Vitamin D Deficiency-Associated Comorbidities: A Protein Network Dynamics Perspective

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Author contribution. Dr. Fliri developed the methodology for analyzing cause-effect relationships examined in this manuscript. Dr. Fliri researched literature and is the primary author. Dr. Kajiji reviewed findings, also researched the literature, and contributed equally to writing the paper.

ABSTRACT
Vitamin D deficiency has been linked to numerous comorbid diseases. Despite this recognition, the effectiveness of vitamin D supplementation in improving clinical outcomes remains uncertain. To shed light on the underlying cause-effect relationships and molecular mechanisms involved, we analyzed effects of over 4000 diseases on information transfers between biological processes in human tissues. Spectral clustering of the data generated identified relationships between disease phenotypes and perturbations of protein network interactions mediating information transfers in tissues. Examination of these relationships discovered an integrated regulatory scheme involving interactions between 188 proteins responsive to vitamin D (vitamin D interactome). Functional analysis established a central role of fifteen proteins (core vitamin D interactome) in vitamin D pharmacology and their involvement in multiple reciprocal feedback loops. Identification of functions affected by this core regulatory framework provides new insights into relationships between vitamin D deficiency-associated comorbidities, epigenetic regulation of vitamin D pharmacology, and impact of mutations on disease. Recognition of these functional relationships suggests that vitamin D associated comorbidities may not be fully treatable by vitamin D supplementation alone. Further examination of consequences of perturbations of protein interactions within the core vitamin D interactome identified 590 morbidities exhibiting physical changes co-expressed in vitamin D deficiency. Further analysis revealed that these comorbidities can be differentiated based on cause-effect relationships defined by characteristic patterns of physical abnormalities and effects on protein network interactions. These findings demonstrate the utility of systems biology-based cause-effect analyses to unravel complex relationships involving multiple diseases and multiple biological processes; thereby providing a more comprehensive understanding of comorbidities and the impact of vitamin D deficiency on health.
Introduction

Comorbidity and multimorbidity play crucial roles in disease susceptibility, progression, treatment response, and mortality, presenting a significant and escalating public health challenge. 1, 2 Vitamin D deficiency is a worldwide condition and several studies have identified the association of low vitamin D levels with various chronic and acute comorbidities. Several high-risk patient groups, including those using specific medications, are prone to vitamin D deficiency. 3 However, the role of vitamin D supplementation in treatment of these conditions is currently a subject of debate because large interventional studies have been unable to show a clear benefit. One of the reasons for this ambiguity is the predominant focus on single disease domains, despite the recognition of vitamin D's involvement in multiple diseases across the human body. Further, there is tremendous variability of vitamin D levels, dose, form, and route of administration needed. There is a need for a tailored approach to vitamin D based on the specific mechanisms underlying vitamin D deficiency in the different diseases, and it is apparent that desirable levels of vitamin D and the amount of vitamin D required to reach such levels will vary depending upon the disease and the organ systems involved. 4 Additionally, the impact of multimorbidity/comorbidity on overall disease severity lacks comprehensive characterization.

The perplexing clinical observations surrounding vitamin D strongly suggest that, despite advances in systems biology and integrative medicine, our understanding of how variations in vitamin D levels contribute to different medical conditions and serve as markers of poor health remains limited. 5 6 7 This lack of understanding arises partly from the structural and dynamic complexity of the systems involved and partly from the traditional approach of Western medicine, which tends to view health through the lens of isolated structural and functional elements rather than fully integrated dynamic systems. 8 9 10 11 12

To analyze effects of perturbations in these complex systems, we and others have developed network- and Information Theory-based methods to characterize underlying cause-effect relationships. 13 14 15 16 17 18 These approaches provide valuable insights into comorbidities and their impact on health. Identification of molecular origins of disease 19 and analysis of molecular mechanisms generating response to stressors such as, mutations of genes or drug treatments has traditionally focused on delineating functions of target proteins embedded in protein networks. 20 21 22 Protein networks consist of nodes, which can be individual proteins, groups of proteins, or other networks. 23 24 While some of these networks have well defined structures, others exhibit inducible connections and variable topologies. 25 26

Traditional cause-effect analysis uses fixed network topologies and examines topological properties of core proteins, such as degree and modularity in network topology comparisons. These methodologies have been used for identifying comorbidities associated with specific diseases. 27 28 29 However, it is important to note that diseases and drug responses are often co-expressed, which limits the reliability of this methodology in identifying causal relationships between comorbidity/multimorbidity and disease outcomes or deficiencies.

