a reduction in day-to-day activities [2]. The pathological profile presents as deposits of fragmented protein beta-amyloid (Aβ plaques) found extraneuronally, and the build-up of intraneronal protein tau twisted fibers (neurofibrillary tangles–(NFTs) [3]. The underlying understanding of the interactive molecular pathways between AD and other causative factors in the body are needed to halt disease progression; and for application of novel therapeutic strategies. Several epidemiologic studies have supported the link between insulin resistance of type 2 diabetes mellitus (DMII) and the increased incidence of AD [4,5,6].

In order to consider an entity as a diagnostic biomarker, one should understand the core pathogenic process. The well-defined biomarkers in AD, viz. amyloid-beta and tau deposition levels, account for the progression of senile plaques and neurofibrillary tangles [7]. However, it is not understood whether amyloid and tau formation and deposition, in the neuronal cells. are etiologically linked to memory discrepancies; or do they mirror secondary effects of a diverse pathogenic mechanism [8]. Systems biology is a developing platform that incorporates multiomics machineries to provide novel biological understanding into several

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pathophysiological states. Additionally, it constructs novel candidate biomarkers that are more suitable than conventional markers [9] AD biomarker identification has generated many research articles [10], however, most of these were validated in the clinical setup for diagnosis, and are mired by this reductionist approach, where a single candidate biological entity is the focus, excluding other causative factors of key importance [7,11,12]. Hence, the identification of a candidate biological entity as a biomarker should integrate health complications, accompanying genes, and biochemical pathways to improve the biomarkers candicacy. Moreover, the pathophysiology of diabetes-induced AD is not merely the result of a single gene defect or its product alone. It is an outcome of to several mechanisms that includes neuropathy, proteopathy, calcium ion homeostasis collectively initiating and resulting in NFT, and Aβ plaques. lesion.
Exosomes, are of emerging interest in diverse pathological conditions due to their key role as signaling entities with characteristic cargo, and have been implicated in the pathology of AD [11] and DM [13]. Understanding the association between AD, diabetes and the exosomes will not only provide a novel view of disease manifestation, but also provide novel liquid biopsy options. The extracellular microviscles i.e. exosomes can be identified in a many number of biological samples including, milk, saliva, serum, plasma, urine and cerebrospinal fluid (CSF). In specific, serum or plasma-derived nanoviscles provide worthwhile alternatives to be explored for biomarker expansion [14]. DMII is the major element of metabolic syndrome (MetS) and a prolonged syndrome leads to brain diabetes or dementia. The well-known pathophysiologic progression pathways of MetS, is a source of blood-based biomarkers of DMII. They provide a minimally invasive method to predict patient outcomes, and the response to early therapeutic, to avoid the development of more severe complications [15]. Patients with different types of diabetes, and complications show alterations in exosomal number cargo, proposing that exosomes contribute to tissue- or stage-specific pathogenic mechanisms of diabetes and other complications [15].

The exosomes linked with amyloid beta, activates microglia along with native exosomes. The exosome-bound amyloid beta travels via the endocytic pathway to microglial lysosomes, where it is degraded. Thus, neuronal exosomes, which can trap amyloid beta, act as couriers of amyloid beta for amyloid beta clearance [11]. TRK (Tyrosine receptor kinase) is located around neuronal cell bodies and along cortical processes. NTRK1, (Human neurotrophic tyrosine kinase receptor, type 1), previously reported as is a single pass transmembrane protein and a receptor tyrosine kinase (RTK); and NTRK1 is located on chromosome 1. The mutation of NTRK1 of a distinct region is associated with the congenital insensitivity to pain with anhidrosis (CIPA) [16]. NTRK 1 is also implicated in neuronal morphology and identity, localization of neurons to precise destinations, and integration into functional neural circuits, as well as synapse formation with specific targets [17]. Moreover the NGF-NTRK1 (nerve growth factor) proliferates tumors with assistance of NGF at the pancreas through upregulation of p38-MAPK in the high glucose condition [18].

