

**Association of regular aerobic exercises and neuromuscular junction variants with incidence of frailty: An analysis of the Chinese Longitudinal Health and Longevity Survey**

Running Title: Aerobic exercises and neuromuscular junction variants with frailty

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## **Abstract**

**Background:** Candidate genes of neuromuscular junction (NMJ) pathway increased risk of frailty, but the extent and whether can be offset by exercises was unclear. The aim of this study was to investigate the association between aerobic exercises and incident frailty regardless of NMJ pathway related genetic risk.

**Methods:** A cohort study on participants from Chinese Longitudinal Healthy Longevity Survey was conducted from 2008 to 2011. A total of 7006 participants (mean age of  $80.6 \pm 10.3$  years) without frailty at baseline were interviewed to record aerobic exercise status, and 4053 individuals among them submitted saliva samples. NMJ pathway related genes were genotyped and weighted genetic risk scores were constructed.

**Results** During a median follow-up of 3.1 years (19,634 person-years), there were 1345 cases (19.2%) of incident frailty. Persistent aerobic exercises were associated with a 26% lessened frailty risk (adjusted  $HR=0.74$ ,  $95\%CI=0.64-0.85$ ). This association was stronger in a subgroup of 1552 longevous participants (age between 90 to 111 years, adjusted  $HR=0.72$ ,  $95\%CI=0.60-0.87$ ). High genetic risk was associated with a 35% increased risk of frailty (adjusted  $HR=1.35$ ,  $95\%CI=1.16-1.58$ ). Of the participants with high genetic risk and no persistent aerobic exercises, there was a 59% increased risk of frailty (adjusted  $HR=1.59$ ,  $95\%CI=1.20-2.09$ ).  $HRs$  for the risk of frailty increased from the low genetic risk with persistent aerobic exercise to high genetic risk without persistent aerobic exercise ( $P$  trend  $<0.001$ ).

**Conclusion:** Both aerobic exercises and NMJ pathway related genetic risk were significantly associated with frailty. Persistent aerobic exercises can partly offset NMJ pathway related genetic risk to frailty in elderly people.

**Key words** regular aerobic exercises; neuromuscular junction; frailty

## Introduction

Both genetic and lifestyle factors, such as frequency of exercises, played roles in building up muscle. Mutants in *Agrin*, an activator on clustering of nicotinic acetylcholine receptors (nAChR) via Muscle-Specific Tyrosine-Protein Kinase Receptor/LDL Receptor Related Protein 4 (Musk/LRP4) signaling, can cause early-onset myasthenia gravis.<sup>1</sup> Cases carried identical homozygous variants in the intracellular domain of MUSK were found to be dyskinesia.<sup>2</sup> Methylation of histone deacetylase 9 (HDAC9), conserved binding motifs of which on myogenin (MyoG) promotor, repressed transcription of MyoG and nAChR, which further caused neuromuscular junction (NMJ) misfunctions.<sup>3-4</sup> Calcineurin, encoded by *PPP3CA* and stabilized NMJ, was associated with endurance capacity and VO<sub>2</sub>max trainability.<sup>5-6</sup> According to previous study, NMJ pathway has been selected as “hallmark of aging” by combined aging and longevity related genes expression databases.<sup>7</sup> Thus, polygenic NMJ pathway risk scores combining multiple related alleles maybe a set of biomarkers in frailty and provide a quantitative measure of genetic frailty risk.

There was existing evidence that elderly people who taking physical activities had a lower frailty risk,<sup>8-9</sup> but the certainty of evidence was not strong enough and the optimal way remained uncertain.<sup>10</sup> It is possible that genetic risk can be offset by regular exercises in older people.<sup>11</sup> Even though molecular biology of aging researches suggested that exercises can moderate HDACs deacetylase activity towards myocyte enhancer factor 2 (MEF2), which interacted with myoblast determination protein (MyoD), synergistically activated *MyoG* expression,<sup>12</sup> little is known about the real-world role of exercises on genetic risk of older people which involved NMJ pathway.

The aim of this study was to use data from a representative aging cohort with a certain number of longevous participants to investigate whether adherence to regular aerobic exercises can offset genetic risk caused by NMJ pathway related variants for frailty.

