Autoantibodies to Low-Density Lipoprotein Receptor-Related Protein 4 in Double Seronegative Myasthenia Gravis: A Systematic Review

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ABSTRACT: *Background:* Myasthenia gravis (MG) is an autoimmune disorder of the neuromuscular junction in which a clinical diagnosis may be confirmed with serological testing. The most common autoantibodies used to support a diagnosis of MG are anti-acetylcholine receptor antibodies and anti-muscle-specific tyrosine kinase antibodies. In cases in which both of these autoantibodies are negative (termed double-seronegative [dSNMG]), other autoantibodies such as low-density lipoprotein receptor-related protein 4 (LRP4) may be used to aid in diagnosis. *Methods:* We have undertaken a systematic literature review to identify studies that have assessed the frequency of anti-LRP4 antibodies in dSNMG patients and the characteristics of anti-LRP4+ dSNMG patients (epidemiology, clinical features, electromyographic findings, or management). PubMed, EMBASE, Medline, and Scopus were searched on January 14, 2017, using the medical subject headings "myasthenia gravis" and "low-density lipoprotein receptor-related protein 4" or "LRP4." *Results:* The initial search identified 367 articles. Fourteen publications met the inclusion criteria. There were ten cross-sectional research studies, three were case series, and one was a case report. The majority of studies were limited by small sample sizes of LRP4+ dSNMG. There has been a wide range of frequencies of anti-LRP4 antibodies detected in different MG patient populations, some involving different laboratory techniques. *Conclusions:* LRP4+ dSNMG is more likely than LRP4− dSNMG to have a younger onset of disease and occur in females. LRP4+ dSNMG most often is mild in severity and often involves isolated ocular weakness. It typically responds well to pyridostigmine or prednisone.

Résumé: Utilisation des auto-anticorps anti-LRP4 chez des patients atteints de myasthénie grave doublement séronégative. *Contexte:* La myasthénie grave (MG) est une maladie auto-immune qui affecte la jonction neuromusculaire et qui peut être diagnostiquée au moyen d'un test sérologique. Pour corroborer un diagnostic de MG, on utilisera le plus souvent deux types d'auto-anticorps : d'une part, les anticorps dirigés contre les récepteurs de l'*acétylcholine* et, d'autre part, ceux qui affectent de façon spécifique les *récepteurs à activité tyrosine kinase*. Dans les cas où ces deux auto-anticorps sont négatifs, on parlera alors de résultats doublements séronégatifs ou «dSNMG». Cela dit, d'autres auto-anticorps comme la protéine 4 en rapport avec la lipoprotéine à faible densité (ou «LRP4» en anglais) peuvent être employés pour faciliter un diagnostic. *Méthodes :* Nous avons effectué une recension systématique des écrits afin de repérer des études ayant évalué la fréquence d'auto-anticorps anti-LRP4 chez des patients de type dSNMG et les caractéristiques des patients séropositifs (épidémiologie, aspects cliniques, résultats à la suite d'un électromyogramme, prise en charge). Le 14 janvier 2017, nous avons ainsi interrogé PubMed, EMBASE, Medline et Scopus en utilisant, à partir d'un vocabulaire normalisé, les descripteurs médicaux suivants : «myasthénie grave» et «protéine 4 en rapport avec la lipoprotéine à faible densité» ou «LRP4». *Résultats:* Notre première recherche nous a permis d'identifier 367 articles. Un total de quatorze publications a ainsi répondu à nos critères d'inclusion. De ce nombre, dix consistaient en des travaux de recherche ayant procédé à une analyse par coupe transversale ; trois, en des études de série de cas ; une, en une étude de cas. Soulignons que la plupart de ces publications était limitée par la taille restreinte de leurs échantillons de patients de type dSNMG chez qui on avait détecté des auto-anticorps anti-LRP4. À cet égard, un large éventail de fréquences d'auto-anticorps a été détecté au sein de diverses populations, certaines études reposant en outre sur différentes techniques de laboratoire. *Conclusions:* Si l'on compare les patients de type dSNMG chez qui l'on a détecté des auto-anticorps anti-LRP4 à ceux chez qui l'on n'en a pas détectés, les premiers se sont avérés plus susceptibles de développer la MG à un stade précoce et d'être de sexe féminin. La MG affectant les patients possédant des auto-anticorps LRP4 est le plus souvent de forme bénigne et sous-tend fréquemment des symptômes isolés de fatigue oculaire. La réponse de cette maladie à un traitement de pyridostigmine ou de prednisone se révèle généralement excellente.