We used a systems biology approach to examine the molecular linkage between Type II diabetes, Alzeimer's disease (AD) and exosomes. This approach integrates data mining and high-throughput interactome examination to reveal a novel pathophysiological basis of disease for the identification of appropriate candidate biomarkers which can be easily obtained as lquid biopsies. An overview of integration of proteome and functional mapping will provides critical and novel insights into pathophysiological processes of diabetes-induced AD.)

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Experimental methods

Identification of genes/proteins through Data Mining

Data mining is the process of examining molecular entities members from large data sets. To distinguish genes/proteins associated to diabetes and AD, the text mining tool, Medline Ranker was employed. Medline Ranker [19] was used to pool or extract the relevant abstracts from PubMed for each of the given queries, viz. "AD" "Alzheimer's disease", "Diabetes", "Exosomes". These abstracts were physically curated to extract genes/proteins associated with diabetes-induced AD. The resulting genes/proteins were examined to eradicate duplicates; and to extract essential gene/proteins that are involved in both pathological conditions.

Protein-Protein Interaction Network

PPI (Protein-Protein interaction) network for diabetes, AD, and exosomes was constructed by the mined seed gene/protein lists. Each and every PPI network was generated using Cytoscape [20]. Bisogenet plug-in [21] was used to retrieve experimentally authenticated protein-protein interactions from BIOGRID, IntAct, MINT, DIP, BIND and HPRD databases [22–27]. Additionally, the networks were protracted from the seed proteins with one neighbor interaction, to ensure direct influence on the disease mechanism and regulation. After eradicating self-loops and repeated edges, the topological boundaries such as betweenness centrality, closeness, clustering coefficients, degree and shortest path lengths were analyzed with the network analyzer. Moreover, the functional relevance of every network was studied using PANTHER database [28].

Functional module identification

The Markov Cluster algorithm (MCL) in Cytoscape was used to identify hubs in the network [29]. MCL creates a flow on the graph by scheming subsequent powers of the associated square matrix, following in high-flow regions (clusters) and precincts deprived of flow [30]. The clusters of a high-flow region with an MCL score of more than 2.5 and at least lowest of three nodes were preferred for further scrutiny.

Prioritization analysis

To confirm the candidate markers, 26 cluster proteins (test set) were used and prioritized using the Moduland weighted degree Cystoscope [31]. Moduland prioritizes the proteins in the test set, by matching with a typical functional gene annotation profile of training set. In the current study, the 214 diabetes, AD, exosomal microarray deferentially expressed genes, were considered to prioritize functionally appropriate proteins (candidate markers) in the test set (system biology-derived proteins). The prioritization procedure was reiterated for 100 duplications by leave one out method in the total set.

Pathway Mapping through KEGG database

The pathway enhancement study were performed to analyse the machinery of sub-network proteins by examining KEGG databank [32]. The KEGG database covers the nature curated pathways of molecular signaling, metabolic, cellular progressions and also regulatory events.

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Understanding these processes pertaining to key molecules allows one to generate a hypothetical model that characterizes the molecular mechanism of AD.

Bioavailability

To aid possible diagnosis, observing disease progression and assessing reactions to therapies, the bioavailability of the topmost-ranked candidates were studied in the databases. In addition, analyzing the bioavailability in recurrently collected body fluids, would be advantageous for clinical applications. Human serum/plasma and urine proteomes were extracted from HPRD and plasma proteome database (PPD) [33].

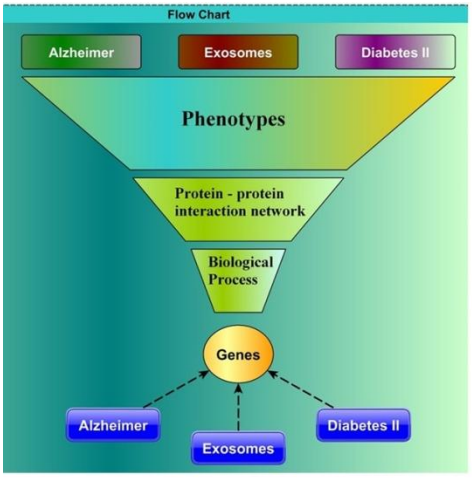

Figure 1. Systems biological framework of linkages of diabetes mediated Alzheimer's disease. The framework describes the linkages between AD, diabetes, and exosomes. These includes mining of biomolecules, construction of interactomes, and identification of functional modules, pathway analysis and prioritization of candidate biomarkers for possible diagnosis.