## Methods

### ***Study design and participants***

Baseline data was obtained from the Chinese Longitudinal Healthy Longevity Survey (CLHLS) 2008-2009 wave. CLHLS was a nationwide cohort study with a randomly selected elderly participants from 23 provinces, which covered nearly 82% of the total population in China in 2010. The inclusion criteria were elderly people aged  $\geq 60$  years old who accepting to participate in the survey. Participants were excluded if they had any of the following: (a) baseline frailty; (b) incomplete key variables or lost to follow-up; (c) death. The next check-up was in 2011-2012 wave. A total of 7006 participants, of whom had their aerobic exercises status checked, were taken into analysis of first step on the association between adherence to aerobic exercises and frailty. After samples with call rate  $< 1$ , minor-allele frequency (MAF)  $< 0.1$ , the inbreeding coefficient  $|F| > 0.1$  and identity by descent (IBD)  $> 0.1$  were excluded, four thousands and fifty three individuals who provided saliva sample and were willing to participate in genotyping assay were included in the analysis of second part, whether NMJ pathway related variants can increase the risk of frailty, and third part, whether persistent aerobic exercises can offset the genetic risk arisen by NMJ variants to frailty. The flow chart can be seen in **Figure 1**.

All participants gave informed consent to take part in the study and for their data to be used. The study protocol was approved by Biomedical Ethics Committee of Peking University (IRB00001052-13074).

### ***Assessment of aerobic exercises status***

Aerobic exercises status was assessed by trained personnel at baseline based on two questions “Do you exercise regularly at present?” and “Did you exercise regularly in the past?”. The exercises referred to purposeful physical activities, such as walking, ball games and running. Participants were regarded as “Persistent aerobic exercises” if the answers of above two questions were “yes”.

### ***Genotyping, imputation and Establishment of NMJ pathway risk score***

Full details of genotyping, imputation and Establishment of NMJ pathway risk score

can be found in **Supplement Methods**. The genotypic data of *HDAC9*, *PPP3CA*, *MUSK* and *MEF2A* were drawn from the CLHLS genome-wide association study (GWAS) dataset.<sup>13</sup> The variants were annotated by HaploReg v2. Single and multiple genotype-phenotype association analyses were performed by *Cox* regressions. Linkage disequilibrium (LD) was also checked by PLINK (1.06) before weighted NMJ pathway risk scores were constructed.

### ***Ascertainment of frailty***

Frailty was ascertained by Frailty Index (FI), which created by health deficits, such as symptoms, signs and disabilities. Variables were comprehensively constituted from Rockwood's Study and Gu Danan 's Study about CLHLS.<sup>14-15</sup> Thirty-four variables were used to construct an FI (detail can be found in **Table S1**). Each variable was recoded as 0 (absence of deficit) or 1 (presence of deficit). For each individual, the FI score was calculated by summing up the deficits and divided by 34 (total number of items). Frailty was defined as  $FI \geq 0.25$ .<sup>16</sup>

### ***Statistical analysis***

GWAS analysis was performed with R language packages, including *snpStats*, *doParallel*, *SNPRelate* and *GenABEL*. Other Data were analyzed using Statistical Package for the Social Sciences (SPSS, Version 22.0, IBM corp., Chicago, IL, USA).

All continuous variables with normal distribution were expressed as the *mean (standard deviation)*. The variables with skewed distribution were expressed as the *median (interquartile range)*. *Independent sample t-test* for normally distributed variables was used to compare different groups. Categorical data were expressed as *count (percentage)*. *Chi-square* tests were used to compare different groups of categorical data. *Cox regressions* were used to examine *HRs* of frailty identified by persistent aerobic exercises and NMJ pathway related homozygous variants. The *HRs* were adjusted for age, sex, smoking, drinking histories, years of schooling, body mass index (BMI), comorbidity (**Table S2**), living arrangement (whether alone) and high protein intake diversity (definition can be found in **Supplement Methods**). Stratified