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Myasthenia gravis (MG) is an autoimmune disorder of the neuromuscular junction, which results in weakness that can range from mild to life threatening. Confirmation of a clinical diagnosis of MG depends on electrophysiological studies and serological testing for autoantibodies directed against postsynaptic components of the neuromuscular junction.

The two autoantibodies most commonly used to aid in the diagnosis of MG are anti-acetylcholine receptor (AChR) antibodies and anti-muscle-specific tyrosine kinase (MuSK) antibodies. AChR antibodies are positive in approximately 85% of generalized MG cases[1]; however, fewer patients with ocular MG are positive for these autoantibodies than generalized MG patients. MuSK antibodies are positive in approximately 40% of patients with MG who are AChR–, although there appears to be geographical variation, with lower prevalence in populations a greater distance from the equator.[2-4] In approximately 9% of cases of MG, both AChR and MuSK antibodies may be negative at presentation, and such patients are referred to as double-seronegative MG (dSNMG).[5] One study has reported that with a follow-up period of 12 months, rates of dSNMG may decrease to 5%.[6] The dSNMG patients are generally assumed to harbor autoantibodies to other components of the post-synaptic apparatus. In addition to being diagnostically useful, the identification of other autoantibodies may provide prognostic information and help guide treatment.

Low-density lipoprotein receptor-related protein 4 (LRP4) is a membrane protein that serves as a receptor for agrin in the neuromuscular junction. The binding of agrin to LRP4 enables the activation of MuSK, which results in the phosphorylation of cortactin, which in turn mediates the clustering of AChR.[7] AChR clusters at sites on the muscle membrane where synapses from motor neurons occur (as opposed to parts of the muscle membrane at which no synapses occur). This AChR clustering results in high receptor availability to synaptic acetylcholine and thereby facilitates muscle excitability.[8]

Because of its role in the neuromuscular junction, autoantibodies to LRP4 have been investigated in MG. Animal studies have previously indicated that anti-LRP4 antibodies may induce a myasthenic state.[9] Anti-LRP4 antibodies may be detected through different techniques, including luciferase immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), and newer cell-based assays (CBAs). Estimates of the prevalence of anti-LRP4 antibodies in dSNMG have varied widely (see the following section); some of this variation may be explained by the methods of detection used.

The aim of this paper is to identify studies in which the frequency of positivity for anti-LRP4 antibodies has been assessed in patients with dSNMG, or in which the clinical characteristics (epidemiology, clinical features, management) of LRP4 + dSNMG patients have been reported.

METHODS

A search of the databases PubMed, EMBASE, Medline, and Scopus was conducted on January 14, 2017, and included results published since the respective commencements of the databases. The searches used the Medical Subject Headings "myasthenia gravis" and "low-density lipoprotein receptor-related protein 4" or "LRP4." Results were then limited to those published in English.

Following the application of this language restriction, the titles and abstracts of the remaining publications were viewed to determine if they met the inclusion criteria. The following inclusion criteria were used: (1) involved human subjects, including individual case reports (excluding reviews); (2) involved patients with MG; (3) assessed the presence of auto-antibodies in these MG patients; (4) the antibodies assessed included LRP4; (5A) presented information on the percentage of individuals negative for AChR and MuSK who were positive for LRP4 or (5B) presented information on the characteristics (epidemiology, clinical features, electromyographic findings or management) of dSNMG patients (negative for AChR and MuSK) who were LRP4 + ; and (6) were available in full-text.

Accordingly, studies presenting information on seropositive MG patients, or patients with other neurological diseases positive for anti-LRP4 antibodies were excluded.

Articles that appeared likely to fit inclusion criteria based on title or abstract were then reviewed in full-text. If it could not be determined whether an article met inclusion criteria based on the title or abstract, it was also retrieved and viewed in full text before inclusion or exclusion. Reference lists of included articles were searched for other potentially eligible studies. Eligibility determination and data extraction were performed in duplicate by SB and PK using a standardized form. Inconsistencies were resolved with discussion until consensus was reached.