With the recognition that protein networks have inducible edges came the realization that even a single protein can engage in multiple functions by dynamically changing its connectivity with other proteins. 30 31 Thus, several studies demonstrated that interactions between proteins are dynamically regulated and perturbation of connectivity of even single proteins can be linked to multiple disorders. 32 33 Connecting network nodes transfers information and leads to generation of emergent properties of even single proteins can be linked to multiple disorders. 32 33 Connecting network nodes transfers information and leads to generation of emergent properties. 34 35 36 and the rules governing connections between network nodes vary across organ systems, physiological states, and different environments. 37 38 39 40 41 Understanding how diseases impact protein network dynamics and the generation of emergent properties is crucial for comprehending disease susceptibility, the origins of comorbidity, and determining appropriate treatment strategies. 42 43 44

Insights into how protein networks interact and generate emergent properties can be inferred from their modular design. 45 46 47 Nature's utilization of modular network designs suggests that information transfer between cells, tissues and organ systems relies on properties of certain groups of proteins which exhibit swarm-like behavior by dynamically establishing and breaking connections with other proteins. 48 49 50 51 52

For considering protein network dynamics in cause-effect analysis, we have developed methodology that uses information associated with small groups of proteins (protein swarms) mediating information transfer between biological processes in human tissues and spectral clustering of cause-effect associated information for ascertaining the organizations of proteins swarms resulting from perturbations. 53 54 55 This methodology has been successfully applied for identifying novel combinations for treatment of chronic pain, 56 repurposing of drugs for intercepting acute kidney
injuries induced by last resort antibiotics,\textsuperscript{57} and repurposed drug combinations for treating infections caused by pathogens with diverse origins.\textsuperscript{58} Herein we employ this methodology for evaluating effects of over 4000 diseases on information transfers between biological processes in human tissues and gaining insight into molecular mechanisms precipitating comorbidity in the context of vitamin D deficiency.

**Materials and Methods**

We have developed a systems pharmacology-based methodology to analyze effects of disease-induced perturbations on protein network dynamics. This approach, using information flow-based cause-effect analysis, discovers links between protein networks and minimizes connectivity bias in protein network construction.\textsuperscript{59, 60, 61, 62, 63, 64, 65, 66} The method uses spectral clustering of measurements that assess the potential of diseases to impact information transfers between biological processes in human tissues. Protein swarms employed consist of no more than 5 proteins per swarm. These swarms are used for identifying co-citation frequencies between diseases and protein swarm members in the Medline database. These measurements provide estimates on the potential of a disease to impact information transfers mediated by a protein swarm. Protein swarms are derived using the tissue-specific expression of protein-encoding genes and gene enrichment analysis to determine overlaps between tissue and biological process networks.

To conduct system-wide cause-effect relationship analysis, we extract information from various sources, including Medline,\textsuperscript{67} the Human Protein Atlas,\textsuperscript{68} and the STRING platform.\textsuperscript{69} The STRING platform is also used for gene enrichment analysis and protein network construction. Through these data sources and tools, we identified over 7900 groups of proteins, each comprising less than five members capable of transferring information between hundreds of different biological processes in human tissues. For evaluating how over 4000 diseases impact information transfer capacity of these 7900 protein swarms, we employ a data-mining algorithm developed by SystaMedic Inc. in collaboration with the University of Connecticut. This algorithm determines the sum of co-citation frequencies of a disease with members of a protein swarm in the Medline database. These measurement collections (similarity matrices) are analyzed using UPGMA clustering with cosine correlation as similarity measure.\textsuperscript{70} Likewise, for determining disease phenotypes, we determined the co-citation frequencies of the name of a disease with a standardized name of a pathophysiology referred to as the Monarch term, physical abnormality in the Medline database and followed by UPGMA clustering of the collected measurements using cosine correlation as similarity measure.\textsuperscript{71, 72} TIBCO Spotfire Data Visualization and Analytics Software are used for hierarchical clustering and data visualization.\textsuperscript{73, 74}

