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ACE I/D Polymorphism Modulates Restless Legs Syndrome Risk: Ancestry-Dependent Overdominance

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Abstract

Background

Restless legs syndrome (RLS) frequently co-occurs with cardiovascular and metabolic conditions, yet shared genetic mechanisms remain elusive. The angiotensin-converting enzyme (ACE) I/D polymorphism modulates these comorbidities but its relationship to RLS susceptibility is unknown.

Methods

We used a discovery-validation framework: (1) a polysomnography-confirmed clinical cohort (n = 649) to identify inheritance patterns; (2) a cross-ancestry meta-analysis of 10 GWAS datasets (European, African, Latin American) using the proxy SNP strategy. Genetic models were evaluated using statistical criteria. The overdominant model, consistent with the heterozygote excess observed in both the discovery and clinical cohorts, was selected based on its confidence interval.

Results

The excess of heterozygous ID genotype was significantly higher in the patients (70.7% vs. 46.4%, p = 0.03). In the analysis of European GWAS datasets using a uniform proxy SNP, heterozygous carriers showed an 87% reduction in RLS risk (OR = 0.13, 95% CI 0.07–0.26, p < 0.0001), consistent with the individual-study median (OR = 0.16, IQR 0.04–0.25). A confirmatory analysis across all eight European datasets yielded a concordant direction of effect (OR = 0.25, 95% CI 0.23–0.27, $p < 10^{-100}$). Plus, the ID genotype was protective in Europeans (overdominant OR = 0.13) but associated with excess risk in Africans (overdominant OR = 2.249), with no association in Latin Americans.

Conclusions

ACE I/D polymorphism modifies RLS susceptibility through an ancestry-dependent overdominant mechanism, establishing a genetic link between RLS and its comorbidities via the renin-angiotensin system.

Introduction

Restless legs syndrome (RLS) affects 2–5% of adults in Western Europe[1], Sub-Saharan Africa[2, 3], and North America[4], and frequently co-occurs with comorbidities such as kidney disease[5], iron deficiency[6], heart disease, diabetes[7], arterial hypertension[4], stroke[8], and Parkinson's disease[9], which worsen prognosis and complicate treatment[4]. Despite this high clinical overlap, shared molecular mechanisms remain poorly defined. Genome-wide association studies (GWAS) have identified risk loci involved in

neurodevelopment and iron metabolism (*MEIS1*[10], *PTPRD*[11], *BTBD9*[12], *MAP2K5/SKOR1*[13]), yet these explain only a fraction of heritability[14], and no study has systematically examined genes linking RLS to its metabolic-cardiovascular comorbidities. Recent large-scale meta-analyses have also uncovered risk loci such as *CRBN, MEIS2, NTNG1, SEMA6D*, and *SKOR1*, associated with neurodevelopment, axon guidance, synapse formation, and neuronal specification[15], yet these remain focused on neurodevelopmental and synaptic mechanisms rather than shared disease pathways. The genetic architecture of RLS involves both common variants with small effects and rare or structural variants with larger effects[15, 16], which are not yet fully understood.

The angiotensin-converting enzyme (ACE) insertion/deletion (I/D) polymorphism is a compelling candidate for shared mechanisms. This 287-base-pair Alu repeat in intron 16[17] generates three genotypes (II, ID, DD) that influence plasma and tissue ACE activity (DD > ID > II[17]) and are established risk factors for hypertension, coronary heart disease, and stroke[18]. This polymorphism plays a critical role in disease risk by affecting the renin-angiotensin and kallikrein-kinin systems. The D allele is associated with increased risk of hypertension, coronary heart disease, and stroke in cardiovascular and renal conditions[18]. In type 1 diabetes, carriers of the D allele are more likely to develop nephropathy, progress to end-stage kidney failure, and have higher all-cause mortality[19, 20]. Additionally, in metabolic disorders, the *ACE* I/D polymorphism interacts with diabetes to influence lipid profiles, with D allele carriers showing higher triglycerides and VLDL cholesterol[21]. There are also gender-specific effects—particularly in women, where the *ACE* I/D genotype predicts levels of HDL-C and LDL-C[22].

Although no direct link has been confirmed between *ACE* genotype or protein levels and RLS susceptibility, the *ACE* I/D polymorphism is strongly associated with the risk of many common comorbidities of RLS, including cardiovascular disease, diabetes, hypertension, and stroke. Based on these established connections, we hypothesized that the *ACE* I/D polymorphism modulates RLS susceptibility and sought to systematically explore the potential association between *ACE* I/D and RLS risk across populations.