Results and discussion

Cognitive impairment is associated with diabetes mellitus (hyperglycemia and hypoglycaemia), abnormalities associated with insulin (insulin deficiency or resistance), vascular abnormalities and oxidative stress in the central nervous system (CNS), linking the pathophysiology of DMII and AD. Cognitive impairment in diabetic patients is two- to three-fold higher than in non-diabetic persons [34]. The study of exosomes is an emerging field in diagnostics,in the form of pliquid biopsies, and which may have relevance for AD and DM II. Evidence collected to date suggests that an elevation of exosome in the brain plays an important role in AD progression [35,36] and that a concomitant elevation of exosomes in the serum, could serve as an early biomarker of AD [37]. Hence exosomes may have a key pathological role in both AD [11] and DM [13] progression.

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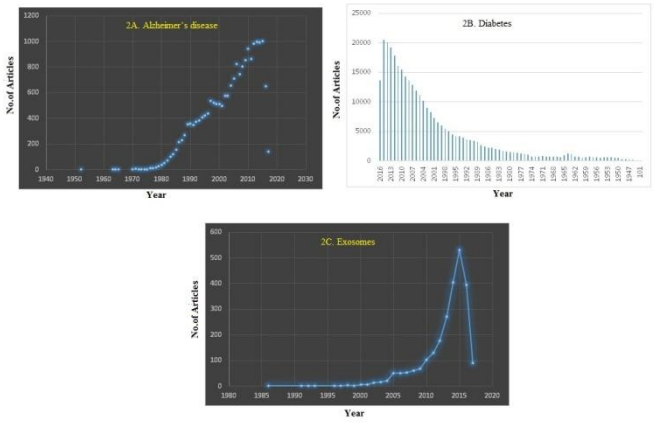

Figure 2. Text mining year wise literature extracted genes/proteins for Alzheimer's and diabetes and exosomes

An integrative systems biological approach (Fig. 1) was used in our investigation to unravel the link between AD and diabetes at the molecular level, to identify valid candidate molecular entities, associated with the pathological processes. We employed Medline Ranker [19] to search for articles from research databases, associated with key words including ''Alzheimers disease'', "Diabetes" and "Exosome". The data mining tool recovered 19,349 abstracts for AD; and also 321179; 2415 for diabetes and exosomes. Moreover, the abstracts were analysed to obtain molecular entities related to AD (Table 1 and Fig. 2ABC).

Table 1. Text mining. Literature extracted genes/proteins for AD, diabetes and pathway involved exosomes

"Alzheimer's disease", "diabetes", "exosome". It recovered 19349 abstracts for AD, and 321179, 2415 and diabetes and exosome respectively. Further, the abstracts were manually examined to extract genes/proteins related to AD and its risk factors (Table 1). Duplicates in the data sets were removed to generate potential seed lists for the construction of PPI networks.

Disease	Abstracts	Genes/proteins
Alzheimer's	19349	79
Diabetes	321179	487
Exosome	2415	31

Interaction networks for Protein

A seed list was created from each and every PPI, and networks were constructed for AD, diabetes mellitus, and exosomes, to scrutinize interactions and produce a framework for pathogenic pathways. Individual networks (Fig. 3) revealed multidimensional interactions explaining complex mechanisms. For example, the AD network entails numerous interactions with 3313 proteins. Using databases and tolls such as PANTHER and DAVID; these network entities were ascribed to a different range of biological functions. [28]. Our findings were in agreement with previous reports which alluded to the contributory role of NTRK1 in the pathogenesis of AD. As expected, diabetes showed complex interactions (8480 nodes, 184620 edges) associated with insulin signaling and resistance, PI3K-Akt, MAPK signaling pathway, which are implicated as key biological role players in the pathogenesis in diabetes-associated AD.