RESULTS

The initial search produced 367 articles, which was reduced to 313 when an English language filter was applied. The titles and abstracts of these articles were reviewed, yielding 41 articles to be viewed in full-text. 14 papers met the inclusion criteria (Figure 1).

Of the 14 included articles, there were ten cross-sectional studies,[5,10-18] three case series,[19-21] and one case report.[22] The majority (11) of these papers used CBA to detect LRP4 antibodies.[10-12,14-21] Other methods used were luciferase immunoprecipitation[13,22] and ELISA.[5]

The primary limitation for the majority of the studies was the small numbers of LRP4 + dSNMG patients (Table 1).[10,11,13-15,19] Sample size varied greatly among the studies, from 635 dSNMG
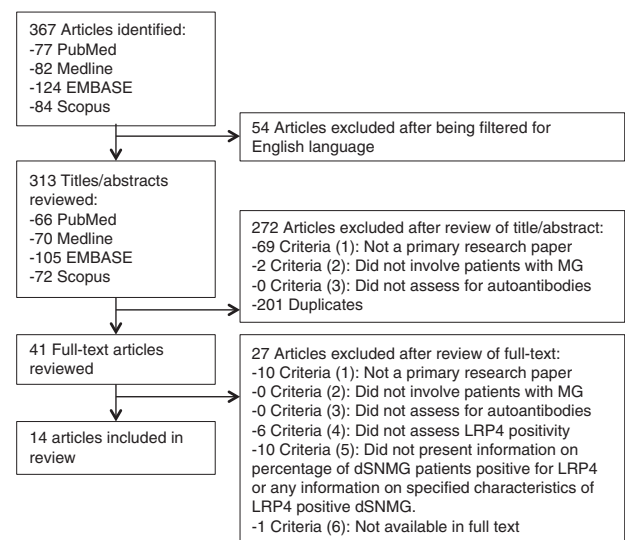
Figure 1: *Flowchart detailing results from the search strategy and application of inclusion and exclusion criteria for a review of articles assessing the frequency and clinical implications of anti-LRP4 positivity in dSNMG.*

Table 1: Results from studies that have assessed the frequency of anti-LRP4 positivity in dSNMG patients

Citation	Means of establishing MG diagnosis	Sample size: no. of dSNMG assessed	Sample size: no. of LRP4 + dSNMG	Frequency of LRP4 + in dSNMG (%)	Type of LRP4 assay	Outcome assessor(s) blinding	No. of outcome assessors
Cross-sectional research studies							
Zisimopoulou et al, 2014	Clinical features and electrophysiological findings	635	119	18.7	CBA	Blinded	NR
Higuchi et al, 2011	Clinical features, edrophonium test, and/or RNS	272	6	2.2	LUCIP	NR	NR
Zhang et al, 2012	Clinical features, response to cholinesterase inhibitors, and/or neurophysiological testing	120	11	9.2	ELISA	Blinded	NR
Gallardo et al, 2014	NR	91	0	0.0	CBA	Blinded	Two outcome assessors
Cossins et al, 2012	NR	73	6	8.2	CBA	NR	NR
Marino et al, 2015	NR	55	8	14.5	CBA	NR	NR
Nikolic et al, 2016	NR	45	8	17.8	CBA	Blinded	One outcome assessor
Pevzner et al, 2012	Clinical features and neurophysiological findings (edrophonium testing and RNS)	38	19	50.0	CBA	Blinded	NR
Rodriguez Cruz et al, 2015	Clinical and EMG criteria; or fatigable weakness and response to treatment with cholinesterase inhibitor/immunosuppression	21	0	0.0	CBA	NR	NR
Tsonis et al, 2015	dSNMG LRP4 results presented were considered likely to represent the same patient group as Zisimopoulou et al, 2014						
Case series							
Tsivgoulis et al, 2014	RNS, SF-EMG, or neostigmine test	NA	3	NA	CBA	NA	NA
Dervenoulas et al, 2014	dSNMG LRP4 presented were considered likely to represent the same patient group as Tsivgoulis et al, 2014						
Zouvelou et al, 2013	RNS and SF-EMG	NA	2	NA	CBA	NA	NA
Case report							
Beck et al, 2016	RNS	NA	1	NA	LUCIP	NA	NA

LUCIP = Luciferase-reporter immunoprecipitation; NA = not available; NR = not reported; RNS = repetitive nerve stimulation; SF-EMG = single-fiber EMG.