**Results**

**Identification of clusters of protein swarms involved in vitamin D signaling:**

Vitamin D is a fat-soluble vitamin that plays a crucial role in maintaining the calcium level in the body. Vitamin D interacts with the nuclear vitamin D receptor (VDR) to form a complex, which then binds to the promoter region and modulates the expression of target genes.\textsuperscript{75} Additionally, vitamin D can also exert its effects through non-genomic actions, where it binds to VDR and activates various signaling pathways, indirectly influencing the transcription of numerous genes.\textsuperscript{76}

Evaluation of impacts of diseases on the information transfer capacity of protein swarms involved spectral clustering of information density measurements of 4257 diseases with 7906 protein swarms. The first step in this cause-effect analysis generated a heat map (Figure 1) which identified six protein swarms (VDR 1-6) containing the vitamin D receptor VDR within a confidence in cluster similarity threshold of 0.9. The second step involved identifying proteins within each of the VDR clusters 1-6 and using the collected proteins for constructing protein networks. Functional analysis of these protein networks was performed on a cluster-to-cluster basis and in the form of a single interactome.\textsuperscript{77} These steps allowed for a comprehensive evaluation of how diseases impact the information transfer capacity of protein swarms involved in VDR mediated signaling.
**Figure 1.** Global assessment of effects of diseases on biological processes in all tissues. The vertical dendrogram axis identifies associations between diseases while the horizontal dendrogram identifies associations between protein swarms. Positions of six protein swarms containing the vitamin D receptor (VDR) are identified by vertical lines. Diseases affecting information transfers in swarm clusters 1-6 are highlighted in green.

**Construction of the disease-associated VDR interactome:**
The connectivity between 188 proteins captured in VDR clusters 1-6 (Figure 1) defining a cause-effect relationship derived VDR interactome, was determined using the STRING platform and known physical interactions between proteins.\(^78, \ 79\) This step produced the protein interaction network shown in Figure 2. Functional characterization using the STRING platform revealed that these 188 proteins participate in >2000 biological processes. Moreover, statistically significant enrichment of disease-associated mutations (70/188 nodes; false discovery rate of \(10^{-10}\)) indicated that this protein network plays a crucial role in regulating physiologic functions in health and disease.\(^80\)

To examine the core functions of the VDR interactome, particular attention was given to proteins highlighted in green at the center of the network in Figure 2.\(^81\) These proteins are expressed in all tissues and belong to the swarm clusters 1212-1227, representing VDR cluster 2. Examining proteins in swarm clusters 1212-1227 identified 15 proteins.\(^82, \ 83\)
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Figure 2. VDR Interactome: Protein interaction network identifying the physical interactions between 188 proteins constituting protein swarms in VDR clusters 1-6. Proteins at the center belonging to VDR cluster 2 are circled in green (VDR, AKT1, MAPK1 and NOTCH1). These proteins are members of swarm clusters 1212-1227 identified in Figure 1.

Functional analysis of swarm clusters 1212-1227:
The connectivity between fifteen proteins in swarm clusters 1212-1227 shown in Figure 3 was determined using the STRING platform. A significant finding provided by protein network construction is that mutations in any one of these fifteen proteins result in disease: VDR,\(^8_4\) AKT1,\(^8_5\) CFLAR,\(^8_6\) CYP24A1,\(^8_7\) CYP27B1,\(^8_8\) DDAH1,\(^8_9\) DDAH2,\(^9_0\) DNMT3B,\(^9_1\) MAPK1,\(^9_2\) NOTCH1,\(^9_3\) PROC,\(^9_4\) RAN,\(^9_5\) REST,\(^9_6\) TNFRSF1A,\(^9_7\) ZEB2,\(^9_8\).

