Article

**rs495139 in the *TYMS*-*ENOSF1* region and risk of ovarian carcinoma of mucinous histology**

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Received: date; Accepted: date; Published: date

**Abstract:** Thymidylate synthase (TYMS) is a crucial enzyme for DNA synthesis. TYMS expression is regulated by its antisense mRNA, ENOSF1. Disrupted regulation may promote uncontrolled DNA synthesis and tumor growth. We sought to replicate our previously reported associations between rs495139 in the *TYMS-ENOSF1* 3′ gene region and increased risk of mucinous ovarian carcinoma (MOC) in an independent sample. Genotypes from 24,351 controls and 15,000 women with invasive OC, including 665 MOC, were available. We estimated per-allele odds ratios (OR) and 95% confidence intervals (CI) using unconditional logistic regression and meta-analysis when combining these data with our previous report. The association between rs495139 and MOC was not significant in the independent sample (OR=1.09; 95% CI=0.97-1.22; P=0.15; N=665 cases). Meta-analysis suggested a weak association (OR=1.13; 95% CI=1.03-1.24; P=0.01; N=1,019 cases). No significant association with risk of other OC histologic types was observed (P=0.05 for tumor heterogeneity). In expression quantitative trait locus (eQTL) analysis, the rs495139 allele was positively associated with ENOSF1 mRNA expression in normal tissues of the gastrointestinal system particularly esophageal mucosa (r= 0.51, P=1.7 x 10-28) and nonsignificantly in five MOC tumors. The association results, along with inconclusive tumor eQTL findings, suggest that a true effect of rs495139 might be small.

**Keywords:** consortia, enolase superfamily member 1, expression Quantitative Trait Locus,genetics,gynecology, ovarian neoplasms, single nucleotide polymorphism, thymidylate synthase

1. Introduction

Ovarian carcinomas of mucinous histology (MOC) are an uncommon type of ovarian cancer characterized by intracellular mucin deposits and relatively favorable prognosis when diagnosed at early stage [1]. Few epidemiologic risk factors are known for these cancers, and the standard risk factors for other types of ovarian cancer do not seem to apply [2]. Increased risk has been associated with current or recent smoking, a higher number of pack years of smoking [3, 4] and with increased body mass index [5]. Recently, we reported the first genome-wide significant susceptibility alleles for MOC at 2q13, 2q31.1 and 19q13.2 [6] and at 3q22.3 and 9q31.1 [7].

Thymidylate synthase (TYMS) is a crucial enzyme for DNA synthesis in both normal and tumor cells. It catalyzes the transformation of dUMP to dTMP and is the only *de novo* source of thymidylate for pyrimidine biosynthesis [8]. Consequently, it is an important chemotherapy target. We previously found an association between the rs495139 single nucleotide polymorphism (SNP) and increased risk of MOC (odds ratio [OR]=1.91; 95% confidence interval [CI]=1.10-3.31; P=0.02; N=80 MOC cases) (unpublished) in an earlier genotyping project [9] that we subsequently confirmed (hereto referred to as “discovery” sample) (OR=1.32; 95% CI=1.07-1.62; P=0.02; N=354 MOC cases) [10]. This SNP is located downstream of the 3′ untranslated region (UTR) of *TYMS* (Entrez Gene ID 7298) and situated intronic to the enolase superfamily member 1 (*ENOSF1*, Entrez Gene ID 55556) gene on chromosome 18. *ENOSF1* encodes an antisense transcript that down-regulates *TYMS* gene expression [11-13]. We hypothesized that genetic polymorphisms could perturb the TYMS mRNA-antisense mRNA auto-regulatory complex by either increasing *TYMS* or decreasing *ENOSF1* gene expression to promote uncontrolled DNA synthesis and tumor growth. Given the SNP’s potential functional role to regulate TYMS expression and because TYMS is an important chemotherapy target, our objective was to replicate the genetic association from our discovery sample [10] using a very large independent sample of MOC from participating studies in the Ovarian Cancer Association Consortium (OCAC) that were genotyped as part of the international Collaborative Oncology Gene-environment Study (iCOGS).