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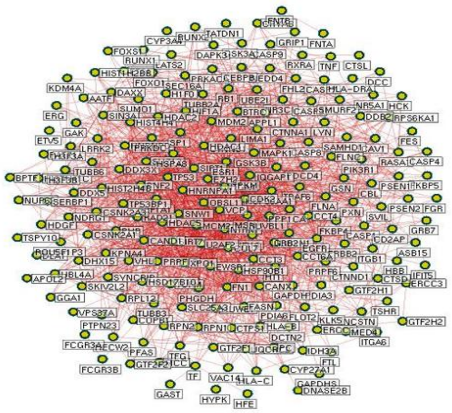

Figure 3.Common network for the experimentally confirmed associated proteins were obtained from the HPRD database. The nodes were classified based on Panther functional classification, where yellow nodes represent functional categories surrounded by corresponding proteins

Topological analysis

Using parameters such as betweenness centrality, closeness, clustering coefficients, degree and shortest path lengths, the interactions and biological mechanisms involved were constructed. Betweenness centrality was used to define crosstalk between proteins, while clustering coefficients refer to functional modules, and shortest path length refers to the path distance between proteins pairs, which establishes quick information transferred within a network. The AD topological analysis showed the following scores for network centralization, characteristic path lengths and heterogeneity 0.167, 3.394 and 2.632, respectively. Diabete mellitus displayed typical scores in a most of the topological structures, supporting multiple molecular regulatory events (Fig 4; Table 2). The collective role of the topological and functional evidence, each network provides, pertaining to the development of plaque and neurofibrillary tangle in neurons is important. The shared common molecules between all (ASCM) with AD were recognized, which resulted in 214 common proteins. And the molecules shared between AD and individual risk factors (ISCMs) were also identified, intimating that individual risk factors could participate in AD progression. The shared common proteins (214) were mapped to PPN to extract a sub-network that determines the diabetes and AD regulation.

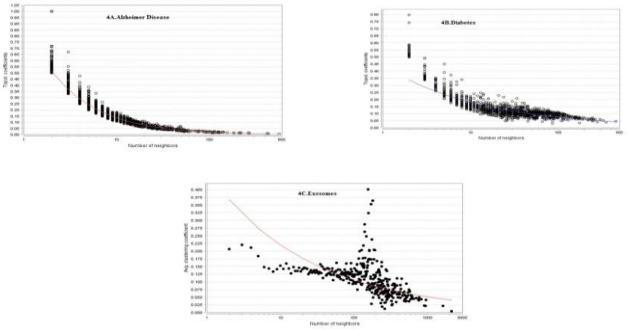

Figure 4. Topological properties of AD, diabtes and exosomes. The degree distribution plotted versus the number of nodes for the networks. The topological coefficient was plotted against number of neighbours of networks

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Table 2 Interactome topology. The topology of interaction networks features of AD and association with diabetes and exosomes

Features	AD	Diabetes	Exosomes
Clustering coefficient	0.123	0.139	0.240
Connected components	6	23	3
Network diameter	8	7	5
Network radius	1	4	3
Network centralization	0.167	0.247	0.302
Shortest paths	10919732(99%)	71529306(99%)	1872792
Characteristics path length	3.394	2.755	2.429
Avg No. of neighbors	8.422	43.537	40.487
No. of Nodes	3313	8480	1371
Network density	0.003	0.005	0.030
Network heterogeneity	2.632	1.621	1.153
Isolated nodes	4	22	2
Analysis time(sec)	115.194	13332.54	176.338

Common network

We used the HPRD database to produce experimentally confirmed seed proteins; and then the common PPI network was formulated, which consists of 13164 nodes. A lower count of nodes were observed in the exosomal network, in comparison with AD and diabetes. This common network was constructed to have connective edges contained by the seed proteins, for demonstration of the common interactome (Fig. 3). The network was produced to match the design of the network topology. The topological strictures, for instance node, clustering coefficient, topological coefficient (Tn), and shortest path length (L) were executed for the network. The topological coefficient specifies that the network is capable of holding significant hubs with narrow common neighbours displaying the property of integrated network organization (Fig. 4 A,B,C). The mean shortest path exploration of the network was 3.39, indicating that the signaling characteristic between the nodes was lower than the other network. On the whole, the topological exploration displayed the significance of scale-free design, modular network organization and also the presence of rapid signaling of the total (diabetes, AD, exosomes) network. We therefore used the common network to create the sub-network for further investigation.

