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No evidence for genetic association with the *let-7* microRNA-binding site or other common *KRAS* variants in risk of endometriosis

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STUDY QUESTION: Is there a contribution of the minor allele at the *KRAS* single nucleotide polymorphism (SNP) rs61764370 in the *let-7* microRNA-binding site to endometriosis risk?

SUMMARY ANSWER: We found no evidence for association between endometriosis risk and rs61764370 or any other SNPs in *KRAS*.

WHAT IS KNOWN ALREADY: The rs61764370 SNP in the 3′ untranslated region of the *KRAS* gene is predicted to disrupt a complementary binding site (LCS6) for the *let-7* microRNA, and was recently reported to be at a high frequency (31%) in 132 women of varying ancestry with endometriosis compared with frequencies in a database of population controls (up to 7.6% depending on ancestry), suggesting a strong effect of this *KRAS* SNP in the aetiology of endometriosis.

STUDY DESIGN, SIZE AND DURATION: This was a case–control study with a total of 11 206 subjects. The study was performed between February 2012 and July 2012.

PARTICIPANTS/MATERIALS, SETTING AND METHODS: We first investigated a possible association between common markers in *KRAS* and endometriosis risk from our genome-wide association (GWA) data in 3194 surgically confirmed endometriosis cases and 7060 controls of European ancestry. Although rs61764370 was not genotyped on the GWA arrays, five SNPs typed in the study were highly correlated with this variant. The rs61764370 and two SNPs highly correlated with rs61764370 were then genotyped in 933 endometriosis cases and 952 controls using the Sequenom MassARRAY platform.

MAIN RESULTS AND THE ROLE OF CHANCE: There was no evidence for an association between rs61764370 and endometriosis risk $P = 0.411$ and odds ratio = 1.10 (95% confidence intervals: 0.88–1.36). We also found no evidence for an association between the highly correlated SNP rs17387019 and endometriosis. Their minor allele frequencies in cases and controls were of 0.087–0.091 similar to the population frequency reported previously for this variant in controls. Analyses of endometriosis cases with revised American Fertility Society stage III/IV disease also showed no evidence for an association between these SNPs and endometriosis risk.

LIMITATIONS AND REASONS FOR CAUTION: The GWA and genotyped data sets were not independent since individuals and cases from some families overlap. Controls in our GWA study were not screened for endometriosis.

WIDER IMPLICATIONS OF THE FINDINGS: The key SNP, rs61764370, was genotyped in a subset of samples. Our results do not support the suggestion that carrying the minor allele at rs61764370 contributes to a significant number of endometriosis cases and rs61764370 is, therefore, unlikely to be a useful marker of endometriosis risk.

STUDY FUNDING/COMPETING INTEREST(S): The research was funded by grants from the Australian National Health and Medical Research Council and Wellcome Trust. None of the authors has competing interests for the study.

Key words: endometriosis / *KRAS* / microRNA / genetic association

Introduction

Endometriosis is a common, chronic gynaecological disease characterized by the development of functional endometrial tissue outside the uterus. The disease is an important contributor to pelvic pain and subfertility. It affects 7–10% of women of reproductive age and up to 50% of women with infertility (Treloar *et al*., 1999; Giudice, 2010). Medical treatment options are limited and surgical intervention may not prevent recurrence (Rogers *et al*., 2009; Giudice, 2010).

Genetic factors contribute to endometriosis and it is inherited as a complex genetic trait (Kennedy, 1999; Treloar *et al*., 1999; Stefanson *et al*., 2002; Simpson and Bischoff, 2003). Numerous candidate gene studies have reported association for markers in candidate genes and endometriosis susceptibility, but results have generally not been replicated in subsequent studies (Simpson and Bischoff, 2003; Bischoff and Simpson, 2004; Guo, 2005; Montgomery *et al*., 2008). This lack of success is likely due to a number of reasons, including differences in disease definitions, case/control population sampling issues and the lack of power in small-scale studies, which detect only variants with large effects and are prone to detection of false-positive results (Zondervan *et al*., 2002).