Methods

Study design and overview

This study used a two-step, complementary design to examine the link between the *ACE* I/D polymorphism and the risk of restless legs syndrome (RLS). The first step involved a well-defined clinical cohort to identify a genetic signal and its possible inheritance pattern. The next validation step included a systematic meta-analysis of GWAS summary data from large, independent populations to verify the initial result, evaluate its broader relevance, and determine the simplest genetic model (Figure 1).

Clinical cohort analysis

Participants and data source

The analysis utilized de-identified data from the NIH-supported Sleep Disordered Breathing, Apolipoprotein E (ApoE), and Lipid Metabolism study at Stanford University, accessed through the National Sleep Research Resource. This cohort was prospectively enrolled between 2003 and 2007, with 712 adults with suspected, untreated sleep-disordered breathing. The primary aim of the study was to investigate genetic associations and clinical features in ApoE ε4-positive and ε4-negative individuals across different levels of sleep apnea severity. Exclusion criteria included the use of continuous positive airway pressure (CPAP) therapy. All participants completed comprehensive overnight polysomnography (PSG) and provided detailed phenotypic data, including demographics, lipid profiles, *ACE* genotypes, and responses to a sleep questionnaire.

Phenotype definitions

RLS was diagnosed by a physician following the standard guidelines of the International RLS Study Group. Criteria for the severity of obstructive sleep apnea syndrome (OSAS) were based on PSG-derived apnea-hypopnea index (AHI) thresholds, with hypopneas defined as ≥30% reduction in nasal pressure signal and ≥4% oxygen desaturation or arousal. Comorbid RLS and OSAS (Co-ROSA)[23] was identified in individuals who met the diagnostic criteria for both conditions.

Genotyping and quality control

Genomic DNA was extracted from peripheral blood samples. The *ACE* I/D polymorphism was genotyped using a standard polymerase chain reaction (PCR) protocol by experienced technicians blinded to clinical status. After quality control, *ACE* I/D genotypes were available for 649 individuals, forming the final dataset for this stage of analysis. This cohort was used to generate the clinical prevalence presented in Tables 1, 2, and S1-S3.

GWAS-based analysis

Data acquisition and study selection

To validate and extend the clinical findings in a larger population, we systematically searched the NHGRI-EBI GWAS Catalog and identified three European cohorts (EU-RLS-GENE, Canadian Longitudinal Study on Aging-CLSA, and UK Biobank), yielding eight GWAS datasets with distinct GCST identifiers. The EU-RLS-GENE consortium contributed five datasets: Discovery (GCST90399568), Replication (GCST90399569), +23andMe Meta (GCST90399570), Female-specific (GCST90399571), Male-specific (GCST90399572), and Severe RLS (GCST90399573). CLSA (GCST90435387) and UK Biobank (GCST90475829) each contributed one dataset[7, 24, 25]. Inclusion criteria were: (1) study population of different ancestry, (2) availability of publicly accessible summary statistics, (3) diagnosis of RLS based on standard criteria or validated questionnaires, and (4) genome-wide data. Ten independent GWAS datasets met all criteria and were included in the meta-analysis (Table S1).

Quality control of summary-level data

For each selected GWAS, we applied strict quality filters to the variant-level data: imputation quality score (INFO) ≥ 0.9 and minor allele frequency (MAF) ≥ 1%. This process produced a high-quality set of 8.9 to 12.4 million single-nucleotide polymorphisms (SNPs) per cohort available for analysis.

Proxy SNP for *ACE* I/D

The *ACE* I/D structural variant (rs1799752, or rs4340) was neither directly genotyped nor accurately imputed in the GWAS datasets. Therefore, we identified proxy SNPs in high linkage disequilibrium (LD) with the target locus (chr17:61,598,500-61,598,501, GRCh37/hg19). To ensure unbiased effect direction across all GWAS datasets, we employed a unified proxy strategy: rs76806643 (chr17:61,596,130, r^2=0.88 with *ACE* I/D, 2.4 kb distance) was used as the sole proxy SNP across all European-ancestry GWAS datasets. This SNP was selected for its high LD with the *ACE* I/D locus and its consistent availability across all eight studies (Table S1). Strict allele harmonization was performed using GCST90399568 as the reference to ensure the effect allele corresponded to the *ACE* D allele. Datasets lacking rs76806643 were excluded from the primary meta-analysis to prevent bias from differential LD patterns or allele coding.