analyses were performed to estimate potential modification effects on regular aerobic exercises according to age (60-79, 80-89, or 90-111), sex (men or women), low BMI (yes (<18.5kg/m<sup>2</sup>) or no (≥18.5kg/m<sup>2</sup>)), one or more self-reported diseases (yes or no), balance on animal-plant proteins intake diversity (yes or no), and high dietary protein intake diversity (yes or no). Trend analysis was applied to explore the association between HRs of frailty and NMJ pathway related genetic risk along with persistent aerobic exercise or not. A two-sided *P* value <0.05 was considered statistically significant. The related protein-protein interaction network was predicted by string analysis (<https://string-db.org>).<sup>17</sup> Conserved Domains (CDS) of *MUSK* isoforms were checked in NCBI database.

## Results

### *Participants' general characteristics*

**Table 1** summarize the baseline characteristics of the participants. The 7006 participants had mean age of 80.6±10.3 years and 49.1% were men. The minimum age was 61 years, the maximum age was 111 years. Based on Rockwood FI cut-off, during a total of 19,634 person-years (median [interquartile range] length of follow-up, 3.1 [2.9-3.2] years), there were 1345 cases (19.2%) of incident frailty. Frailty was significantly more prevalent in older age and in women than in men (all *P*<0.001). Among the lifestyle factors, individuals with frailty had significantly lower proportions of persistent aerobic exercises (*P*=0.034), and there were no significant differences in categories of dietary protein intake diversity (*P*=0.626).

### *Association between persistent aerobic exercises and frailty*

In whole population, compare to elderly people without persistent aerobic exercises, those with persistent aerobic exercises showed lessen frailty risk, with adjusted *HR* of 0.74 (95%*CI*=0.64-0.85) (**Table 2**). This reverse association was stronger in a subgroup of 1552 longevous participants (age between 90 to 111 years, adjusted *HR*=0.72, 95%*CI*=0.60-0.87). The stratified analyses were uploaded in **Figure S1**.

### ***Association between NMJ pathway related variants and frailty***

High NMJ pathway related genetic risk was associated with a 35% increased risk of frailty in elderly individuals (adjusted  $HR=1.35$ ,  $95\% CI=1.16-1.58$ , **Table 2**). Single-SNP analysis and LD distant analysis of each in the discovery sample of NMJ pathway variants related to frailty can be found in **Table S3** and **Table S4**, respectively.

### ***Joint association of aerobic exercises and genetic risk with frailty***

Of the participants with high genetic risk, persistent aerobic exercises were associated with a 25% lower risk of frailty (adjusted  $HR=0.75$ ,  $95\%CI=0.57-0.99$ ). Of the participants with no persistent aerobic exercises, high genetic risk was associated with a 40% increased risk of frailty (adjusted  $HR=1.40$ ,  $95\%CI=1.17-1.67$ ) (**Figure 2**). Of the participants with high genetic risk and no persistent aerobic exercises, there was a 59% increased risk of frailty (adjusted  $HR=1.59$ ,  $95\%CI=1.20-2.09$ ).  $HRs$  for the risk of frailty increased from the low genetic risk with persistent aerobic exercise to high genetic risk without persistent aerobic exercise ( $P$  trend<0.001, **Figure 3**). There was no significant interaction between genetic risk and regular aerobic exercises ( $P$  for interaction=0.309).

## **Discussion**

This study, to the best of our knowledge, was the first report of genetic risk caused by NMJ pathway variants to frailty in a relatively large elderly population which can be partly offset by persistent aerobic exercises.

Many years ago, one classic study showed that 8 weeks of high intensity resistance training improved muscle mass and functional mobility among 9 people with an average age of 90.3.<sup>18</sup> More recently, another study demonstrated that an 8-week low to moderate intensity resistance exercises increased muscle strength in 40 residents aged 90-97 years. What counts was the authors reported no major adverse effects associated with the intervention despite the advanced age and frailty

of the participants.<sup>19</sup> A recent meta-analysis in older adults (n=28 523, mean age 74.2 years) also found that long term exercises ( $\geq 1$  year), independent of exercise frequency, improved physical function but were not associated with an increased risk of dropout due to health issues and mortality.<sup>20</sup> However, the validation of persistent aerobic exercises in oldest old to prevent frailty has not been fully proof in these studies. In our study, with a subgroup of 1552 longevous participants (age between 90 to 111 years), we found a significantly association between persistent aerobic exercises and frailty (**Figure S1**), which had larger sample size of nonagenarians and centenarians with a stronger statistical power.