2. Results

2.1. Association Testing

The association between rs495139 and MOC in the independent iCOGS sample was not significant (OR=1.09; 95% CI=0.97-1.22; P=0.15, N=665 cases). The meta-analysis combining the iCOGS sample with the discovery sample [10] suggested an increased risk (OR=1.13; 95% CI=1.03-1.24; P=0.01, N=1,019 cases) (**Figure 1, Table 1**). The between-group heterogeneity was low to moderate for the discovery studies (*I2*=37.6) and low for the iCOGS studies (*I2*=0) and for all studies in the meta-analysis (*I2*=14.9). The ORs did not differ appreciably using a pooled analysis approach of discovery [10] and iCOGS samples adjusted for study (OR=1.12; 95% CI=1.02-1.22; P=0.02, N=1,021 cases) (**Table 1**). Thus, we proceeded with additional analyses using the pooled data. rs495139 was not associated with ovarian cancer overall or with the other histologic types (**Table 1**, P=0.05 for tumor heterogeneity, 4 df). Because molecular evidence suggests that many invasive MOC evolve along a multistep model of progression from benign to atypical proliferative (borderline) epithelium [1] similar to colorectal cancer [14], we evaluated the association between rs495139 and borderline MOC only (OR=0.97; 95% CI=0.86-1.09; P=0.59, N=621) and with combined borderline and invasive MOC (OR=1.06; 95% CI=0.99-1.14; P=0.11, N=1,642 cases). The weakened associations suggested the SNP might contribute only to the invasive phenotype. Women diagnosed with MOC had an earlier age at diagnosis (median age=54 years) than women with the more common serous histology (median age=60 years). To assess whether or not potentially different age distributions between MOC cases and controls influenced ORs, we matched each case to within 5-year age categories to three controls, where possible, and performed an age-stratified logistic regression. The OR was slightly larger (seven cases could not be matched due to young age of diagnosis): OR=1.16 (95% CI=1.04-1.28; P=0.006, N=1,013 cases and 3,014 controls) when the median age was similar (54 years for cases and 54.7 years for controls).



**Figure 1.**  Forest plot of the study-specific (filled diamonds) and pooled (open diamonds) odds ratios and 95% confidence intervals (solid horizontal lines) for the association between rs495139 and risk of mucinous ovarian carcinoma among 1,019 cases and 23,666 controls of European ancestry under the ordinal genetic risk model. Dashed vertical line represents overall summary estimate odds ratio. Individual study estimates are adjusted for ancestry and pooled estimates are adjusted for ancestry and study. See Table 1 footnote for details regarding sample size.

**Table 1.** Associations1 between rs495139 and ovarian carcinoma among European subjects.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Tumor histology | Analysis Method | Cases, N | Controls,N | OR | 95% CI | P-value |
| Mucinous invasive only | Meta-analysis | 1,0192 | 23,6663 | 1.13 | 1.03-1.24 | 0.01 |
| Mucinous invasive only | Pooled | 1,0212 | 24,351 | 1.12 | 1.02-1.22 | 0.02 |
| Mucinous borderline | Pooled | 621 | 24,351 | 0.97 | 0.86-1.09 | 0.59 |
| Mucinous invasive and borderline combined | Pooled | 1,642 | 24,351 | 1.06 | 0.99-1.14 | 0.11 |
| Serous invasive | Pooled | 8,889 | 24,351 | 1.01 | 0.98-1.05 | 0.53 |
| Endometrioid invasive | Pooled | 2,164 | 24,351 | 0.97 | 0.91-1.04 | 0.40 |
| Clear cell invasive | Pooled | 1,046 | 24,351 | 0.93 | 0.85-1.02 | 0.11 |
| All invasive tumors4 | Pooled | 15,000 | 24,351 | 1.00 | 0.97-1.03 | 0.84 |

1Studies in the meta-analysis were restricted to non-Hispanic whites (Discovery set) as in the original report [10] or adjusted for European ancestry using principal components analysis (iCOGS). Studies in the pooled analysis were also adjusted for study set.

2Two studies (HAW and STA) in iCOGS each contributed one mucinous ovarian cancer case: these were dropped from the meta-analysis but were retained in the pooled analysis.

3The control subjects from four studies were dropped from the meta-analysis but retained in the pooled analysis: HAW (n=17), STA (n=6), NHS (n=69) and MSK (n=593). NHS and MSK did not contribute any mucinous ovarian carcinoma cases in iCOGS.