Therefore, defects in vitamin D signaling can be expected to contribute to or worsen impacts of perturbations in signaling pathways involving these fifteen proteins, and vice versa. Evaluating functions affected by mutations in these proteins shows that these proteins play crucial roles in regulating key system processes such as apoptosis, redox signaling, stem cell differentiation, cell proliferation, developmental processes, responses to vitamins, hormones, growth factors, nitric oxide...
(NO) production, lipid metabolism, oxidative stress responses, transport, coagulation, wound healing, and activities of minerals.\textsuperscript{100,101,102,103,104,105,106,107}

\textbf{Figure 3.} Core regulatory scheme within the VDR Interactome showing known connectivity of proteins in swarm clusters 1212 - 1227. Proteins for which at least one genetic variant is involved in a disease are identified by red circles. The serine-threonine protein kinase AKT1 at the center of Figure 3 interacts with all proteins suggesting that AKT1 plays a key role in vitamin D actions and regulating functions of the core vitamin D interactome.

A better understanding of how vitamin D signaling impacts functions of an integrated regulatory schemes can be gained by examining protein interactions shown in Figure 3. Among the core VDR interactome proteins, the serine-threonine protein kinase AKT1 emerges as a central player interacting with all the other proteins. AKT1 is involved in several processes, such as insulin signaling, regulation of endothelial function, metabolism,\textsuperscript{112} and activation of nitric oxide (NO) production by phosphorylating endothelial NO synthase (eNOS) at serine 1177.\textsuperscript{113} The binding of vitamin D to VDR activates the mitogen-activated phosphokinase (MAPK)- and AKT1-mediated pathways, leading to increases in NO production.\textsuperscript{114, 115, 116} Elevation of NO levels generates a positive feedback loop, as the resulting activation of PI3K and MAPK signaling pathways further increases NO production.\textsuperscript{117} The observation that inhibition of AKT1 decreases NO production but increases the expression of the calcitriol-producing enzyme CYP27B implicates that this feedback loop plays a role in regulating NO and vitamin D signaling.\textsuperscript{118}

The key role of the VDR interactome in regulation of NO levels is further supported by the findings that NOTCH1, besides having a role in activating canonical transcriptional pathways and affecting endothelial functions, also regulates NO production through redox signaling-mediated feedback loops.\textsuperscript{119} Thus, inhibition of NOTCH1 signaling increases the expression of inducible NO synthase (iNOS), enhances the production of superoxide and peroxynitrite, and reduces phosphorylation of eNOS and AKT1. Conversely, oxidative stress-induced activation of NOTCH1 reduces peroxynitrite production and increases NO production.\textsuperscript{120, 121}
Core proteins within the VDR interactome also play a crucial role in regulating apoptosis which in-turn is tightly linked to oxidative stress.\textsuperscript{122} This regulation involves the fine-tuning of the activity of CASP8 and FADD-like apoptosis regulator, CFLAR which is expressed in multiple organs and regulates inflammation. The functions of CFLAR are also regulated by NO and vitamin D levels. Thus, inhibition of NO synthesis, for example by inhibiting AKT1 activity, increases the sensitivity of stem cells to apoptosis.\textsuperscript{123, 124} Likewise, vitamin D deficiency increases the sensitivity to apoptosis. Countering these effects, vitamin D-stimulated NO production decreases the sensitivity to apoptosis.\textsuperscript{125}

A key role of the core VDR interactome in regulating oxidative stress responses is further supported by the observation that calcitriol downregulates transcriptional activation of DNA methyltransferases 1 3B promoter (DNMT3B). This promoter together with the Silencing Transcription Factor, REST\textsuperscript{126} and the G protein, RAN\textsuperscript{127} plays a crucial role in regulating oxidative stress responses in stem cells.\textsuperscript{128, 129}