(yielding 119 LRP4+ patients) to an individual case report.[18,22] Six of the ten studies explicitly stated that the outcome assessor(s) were blinded.[5,12,15-18] Only two of the ten studies stated the number of outcome assessors.[12,15] Five of the cross-sectional studies reported the methods by which a diagnosis of MG was established.[5,11,13,16,18]

The data presented on the dSNMG LRP4+ group by Tsonis et al[17] were considered likely to be the same patient population as that presented in Zisimopoulou et al[18] because of the similarities between clinical features and thymic pathology and that the same research group performed the two studies. The results presented in the abstract by Dervenoulas et al[19] were considered likely to represent the same patients as those reported in Tsivgoulis et al.[20] Accordingly the results for the LRP4+ dSNMG groups from Tsonis et al and Dervenoulas et al are not presented independently in the following section.

Frequency of Anti-LRP4 Antibody Positivity in dSNMG

There was a wide range of results regarding the frequency of LRP4 antibodies in dSNMG patients (Table 1), from 50% in Pevzner et al (n = 38 dSNMG) to 2% in Higuchi et al (n = 272 dSNMG).[13,16] There were also two studies that failed to identify any LRP4+ dSNMG patients: Gallardo et al[11] (n = 91 dSNMG) and Cruz et al[12] (n = 21 dSNMG).

The largest study to date that has assessed patients from multiple countries did not identify an obvious pattern in the geographical variation.[18] For example, this study identified a prevalence of 32.8% in Poland (19/58) and 7% in Norway (3/43). This is in contrast to MuSK antibodies, which appear to have increasing prevalence closer to the equator.[4]

Differences in the LRP4 antibody assays may also account for the varying prevalence. CBA appear, in general, to have a higher sensitivity for detecting anti-LRP4 antibodies. CBA studies have reported frequencies of 50%, 18.7%, 17.8%, 14.5%, and 8.2%.[10,14-16,18] However, the two studies that failed to identify any LRP4+ dSNMG also both used CBA,[11,12] although one of these studies had a sample size of only 21.[11] Zhang et al used ELISA and found a prevalence of 9.2%.[5] Higuchi et al used luciferase immunoprecipitation and found a prevalence of 2.2%.[13]

Epidemiology of LRP4+ dSNMG

Three cross-sectional studies indicated that patients who have LRP4+ dSNMG seem to be more likely to have a younger age of onset of the disease and are more likely to be female than LRP4- dSNMG.[14,16,18]

The study with the largest sample of LRP4+ dSNMG patients (119 patients) identified an average age of onset of 34.9 years. This study also reported that 84% of these patients had an onset of the disease earlier than the age 50.[18] Other studies with smaller sample sizes have supported this result. For example, Marino et al[14] found that 87.5% of LRP4+ dSNMG patients had the onset of their disease before age 50, compared with 74.5% of LRP4– patients (in a sample including 8 LRP4+ and 55 LRP4– dSNMG patients). Similarly, in the initial sample in Pevzner et al, all seven of the LRP4+ dSNMG patients experienced disease onset before 50 years of age[16]; however, new-onset cases of LRP4+ dSNMG have been reported in individuals from ages 26 to 83.[13,20]

Zisimopolou et al found LRP4+ dSNMG to be more common in women (female:male [F:M] ratio of 2.5:1) compared with

LRP4– dSNMG.[18] This was supported by Marino et al, in which the tendency for LRP4+ to occur in women was stronger (LRP4+dSNMG F:M 7:1 vs LRP4- dSNMG F:M 2.1:1), although with a much smaller sample size (n = 8 LRP4+ dSNMG).[14] Nikolic et al identified a higher proportion of male LRP4+ dSNMG patients than other studies (F:M 1.7:1), but had no LRP4– dSNMG comparator group.[15] Pevzner et al[16] 2012 found the opposite trend, with LRP4– dSNMG being more common in females; however, the sample size was small (n = 7 LRP4+dSNMG and n = 6 LRP4– dSNMG).

There has been significant variation in the prevalence of LRP4+ in dSNMG by the country of residence of the patient (see previous section).