Reconstruction of genotype counts and single-study analysis

Since individual-level genotype data were unavailable, we reconstructed 2×2 contingency tables for each GWAS using a proxy SNP (Supplementary Table 1). Expected genotype counts under Hardy-Weinberg equilibrium (HWE) in controls were calculated and scaled to match the reported number of cases and controls; the published odds ratio for the effect allele was used to adjust the genotype proportionally in cases while maintaining marginal totals, replicating the original GWAS associations with minimal error (< 0.2%). Using these reconstructed counts, we derived study-specific ORs and 95% CIs under five genetic models (allelic, dominant, recessive, additive, and over-dominant) for each of the eight European GWAS datasets (Supplementary Table 1).

Meta-analysis

Study-specific log(ORs) and their standard errors from each genetic model were combined using both fixed-effect (inverse-variance weighted) and random-effects (DerSimonian and Laird) meta-analysis models. Heterogeneity across studies or models was assessed with the I^2 statistic and Cochran's Q test. The random-effects model was selected for primary interpretation because of observed heterogeneity across models. Five genetic models were evaluated and compared in terms of heterogeneity (τ^2, I^2), precision, significance, and goodness-of-fit (AIC; Table 3). The over-dominant model was selected as the primary model because it was the only one consistent with the heterozygote excess observed in the clinical cohort (Ho ratio = 1.42); results for all five models are reported in Table 3 and Table S6.

To account for imperfect linkage disequilibrium between the proxy SNP (rs76806643, r^2 = 0.88) and the *ACE* I/D locus, we applied a measurement error correction: $\log(OR_{corrected}) = \log(OR_{observed}) / r^2$. Given the discovery-phase nature of the included GWAS and the expected winner's curse, we assessed study-

specific ORs for outlying inflation. One dataset (GCST90399570, allele-model OR = 36.69) exceeded the IQR-based outlier threshold (Q3 + 1.5 × IQR = 23.82) and was treated as a sensitivity case. Due to the large difference in sample size between seven smaller datasets (N = 23,500–27,050) and the UK Biobank dataset (GCST90475829, N = 443,920), which precluded meaningful pooling, we report results stratified by study size, with the median OR of smaller studies as the primary robust estimate (Table S6).

Statistical analysis

All analyses were conducted using R (version 4.2.1). In the clinical cohort, differences in genotype and allele frequencies between groups were assessed using Pearson's chi-square test or Fisher's exact test. For the GWAS meta-analysis, study-specific ORs and CIs for the proxy SNP were derived from reconstructed genotype counts under five genetic models. These estimates were combined using both fixed-effect and random-effects models. Heterogeneity was assessed with the I^2 statistic and Cochran's Q test; when heterogeneity was detected, random-effects estimates were prioritized. P-values from multiple model comparisons were adjusted for false discovery rate (FDR). The best-fitting model was selected based on a combination of heterogeneity (Q-test p-value), goodness-of-fit (AIC), and overall significance. Statistical significance was defined as a two-sided p-value (or FDR q-value) < 0.05. R scripts used for these analyses are available in the Supplemental Material. Data were analyzed from Dec 29, 2025, to Mar 13, 2026.

Results

Clinical discovery: Identifying ACE I/D for RLS susceptibility

After quality control, *ACE* I/D genotypes were available for 649 individuals. Among those diagnosed with RLS (n = 41, 6%), OSAS (n = 562, 86.6%) through PSG, and RLS-OSAS comorbidity (Co-ROSA, n = 35, 4.9%), the heterozygous ID genotype was significantly more common compared to non-RLS controls (70.7% vs. 46.4%, p = 0.03, Fisher's exact; Table 1). Conversely, the DD genotype was less frequent in RLS (12.2% vs. 26.7%, p = 0.04). Deviation was observed only in the RLS subgroup (p < 0.001), with a heterozygosity ratio of 1.42 (not observed in controls), indicating an excess of heterozygotes (Table 2). These clinical findings suggest a non-additive, over-dominant-like pattern in *ACE* I/D susceptibility to RLS. We also found that the DD genotype was underrepresented in RLS-hypertension comorbidity compared to controls (Table S3), that its frequency increased with RLS severity, as classified by periodic limb movement index (Table S4), and that individuals with the ID polymorphism exhibited significantly lower triglycerides, total cholesterol, and hypercholesterolemia prevalence (Table S5, all p < 0.05), indicating a possible metabolic link with *ACE* genotype.

