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Investigating The Causal Effect of Calcium Level On Motor, Cognitive, Composite Symptoms of PD, and PD Itself Using Mendelian Randomization

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ABSTRACT

Parkinson's Disease (PD), the second-most common neurodegenerative disorder, is increasing in prevalence worldwide with the phenomenon of world population ageing. Common symptoms of PD including rest tumor, dementia, and Lewy bodies can severely deteriorate patients physically and mentally; however, factors causing PD and the treatment to cure PD is still under investigation. Calcium, which is a chemical responsible for muscle contractions, has been associated with some PD symptoms such as formation of Lewy bodies and muscle rigidity. While studies that show association between calcium and PD are abundant, there is no study that specifically focuses on the causal relationship between them: hence, in this study, we aimed to examine the causal relationship between calcium level in patients and motor, cognitive, composite symptoms of PD, and PD itself using the Mendelian Randomization (MR) method. Other studies that used calcium-blocking drugs such as isradipine, mitochondrial calcium inhibitors, and MUC blockers to demonstrate the impact of calcium-blocking treatment on PD patients led us to take calcium as an exposure in our study as well. After constructing MR under both highly stringent and less stringent conditions using different p-values, we were able to observe whether there is a causal relationship identified. The result of our study reveals that there is no direct causal relationship between calcium and PD or PD symptoms, even though there might be a possibility that calcium indirectly induces PD.

Introduction

Parkinson's Disease (PD) is the second-most common neurodegenerative disorder, and its prevalence increases with age. Symptoms of PD involves progressive motor and nonmotor impairment as PD affects dopaminergic, meaning dopamine-producing, neurons. Parkinson's Disease is named after James Parkinson, an English surgeon, who described the condition of shaking palsy. PD was first described in 1817 with its symptoms of paralysis by James Parkinson [1]. Even though approximately 90% of PD patients got the disease unrelated to genetics, 10% of the patients do carry disease-causing genetic mutations [2]. Regions such as SNCA, LRRK2, GBA, and VPS13C are loci where coding variants, although rare, can cause Parkinson's disease [3].

Risk factors including aging, family history, and exposure to environmental chemicals (e.g. pesticides) are associated with idiopathic PD. PD affects male 1.5-2 times more than female, but female patients appear to have faster disease progression and higher mortality compared to male patients [4]. Therefore, sex is considered as one of the consequential factors that has a connection with PD development, along with age, genetic features, and environmental factors. As the United States population ages, the prevalence of PD and importance of figuring out therapies for PD is predicted to rise rapidly over the next 20 years [3].

PD patients experience both motor and non-motor symptoms. Motor symptoms of PD include rest tremor, rigidity, bradykinesia, and stooping posture. Symptoms such as rest tremor is experienced by over 70% of PD patients, and rigidity is experienced by almost all PD patients. Other symptoms including hypomimia,

decreased eye blink rate, dystonia, blurred vision, and speech impairment like hypophonia and palilalia are uncommon motor signs of PD. Motor symptoms only occur after PD develop to a certain level and over 50-70% neurons in substantia nigra lose their ability. However, other neurodegenerative disorders such as Corticobasal Degeneration (CBD) have overlapping symptoms with idiopathic PD such as Dementia, Lewy Bodies (DLB), and Corticobasal Degeneration (CBD) [4].

Non-motor symptoms of PD can be divided into four categories: cognitive changes, hallucinations, mood disorders, and sleep disturbance. Almost 90% of PD patients experience non-motor symptoms, but those non-motor symptoms usually do not respond to dopamine therapy, therefore, difficult to treat. Therapeutic treatments used to patients' motor symptoms might exacerbate the situation or even cause some non-motor symptoms [4]. Cognitive changes due to PD commonly results in dementia, including problems with decision-making, multitasking, memory, and visuospatial perception. Dementia occurs later in disease progression and about 60% of PD patients experience dementia.

Symptoms like hallucinations, sleep disturbance, and rest tremor which causes early morning awakening and frequent awakening at night also disturbs patients from having deep sleeps. Mood disorders that come with PD make patients feel anxious and become apathetic. Autonomic disturbance during the development of PD disables patients from doing things that they were supposed to do by themselves which include orthostasis, decrease in blood pressure soon after standing up; constipation, problem with passing objects using hands; dysphagia, swallowing difficulties; urinary difficulties, inability to completely empty bladder; and sexual dysfunction [4].