Clinical Features of LRP4+ dSNMG

Four studies found that LRP4+ dSNMG often presents with isolated ocular weakness and is most commonly mild in severity (Myasthenia Gravis Foundation of America [MGFA] I-II).[14,15,18,20] One case series described patients with LRP4+ dSNMG presenting with isolated ptosis and diplopia, with the duration of symptoms ranging from 12 to 36 months.[20] Nikolic et al and Zisimopoulou et al identified a significant proportion of LRP4+ dSNMG patients presenting with isolated ocular symptoms (MGFA I 50% and 29.9%, respectively).[15,18] In some case reports, LRP4+ dSNMG initially presented with primarily neck extensor weakness.[21,22]

Moderate to severe disease (MGFA III-IV) appears to be less common than mild disease in LRP4+ dSNMG. The largest study (Zisimopoulou et al) had clinical data on 67 patients with LRP4+, of which 57 (85.1%) had MGFA I-II disease and ten (14.9%) had MGFA III-IV disease.[18] Two smaller studies found rates of MGFA I-II disease of 75% and 62.5% (vs MGFA III-IV 25% and 25%, respectively).[14,15] Higuchi et al and Pevzner et al reported a different pattern to that described previously, with generalized disease and moderate severity being a more common presentation than isolated ocular weakness and mild disease; however, these studies are small (n = 6 and 7 LRP4+ dSNMG cases, respectively).[8,13]

Although uncommon, cases have been documented of LRP4+ dSNMG requiring intubation (MGFA V).[13,15,22]

EMG in LRP4+ dSNMG

Only one paper specifically studied electromyography (EMG) findings in LRP4+ dSNMG patients. It was found that repetitive nerve stimulation was infrequently abnormal (abnormal in at least one nerve-muscle pair in only 12.5%) and that jitter values were lower than in the AChR+ and MuSK+ MG patients.[14]

Thymic Pathology in LRP4+ dSNMG

Zisimopolou et al presented the most comprehensive assessment of thymic changes, with hyperplasia in 13/42 (31%), an involuted thymus in 12/42 (28.6%), and thymus atrophy in 3/42 (7.1%), with all of the remaining being normal. Note that these 42 individuals for whom thymus pathology was available form a small subset of the patients involved in this study, and it is unclear whether they differed from the other patients in terms of age, gender, or presentation. However, it was reported that thymic abnormalities appear to be less frequent in the LRP4+ dSNMG patients than in AChR antibody positive MG patients (p < 0.05).[18]

There was only one reported instance in the identified publications of an LRP4+ dSNMG patient having a thymoma.[14] Higuchi et al reported that there was no evidence of thymoma in the six dSNMG LRP4+ patients that they identified.[13] Tsivgoulis et al reported no thymoma or thymic hyperplasia in any of the three patients in their case series.[20] The patient reported on in Beck et al was found to have no evidence of thymoma on chest computed tomography.[22] Zouvelou et al observed residual thymic tissue, which revealed thymus hyperplasia on thymectomy.[21]

Treatment of LRP4+ dSNMG

Several cross-sectional studies and case series reported that the majority of patients with LRP4+ dSNMG responded well to cholinesterase inhibitors (such as pyridostigmine) and that, when used, the response to prednisone was good.[16,18,20,21] Zisimopoulou et al reported that 40/52 (76.9%) LRP4+ dSNMG patients had a good response to pyridostigmine and 7/52 (13.5%) had a moderate response. Similarly, they found that with prednisone 28/39 (71.8%) had a good response and 7/39 (18%) a moderate response.[18] However, there are several reported instances of further treatments, such as azathioprine, intravenous immunoglobulin, plasma exchange, tacrolimus, and thymectomy being required to control symptoms.[15,18,22] The outcomes reported in the Zisimopoulou et al study suggest that many LRP4+ dSNMG patients have good outcomes, with complete or pharmacologic remission in 34.6% (19/55), minimal manifestations in 5.5% (3/55), improved symptoms in 38.2% (21/55), and unchanged or worse in 16.4% (9/55) and 5.5% (3/55), respectively.[18]

DISCUSSION

We have reviewed the literature reporting the prevalence of anti-LRP4 antibodies in patients with dSNMG and the implications of anti-LRP4 positivity for such patients. A wide range of anti-LRP4 antibody positivity in dSNMG has been reported, which may reflect geographical location, assay technique, or other factors. Patients with dSNMG who are LRP4+ appear to be more likely to have early disease onset and to be female than LRP4– dSNMG patients. LRP4+ dSNMG patients seem likely to have mild disease, particularly with isolated ocular weakness, although more severe manifestations, including respiratory failure, are reported. Electrophysiological abnormalities appear to be milder, and thymoma is uncommon in anti-LRP4+ dSNMG patients. The majority of LRP4+ dSNMG patients appear to respond well to pyridostigmine, prednisone, or both; however, some patients have required long-term immunosuppression. There is little evidence to guide the use of thymectomy in LRP4+ dSNMG patients.