Two genome-wide association (GWA) studies have reported significant associations with endometriosis risk in three genomic regions on chromosomes 9p21, 7p15.2 and 1p36 (Uno *et al*., 2010; Painter *et al*., 2011). The regions showed strong evidence for association with disease risk and were replicated in independent data sets, demonstrating that the studies had the power to detect common variants contributing to disease risk. However, effect size estimates were small [odds ratios (ORs) $\leq$1.22] and the proportion of variance explained by the key single nucleotide polymorphism (SNP) on chromosome 7p15.2 (rs12700667) was 0.36, or 0.69% of the estimated 51% heritability of endometriosis (Painter *et al*., 2011).

Recently, the minor allele at a SNP (rs61764370) within the 3′ untranslated region (UTR) of the Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene was reported to be at a high frequency (31%) in women with endometriosis, much higher than frequencies reported in unselected population samples (Grechukhina *et al*., 2012). *KRAS* is a strong candidate gene for endometriosis. The activation of *Kras* induced peritoneal endometriosis in 47% of mice, and 100% of mice developed benign endometriosis-like lesions within the ovarian surface epithelium (Dinulescu *et al*., 2005). Endometriosis-like lesions also developed and survived over a prolonged period in wild-type mice after transplanting endometrial tissue from mice with the activated *Kras* mutation (Cheng *et al*., 2011). The rs61764370 SNP is predicted to disrupt a complementary binding site (LCS6) for the *let-7* microRNA (miRNA) (Johnson *et al*., 2005; Esquela-Kerscher and Slack, 2006). The minor allele at rs61764370 was shown to alter mRNA and protein expression levels in cultured endometrial stromal cells (Grechukhina *et al*., 2012), and stromal cells from women carrying the variant allele also showed increased proliferation and invasion. The authors concluded that carrying the minor allele at the rs61764370 variant might account for up to one-third of endometriosis cases (Grechukhina *et al*., 2012).

Previous studies have examined an association between variants in *KRAS* and endometriosis risk. Mutational screening of *KRAS* identified *KRAS* mutations in endometrioid carcinoma, but not in endometriosis lesions (Amemiya *et al*., 2004). Genotyping 32 tagging SNPs across *KRAS* in 958 endometriosis cases and 959 controls showed no evidence for common variants contributing to disease risk in a study that had 80% power to detect a risk variant with a minor allele frequency (MAF) of 5% at a genotype relative risk of 1.7 (Zhao *et al*., 2006). We would have expected the association signal for rs61764370 to have been detected by correlation with tagging SNPs (Zhao *et al*., 2006) or in the GWA studies (Uno *et al*., 2010; Painter *et al*., 2011).

We therefore re-examined evidence for an association between common markers in *KRAS* and endometriosis risk from our GWA data (Painter *et al*., 2011) and genotyped rs61764370 in endometriosis cases and controls.

Materials and Methods

Study samples

We examined data from our previous GWA study including 3194 endometriosis cases and 7060 controls, with 2270 endometriosis cases from Australia (QIMR, Australian data set) and 924 cases from the UK (Oxford, UK data set) (Painter *et al*., 2011). The QIMR samples were unrelated cases (one individual per family) drawn from 3908 affected women from multiple case families and single cases previously recruited into our endometriosis study. Women included in the study completed questionnaires, provided a blood sample and gave access to their medical records, allowing retrospective confirmation of diagnosis for affected individuals (Treloar *et al*., 2005; Painter *et al*., 2011). UK cases included unrelated cases drawn from 245 families with $\geq$2 affected sisters with surgically confirmed endometriosis (Treloar *et al*., 2002), and 785 singleton cases. We obtained medical records for all the cases and diagnosis of endometriosis was confirmed from surgical records in 100% of cases in Australia and 97% of cases in the UK (Painter *et al*., 2011). Disease severity was retrospectively assessed using the revised American Fertility Society (rAFS) classification system (American Fertility Society, 1985); A total of $n = 1686$ (52.7%) were diagnosed with stage A disease (rAFS I/II or some ovarian disease with few adhesions), $n = 1364$ (42.7%) had stage B (stage III/IV disease) and $n = 144$ (4.6%) had an unknown stage. In both the Australian and UK data sets, affected women completed a self-report questionnaire including questions about fertility history and pain symptoms; however, as questions were phrased differently, sub-phenotype analysis was limited to data from the largest case data set (QIMR, 2078 cases).

The controls consisted of 1870 individuals recruited by QIMR and a further 5194 individuals provided by the Wellcome Trust Case Control Consortium 2 (WTCCC2) (Painter *et al*., 2011). Ethics approval for the studies was obtained from the QIMR Human Ethics Research Committee, the Australian Twin Registry and the Oxford regional multi-centre and local research ethics committees. All the participants gave informed consent prior to testing.