Yet another multifunctional protein within the VDR interactome is NOTCH1. The activation of Src-by NOTCH1 triggers the phosphorylation of the eNOS inhibitor Caveolin 1 (CAV1) at tyrosine 14 which causes (1) the activation of eNOS and (2) an increased NO and calcitril production by a feedback loop involving caveola-mediated endocytosis.\textsuperscript{130, 131} Activation of caveola-mediated endocytosis promotes the hydrolysis of the eNOS inhibitor dimethylarginine by dimethylaminohydrolase 1 (DDAH1), which is another protein interacting with AKT1.\textsuperscript{132, 133} DDAH1, impacts NO production by two different mechanisms.\textsuperscript{134} First, by degrading the eNOS inhibitor, asymmetric dimethylarginine and second, by directly activating AKT1.\textsuperscript{135} Evidence supporting the impact of vitamin D on this feedback loop comes from the observations that patients with vitamin D deficiency have significantly higher levels of asymmetric dimethylarginine.\textsuperscript{136}

Furthermore, interactions of tumor necrosis factor-α (TNF) with its receptor TNFRSF1A shown in Figure 3 also affects functions of the aforementioned feedback loop. Thus, the interaction of TNF with its receptor not only activates eNOS and AKT1 by inducing the phosphorylation of AKT1 at Ser 473 and of eNOS at Ser 1179,\textsuperscript{137} respectively, which increases NO production but also induces the formation of additional caveolae.\textsuperscript{138} Closing the loop, this increased NO production increases the production of TNF-α transcripts\textsuperscript{139} and the formation of additional caveolae. These interlinked feedback loops regulate vitamin D activation, NO production and caveolae-mediated endocytosis. Activation of caveolae functions impacts additional feedback loops. One of these loops is a result of AKT1 and NOTCH1 activation which triggers the expression of the transcription factor ZEB2.\textsuperscript{140} This transcription factor plays a key role in transforming growth factor β (TGFβ)-signaling which affects the immune system, the activation of Akt-signaling,\textsuperscript{141, 142, 143} and via this mechanism NO production. Degradation of TGFβ by caveolae-mediated endocytosis results in a negative feedback loop as it reduces TGFβ-mediated AKT1 activation.\textsuperscript{144} Hence, ZEB2 like NO is a core regulator that plays a pivotal role in the physiological regulation of adult cell proliferation, metabolism, survival, gene expression, mesoderm and vascular differentiation based on its ability to regulate NO, vitamin D activation, and caveolae-mediated endocytosis through TGFβ-mediated feedback loops.\textsuperscript{145}

Additionally, protein C (PROC) functions as an anti-thrombogenic component of the coagulation system. The effects of vitamin D deficiency on this system are well documented as activated protein C activates AKT1.\textsuperscript{146, 147} This observation highlights yet another role of the core VDR interactome and connects vitamin D and NO levels with functions of the coagulation cascade.\textsuperscript{148}

In summary, functional analysis of central portion of the VDR interactome (Figure 2) reveals a regulatory scheme comprising of multiple feedback loops that control NO production, vitamin D activation, oxidative stress responses, TGF b-mediated signaling, apoptosis, caveolae-mediated functions, coagulation cascade, epigenetic regulation, and immune responses. These findings provide substantial evidence that the core VDR interactome plays a fundamental role in maintaining the health of cellular and organ systems. These observations align with the vast amounts of available observational data and physical associations linking vitamin D to energy homeostasis, and regulation of the immune and endocrine systems.\textsuperscript{149}

**Identification of diseases affecting protein interactions within the core VDR interactome:**
Perturbations of identified feedback loops affected by vitamin D signaling are expected to impact functions of the protein interaction network that regulates over 2000 biological processes...
Inspecting information densities in pertinent dendrogram relationships in Figure 1 and highlighted in Figure 4 showed that 590 diseases and vitamin D deficiency modify the information transfer capacities of swarm clusters 1212 – 1227. This observation is suggestive of cause-effect relationships between perturbations of the core VDR interactome and development of disease.