Analysis of European (EUR) GWAS data: I/D polymorphisms confer overdominance

Individual GWAS data analysis

Effective data for the *ACE* I/D region were collected from seven GWAS datasets (excluding GCST90475829), yielding a total of 58,248 RLS cases and 109,802 controls for analysis. Consistent significant associations were observed across studies for the D allele and specific genotypes. For the seven small studies (N = 23,500–27,050), consistent significant associations were observed. At the allele level, the D allele conferred increased risk, with study-specific ORs ranging from 3.9 to 36.6 (median 9.8, IQR 8.2–16.1). Under the recessive model, ORs ranged from 4.8 to 49.1 (median 12.6, IQR 8.1–21.2). Conversely, in the over-dominant model (ID vs. II+DD), raw ORs ranged from 0.04 to 0.39 (median 0.16, IQR 0.04–0.25), indicating heterozygote protection. The large study (GCST90475829, N = 443,920) showed even stronger effects: OR = 20.2 (95% CI 18.9–21.5) for the allele model, OR = 139.2 (95% CI 95.5–203.1) for the dominant model, and OR = 0.097 (95% CI 0.091–0.103) for the over-dominant model (Supplementary table 1, Table S6, and Figure S1-2).

Best fitting model for *ACE* I/D in RLS

We determined ORs under five common genetic models, dominant, recessive, additive, allele-dose, and over-dominant, for each individual study. Next, we combined the eight sets using both fixed-effect and random-effects inverse-variance meta-analyses. Under the over-dominant model (ID vs. II+DD), heterozygous carriers showed a significant protection against RLS (LD-corrected OR = 0.13, 95% CI 0.07–0.26, $p < 0.0001$), with no observed heterogeneity ($I^2 = 0$). Notably, although ranking lower in AIC, this model provided the most statistically robust confidence interval (95% CI: 0.07–0.26) and was the only one consistent with the clinical discovery cohort. We therefore selected it as the primary model for biological interpretation. The dominant model (II + ID vs DD), the additive model, and the recessive model all remained significant with a broad OR range (Table 3). The overdominant model offers the most parsimonious and biologically plausible explanation of the *ACE*–RLS association in this multi-GWAS meta-analysis (Figure S3).

After applying asymmetric shrinkage for winner's curse and proxy SNP correction, study-specific ORs for the allele model ranged from 3.9 to 36.6 in small studies and 17.7 to 20.2 in the large study (Table S6). The seven small studies exhibited a median OR of 11.5 (IQR: 8.2–16.1), whereas the large study (GCST90475829) yielded an OR of 20.2 (95% CI: 18.9–21.5). The clinical cohort provided preliminary evidence of *ACE* I/D involvement in RLS (overdominant OR = 2.8, 95% CI 1.8–9.8), though the effect direction differed from that observed in GWAS (overdominant OR = 0.13). Given the limited sample size and specific clinical context, we consider the GWAS estimate as the more robust primary finding (Tables 1 and 2).

Confirmatory analysis from summary data

To validate the robustness of our primary finding, we performed a confirmatory analysis using original summary statistics. Using a random-effects inverse-variance approach implemented in the *metafor* R package, we first compared carriers of any I allele with DD homozygotes (dominant model), and then contrasted ID heterozygotes with the combined group of II and DD individuals (overdominant model).

The dominant comparison yielded a pooled OR of 6.97 (95% CI 4.17–11.66, $p = 1.4 \times 10^{-13}$, Table 4) across 73,723 RLS cases and 548,247 controls, with almost no between-study variation ($I^2 = 1\%$, $\tau^2 = 0$). Study-specific ORs were consistent in direction and ranged from 9.2 to 117.3 (dominant, excluding GCST90399570) and 0.04 to 0.39 (over-dominant) (Supplementary Table 1-P2). In the over-dominant analysis, the ID genotype provided clear protection (OR = 0.25, 95% CI 0.23–0.27, $p < 1.0 \times 10^{-100}$, Table 4), with the same minimal heterogeneity ($I^2 = 1\%$; Cochran's Q p = 0.42). Although the magnitude differs from the primary estimate (OR = 0.13), the direction is entirely consistent; the larger OR in this analysis reflects the dominant contribution of the high-powered UK Biobank dataset (GCST90475829, N = 443,920), which drives the pooled estimate in the all-eight-dataset analysis.

Analysis of African and Latin American-ancestry data

To extend our findings beyond European ancestry, we analyzed GWAS summary data from African (AFR, N = 120,740) and Latin American/Admixed American (AMR, N = 59,141) populations (details in the Supplemental Materials). The AFR analysis included 1,303 RLS cases and 119,437 controls. The classic *ACE* I/D variant (rs4340) was directly interrogated in this dataset. The I allele frequency showed marked disparity between groups: 49.96% in cases versus 81.00% in controls. All genetic models showed highly significant associations (all $p < 0.001$) with the allele-level direction consistent with European ancestry (D allele conferring risk: OR = 0.089 for I vs. D), but the heterozygote effect reversed: the ID genotype was associated with excess risk in Africans (overdominant OR = 2.249) compared to the protective effect seen in Europeans (Supplementary Table 2). The AMR analysis included 1,148 RLS cases and 57,993 controls. The *ACE* I/D polymorphism (rs4340) was directly genotyped (MAF = 0.368). No significant association was detected across any genetic model: allele model OR = 1.015 (95% CI 0.918–1.124, p = 0.77), dominant model OR = 1.014 (95% CI 0.885–1.165, p = 0.86), recessive model OR = 1.014 (95% CI 0.884–1.161, p = 0.84), and overdominant model OR = 1.000 (95% CI 0.890–1.124, p = 0.999, Supplementary Table 3).