MCL (Markov Clustering Algorithm)

We used the MCL algorithm to extract the subnetwork, which showed 27 clusters for ASCM network, of which, 10 clusters were designated with minimum of three nodes (Fig.5 (1–10)). The cluster centered with NTRK1 containing ninety-five interacting proteins. Of these FN1, MCM2, TP53, and GSK 3β were previously reported for their role in the formation of neurofibrillary tangles expression proteins that are connected to the activation of cell cycle mechanisms and the regulation of chromosomal duplication, observed in glial cells and neurons in the hippocampus,

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entorhinal cortex, and white matter in aging human brains with different ranges of AD-type pathology [38,39]. The cluster 2 with TP53, is connected with proteins that are strongly associated with PI3K-Akt and MAPK signaling pathway, apoptosis, cell cycle, neurotrophin; NOD-like receptor and Wnt signaling pathways. For instance, p53-evoked insulin resistance in neurons; enhanced transcription of pro-oxidants, toxic metabolite accumulation (e.g. ceramide and products of advanced glycation) and ROS-modified cellular components, together with the activation of proapoptotic genes, could collectively facilitate a suicide death program of neuronal autophagy/apoptosis [40]. The cluster 3 contains 14 interacting proteins that were attributed to NOD-like receptor signaling pathway, ubiquitin mediated proteolysis, insulin signaling pathway. Additionally, cluster 4, 5, 6, 7 and 8, 9,10 with central nodes, HIST2H4B, PC, CASP9, CYP27A1, ERCC2, CTNNA1 were found to regulate cholesterol hydroxylases, apoptosis, MAPK, PI3K and insulin signaling. Overall, the clusters showed vital regulatory molecular mechanisms of tangle formation in the neurons that was initially attributed from the association of risk factors and AD network.

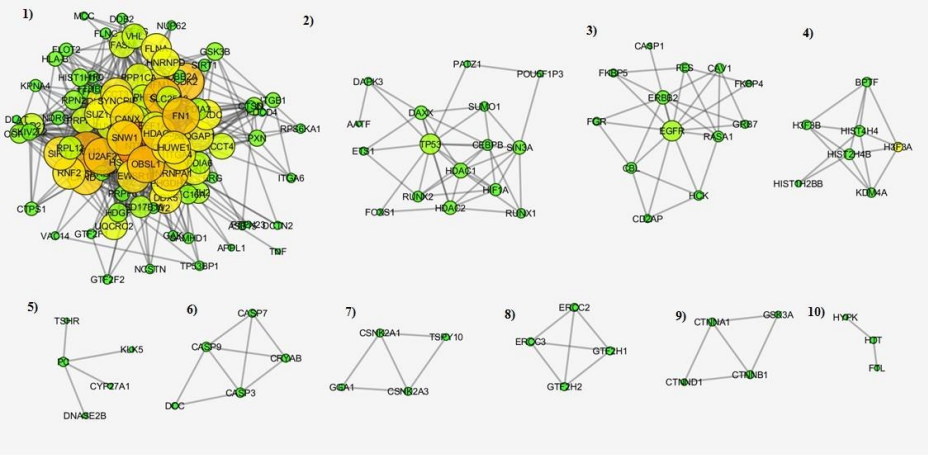

Fig. 5. Functional modules. The MCL algorithm identified eight significant clusters from the ASCM network. These clusters represent core mechanisms that aid in pathway analysis to understand the risk factor mediated pathophysiology of dibetes mediated AD.