Low dietary protein intake, especially when accompanied with low physical activity, has been linked to progressive loss of muscle mass leading to low muscle strength, low walking speed, and feeling of exhaustion, all of which were associated with the physical frailty phenotype.<sup>21-23</sup> Indeed, several randomized controlled trials observed that the combination of physical activity and protein intake have a beneficial effect on muscle function and can help to prevent frailty.<sup>24-25</sup> But till now, far less is known on the association between appropriate protein intake, including protein source and timing, with physical activity and the broader concept of frailty.<sup>26</sup> In our study, with subgroups of 3574 participants who balanced on animal-plant protein intake diversity and 2068 who were in high dietary protein intake diversity, we found a stronger inverse relationship between persistent aerobic exercises and frailty (**Supplement Methods** and **Figure S1**), which provided better evidence on positive effect of exercises in combination with protein intake on physical frailty.

Wnt signaling may contribute to impaired aged skeletal muscle repair. However, the role of Wnt signaling in impaired aged skeletal muscle repair is controversial. Some suggested that there was an exacerbation of Wnt signaling in aged skeletal muscle. Although Wnt signaling is required for completion of muscle repair, the incongruous elevation of Wnt signaling in aged skeletal muscle may result in dysfunctional myogenic capability and acceleration of aging. Besides, increased Wnt signaling in aged skeletal muscle may inhibit myogenicity.<sup>27</sup> On one side, one of Wnts family, Wnt 3a, was over-activated to upregulate the expressions of p53,<sup>28-29</sup> a cell



arrest protein, and significantly downregulated MyoD1 and MyoG expression in muscular cells by activating HDAC9/MEF2, a transcription repressor on the MyoG promoter, which further impaired proliferation and differentiation of muscle progenitor cells and contributed to the development of muscle cell senescence and loss of muscle mass.<sup>30-31</sup> On the other side, over-activation of canonical Wnt/ $\beta$ -catenin signaling followed when soluble Wnt ligands interacted with postsynaptic membrane receptor, frizzled (one of MuSK CDS) and LRP4 on the muscle fiber.<sup>32</sup> Upon Wnt overactivation, signal transduced by segment polarity protein dishevelled homolog (DVL1) binding to C-terminus of frizzled, GSK3 $\beta$ 's phosphorylation of  $\beta$ -catenin was activated, which did not allow nuclear translocation of  $\beta$ -catenin. Within the nucleus, inhibited  $\beta$ -catenin reduced the binding to transcription factors such as T-cell factor 1 (TCF1) and lymphoid-enhancing factors 1 (LEF1), resulting in MyoD1 and MyoG inhibited.<sup>33</sup> Deprivation of calcineurin decreased MyoG expression by regulating MEF2, which possibly leading to abnormal NMJ formation.<sup>34</sup> Genetic variation in exons 10 and 18 of the *LRP5* modulates canonical Wnt signaling and the relationship between physical activity and bone mineral density (BMD) in men. Wnt-LRP5 may play a role in the adaptation of bone to mechanical load in humans, which also implied the potential relationship between Wnt signaling and frailty.<sup>35</sup> In our study, we found that multiple variants, including the SNPs of *HDAC9*-rs2074633, *PPP3CA*-rs17030795, *MUSK*-rs2298492, and *MUSK*-rs2274419, increased risks of frailty. Clusters of HDAC9, calcineurin and MuSK were linked to the core protein, wnt3a, a ligand for members of the frizzled family of seven transmembrane receptors, had functions in the canonical Wnt signaling pathway that associated with inhibition of MyoG and participated in the modulation of chemical synaptic transmission (GO: 0050804, **Figure S2**). Especially, homozygous variant in rs2274419 changed encoded AA from Met to Ile in frizzle domain of MuSK. The detailed analysis of rs2274419 location in CDS of different MuSK isoforms can be found in **Supplement Results** and **Figure S3**. The possible reason was as followed: In the older people, even though variants of rs2074633, rs17030795, and rs2298492 happened in 3'utr or introns which did not encode proteins, the synergistic effect,

together with rs2274419 changed, may enlarge adverse impact of Wnts and further inhibit MyoD1 and MyoG in NMJ pathway, leading to muscle weakness, as a feature of frailty in muscular system.