Experts in the area are trying to develop biomarker test and clinical diagnostic criteria for PD to enhance the accuracy of Parkinson's Disease diagnosis [5]. However, accurate diagnosis of PD still remains a challenge since its clinical features overlap with other neurodegenerative conditions, and better defining of PD subtypes is still needed [6]. Loss of dopaminergic neurons in substantia nigra and development of neuronal Lewy Bodies are pathophysiologic features of PD. However, the exact cause why dopamine loss and Lewy Bodies development take place in PD patients is unknown [4]. There are branches of therapies for PD: pharmacotherapy and non-pharmacotherapy. Pharmacotherapy refers to therapies to resolve dopamine deficit or inappropriate dopamine imbalance problems, while non-pharmacotherapy refers to slow exercise, education about PD, speech therapy to cure hypophonia (excessive softness while talking), and dietary control, mainly giving high fiber food [2, 4].

Pharmacotherapy that deals with dopamine level in PD patient's body is able to reduce motor symptoms of the patient, but cannot cure. Gene therapy, which targets a specific gene associated with PD, is a potentially useful method that is not often being used currently. Gene therapies of the future may also offer a complete cure to PD patients with genetic causes [2].

The significance of treating PD is clear: PD is the second most common neurodegenerative disorder that affects Americans at age 65 or above most significantly, followed after the Alzheimer's Disease which is the most common neurodegenerative disorder. Non-motor symptoms such as sleep disturbance and mood disorder of PD affects patients emotionally, bringing frequent anxiousness and apathy. Both apathy and constant anxiety seriously erode quality of life in PD patients. Chance of getting PD appears to be higher among older people, but it may also appear to people at much younger age group [4]. However, the possibility is very low for younger people, especially those under age 40. Approximately 3% of people at age above 65 are affected by PD while about 5% of people at age about 85 are affected by PD. Majority of the PD patients experience the symptoms without any genetic connection, so only about 10% of the people affected by PD have a certain genetic mutation [2].

Among the motor symptoms of PD, rest tremor of muscle can be explained through the connection between the release of calcium to muscle fibres, which is described in the as a section of IB biology course. Skeletal muscle fibres have striped appearance due to an interlocking arrangement of two types of protein filaments: the thick filaments made of myosin and the longer, thinner filaments are made of actin. Two filaments

are held together by bands called Z lines, and when nervous stimulation causes skeletal muscle contraction, myosin and actin filaments slide and reposition themselves in a more closely packed way, causing the shortening of sarcomeres. Muscle contraction occurs in several processes; therefore, certain amount of ATP is necessary for the muscle contraction to happen. Along the actin filament, there are series of binding sites where bulbous heads can fit into for the muscle contraction. However, when the muscle fibres are at rest, a protein called tropomyosin blocks the binding sites, hindering the muscle contraction. Here, calcium ions play an essential role along with proteins called tropomyosin and troponin. When the action potential arrives to the myofibril, it releases calcium ions from the sarcoplasmic reticulum. Once calcium ions are released, they react with the protein troponin, removing the blocking molecule tropomyosin. Each bulbous head of myosin reacts with a binding site on the actin molecule beside it, shortening the myofibril. Finally, when a molecule of ATP binds to the bulbous head with the enzyme ATPase, bulbous head detaches from the binding site and straightens. Likewise, the release of calcium controls the muscle contraction mechanism, playing a significant role to remove the tropomyosin, which is the blocking molecule. Therefore, irregular release of calcium ions to the muscle fibres can cause irregular muscle contraction which leads to the rest tremor, a major symptom of PD.

There are many known risk factors for PD. Amongst them, calcium is one of the promising risk factors with its clear connection to the motor symptoms of PD like rest tremor as explained earlier. Many researches show the association between calcium level and PD [7-9]. Specifically, the associations between calcium level in a type of protein called alpha-synuclein and organelles such as mitochondria, lysosomes, and mitochondria-associated ER membranes and PD are explained thoroughly in various research papers. One of the research papers shows that calcium homeostasis in alpha-synuclein has connection with pathophysiology of PD. Calcium can bind to alpha-synuclein and promote the abnormal aggregation of alpha-synuclein, which is one of the major risk factors of Parkinson's Disease (PD). There are two types of terminuses where alpha-synuclein interacts with isolated synaptic vesicles: C-terminus and N-terminus. Among the two terminuses, calcium regulates the binding with C-terminus. C-terminus binding of calcium to alpha-synuclein promotes the misfolding of alpha-synuclein, causing the aggregation of it. The aggregation of alpha-synuclein causes toxic Lewy bodies, which is thought to be one of the core symptoms in PD. In addition, misfolding or unfolding of alpha-synuclein induces increase in intracellular calcium, which might lead to an abnormal calcium influx or calcium-dependent cell deaths [8].