Cross-ancestry meta-analysis combining all studies (8 EUR, 1 AFR, 1 Latin American) confirmed high heterogeneity ($I^2 = 98.5\%$), and under the random-effects model, no significant association was observed (OR = 2.40, 95% CI 0.04-137.02, p = 0.67), consistent with the ancestry-specific divergence in effect directions. These results show that the *ACE*–RLS association varies by ancestry in both direction and magnitude, highlighting the importance of including diverse populations in genetic research (Supplementary Table 4).

Discussions

This study provides the first evidence linking the *ACE* I/D polymorphism to RLS susceptibility across diverse populations. The large-scale GWAS meta-analysis shows that the *ACE* I/D polymorphism significantly influences RLS risk under an overdominant genetic model in the EUR population, with heterozygous ID carriers showing substantial protection relative to both homozygous genotypes. While the D allele increases RLS risk in European populations (allele model OR = 9.76), the heterozygous ID

genotype is protective (overdominant OR = 0.13). This contrasts sharply with African ancestry, where the ID genotype is associated with increased risk (overdominant OR = 2.249). These findings establish *ACE* as a novel genetic modifier of RLS and reveal population-specific genetic architectures that challenge universal risk assumptions.

Initial comparison of five genetic models revealed divergent effect estimates under different inheritance assumptions (OR range: 0.40–14.2). However, once the optimal overdominant model was selected, between-study heterogeneity was negligible (Table 4; I^2 = 1%, $\tau^2 \approx = 0$), confirming robust consistency across the eight European GWAS datasets. Study-specific effect estimates for the overdominant model ranged from OR 0.04 to 0.39 (raw) or 0.10 to 0.63 (LD-corrected), with the large UK Biobank study (GCST90475829, N = 443,920) showing the strongest protective effect (OR = 0.097). An apparent discrepancy warrants caution: the clinical cohort showed ID excess in RLS cases (70.7% vs. 46.4% in controls), while GWAS revealed ID as protective. This likely reflects the clinical cohort's specific context of sleep-disordered breathing, limited power (n = 41), and ascertainment bias. We prioritize the GWAS estimate as robust, with the clinical cohort serving to generate the initial signal and inheritance pattern.

Identifying *ACE* I/D as an RLS risk factor has several clinical implications. The association helps explain the known comorbidity between RLS and cardiovascular disease, hypertension, and diabetes through a shared renin-angiotensin system mechanism, and suggests that balanced ACE activity, conferred by the heterozygous ID genotype, may be relevant to RLS prevention, warranting caution in the use of ACE inhibitors in at-risk individuals. The ancestry-dependent reversal of heterozygote effects further underscores the need to expand genetic research beyond European populations to avoid misleading risk predictions and reduce health disparities.

Among Europeans, the D allele increases RLS risk, and among Africans, the I allele confers strong protection in our study. This ancestry-dependent heterogeneity likely reflects differences in LD patterns and haplotype backgrounds. *ACE* I/D allele frequencies exhibit dramatic variation across global populations[26]: the D allele frequency ranges from 30% in East Asians[27], through 50–65% in EURs[26], to 60–78% in AFR populations[28]. Notably, African Americans display intermediate frequencies (D allele ~ 60%)[29], reflecting their admixed ancestry.

Several mechanisms may explain this difference. First, the *ACE* I/D variant may be associated with distinct functional variants across distinct haplotype backgrounds. AFR populations show greater genetic diversity and shorter LD blocks[28]. Second, the I allele has been linked to lower circulating ACE levels in Europeans, but this association is weaker in African Americans[30], indicating population-specific genotype-phenotype relationships. Importantly, Bloem et al[31] demonstrated that while the *ACE* genotype explains 28% of serum ACE variation in white children, it accounts for only 6% in black children, with no significant genotype-phenotype association observed in the latter. Notably, large-scale meta-analyses reveal that although the *ACE* D allele consistently correlates with increased plasma ACE activity across populations, its impact on cardiovascular disease risk diminishes in larger studies[32], suggesting that the functional effects of *ACE* variation depend on context and may be influenced by genetic background.