The effect of exercises on several SNPs of genes associated with frailty has been reported before. Older individuals (aged 70 to 79 years) who exercised, with the angiotensin-converting enzyme (ACE) Insertion/Deletion (DD/ID) genotypes, were less likely to develop mobility limitation than those with the II genotype, which was associated with higher levels of intermuscular fat (IMF).<sup>36</sup> Another study showed that exercises reduced IMF in those aged between 50 to 83 years who are adrenergic receptor (ADR)  $\beta$ 2 Glu27 carriers and Glu27 carriers who also carry the ADR $\alpha$ 2b Glu9 allele.<sup>37</sup> To the SNP of TNF $\alpha$ -rs1800629, individuals aged over 65 years homozygous for the G allele presented a higher percentage of change with exercise training, with better functional performance in comparison to individuals with genotypes AA + AG. Besides, individuals with combined genotypes TNF $\alpha$ -rs1800629 GG and IL6-rs1800795 CC + CG showed greater improvement in physical performance after exercise training in comparison to other genotypes.<sup>11</sup> Middle-aged men (aged 50 to 60 years) with estrogen receptor  $\alpha$  (*ER $\alpha$* ) PP or Pp genotypes appear to have increased BMD values in the lumbar spine after aerobic exercises training.<sup>38</sup> Catechol-o-methyltransferase (COMT) metabolized hydroxylation of estrogens into more inactive methoxy-estrogens which would not bind to ER. Sedentary old women (aged 63 to 76 years) with the COMT polymorphism HH genotype had lower muscle mass, strength and power, but they benefited the most from physical activity.<sup>39</sup> The  $\alpha$ -actin3 gene (*ACTN3*) genotype exhibits a potentially modifying effect on muscle mass, maintenance of muscle function, and sarcopenia risk in elderly athletic populations, with the R allele associated with an increase in type-II muscle fibers.<sup>40</sup> Physically inactive participants (aged 50 to 85 years) with the IGF1 CA repeat polymorphism was significantly influenced on the change in strength and muscle volume combined with strength training.<sup>41</sup> But till now, whether persistent aerobic exercises can offset the risk of frailty partly caused by overactivated Wnt signaling in NMJ, especially at a very advanced age, was still unclear.<sup>42</sup> In our study, with a certain number of oldest old people, we found that, of the participants with high Wnt signaling related genetic risk in NMJ, persistent aerobic exercises were associated with a partly lessen risk of frailty. However, little was known about the effects of exercises on Wnt signaling pathways in aged muscle, but because exercises elevated circulating pro-myogenic factors, it appeared reasonable to consider that appropriate aerobic exercises may release systemic factors that returned the balance of Wnt signaling in aged skeletal muscle.<sup>43</sup>

This study has several strengths. First, we reported a prospective association between persistent aerobic exercises and frailty, especially in nonagenarians and centenarians with a certain number. Second, we further proved that NMJ pathway was one of potential mechanisms in frailty. Third, we found that, of the participants

with high Wnt signaling related genetic risk in NMJ, persistent aerobic exercises were associated with a lessen risk of frailty.

However, the work had several inevitable limitations. First, the results of genotyping assay were not identified by quantitative real-time polymerase chain reaction (qRT-PCR) from human cells. Second, due to saliva samples were voluntarily collected, people who did SNPs genotyping were not randomly assigned. Third, because of relatively small parts of elderly people persisting aerobic exercise, the function of persistent exercises in high genetic risk may be underestimated in the association analysis with frailty. Fourth, persistent aerobic exercises and dietary protein intake diversity were self-reported which may arise recall biases. Fifth, the detailed varieties of sports and amount of foods were not recorded in this study. Sixth, although analyses were adjusted for a number of key confounding, such as age, sex, BMI, comorbidities and protein intake diversity, the possibility of unmeasured determinants and reverse causation remained. Seventh, additional NMJ pathway variants associated with frailty are likely to be identified in future studies, and these variants may prove useful in further refining estimates of genetic risk. The validation of NMJ pathway risk scores also need to be reappraised in another ascertained population.