GWA association data

Samples from the GWA study were genotyped on Illumina genotyping platforms. We reviewed a total of 97 common SNPs (MAF range from 2.4 to 47.7%) located between 150 kb upstream and 150 kb downstream of *KRAS* in all the endometriosis cases and in stage III/IV cases compared with the controls. The *KRAS* variant rs61764370 was not present on the chips, so we searched for correlated SNPs using the SNP Annotation and Proxy Search (SNAP) software available through the Broad Institute (http://www.broadinstitute.org/mpg/snap/ldsearch.php). Linkage disequilibrium (LD) is the non-random association of alleles at different loci

and indicates to what extent the SNPs are correlated in a particular ethnic population. Using the 1000 Genome pilot 1 data generated on 179 individuals including 60 of European origin ('CEU'), the SNAP program compared the genotypes of SNPs within this region and identified those present on Illumina Human 610 and 1 M chips highly correlated (in strong LD) with rs61764370. We identified five SNPs typed that were strongly correlated with rs61764370 ($r^2 > 0.5$) (Table I) and reviewed the association results previously calculated in our GWA study (Painter *et al.*, 2011). The effect of SNP genotype on the distributions of women with stage B disease or who answered 'Yes' to questions on subfertility and pain were tested by Pearson's chi-squared test.

Genotyping

To confirm results from the GWA data, we genotyped rs61764370 and two correlated SNPs in the same sample of 958 endometriosis cases and 959 unrelated controls examined in our previous KRAS study (Zhao *et al.*, 2006) using the Sequenom MassARRAY Genomics Platform. SNPs were genotyped as part of multiplex assays designed using the Sequenom MassARRAY Assay Design software (version 3.0: Sequenom Inc.) and samples were genotyped using standard methods (Zhao et al., 2007, 2008). Departures from Hardy-Weinberg equilibrium and statistical analysis for association between SNPs and endometriosis risk were tested using the PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/). Quality control of case samples from the GWA data analyses had identified case families with varying degrees of non-Caucasian ancestry. These were removed from the analyses of this data set and genotype data from 933 cases and 952 controls were included in the association analyses.

Results

We identified five SNPs present on Illumina HumanHap610-Quad and 1M-Duo Chips that were highly correlated with the SNP rs61764370 genotyped in the Grechukhina *et al.* (2012) study. These SNPs were in strong LD with rs61764370 ($r^2 \geq 0.568$, Table I) and were all located within 140 kb of the 3′UTR for *KRAS*. One SNP rs17387019 was reported to be in perfect ($r^2 = 1$) LD with rs61764370. There was no evidence for an association between this SNP and endometriosis risk (Table I) with $P = 0.305$ [OR = 0.95; 95% confidence intervals (CIs): 0.85–1.05]. The MAF for this SNP in our GWA data was 0.087 in the cases, consistent with published estimates in other Caucasian populations (Hapmap-CEU and EUR—interim phase 1–1000 Genomes) (Chin *et al.*, 2008).

There was no evidence for any association between endometriosis risk and other correlated SNPs, including rs859141, which was in high LD with rs61764370 ($r^2 = 0.789$) (Table I) with $P = 0.067$ and OR = 0.90 (95% CIs: 0.82–1.02). The latter SNP also has an MAF of 0.090. In addition, we checked all other common SNPs genotyped across the region, from 150 kb upstream to 150 kb downstream of *KRAS* in our GWAS data. There was no evidence for an association between any of the genotyped SNPs in the *KRAS* region and endometriosis risk (Fig. 1).

As cases in Grechukhina *et al.*'s analysis included 89% with a rAFS stage III/IV diagnosis, we restricted our analysis to cases diagnosed with rAFS stage III/IV (stage B) disease, and the results showed that the minor allele at each correlated SNP was slightly lower in the cases compared with the controls (Table I). We also found no evidence for an association between endometriosis risk and any correlated SNPs (Table I). We further analysed the genotypic association between SNP rs17387019 and sub-phenotypes of endometriosis collected in the Australian data set (see methods). There was no evidence that genotypes at rs17387019 influenced the proportions of women with stage B disease, infertility or pain (Table II).