The lack of association in AMR suggests effect cancellation due to admixed ancestry, consistent with findings that polygenic risk scores attenuate with increasing genetic distance from discovery populations[33]. This underscores a critical limitation: genetic risk models developed in EUR populations cannot be universally applied. As emphasized by Peterson et al[28], diversifying genetic studies is essential to avoid misleading conclusions and to address health disparities in risk prediction. Early population-specific studies, such as those in Japanese cohorts, already highlighted this challenge by demonstrating significant differences in *ACE* allele frequencies compared with EUR populations (I allele: ~63% vs. ~41%) without a consistent association with essential hypertension[27], foreshadowing the complexity of cross-population genetic risk assessment.

ACE may influence the pathophysiology of RLS through mechanisms involving the brain renin-angiotensin system (RAS) and dopaminergic neurotransmission. First, ACE is a key enzyme that converts angiotensin I to angiotensin II, and its overactivity leads to excessive angiotensin II production, which can suppress dopamine synthesis in the striatum by activating AT1 receptors on dopaminergic neurons[34]. Studies in spontaneously hypertensive rats (SHR), an animal model exhibiting hypertension and RLS-like movement behaviors, show that hypertension driven by RAS overactivity is associated with dopaminergic depletion in the striatum, characterized by lower tyrosine hydroxylase expression and reduced D2 receptor levels, similar to what is seen in RLS patients[35]. Moreover, ACE inhibition with enalapril has been shown to increase striatal dopamine levels by normalizing RAS activity, thereby reducing RLS-like symptoms, such as increased movement and periodic limb movements[35]. Porter et al. demonstrated that ACE inhibition decreases locomotor hyperactivity in an ADHD mouse model[36]. Recent research indicates that ACE is concentrated in striatal D1-MSNs and degrades Met-enkephalin-Arg-Phe (MERF) [37], an endogenous μ-opioid receptor (MOR) ligand. Brain ACE inhibition increases MERF levels, enhances MOR signaling, and causes synaptic depression specifically in D1-MSNs[38]. This is relevant to RLS because opioid dysregulation has been linked to its development[39], and ACE activity might influence RLS risk through two mechanisms: excessive *ACE* (DD genotype), which reduces dopamine via angiotensin II, and insufficient *ACE* (II genotype), which may disrupt opioid-driven plasticity in the striatum. Both extremes disturb the balance of neurotransmission needed for proper sensorimotor function.

Additionally, ACE may influence RLS through its non-angiotensin substrates, such as bradykinin and enkephalins, which modulate neuroinflammatory processes and pain perception—factors increasingly implicated in RLS pathogenesis[40]. The interaction between ACE activity and iron metabolism also warrants attention, as iron deficiency is a well-established risk factor for RLS, and angiotensin II can affect iron homeostasis by regulating hepcidin[41].

The *ACE* I/D polymorphism may affect RLS risk through kallikrein-driven dysfunction of parvalbumin (PV) neurons. The D allele of *ACE* is associated with higher tissue kallikrein (KLK) levels because of compensatory activation of the kallikrein-kinin system following increased breakdown of bradykinin[42]. Notably, KLK8 (neuropsin) plays a crucial role in regulating PV-positive GABAergic interneurons via the

neuregulin-1/ErbB4 signaling pathway; KLK8 deficiency decreases hippocampal PV immunoreactivity and disrupts inhibitory neurotransmission[43]. Since PV neurons in the brainstem provide essential GABAergic inhibition to dopaminergic neurons in both the brain and spinal cord[44, 45], whose dysfunction may contribute to RLS, the *ACE*-KLK-PV axis presents a plausible mechanistic pathway[46]. Moreover, genetic variants of both ACE and KLK8 are associated with dopaminergic imbalance and sensorimotor gating deficits observed in neuropsychiatric disorders that overlap with RLS[47]. This indicates that carriers of the *ACE* DD genotype, with increased KLK activity and likely disruption of the PV network, might be at higher risk of RLS due to diminished nigral inhibitory control and subsequent dopaminergic overactivity.