4Includes invasive tumors with the following histology: serous, mucinous, endometrioid, clear cell, mixed cell, other specified epithelial ovarian cancer, undifferentiated or poorly differentiated epithelial and unknown histology, but known to be epithelial

2.2. expression Quantitative Trait Locus (eQTL) Analysis

We tested for eQTL association between rs495139 genotypes and gene expression in normal tissues in the Genotype Tissue Expression (GTEx) project. The most significant eQTL was with increased ENOSF1 mRNA expression in normal esophagus mucosa (r=0.51, P=1.7 x 10-28) (**Figures S1 and S2**). There was no reported association with TYMS mRNA expression in esophageal mucosa. In 316 women with ovarian cancer (five were MOC tumors), eQTL analysis between germline genotypes of rs495139 and tumor gene expression showed positive associations for two probes targeting ENOSF1 (P15906: beta=0.17, P=0.03 and P4503: beta=0.15, P=0.05) but not with the probe targeting TYMS (P50096: beta=0.12, P=0.12) (**Table S1**). Among the five MOC tumors, we observed positive eQTL associations with one probe targeting ENOSF1 (P4503: beta=0.87, P=0.32) but not the other (P15906: beta=0.08, P=0.94) and an inverse eQTL association with the probe targeting TYMS (P50096: beta=-0.70, P=0.45) but none were statistically significant given the small sample size.

3. Discussion

We previously observed a statistically significant risk association of rs495139 [10], which was not replicated by this larger analysis of MOC. Combining the two independent datasets of 1,019 MOC samples suggested a weakly positive association between rs495139 and risk of MOC, particularly in age-matched analysis. The association observed from the combined analysis was attenuated with the inclusion of borderline cases.

Few genetic risk factors for MOC are known. We recently published results from genome-wide association studies reporting five susceptibility variants for MOC at 2q13, 2q31.1, 19q13.2 [6], 3q22.3 and 9q31.1 [7]. The likelihood that rs495139 is a genetic risk factor for MOC is small. Summary-level statistics from a larger OCAC genotyping project [7], which included many of the participants in the current report, do not support significant associations between rs495139 and invasive MOC (OR=0.93; 95% CI=0.86-1.01; P=0.07, 1,417 cases) or combined borderline and invasive MOC (OR=0.97; 95% CI=0.91-1.03; P=0.29, 2,566 cases). Our results suggest the previously published significant association [10] was likely driven by heterogeneity from a few studies with extreme ORs and that balanced age matching between MOC cases and controls might reduce some bias. A strong functional influence of rs495139 on increased *ENOSF1* gene expression was found in GTEx normal gastrointestinal tissues and, in particular, esophageal mucosa. This is interesting because the origin of normal tissue from which MOC arises is unclear but may be similar to the mucosa of the gastrointestinal system [1, 15]. For example, small array-based studies (N = 3-9) of MOC relate them more closely to colonic epithelium or colorectal cancers than to normal ovarian surface epithelium [15, 16]. Further, some MOC may arise from a morphologically-analogous transition observed in gastro-esophageal adenocarcinoma. The metaplasia seen in MOC is a change in cell type from a monolayer of normal nondescript, poorly differentiated mesothelial cells [17] to a single layer of tall, columnar epithelial cells with mucin-containing cytoplasm [18]. This is analogous to the metaplasia seen in the development of Barrett’s esophagus, the precursor lesion to gastro-esophageal adenocarcinoma, where there is a change in cell type from a flat squamous cell layer to columnar-like epithelium with visible mucus [19]. We also observed a non-significant but positive eQTL association between rs495139 and a probe for ENOSF1 mRNA in MOC, as well as an inverse eQTL association between rs495139 and TYMS mRNA in MOC tumors, which contradicts our hypothesis that rs495139 might decrease ENOSF1 antisense mRNA to possibly increase TYMS mRNA availability for DNA synthesis and tumor growth. The small number of MOC tumors in this eQTL analysis precluded any definitive interpretation of results. Given that TYMS is a chemotherapy target, an rs495139-associated increase in ENOSF1 and decrease in TYMS expression might suggest a favorable tumor profile for patient survival.

This investigation was strengthened by including large numbers of women with MOC that contributed individual genotype data in iCOGS and by the ability to conduct an age-matched analysis to control for differences in the distribution of age among cases and controls, which could potentially bias associations. A further advantage was our centralized genotyping and rigorous quality control standards. We were also able to query data *in silico* as well as in women with corresponding germline SNP and tumor gene expression data from the Mayo Clinic in order to provide contextual information on potential functional influences between genotypes and tumor gene expression.