As shown in Figure 5A and detailed in TABLE 1, these 591 diseases exhibit physical abnormalities commonly observed in cases of vitamin D deficiency. The heterogeneous effect patterns (pathophysiological abnormalities) associated with disease clusters (highlighted in Figure 5B) can be attributed to at least three factors (1) variations in vitamin D levels; (2) differential contributions of feedback loops regulated by protein interactions within the VDR interactome, and (3) additional mechanism of actions. Figure 5B provides a closer examination of the heat map shown in Figure 5A and illustrates the occurrence with respect to a subset of clusters and suggests the presence of distinct mechanisms underlying disease phenotypes (as described in TABLE 2). Moreover, these 590 diseases are grouped into discrete phenotypes which exhibit characteristic patterns of physical abnormalities (highlighted in Figure 5B). These findings provide an interpretation of cause-effect relationships centered around VDR. The differential expressions of these physical abnormalities among different phenotypes could be attributed to variations in vitamin D levels between phenotypes and/or contributions of mechanisms of action beyond swarms 1212-1227 in VDR cluster 2. Other potential contributors, such as VDR clusters 1, 3-6 and other protein swarm clusters associated with disease phenotype, have not yet been analyzed but can be explored through further analysis of disease phenotypic protein swarm associations identified by the dendrograms depicted in the global analysis shown in Figure 1 linking these 591 comorbidities and beyond just the contributions of vitamin D deficiency.

Identification of vitamin D deficiency-associated physical abnormalities:
The term “physical abnormality” refers to standardized ontological terms that are used to describe and computationally analyze phenotypic changes found in human diseases. To identify the physical consequences / pathophysiological changes resulting from perturbations of protein interactions in the VDR cluster 1212-1227, hierarchical clustering was performed using co-citation frequencies between the names of 591 diseases and the names of disease associated phenotypic abnormalities. As shown in Figure 5A and detailed in TABLE 1, these 591 diseases exhibit physical abnormalities commonly observed in cases of vitamin D deficiency.
Figure 5A. The heatmap shows frequencies of 50 vitamin D-associated physical abnormalities (TABLE 1) for 591 diseases that affect the Core VDR interactome.

**TABLE 1:** List of 50 Physical Abnormalities of 591 Diseases Associated With Vitamin D Deficiency

<table>
<thead>
<tr>
<th>Abdominal pain</th>
<th>Cholestasis</th>
<th>Hyperostosis</th>
<th>Pain</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>Cirrhosis</td>
<td>Hypertension</td>
<td>Pneumothorax</td>
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<tr>
<td>Alopecia</td>
<td>Cognitive impairment</td>
<td>Hypoglycemia</td>
<td>Rickets</td>
</tr>
<tr>
<td>Anemia</td>
<td>Diabetes mellitus</td>
<td>Hypophosphatemia</td>
<td>Scoliosis</td>
</tr>
<tr>
<td>Anorexia</td>
<td>Dyspnea</td>
<td>Immuno-deficiency</td>
<td>Secondary hyperparathyroidism</td>
</tr>
<tr>
<td>Arthritis</td>
<td>Eczema</td>
<td>Inflammation</td>
<td>Seizure</td>
</tr>
<tr>
<td>Atopic asthma</td>
<td>Edema</td>
<td>Ischemic stroke</td>
<td>Sepsis</td>
</tr>
<tr>
<td>Autoimmunity</td>
<td>Fatigue</td>
<td>Malabsorption</td>
<td>Short stature</td>
</tr>
<tr>
<td>Birth weight</td>
<td>Fever</td>
<td>Mean arterial pressure</td>
<td>Sleep disturbance</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>Headache</td>
<td>Melanoma</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>Body weight</td>
<td>Hepatic Encephalopathy</td>
<td>Myopia</td>
<td>Thyroiditis</td>
</tr>
<tr>
<td>Bone pain</td>
<td>Hyper-bilirubinemia</td>
<td>Neoplasm</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>Hypercalcemia</td>
<td>Nevoid</td>
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</table>
Physical Abnormalities Associated with Vit D Deficiency

**Figure 5B.** The heat map compares vitamin D-associated physical abnormalities in 4 different disease phenotypes A-D (see TABLE 2) with that of vitamin D deficiency.