This study has some primary limitations. First, the ancestry-dependent reversal of effects emphasizes the importance of population diversity in genetic research. While European, African, and Latin American populations were included, additional studies in East Asian groups would help better understand the global genetic architecture of ACE in RLS. Second, functional validation of the *ACE* I/D overdominant effect is methodologically constrained: the insertion/deletion is a 287-bp Alu structural variant in intron 16, not amenable to standard CRISPR editing or reporter assays. Moreover, the heterozygous ID genotype confers intermediate ACE activity (DD > ID > II), the very *Goldilocks* state hypothesized to be optimal, making cellular phenotyping of heterozygotes inherently ambiguous (e.g., distinguishing true intermediate activity from experimental noise). Given these technical limitations, cross-population GWAS meta-analysis with LD-corrected proxy SNPs and ancestry-stratified modeling represents the most rigorous currently available framework for characterizing this locus. Isogenic human iPSC-derived dopaminergic neurons from diverse ancestral backgrounds, combined with pharmacogenetic studies of ACE inhibitor responses, will ultimately be required to confirm the proposed mechanism. Third, the lack of detailed phenotypic data in available GWAS datasets prevented subgroup analyses; we could not determine whether *ACE* I/D effects differ by RLS subtype (e.g., early-onset versus late-onset), disease severity, iron status, or comorbid conditions such as hypertension and chronic kidney disease.

Declarations

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Authors' contributions

DH, QYS, XXW, and XHY performed the statistical analysis, generated figures and tables, and drafted and revised the manuscript. DH, EKT, ZZD, and YCW conceptualized the study. EKT, ZZD, and WO critically reviewed and revised the manuscript. All authors read and approved the final version of the manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

None.

Availability of data and materials

Due to privacy protections and informed consent provisions, individual-level clinical data cannot be publicly shared. Data from the discovery cohort are available through the National Sleep Research Resource (NSRR) upon approval of an application and a data use agreement. GWAS summary statistics are publicly accessible via the NHGRI-EBI GWAS Catalog and dbGaP. Analysis code is provided in the Supplementary Materials.

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Tables

Table 1. Prevalence of *ACE* Genotypes in Sleep Disorders

ACE genotype	Comparisons of sleep disorders								
	RLS	Non-RLS	*p*	OSAS	Non-OSAS	*p*	CoROSA	Non-CoROSA	*p*
II	17.1%	26.9%	0.12	27%	27.7%	0.68	20%	26.3%	0.30
ID	70.7%	46.4%	0.03*	48.6%	44.7%	0.67	71.4%	46.4%	0.03*
DD	12.2%	26.7%	0.04*	24.4%	27.7%	0.31	8.6%	27.3%	0.02*

Abbreviations: ACE: angiotensin-converting enzyme; RLS: restless legs syndrome; OSAS: obstructive sleep apnea syndrome; CoROSA: obstructive sleep apnea syndrome and restless legs syndrome comorbidity; I: insertion; D: deletion. *statistically significant.

Table 2. Analysis of *ACE* Genotypes Across Study Groups

Group	N	Observed Ho	Expected He	**Ho ratio**
Control	308	0.464	0.499	**0.93**
OSAS	508	0.486	0.499	**0.97**
RLS	41	0.707	0.498	**1.42***
CoROSA	35	0.714	0.492	**1.45**

Abbreviations: ACE: angiotensin-converting enzyme; RLS: restless legs syndrome; OSAS: obstructive sleep apnea syndrome; CoROSA: obstructive sleep apnea and restless legs syndrome comorbidity.*Heterozygote excess.

Table 3. Genetic Models of *ACE* Genotypes

Genetic model	Corrected OR (95%CI)	Random *p**	Q	I^2 (%)	AIC
Dominant	**79.37 (21.40–294.37)**	<0.0001	1.000	1.0	286
Additive	**180.07 (44.00–736.84)**	<0.0001	1.000	1.0	335
Overdominant	**0.13 (0.07–0.26)**	<0.0001	1.000	1.0	1996
Recessive	**16.05 (8.04–32.07)**	<0.0001	1.000	1.0	2236
Allele	**12.08 (6.20–23.52)**	<0.0001	1.000	1.0	2525

Abbreviations: ACE: angiotensin-converting enzyme; GWAS: genome-wide association study. *Statistically significant. Note: Analysis restricted to rs76806643 exclusively across 7 European GWAS

datasets. ORs corrected for imperfect LD (r^2=0.88) using $\log(OR_{corrected}) = \log(OR_{observed})/r^2$. Notably, heterogeneity was small under the uniform proxy strategy after excluding GCST90475829.

Table 4. Genetic Models of *ACE* Genotypes Using Summary Data

Model[#]	Cases	Controls	Pooled OR (95% CI)	*p*[*]	I^2
Dominant (II+ID vs DD)	73 723	548 247	6.97 (4.17–11.66)	1.4×10^{-13}	1%
Overdominant (ID vs II+DD)	73 723	548 247	0.25 (0.23–0.27)	1.0×10^{-100}	1%

Abbreviations: RLS: restless legs syndrome; GWAS: genome-wide association study; FDR: false discovery rate. *Statistically significant. #Random-effects inverse-variance meta-analysis; heterogeneity was negligible (I^2 = 1%).