Mendelian Randomization (MR) uses genetic variant from genome-wide association study (GWAS) data to infer the cause-and-effect relationship between the exposure and the outcome [10]. Several studies used Mendelian Randomization to investigate the causal relationship between calcium and motor actions: some notable findings include correlational relationship between calcium deficiency and the abnormalities in muscle contractions [9].

It is well known that there is an association between calcium level and PD as several studies have shown that calcium may induce PD symptoms [8]. However, the studies have not yet investigated the cause-and-effect relationship between calcium level and PD. The aim of this research is to present the cause-and-effect relationship between calcium level and PD found through MR analysis. In addition, to explore the causal relationship between calcium and other PD symptoms including cognitive and composite symptoms. Based on the evidence that calcium ion is a key molecule that triggers the muscle contraction which was provided by Oxford IB Biology Textbook and study by Berchtold et al [9], we thought that the calcium ion present in individuals contributes as another causation of motor symptoms of PD. In Berchtold's study, he points out the fact that all muscle fibres use calcium ion as their main regulatory and signaling molecule, which is responsible for controlling muscle plasticity, contraction, disease, and every movement. Hence, we hypothesized that there will be a cause-and-effect relationship between calcium level and PD.

Methods

GWAS datasets for the calcium level and motor, cognitive, and composite symptoms of the PD and PD itself were sourced from the GWAS catalog. For calcium level, we used appropriate dataset with the biggest sample size of 315153 Europeans and 83980 East Asians [11], which was driven from a cross-population atlas of genetic associations. For all motor, cognitive, and composite symptoms of the Parkinson's Disease, we used datasets published by the same author in recent years [12]. Each dataset has sample size of 2848, 2788, and 2755 individual.

In this study, we aimed to reveal the relationship between calcium level that the individual's body owns and the chance of having motor, cognitive, or composite symptoms of Parkinson's Disease using Mendelian Randomization (MR) analysis. In order to perform a MR analysis, these three assumptions were met: first, association between the genetic variants included in the analysis and the exposure of interest is significant; second, exposure of interest is the only factor that is associated with the outcome; and third, the genetic variants only affected the outcome through the exposure of interest [10].

To satisfy the first assumption, only SNPs that are considered to be significant (p-value <0.00005) were extracted from the resulted datasets. Next, we analyzed the p-values of Inverse Variance Weighed (IVW) method, MR Egger method, and Weighted Median (WM) method to eliminate the possibility that the certain causal relationship occurred by chance and to satisfy the seconds assumption. Lastly, we eliminated any resulted datasets with y-intercept value that significantly deviates from zero, as y-intercept that is not equal to zero suggests the possibility of existence of another association other than exposure of interest; this step allowed us to satisfied the third assumption during our MR analysis.

Moreover, throughout the experiment, we used different LD values: 0.01, 0.05, 0.1, and 0.3. Utilization of different LD values means we investigated the causal relationship between calcium level and PD symptoms and PD itself in both loose and stringent conditions, which allows us to investigate if there are any causal relationship that is not identified under a stringent condition can be identified under a less stringent condition. MR results using LD values of 0.01, the most stringent condition, and LD value of 0.3, the least stringent condition, are presented in Table 1.

We used Two Sample MR and the Mendelian Randomization packages and R to perform all of the statistical analyses. p-values of <0.00005 were considered to be significant.

Results

When the MR was held in a strict condition with LD of 0.01 and p-value threshold of 0.00000005, with p-value of 0.878 and 0.599 respectively for inverse variance weighted (IVW) and weighted median (WM) method. High p-value, which tells that there is more than 50 percent possibility that the cause-and-effect relationship found in MR analysis was by chance, made us to conclude that there is no meaningful cause-and-effect relationship between calcium level and PD motor symptoms. When the same analysis was held in a loose condition with LD of 0.3 and p-value threshold of 0.00005, the p-value for inverse variance weighted method was still 0.847 and that of weighted median method was 0.104, significantly higher than the p-value threshold which is 0.00005. Using the results driven from the strict and loose conditions, we concluded that there is no causal relationship between calcium level and PD motor symptoms.