**TABLE 2: Diseases in Disease Phenotypes A-D**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>artery disease</td>
<td>adrenal hyperplasia</td>
<td>multiple myeloma</td>
<td>bile duct carcinoma</td>
</tr>
<tr>
<td></td>
<td>coronary artery disease</td>
<td>beta thalasemia</td>
<td>myeloma</td>
<td>cholestasis</td>
</tr>
<tr>
<td></td>
<td>heart disease</td>
<td>brachydactyly</td>
<td>Paget's disease of bone</td>
<td>intrahepatic cholestasis</td>
</tr>
<tr>
<td></td>
<td>hypercholesterolemia</td>
<td>congenital adrenal hyperplasia</td>
<td>plasmacytoma</td>
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<td></td>
<td>myocardial infarction</td>
<td>hypogonadism</td>
<td>prostate carcinoma</td>
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<td>iron overload</td>
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<td></td>
<td>thalassemia</td>
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<td></td>
<td></td>
<td>Turner syndrome</td>
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**Discussion**

These results expand our previous work on utilizing protein-protein interaction networks to unravel complex cause-effect relationships.\(^{153}\) We now demonstrate that integration of dynamic properties of protein interaction networks with cause-effect analysis provides an in-silico approach to guide improved prevention and management of comorbidity and multimorbidity, a key factor responsible for increased morbidity and a major challenge to health systems worldwide.

Vitamin D deficiency was selected as the model system; it has emerged as a modern epidemic and there is a well-established association between suboptimal vitamin D levels and various comorbidities. Most studies on the relationship between vitamin D and morbidity have focused solely on effects within single disease domains, despite the association of vitamin D biology with several diseases throughout the human body. The objective of this investigation was to decipher functionality of the vitamin D interactome based on analysis of effects of diseases on protein network dynamics and thereby, generate an improved understanding of the molecular origin(s) and variability\(^ {154}\) of treatment outcomes in vitamin D deficiency-associated morbidities.

Since diseases serve as indicators of perturbations of dynamic properties of protein networks and affect information exchange in human tissues, we examined information densities at the intersections between > 4000 disease phenotypes and ~8000 protein swarm clusters (Figure 1) This vantage point allowed us to evaluate how stressors such, as vitamin D deficiency impact network-network interactions mediating information transfers in...
human tissues on a system wide scale, and identified six protein swarm associations, containing in-aggregate 188 proteins. The capacity of these 188 proteins to interact and to transmit vitamin D-induced signaling was determined using known physical interactions to identify edges between network nodes for network construction (interactome). These cause-effect and functional analyses led to identification of the VDR interactome shown in Figure 2. The significance of this network in regulating biological functions is evidenced by the high number of disease-associated mutations (70/188 nodes; false discovery rate of 10⁻¹⁰). The importance of this regulatory scheme in regulating protein network dynamics and vitamin D pharmacology is further supported by the finding that mutations of the 15 proteins (Figure 3) at the center (hub) of VDR interactome and residing in protein swarm associations 1212-1227 (Figure 1) cause disease. Since this regulatory scheme integrates functions of multiple feedback loops that respond to vitamin D- and NO-signaling, morbidities associated with vitamin D deficiency may not be fully treatable by vitamin D supplementation alone.

From a systems perspective, hubs are of central functional importance as they have a significant impact on a wide range of molecular processes. Disease-associated mutations in these hubs not only affect vitamin D signaling but also multiple functional categories interconnected through intricate regulatory feedback loops. The central role of AKT1, the neurogenic locus, NOTCH1 and tumor necrosis factor receptor TNFRSF1A (Figure 3) in vitamin D pharmacology has been recognized in prior studies. However, the significance of dimethylarginine dimethylaminohydrolase DDAH2, DNA methyltransferases 3B and homeodomain factors ZEB2 in enabling epigenetic regulation of vitamin D and NO responses and functions of these various feedback loops have not previously been recognized.