To directly examine the association between rs61764370 and endometriosis risk, we genotyped this SNP, together with rs17387019 and rs859141 in our subset of endometriosis cases with a family history of disease and controls used in previous studies. There was no evidence for association between rs61764370 and

Table I Results of the tests of association for SNPs correlated with rs61764370 in all endometriosis and stage B disease.

SNP[a]	Position	r^2 with rs61764370*	A1	A2	Allele frequency in cases	Allele frequency in controls	Chi-square	*P*-value	OR (95% CIs)
All Cases									
rs859141	25113291	0.789	G	A	0.090	0.098	3.346	0.067	0.90 (0.82–1.02)
rs7303889	25146242	0.571	C	A	0.173	0.179	0.877	0.350	0.96 (0.89–1.04)
rs17387019	25155526	1.000	G	A	0.087	0.091	1.053	0.305	0.95 (0.85–1.05)
rs17388893	25285132	0.568	A	C	0.060	0.064	0.790	0.375	0.95 (0.84–1.07)
rs17329975	25290239	0.568	C	T	0.060	0.064	1.145	0.285	0.93 (0.83–1.06)
Stage B Cases									
rs859141	25113291	0.789	G	A	0.094	0.098	0.441	0.506	0.95 (0.82–1.10)
rs7303889	25146242	0.571	C	A	0.179	0.179	0.000	0.984	0.99 (0.90–1.11)
rs17387019	25155526	1.000	G	A	0.089	0.091	0.080	0.777	0.98 (0.85–1.13)
rs17388893	25285132	0.568	A	C	0.062	0.064	0.067	0.795	0.98 (0.83–1.16)
rs17329975	25290239	0.568	C	T	0.061	0.064	0.302	0.583	0.95 (0.80–1.13)

r^2: A measure of LD or non-random association for the observed frequencies of alleles at two markers measured as the square of the correlation coefficient.
LD was assessed using the SNAP proxy search program (http://www.broadinstitute.org/mpg/snap/ldsearch.php).
A1, reference allele; A2, alternative allele.
[a]SNPs present on Illumina HumanHap610-Quad BeadChip and Illumina Human1M-DuoChip that are highly correlated with SNP rs61764370 in 1000 Genomes Pilot 1 data of 60 Utah residents with European ancestry (CEU) individuals.