Same process was used to explore the causal relationship between calcium level and PD cognitive and composite symptoms. With all results not making the p-value threshold, we concluded that there is no cause-and-effect relationship between calcium level and PD cognitive and composite symptoms.

Table 1. Summary of Mendelian Randomization results, LD = Linkage disequilibrium, IVW = Inverse variance weighted, WM = Weighted median, SE = Standard error.

Exposure	Outcome	LD	Method	p-value threshold	Beta	SE	p-value
Motor	Calcium	0.01	IVW	5×10^{-8}	-0.35	0.55	0.87
Motor	Calcium	0.01	WM	5×10^{-8}	-0.08	0.68	0.59
Motor	Calcium	0.30	IVW	5×10^{-5}	-0.06	0.31	0.84
Motor	Calcium	0.30	WM	5×10^{-5}	-0.71	0.43	0.10
Cognitive	Calcium	0.01	IVM	5×10^{-8}	0.71	0.62	0.25
Cognitive	Calcium	0.01	WM	5×10^{-8}	0.30	0.70	0.66
Cognitive	Calcium	0.30	IVW	5×10^{-5}	0.29	0.32	0.36
Cognitive	Calcium	0.30	WM	5×10^{-5}	-0.02	0.45	0.96
Composite	Calcium	0.01	IVM	5×10^{-8}	-0.57	0.69	0.40
Composite	Calcium	0.01	WM	5×10^{-8}	-0.51	0.81	0.52
Composite	Calcium	0.30	IVW	5×10^{-5}	-0.28	0.37	0.45
Composite	Calcium	0.30	WM	5×10^{-5}	-0.31	0.52	0.55

Discussion

In this study, we explored the causal relationship between calcium level and PD symptoms using the Mendelian Randomization utilizing SNP-trait association data from large scale GWAS studies. After conducting the analysis using various criteria, both stringent and loose, we failed to detect a causal relationship between calcium levels and the motor, cognitive and composite symptoms of PD. This result may change when larger sample sizes become available, or may be possible that calcium does not causally affect PD symptoms by itself but together with other factors.

A few studies suggested the relationship between calcium and PD, leading calcium to be a suitable exposure for our study as well. For instance, the study by Jingxian Zhang [8] suggests the association between calcium homeostasis in mitochondria and PD – Zhang emphasizes that mitochondrial calcium signaling is connected with the death of nigral dopaminergic neurons. The regulation of production of adenosine triphosphate (ATP) and mitochondrial oxidant stress are the two factors that contribute to the death of nigral dopaminergic neurons in PD. The elevation of mitochondrial calcium is caused by the increase in alpha-synuclein-dependent calcium in the neurons. The rise in mitochondrial calcium promotes mitochondrial ATP synthesis, which causes controlling of mitochondrial calcium particularly important in terms of high-pacing cells like neurons. Moreover, mitochondrial oxidative stress is also enhanced by increased calcium level. The system inside the brain reduces mitochondrial oxidative stress to protect itself from mitochondrial calcium influx [8].

Furthermore, decreased lysosomal calcium is observed in the fibroblasts of PD patient, getting an attention as another factor of PD, suggesting the connection between calcium homeostasis in lysosomes and PD. There are several calcium-releasing channels and regulators in lysosome such as Lsm12, TRPML1, and LRRK2. For instance, TRPML1 plays an important role in lysosomal calcium regulation by controlling alpha-synuclein secretion and even preventing the alpha-synuclein aggregation in PD [8].

Additionally, even though minor, calcium homeostasis in mitochondria-associated ER membranes (MAMs) is also reported to have certain connection to PD due to MAMs' interlocking connection between

other organelles such as alpha-synuclein and mitochondria. MAMs have the calcium-transfer system which the transfer of calcium between ER and mitochondria mainly depends on. MAMs are closely related to mitochondria and alpha-synucleins are also located on it, which make MAMs to be associated with PD as well. Recently, a report shows that the regulation of MAMs also negatively influenced the regulation of alpha-synuclein aggregation by affecting ER-mitochondrion calcium transport [8].