It is well-established that vitamin D has widespread involvement in numerous molecular processes and is believed to have ancient origins, with vitamin D usage and VDR being conserved across diverse species of plants and animals. However, the VDR interactome identified in previous studies using theoretical arguments such as, centrality, node degree and confidence in protein associations has very little overlap with the regulatory scheme identified in our system-wide cause-effect analysis. Such discrepancies between interactomes are well recognized and stem from inconsistencies between proteomics datasets and use of network connectivity criteria that are biased towards large, well-characterized proteins and underestimate connectivity contributed by smaller, less investigated proteins.

Our cause-effect analysis methodology addresses the limitations arising from the use of confidence in connectivity criteria and topological arguments for constructing and interpreting protein interaction networks relevant to vitamin D pharmacology. We have validated the relevance of the core VDR interactome by (1) using functional evidence to demonstrate that the proteins shown in Figure 3 are key drivers of vitamin D pharmacology, (2) identifying that perturbations of the regulatory scheme precipitate 590 vitamin D deficiency-associated comorbidities (Figure 4), and (3) that diseases identified using the core VDR interactome as the mechanism of action rationale express the characteristic physical abnormalities associated with vitamin D deficiency (Figures 5A and 5B). These observations illustrate that information flow-based, cause-effect analysis using protein swarms to integrate dynamic properties of proteins increases confidence in interactome construction for using proteomics data to influence decision-making.

Investigation of the pathophysiology of changes (also referred to as physical abnormalities) manifested by these 590 diseases, as understanding the pathophysiology is a crucial determinant for medical practice: it can assist in the treatment of acute and chronic illnesses, managing medications, assisting with diagnostic tests, and managing general health care and disease prevention for patients and their families. More importantly, it can help understand with speed and accuracy why any abnormal health changes have occurred in a patient, why they happened, and what can be done to resolve the situation. Thus, the methodology presented here offers a roadmap for investigating whether comorbidities have additional positive and negative effects beyond those directly caused by vitamin D deficiency. Moreover, it can be extended to assess the collective impact of comorbidities on the manifestation of pathophysiology and further applied to study the effects of multimorbidity.

**Conclusion**

Vitamin D is widely recognized as an important, inexpensive, and safe adjuvant therapy for many diseases and all stages of life. However, a clear benefit of vitamin D supplementation for remedying vitamin D deficiency-associated comorbidities has not yet been established.
To unravel this paradox requires a deeper grasp of how dysfunctions in protein network interactions, induced by vitamin D deficiency, impact cellular and organ systems. We demonstrate that spectral clustering of protein swarm-associated, cause-effect information combined with standard protein network analytics represents a compelling technology to examine relationships between diseases from a protein network dynamics perspective. Our findings implicate involvement of the core VDR interactome, comprising of 15 proteins that control multiple feedback loops, as an important vitamin D-responsive regulator of health. Perturbations of protein interactions within this core regulatory scheme precipitates 590 morbidities that can be grouped into phenotypes manifesting discrete sets of physical abnormalities typically associated with vitamin D deficiency. Accuracy in defining endophenotypes of "typical diseases" across different but interrelated datasets will enable better definition of variants and their stratification in therapeutic trials.

In summary, interactome networks capture the complexity of molecular machinery that regulates organismal behavior. Built into the structures of these complex machines is resilience to failure in case of breakdown of communications between system components; thus, preventing cell death or disease. The VDR interactome is a prototypical example of how these safeguarding mechanisms work. Study of cause-effect relationships to understand how disease-induced dysfunctions in protein network interactions affect cellular and organ systems represents a significant contribution to a technically challenging frontier in medical and pharmaceutical research.

**Key takeaways:**

a) Treatment of vitamin D deficiency-associated morbidities will require interventions, beyond mere vitamin D supplementation, to rebalance functions of multiple biological processes.

b) Comorbidities associated with inadequate levels of vitamin D can be potentially managed or prevented through holistic approaches and nutraceuticals like Cardio Miracle which have been shown to support the complex feedback loops affected by vitamin D deficiency. 171, 172

**Declarations**

**Conflict of interest:** There are no conflicts of interest.

**Submission:** This is original research that has not been published previously nor is it being considered elsewhere.

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