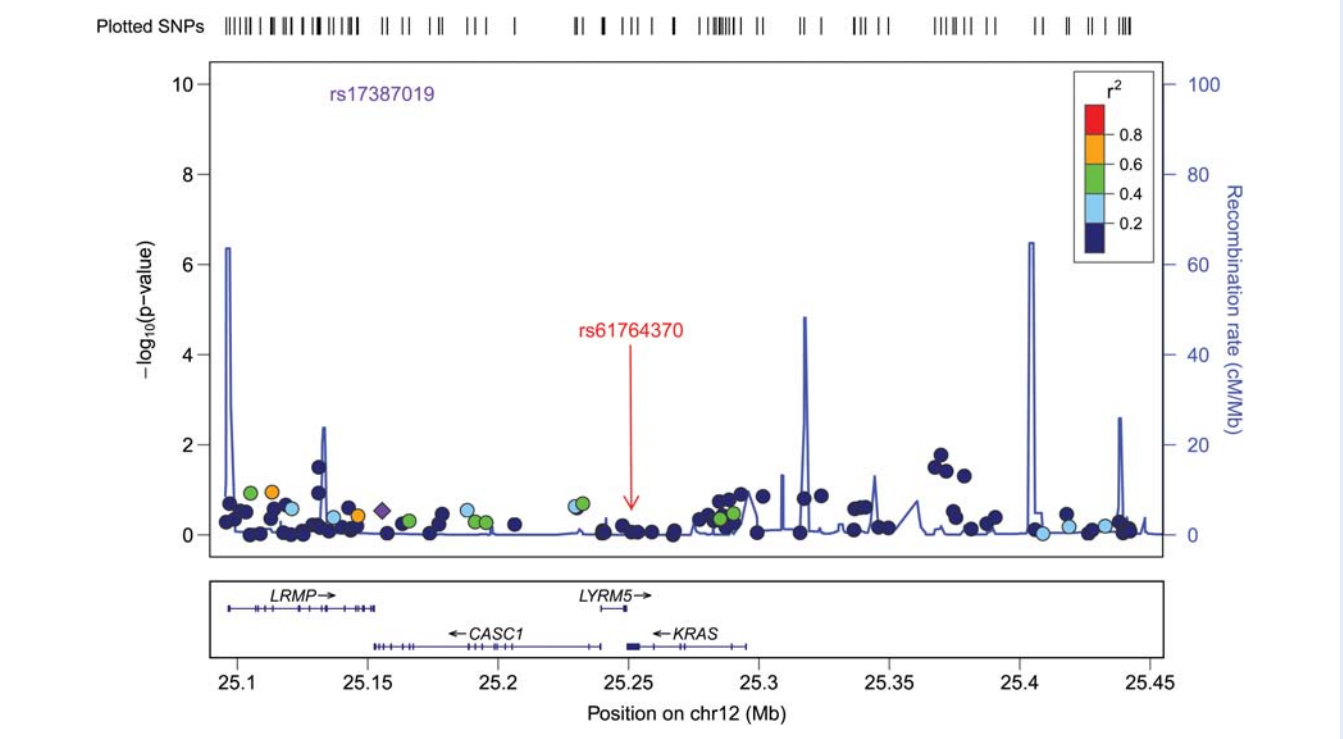

Figure 1 Evidence for association (−log10 *P*-values, *Y*-axis) between genotyped SNPs and endometriosis across the chromosome 12 region ~150 kb from the 3′UTR KRAS variant (rs61764370). In this Locus Zoom association plot SNP rs17387019 is represented by a purple diamond (http://csg.sph.umich.edu/locuszoom/) and is perfectly correlated with the 3′UTR KRAS variant rs61764370. The red arrow shows the position of the 3′UTR KRAS variant rs61764370. Other SNPs are colour coded according to the strength of their correlation (LD) with rs17387019 (measured by r^2).

Table II Genotype at rs17387019 in the Australian cases and the number (and proportions) with moderate/severe (stage B) disease or who answered 'Yes' to questions on subfertility or pain.

	Genotype at rs17387019			*P*-value
	AA	AG	GG	
Stage B	7 (0.39)	149 (0.42)	754 (0.39)	0.74
Subfertility[a]	5 (0.29)	129 (0.37)	717 (0.38)	0.64
Menstrual pain[b]	17 (1.00)	332 (0.94)	1729 (0.91)	0.13
Pelvic pain[c]	16 (0.94)	294 (0.83)	1540 (0.82)	0.40
Emergency treatment[d]	10 (0.58)	156 (0.45)	792 (0.42)	0.30
Dysperunia[e]	11 (0.69)	276 (0.79)	1433 (0.77)	0.48

The number of women who answered 'Yes' to the following questions:
[a]Have you tried for 12 months or more on any occasion to conceive without success?
[b]Have you EVER experienced severe menstrual pain?
[c]Have you EVER experienced severe pelvic pain?
[d]Have you ever had to seek emergency treatment because of pain?
[e]Have you ever experienced pain during sexual intercourse?

endometriosis risk (Table III) with $P = 0.411$ and OR = 1.10 (95% CIs: 0.88–1.36). The MAF for this SNP was 0.099 in endometriosis cases. In agreement with the GWA data, we did not find any evidence for association between endometriosis risk and rs17387019 and rs859141 (Table III). These two SNPs were also in high LD with the *KRAS* SNP rs61764370 in our data set ($r^2 = 0.858$ and 0.537, respectively), although the estimates were slightly lower than in the 1000 Genome pilot 1 data in our larger sample.

Discussion

Ras proteins have an important role as binary molecular switches in controlling signal transduction pathways. In mouse models, the activation of *Kras* triggers the development of tissue resembling endometriosis and endometrioid ovarian cancer (Dinulescu *et al.*, 2005; Cheng *et al.*, 2011). In women with endometriosis, the frequency of the minor allele at rs61764370 in the 3′ UTR of *KRAS* gene was reported to be 31% (41/132 cases), a much higher frequency than expected in the general population (Grechukhina *et al.*, 2012). The authors concluded that rs61764370 is a marker of endometriosis risk, potentially contributing to nearly one-third of all endometriosis cases and that it provides a novel method for diagnosis (Grechukhina *et al.*, 2012). However, the results from our analyses in two large data sets provide no support for an association between rs61764370 and endometriosis risk.