In summary, both aerobic exercises and NMJ pathway related genetic risk were significantly associated with frailty. Persistent aerobic exercises can partly offset genetic risk caused by NMJ pathway variants to frailty. This is an important finding given that regular aerobic exercise was a personal and economical habit without major adverse effect that can be easily developed to cope with accelerating global burden of frailty in elderly people.

**Author Contributions** ZYJ contributed to the conception of the study design and drafted the manuscript. YY did the genotyping assays and provided the basic data. ZPD, LZH, ZP and LFR helped with the interpretation of data and the statistical

analysis. WZH, LD, LYB, KL and CLK helped with the revision of manuscript. SXM and MC participated in the design of the study and were the guarantors of the work. All authors read and approved the final manuscript.

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**Conflicts of interest** The authors declare that they have no competing interests.

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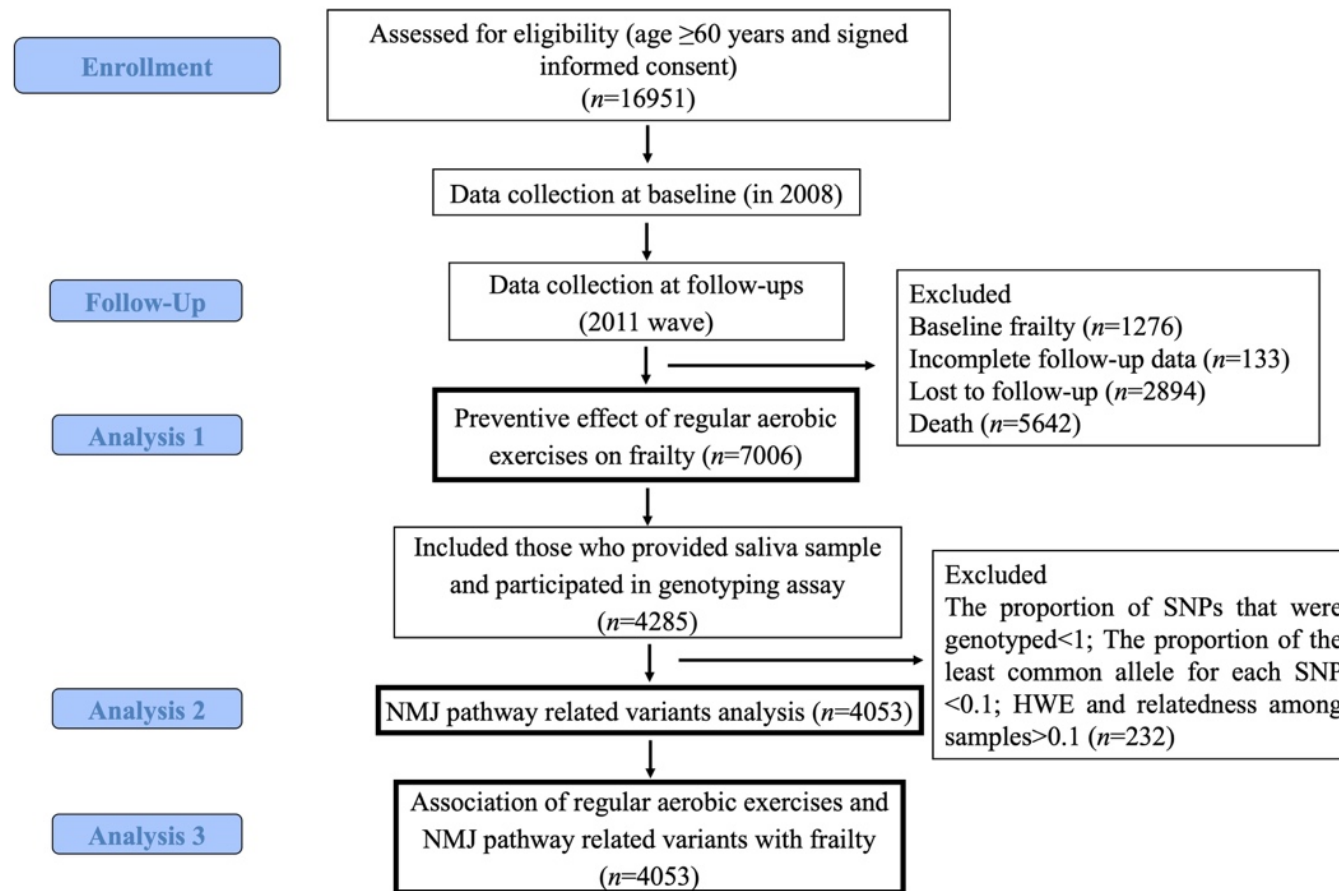
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**Figure 1** Flowchart of study population. Notes: SNP: single nucleotide polymorphism; HWE: Hardy-Weinberg Equilibrium; NMJ: neuromuscular junction.