In summary, the evidence in this study to support a role of rs495139 in the *TYMS-ENOSF1* region as a genetic risk factor for MOC is weak and suggests that any true effect is likely to be small.

4. Materials and Methods

4.1. Study Subjects and Genotyping

Subjects (n=47,630) represented multiple individual studies participating in the OCAC [20]. Informed consent was obtained in individual studies and local human research investigations committees approved each study. *TYMS- ENOSF1* rs495139 was genotyped on an Illumina Infinium custom iSelect BeadChip developed for the international Collaborative Oncology Gene-environment Study (“iCOGS” Beadchip) [21]. Among 44,634 subjects whose DNA passed centralized genotyping quality control criteria [20], the call rate for rs495139 was 99.98%. We restricted the analysis to subjects with European ancestry and invasive tumors, leaving 37,792 eligible subjects. Of these, the effect allele frequency (EAF) among 23,444 controls was 0.41 and no departure from Hardy-Weinberg equilibrium was seen (P=0.32).

To obtain the independent sample (referred to as the “iCOGS” sample), we excluded genotypes from women who were in our earlier analysis [10] (referred to as the “discovery” sample). Individual-level genotypes were available on 10,019 invasive ovarian cancer cases (667 with invasive MOC) and 15,941 controls from 31 studies in iCOGS for replication analysis. We also re-evaluated the rs495139 association by combining genotypes from the iCOGS sample with the discovery sample (4,981 invasive ovarian cancer cases, of which 354 were invasive MOC, and 8,410 controls from 14 OCAC studies) [10], resulting in a total of 15,000 invasive ovarian cancer cases (1,021 with invasive MOC) and 24,351 controls (**Table S2**). This larger sample allowed additional subset analyses to be performed.

4.2. In Silico and Expression Quantitative Trait Locus (eQTL) Analysis

 We used the GTEx (Genotype Tissue Expression) project portal (V7 data release, 2017-09-18) [22] for *in silico* eQTL analysis between rs495139 and TYMS and ENOSF1 gene expression in 10,294 eQTL normal tissues representing over 50 different tissue types. We evaluated eQTL between rs495139 genotypes from lymphocytes with gene expression from patient-matched tumors among 316 women with ovarian cancer (including five MOC tumors) from the Mayo Clinic. Gene expression was assessed using a 4x44K Agilent array (Agilent Technologies, Santa Clara, CA) and measured as log2(tumor/reference) probe intensity signals as described previously [23] based on a mixed tumor cell type reference of 104 tumor samples including papillary serous (n = 67), endometrioid (n = 5), mucinous (n = 3), clear cell (n = 3), malignant mixed Müllerian tumor (n = 5), goblet cell (n = 1), squamous (n = 1), transitional cell (n = 1), benign (n = 4), normal (n = 7), and unknown epithelial (n = 7). Analyses included one Agilent probe for TYMS (P50096) and two probes for ENOSF1 (P15906 and P4503).

4.3. Statistical Analysis

 ORs and 95% CIs were estimated using unconditional logistic regression assuming an ordinal (log-additive) genetic model and adjusting for study and for the first five eigenvalues from principal components analysis to account for sub-strata of European ancestry [20]. Using fixed-effects meta-analysis, we combined study-specific effect estimates from our previous report [10] with study-specific effect estimates from unique iCOGS subjects using the inverse variance method to weight the overall summary OR. The degree of statistical heterogeneity between studies was estimated by *I2*, the between-group variance [24]. Studies with statistically homogeneous ORs yield an *I2* value of zero. ORs were derived for ovarian cancers overall and simultaneously for the different histologic types (serous, mucinous, endometrioid and clear cell) using polytomous logistic regression. Statistical heterogeneity of the SNP-ovarian carcinoma histology associations was tested using the type 3 analysis of effects with 4 degrees-of-freedom [25]. Van der Waerden rank transformation was applied to tumor gene expression values from the Agilent array, adjusting for age in linear regression and treating the number of variant alleles carried as ordinal on the log-additive scale. Statistical tests were two-sided and implemented with SAS version 9 (SAS Institute, NC). Meta-analysis was performed with Stata/SE (version 13.1, StataCorp, TX).

**Supplementary Materials:** Supplementary materials can be found at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1).

**Figure S1:** Correlation between rs495139 and ENOSF1 mRNA expression in normal esophageal mucosa from the Genotype-Tissue Expression project.