Signaling Pathway Analysis

Pathway analysis of the ASCM and ISCMs sub-network previously provides a core mechanism of AD. The cluster proteins in ASCM, such as NTRK1, MCM2, TP53, ESR1, EGFR, CDK2, HSP90AA, are involved in MAPK, PI3K-Akt, insulin resistance and insulin signaling. These pathways have been previously reported in the pathophysiology of DMIII. p53 signaling pathway, Notch signaling, T-cell receptor signaling pathway, apoptosis, endocytosis, focal adhesion was also implicated. For example, the involvement of Notch signaling and essential ligands, Delta and Jagged, and additional type I membrane proteins, raises concerns about mechanism-based toxicities that might arise due to inhibition of gamma-secretase. It has been reported that Notch signaling is implicated in tumorigenesis and neural and non-neural cells during development and in adults. Apart from this, it controls several other molecular pathways and gamma-secretase could be involved in the pathogenesis of AD through proteolytic

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modification of Notch. In conclusively, several latest studies have indicated that enhanced Notch signaling and expression could cause neurodegeneration in AD [41].

Prioritization

Prioritization strategy was used to identify valid candidate biomarkers from the cluster proteins. The Cytoscape moduland tool was used to analyse the cluster proteins using a weighted degree algorithm, where the test set was prioritized based on the functional significance of a training set. In this study, the training set was the initially identified genes/protein and cluster proteins was the test set (196 proteins). The leave-one out method was used to identify the 8 proteins (Table 3) followed by ranking. Most of these were previously associated with in AD disease. Amongst these, the high ranked NTRK1 contributed as a membrane-bound receptor kinase that binds to neurotrophin, resuting in phosphorylation of itself and the MAPK pathway. It leads to cell differentiation and may be involved identifying sensory neuron subtypes. Mutations in NTRK1 have been related to congenital insensitivity to pain, anhidrosis, self-mutilating behavior, cognitive disability and cancer. The MCM2 (minichromosome maintenance complex component 2 gene) was the second top-ranked protein, that acts as a key role player in the IDE (Insulin degrading enzyme) in knockdown cells. Further, the TP53 was the third ranked protein, p53 (TP53) whose protein levels and post-translational modification process alters in response to cell stress (e.g. hypoxia, DNA and spindle damage). Activation of p53 occurs by several mechanisms including phosphorylation by ATM, ATR, Chk1 and MAPKs [42,43]. Similarly, the markers such as ESR1, EGFR, CDK2, HSP90AA1, VCP, FN1 had been reported for their essential role in the development of DMIII and could serve as potential targets for diagnostic applications. In order to facilitate possible diagnosis, the bioavailability of top eight proteins was mapped with plasma and urine proteome (Table 4), retrieved from proteome databases by HPRD (Human protein reference database) and PPD (Plasma proteome database).

Table 3 Prioritization and of the top 8 prioritized candidate biomolecules.

Gene/protein	Moduland overlap	Moduland weighted degree
NTRK1	1	93
MCM2	1	69
TP53	1	69
ESR1	1	65
EGFR	1	61
CDK2	1	60
HSP90AA1	1	59
VCP	1	59

Table 4 Bioavailability. The presence of contender biomolecules in body fluids.

Protein	Plasma/ Blood	Urine

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NTRK1 Human neurotrophic tyrosine kinase receptor, type 1	✓	-
MCM2 minichromosome maintenance deficient 2	✓	-
TP53 Tumor protein 53	✓	-
ESR1 estrogen receptor 1	✓	
EGFR epidermal growth factor receptor	✓	-
CDK2 cyclin-dependent kinase 2	✓	-
HSP90AA1 heat shock protein 90kDa alpha (cytosolic), class A member 1	✓	-
VCP valosin containing protein	✓	-

Conclusion

This approach and strategy using system biology shows how integrating, and functional annotations of different conditions support previously studies on diabetes mediated AD. Furthermore we are able to elucidate pathophysiological processes of AD, leading to the identification of candidate biomarkers for AD for early diagnosis. Moreover, future studies are warrneted for the confirmation of NTRK1 gene association of prolonged diabetes and AD systemic samples.

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Conflict of Interest

The authors declare that there is no conflict of interest

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