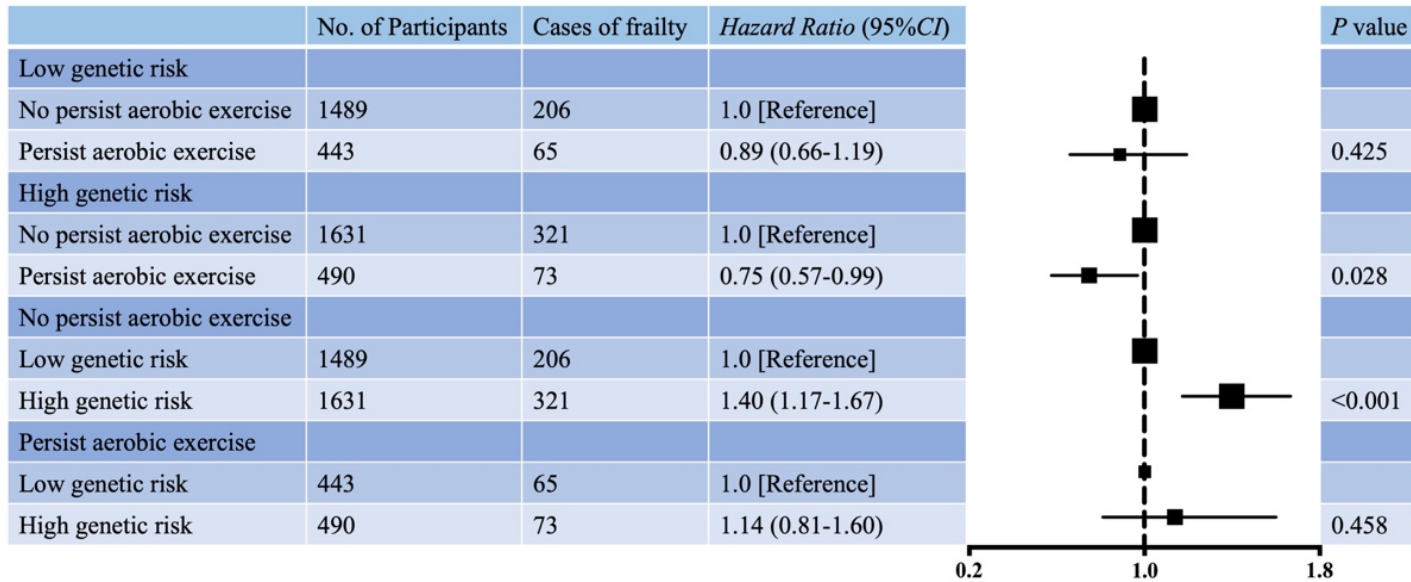
**Table 1** Participants' clinical characteristics