**Figure S2:** P values for expression quantitative trait locus analysis between rs495139 and ENOSF1 mRNA expression in different tissues in the Genotype-Tissue Expression project.

**Table S1:** Age-adjusted expression quantitative trait locus associations between rs495139 and TYMS and ENOSF1 gene expression in 316 ovarian tumors, Mayo Clinic.

**Table S2:** Contributing studies of invasive ovarian cancer cases and controls of European ancestry, OCAC.

**Author Contributions:** Conceptualization, L.E.K.; Overall study design, L.E.K., G.C.-T., K.B.M., M.T.G., T.A.S., J.M.S., P.D.P.P., C.M.P., C.L.P., E.L.G., A.B.; Methodology, L.E.K., T.A.S., P.D.P.P., E.D., H.S., J.P.T., B.L.F., E.L.G., A.B.; Formal Analysis, L.E.K., M.E., P.D.P.P., E.D., H.S., J.P.T.; Project administration, T.A.S., P.D.P.P., A.B.; Resources, All authors; Data Curation, All authors; Writing-Original Draft Preparation, L.E.K.; Writing-Review & Editing, All authors; Funding Acquisition, L.E.K., G.C.-T., K.B.M., M.T.G., T.A.S., J.M.S., P.D.P.P., C.M.P., C.L.P., E.L.G., A.B.

**Funding:** The Collaborative Oncology Gene-environment Study project is funded through a European Commission's Seventh Framework Programme grant (agreement number 223175 - HEALTH-F2-2009-223175). The Ovarian Cancer Association Consortium is supported by a grant from the Ovarian Cancer Research Fund thanks to donations by the family and friends of Kathryn Sladek Smith (PPD/RPCI.07). The scientific development and funding for this project was supported by the Canadian Institutes of Health Research (MOP-86727) and the US National Cancer Institute GAME-ON Post-GWAS Initiative (U19-CA148112). Funding of the constituent studies was provided by the American Cancer Society (CRTG-00-196-01-CCE); the California Cancer Research Program (00-01389V-20170, N01-CN25403, 2II0200); the Canadian Institutes for Health Research (MOP-86727); Cancer Council Victoria; Cancer Council Queensland; Cancer Council New South Wales; Cancer Council South Australia; Cancer Council Tasmania; Cancer Foundation of Western Australia; the Cancer Institute of New Jersey; Cancer Research UK (C490/A16561, C490/A6187, C490/A10119, C490/A10124, C536/A13086, C536/A6689); the Celma Mastry Ovarian Cancer Foundation; the Danish Cancer Society (94-222-52); the ELAN Program of the University of Erlangen-Nuremberg; the Eve Appeal; the Helsinki University Hospital Research Fund; Helse Vest; Imperial Experimental Cancer Research Centre (C1312/A15589); the Norwegian Cancer Society; the Norwegian Research Council; the Ovarian Cancer Research Fund; Nationaal Kankerplan of Belgium; Grant-in-Aid for the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health Labour and Welfare of Japan; Grants-in-Aid for Scientific Research on Priority Areas and Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports, Culture and Technology of Japan; Takeda Science Foundation; the L & S Milken Foundation; the Polish Ministry of Science and Higher Education (4 PO5C 028 14, 2 PO5A 068 27); Malaysian Ministry of Higher Education (UM.C/HlR/MOHE/06) and Cancer Research Initiatives Foundation; the Roswell Park Cancer Institute Alliance Foundation; the US National Cancer Institute (K07-CA095666, K22-CA138563, N01-CN55424, N01-PC067010, N01-PC035137, P01-CA017054, P01-CA087696, P30-CA15083, P30-CA008748, P50-CA105009, P50- CA136393, P50-CA159981, R01-CA014089, R01-CA016056, R01-CA017054, R01-CA049449, R01-CA050385, R01-CA054419, R01- CA058598, R01-CA058860, R01-CA061107, R01-CA061132, R01-CA063678, R01-CA063682, R01-CA064277, R01-CA067262, R01- CA071766, R01-CA074850, R01-CA076016, R01-CA080742, R01-CA080978, R01-CA083918, R01-CA087538, R01- CA092044, R01-095023, R01-CA106414, R01-CA122443, R01-CA112523, R01-CA114343, R01-CA126841, R01- CA136924, R01-CA149429, R03-CA113148, R03-CA115195, R37-CA070867, R37-CA70867, U01-CA069417, U01- CA071966, UM1 CA186107, UM1 CA176726 and Intramural research funds); the US Army Medical Research and Material Command (DAMD17-98-1- 8659, DAMD17-01-1-0729, DAMD17-02-1-0666, DAMD17-02-1-0669, W81XWH-10-1-0280, W81XWH-07-0449); the National Health and Medical Research Council of Australia (199600 and 400281); the German Federal Ministry of Education and Research of Germany Programme of Clinical Biomedical Research (01 GB 9401); the German Cancer Research Center (DKFZ); the Minnesota Ovarian Cancer Alliance; the Mayo Foundation; the Fred C. and Katherine B. Andersen Foundation; the Lon V. Smith Foundation (LVS-39420); the Oak Foundation; the OHSU Foundation; the Mermaid I project; the Rudolf-Bartling Foundation; the UK National Institute for Health Research Biomedical Research Centres at the University of Cambridge, Imperial College London, University College Hospital “Womens Health Theme”, the Royal Marsden Hospital; WorkSafeBC, and OvCaRe: British Columbia’s Ovarian Cancer Research Team.