The *KRAS* polymorphism rs61764370 was not present on the Illumina 610 chip used for our GWAS. We therefore examined LD between rs61764370 and the genotyped SNPs. SNP rs17387019

Table III Results of the tests of association for rs61764370 and SNPs correlated with rs61764370 in multiplex genotyping of endometriosis cases and controls.

SNP	Position	r^2 with rs61764370	A1	A2	Allele frequency in cases ($n = 933$)	Allele frequency in controls ($n = 952$)	Chi-square	*P*-value	OR (95% CIs)
rs61764370	25360224		G	T	0.099	0.091	0.677	0.411	1.10 (0.88–1.36)
rs17387019	25155526	0.858	A	G	0.089	0.082	0.509	0.476	1.09 (0.86–1.37)
rs859141	2511329	0.537	T	C	0.097	0.090	0.493	0.483	1.08 (0.87–1.35)

LD was assessed using the PLINK program which was run on our multiplex genotyping data set.

which was typed on the chips is in strong LD with rs61764370 and genotyping of this SNP provides almost complete information on the other. We also identified four other SNPs typed on the Illumina 610 chip and in moderate to high LD ($r^2 > 0.57$) with the *KRAS* 3′UTR SNP. There was no evidence for an association with endometriosis for any of these correlated SNPs; allele frequencies in the cases and the controls were similar in our GWA sample including 3194 endometriosis cases.

Since rs61764370 was not on the Illumina chips, we genotyped this SNP in a sample of endometriosis cases with a family history of disease. The studies were not independent since individual cases and some families overlap between the two sample sets. There was no evidence for an association as the allele frequency for rs61764370 in our genotyped sample was 0.099 for the cases and 0.091 for the controls, similar to the population frequency reported previously for this variant (Grechukhina *et al.*, 2012). The results also agree with those of our previous study, where we found no association between common variants in *KRAS* and endometriosis risk from genotyping 32 tagging SNPs across the *KRAS* gene (Zhao *et al.*, 2006). Analysis in our GWA data for 97 SNPs located within the region from 150 kb upstream and downstream of *KRAS* gene showed no evidence for different allele frequencies between the cases and the controls and no association signal between any SNP in *KRAS* and endometriosis risk.

Of 132 women with endometriosis typed for the *KRAS* SNP rs61764370 by Grechukhina *et al.* (2012), 89% ($n = 117$) of the cases were assigned stage III/IV disease. When we limited our analysis to cases with stage B (III/IV) disease, our data set showed no evidence of any association between endometriosis risk and SNPs highly correlated with rs61764370 [proxy SNP rs17387019: OR = 0.98 (95% CIs: 0.85–1.13)]. MAFs in stage B cases were slightly lower in the cases compared with the controls, very similar to those in all endometriosis cases and our Australian and UK control population samples. Taking these results together, our data provide no support for the minor allele of rs61764370 conferring risk of endometriosis.

Our analysis of *KRAS* variants is based on a sample of 2270 endometriosis cases recruited in Australia and 924 cases recruited in the UK (Painter *et al.*, 2011). The diagnosis of endometriosis was confirmed from surgical records in 100% of cases in Australia and 97% of cases in the UK (Painter *et al.*, 2011). The controls in our GWAS were not screened for endometriosis. However, Grechukhina *et al.* used data from public databases for control allele frequency information and it is also unlikely that these individuals were screened for disease.

In summary, we find no evidence for an association between risk of endometriosis and variants in *KRAS* including the rs61764370 SNP in the *let-7* miRNA-binding site in the 3′UTR of *KRAS*. Our results do not address the physiological consequences of carrying the variant allele in the *let-7* miRNA-binding site. However, our study provides no support for suggestions that carrying the minor allele of rs61764370 contributes to a significant number of endometriosis cases and rs61764370 is unlikely to be a useful marker of endometriosis risk.

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Authors' roles

H.T.T.L, G.W.M., and D.R.N.: Study design and manuscript preparation; S.K., G.W.M., D.R.N., J.N.P., S.A.T., and K.T.Z.: GWAS data and clinical phenotyping; B.C.: Replication genotyping; H.T.T.L., G.W.M. D.R.N., J.N.P.: Data analysis; B.C., H.T.T.L, S.K., G.W.M., D.R.N., J.N.P., S.A.T., and K.T.Z.: Revision for critical content and approval of the final version of the manuscript.

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Conflict of interest

None declared.

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