	Non-frailty ( <i>n</i> =5661)	Frailty ( <i>n</i> =1345)	<i>P</i> -value
Age (years)	78.7 (9.7)	88.7 (8.8)	<0.001
Sex			
Men ( <i>n</i> , %)	2934 (51.8)	509 (37.8)	<0.001
Women ( <i>n</i> , %)	2727 (48.2)	836 (62.2)	
Smoking ( <i>n</i> , %)	1386 (24.5)	187 (13.9)	<0.001
Drinking ( <i>n</i> , %)	1301 (23.0)	194 (14.4)	<0.001
Illiteracy ( <i>n</i> , %)	2790 (49.3)	923 (68.6)	<0.001
Widowed ( <i>n</i> , %)	1001 (17.7)	218 (16.2)	0.200
BMI (kg/m <sup>2</sup> )	21.05 (3.57)	20.21 (3.56)	<0.001
Comorbidity ( <i>n</i> , %)	1361 (24.0)	327 (24.3)	0.835
Dietary protein intake diversity (cat.)	2.6 (1.4)	2.6 (1.4)	0.626
Persistent aerobic exercises ( <i>n</i> , %)	1239 (21.9)	259 (19.3)	0.034

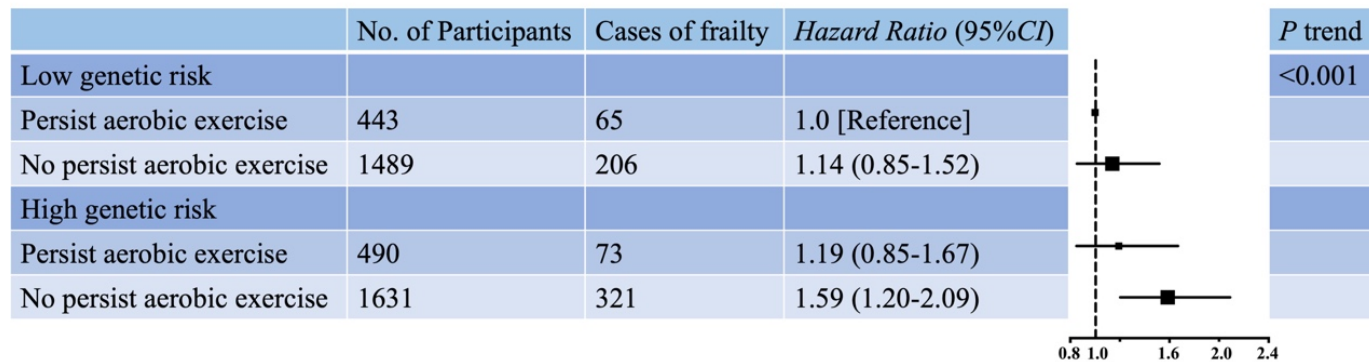
**Table 2** Risk of incident frailty according to persistent aerobic exercises and NMJ pathway related genetic risk levels.

	No. of Participants	No. of Cases of Frailty	HR (95%CI)	P value
<b>Persist aerobic exercises</b>				
No	5508	1086	1.0 [Reference]	
Yes	1498	259	0.74 (0.64-0.85)	<0.001
<b>Genetic risk</b>				
Low	1932	271	1.0 [Reference]	
High	2121	394	1.35 (1.16-1.58)	<0.001

Notes: The *Hazard Ratio* of persist aerobic exercise was adjusted for age, sex, smoking, drinking histories, years of schooling, BMI, comorbidity, whether alone and high protein intake diversity. Diseases were listed in Table S2.



**Figure 2** Risk of incident frailty according to NMJ pathway related genetic risk levels or whether persisting aerobic exercises or not. Notes: NMJ: neuromuscular junction; The *Hazard Ratios* of persist aerobic exercise were adjusted for age, sex, smoking, drinking histories, years of schooling, BMI, comorbidity, whether alone and high protein intake diversity.



**Figure 3** Risk of incident frailty according to NMJ pathway related genetic risk levels and persist aerobic exercises. Notes: NMJ: neuromuscular junction; The *Hazard Ratios* of persist aerobic exercise were adjusted for age, sex, smoking, drinking histories, years of schooling, BMI, comorbidity, whether alone and high protein intake diversity.