 L.E.K. was supported by a Canadian Institutes of Health Research Investigator award (MSH-87734). G.C.-T. is supported by the National Health and Medical Research Council. B.K. is supported by an American Cancer Society Early Detection Professorship (SIOP-06-258-01- COUN) and the National Center for Advancing Translational Sciences (NCATS) Grant (UL1TR000124). F.M. is supported by a K-award from the National Cancer Institute (K07-CA080668). A.L. was supported by a T32 training grant from the US National Institute of Environmental Health Sciences (T32ES013678).

**Acknowledgments:** This study would not have been possible without the contributions of the following: P. Hall, (COGS); D. F. Easton, A. M. Dunning and A. Lee (Cambridge); J. Benitez, A. Gonzalez-Neira and the staff of the CNIO genotyping unit; D. C. Tessier, F. Bacot, D. Vincent, S. LaBoissière and F. Robidoux and the staff of the Genome Quebec genotyping unit; S. E. Bojesen, S. F. Nielsen, B. G. Nordestgaard, and the staff of the Copenhagen DNA laboratory; and S. A. Windebank, C. A. Hilker, J. Meyer and the staff of Mayo Clinic Genotyping Core Facility. We thank all the individuals who took part in this study and all the researchers, clinicians and technical and administrative staff who have made possible the many studies contributing to this work. In particular, we thank: D. Bowtell, A. deFazio, D. Gertig, A. Green, P. Parsons, N. Hayward, P. Webb and D. Whiteman (AUS); G. Peuteman, T. Van Brussel, and D. Smeets (BEL); L. Gacucova (HMO); P. Schurmann, F. Kramer, W. Zheng, T.W. Park-Simon, K. Beer-Grondke, and D. Schmidt (HJO); J. Vollenweider (MAY); the MD Anderson Center for Translational and Public Health Genomics (MDA); the state cancer registries of AL, AZ, AR, CA, CO, CT, DE, FL, GA, HI, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, and WY (NHS); L. Paddock, M. King, L. Rodriguez-Rodriguez, A. Samoila, and Y. Bensman (NJO); M. Sherman, A. Hutchinson, N. Szeszenia-Dabrowska, B. Peplonska, W. Zatonski, A. Soni, P. Chao, and M. Stagner (POL); C. Luccarini, P. Harrington, the SEARCH team and ECRIC (SEA); the Scottish Gynaecological Clinical Trails group and SCOTROC1 investigators (SRO); I. Jacobs, M. Widschwendter, E. Wozniak, N. Balogun, A. Ryan, J. Ford and C. Karpinskyj (UKO); and Carole Pye (UKR).

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

|  |  |
| --- | --- |
| dTMP | Deoxythymidine monophosphate |
| dUMP | Deoxyuridine monophosphate |
| ENOSF1 | Enolase superfamily member 1 |
| eQTL | Expression Quantitative Trait Locus |
| GTEx | Genotype Tissue Expression project |
| iCOGS | international Collaborative Oncology Gene-environment Study |
| MOC | Mucinous ovarian carcinoma |
| mRNA | Messenger ribonucleic acid |
| OCAC | Ovarian Cancer Association Consortium |
| SNP | Single nucleotide polymorphism |
| TYMS | Thymidylate synthase  |
| UTR | Untranslated region |
|  |  |

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