Figures

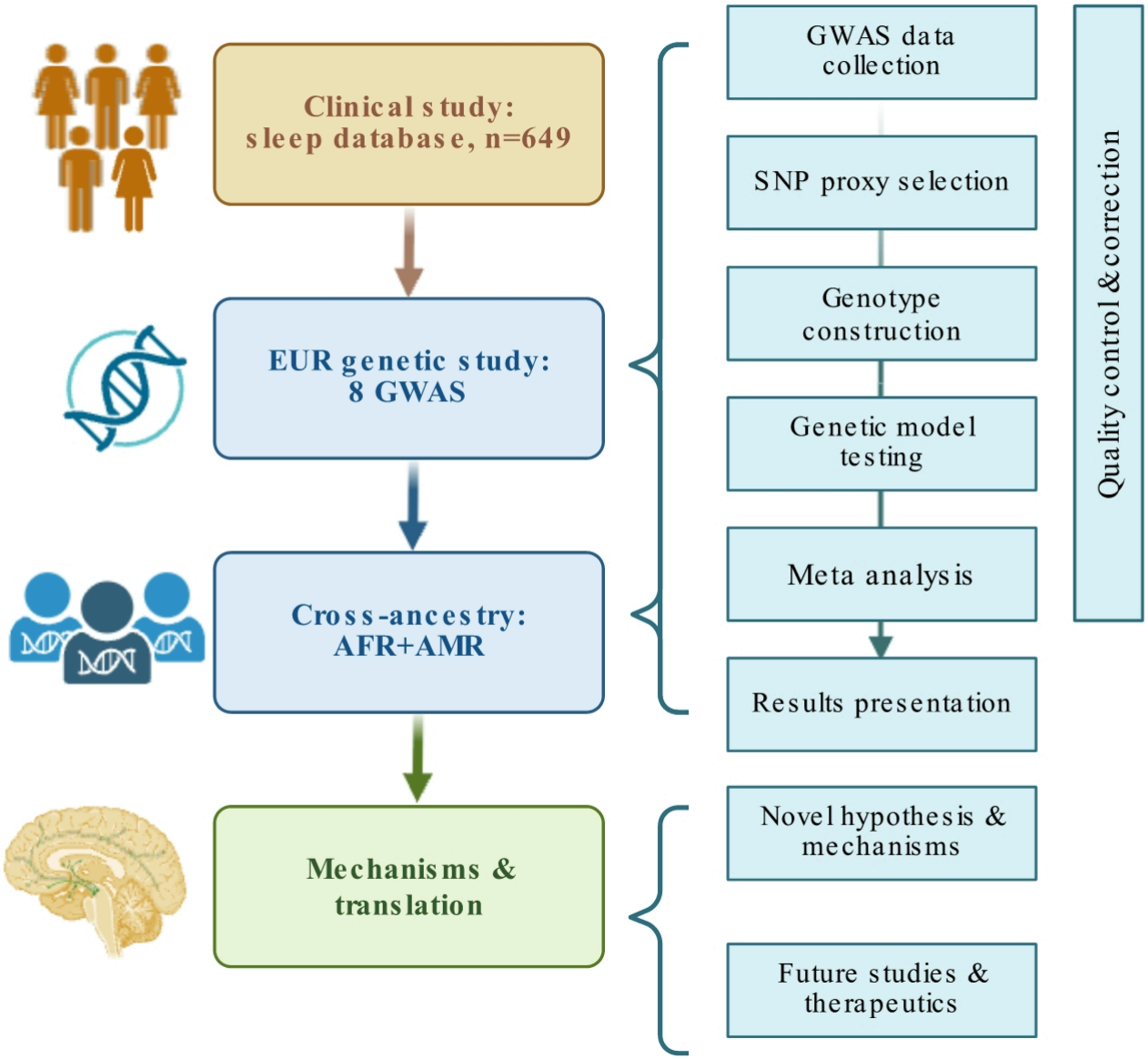


Figure 1

Study design flowchart. (A) Clinical discovery cohort (n=649) with polysomnography-confirmed RLS and ACE genotyping to identify genetic signal and inheritance pattern. (B) Systematic meta-analysis of 10 GWAS datasets (8 European, 1 African, 1 Latin American), involving tens of thousands of RLS cases, using proxy SNPs for ACE I/D to validate findings and determine the optimal genetic model.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementalmaterial.docx

- SupplementaryTable1EURanalysis.xlsx
- SupplementaryTable2AFRACEfinalanalysis.xlsx
- SupplementaryTable3AMRACEfinalanalysis.xlsx
- SupplementaryTable4CrossAncestryMetaanalysis.xlsx