A number of recent observational studies have looked at the role of calcium in PD, pointing out calcium regulation as a therapeutic target for PD. Trails of a calcium channel inhibitor called isradipine in the treatment of PD carried about by Venuto et al [13], showed that isradipine does not reduce the risk of PD nor reduce severity of PD symptoms. In this study the researchers compared 5mg intravenous injection of isradipine twice a day versus placebos over the course of 36 months after which correlation between serum isradipine concentration and Unified Parkinson's Disease Rating Scale (UPDRS) score was measured. There was no significant correlation between the treatment and changes in UPDRS score. However, this study allowed the researchers to investigate the relationship between plasma exposure to a calcium channel inhibitor with PD progression: even though isradipine exposures turn out to not have a causal relationship between most clinical measures of PD including severity, function, disability, and quality of life, there were a few significant and moderate correlation between the two factors. Firstly, there was a statistically significant positive exposure-response relationship between them, showing that higher the isradipine exposures, lower the hazard for need for symptomatic treatment in PD. Secondly, there was also a significant, but weak, positive correlation between isradipine exposure and time to need antiparkinson medication after 36 months in both male and female, reporting the association between calcium channel inhibitor and PD in both sexes. Lastly, the research also figured out a modest correlation between two factors, slower clearance rate of isradipine in participants noting less worsening of PD symptoms. Likewise, these correlations show that even though isradipine does not causally influence the PD symptoms, supporting our result about the same topic, calcium does play a certain role [13].

The study conducted by Kuntal et al [14] also supports our results, they reported an absence of a causal relationship between calcium levels and risk factors of PD symptoms, however they suggest that mitochondrial calcium regulation is a possible treatment for PD. Zebrafish that were used already had 20% loss of dopaminergic neurons and were treated with four different drugs: morpholino, MCU-i4, MCU-i11, and VDAC, which contains mitochondrial calcium inhibitors that can prevent mitochondrial calcium overload. The result revealed that three of four exposures (MCU-i4, MCU-i11, and VDAC) were able to reduce the dopaminergic neuronal death. However, similar research conducted using MCU-i4 and MCU-i11 suggests that reduced loss of dopaminergic neurons after the treatment with two drugs were not due to drugs' ability to modulate mitochondria calcium, but another factor unrelated to calcium. Taken together both of the studies suggest that calcium levels are associated with PD in some way, and it may be that calcium fluctuations within the mitochondria, rather than serum calcium levels may play a causal role in PD [15].

The idea that calcium abnormalities are present in the mitochondria rather than in serum is also supported by the work of Scorza et al [15], who have shown that there is a significant relationship between mitochondrial levels of calcium and PD symptoms. Furthermore, more they go on to state that the PD-related cardiac dysfunction may be due to abnormal activity of proteins and organelles responsible for the precise adjustment of calcium level, which causes irregular calcium concentration in cells and abnormality in calcium channels. The team thought irregular muscle contraction in muscles including cardiac muscles in PD patients is due to calcium overload in mitochondria. To investigate this, an experiment was conducted, using a calcium blocker on the rats with same genetic mutations to PD patients' genes. The result revealed that the chance of getting common cardiac disorders in PD patients due to abnormal muscle contraction was much lower in rats that were treated with calcium blocker compared to those that didn't. Specifically, the specific type of calcium-blocking drug used during the experiment (MUC blockers) decreased the opening probability of calcium channel, thereby reducing the entering of calcium into mitochondria and lowering the frequency of abnormal muscle contractions. Having their hypothesis that mitochondrial calcium blocker will reduce the risk of PD symptoms proven,

the team of researchers suggest that selective calcium blockers can be a therapeutic target for PD even though it is too reductive to conclude that calcium blocking treatment can be applied to humans immediately. This study, therefore, shows a result that is in convergence with our result: calcium level is a crucial factor that affects the PD symptoms. Furthermore, like our study, even though the experiment was not able to reveal the causal relationship between calcium exposure and PD symptoms, it clearly demonstrated that there is a significant association between two factors.

A major limitation of our research is small sample size and narrow scope of variety in the sample population. The Parkinson's Disease genetic variant datasets from GWAS catalog that we used in the investigation have sample sizes of 2848, 2788, and 2755 individuals respectively for PD motor, cognitive, and composite symptoms. The datasets did not divide the measurement results based on gender and ethnicity, which did not allow us to explore the relationship in a more controlled condition. To further expand this research in the future, researchers should aim to use genetic variant datasets with larger sample size that classify the sample based on gender and ethnicity. This way, researchers will be able to determine the causal relationship between calcium and PD symptoms in specific gender or specific ethnic groups.

In summary, we performed mendelian randomization analysis of serum calcium levels and different PD symptoms. Our results showed that there is no significant causal association between serum calcium levels and PD symptoms. However, this may be because the genetic instrument used cannot account for calcium levels in the mitochondria, which are independent of the levels in the serum. Future research may be required to ascertain the causal role of calcium in PD.

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