We limited our review to anti-LRP4 positivity in dSNMG patients only, and thus cannot draw detailed conclusions about anti-LRP4 positivity in other groups, such as seropositive MG and other neurological diseases. Five of the studies that were identified in this review also assessed anti-LRP4 status in seropositive (for anti-AChR or anti-MuSK) MG patients. Reports on patients who are seropositive for anti-AChR or anti-MuSK *in addition* to anti-LRP4 (dSNMG) suggest that double seropositive patients may have more generalized and severe disease than patients who are seropositive for one autoantibody alone.[18,23] It has been found that, generally, there are low rates of anti-LRP4 positivity in patients positive for another MG autoantibody, ranging from 0% to 10% (seropositive MG patient sample sizes, 97-174).[5,13,18]

However, there is also variation in the frequency of LRP4+ in seropositive MG because two studies identified rates of LRP4+ in AChR/MuSK seropositive patients of up to 22% (although with comparatively smaller sample sizes of 41-46 seropositive MG patients).[14,15] As for seronegative MG, antibody assay sensitivity may contribute to variability in the results of different studies assessing the frequency of autoantibody positivity in seropositive MG. A study published after the systematic search for this review was conducted has reported that in previously seronegative patients, highly sensitive radioimmunoassays and CBA can identify autoantibodies in an additional 37% of patients (30/81).[24]

There have also been documented cases of anti-LRP4 positivity in diseases other than MG. The diseases in which anti-LRP4 antibodies have been identified include polymyositis,[14] neuromyelitis optica,[5] multiple sclerosis,[18] and amyotrophic lateral sclerosis (ALS).[25,26] In particular, it has been reported that anti-LRP4 may be positive in up to 23.4% of ALS patients.[25,26] The study that produced this result used CBAs in a sample of 104 ALS patients.[26] This finding has implications for the specificity of anti-LRP4 for the diagnosis of dSNMG, particularly given that ALS may be a part of the differential diagnosis for patients presenting with weakness.

There have been some investigations of anti-LRP4 antibody subtypes, although none that specifically focused on dSNMG patients. Rather, these studies either did not specify whether the subjects were dSNMG or seropositive, or included both categories of MG patients in the same analysis. The anti-LRP4 antibodies identified were primarily immunoglobulin (Ig) G [13] and IgG2.[18] This is similar to anti-AChR, which is primarily IgG1,[27] and in contrast to anti-MuSK, which is most commonly IgG3 or IgG4.[28-30] This finding implicates anti-LRP4 as possibly playing a role in activating the complement cascade and resulting in neuromuscular junction dysfunction via this pathway.

We acknowledge that the exclusion of non-English language publications is also a potential limitation of this review. The possibility of publication bias or selective outcome reporting may also influence our conclusions.

Future research in this area should clearly define the criteria by which participants are diagnosed with MG, endeavor to standardize laboratory procedures between different geographic locations and assess larger samples of dSNMG patients. This may help to clarify the true frequency of anti-LRP4 antibodies in dSNMG. Further research into the role of thymectomy for LRP4+ dSNMG patients is required.

CONCLUSION

Seronegative MG is an ongoing diagnostic challenge. Reported anti-LRP4 antibody prevalence varies widely between studies. Characteristics of reported LRP4+ dSNMG patients include younger age at onset, female predominance, mild disease severity (frequently isolated ocular weakness), and a good response to pyridostigmine, prednisone, or both.

DISCLOSURES

None.

STATEMENT OF AUTHORSHIP

SB and PK were involved in the development of the concept for the project, eligibility determination, data extraction, and manuscript preparation. CC was involved in the development of the concept for the project and manuscript preparation.

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