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# Linking TP53 codon 72 and P21 nt590 genotypes to the development of cervical and ovarian cancer

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ABSTRACT

*TP53* and its downstream effector gene *P21* are two important genes in cell cycle regulation. Genetic alterations on p53 and attenuation of p21 expression result in progression through cell cycle G1 checkpoint, which can lead to cancer development. We analysed the frequency of *TP53* codon 72 and 3′UTR *P21* polymorphisms in 681 blood samples from 371 cervical cancer patients, 122 ovarian cancer patients and 188 healthy controls using AS-PCR and PCR–RFLP. Approximately twofold increased risk of ovarian cancer (OC) was observed for *TP53* Pro carriers ($P = 0.038$), with a significantly higher risk for advanced OC ($P = 0.018$). Furthermore, among the *P21* CC genotypes, *TP53* P allele was also associated with a twofold increased risk of OC ($P = 0.014$) and to a threefold increased risk for advanced OC ($P = 0.003$) with an attributable proportion of 44.2%. These results were confirmed in an age-adjusted logistic regression analysis. No association was found between these polymorphisms and cervical cancer. Our results suggest that the *TP53* codon 72 genotypes may be considered as a molecular marker, contributing to a genetic profile for ovarian cancer in women.



## 1. Introduction

Cervical cancer (CC) and ovarian cancer (OC) are the most frequent gynaecologic malignancies among women worldwide. In Portugal, CC has a relatively high incidence rate (standardized European rate) of 17/100,000 with around 950 new cases each year, whereas OC has approximately 600 new cases each year, with an incidence rate of 9.9/100,000. Mortality rates due to CC and OC are 5.9/100,000 deaths 5.4/100,000, respectively.[1,2]

Persistent infection with high risk *Human papillomavirus* (HPV), a sexually transmitted agent, was established as a necessary but not sufficient cause for CC development.[3,4] However, less is known regarding the etiologic factors in the development of OC.[5] Nonetheless, as cancer is a multistep disease, it is believed that genetic variations, as polymorphisms, may be associated to different cancer susceptibilities, as well as important prognostic and predictive factors.[6–8]

*TP53* is a tumour suppressor gene, located at chromosome 17p13, referred as altered in 50–55% of cancer cases.[9,10] The p53 protein, encoded by the *TP53* gene, is known as the cellular gatekeeper for growth and division, as it plays an essential role in safeguarding the integrity of the genome.[11] This protein is involved in processes as cell cycle arrest, gene transcription, DNA repair and apoptosis. If a mutation occurs, normal p53 functions may not be activated leading to cell cycle pathways or loss of apoptosis control and, as a consequence, to unchecked cell proliferation. High risk HPV oncoprotein E6 binds to p53 to promote its degradation via ubiquitin dependent proteolysis.[12] Thus, inactivation of p53 by HPV-E6 is analogous to a functional p53 mutation.[13]

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A common polymorphism on *TP53* codon 72, exon 4, encodes either arginine (CGC) or proline (CCC) amino acid. Some reports suggest that the arginine p53 form is more susceptible to HPV-E6 mediated degradation and thus is associated to a higher risk of developing CC.[14,15] Nonetheless, this finding was refuted by other research studies that did not confirm this association.[6,13,16] Regarding OC, a significantly higher frequency of the Pro allele in patients with this neoplasia has been described.[17] Also, Hogdall and colleagues reported that this allele was associated with a worse response to chemotherapy.[18] On the other hand, Agorastos and colleagues suggest that the Arg/Arg genotype may represent a risk factor for OC.[19]

It is known that in normal cell conditions, p53 protein is present at low concentration levels, but an external signal as such DNA damage, hypoxia or deregulated cell cycle progression can activate its function on the transcription of one of its downstream genes.[11] *P21*, one of p53 target genes, is responsible for inactivating G1 cyclin-dependent kinases blocking cell cycle progression through G1. It is located at chromosome 6p21.2 and encodes the p21 protein, also known as the cyclin kinase inhibitor WAF1/CIP1.[10] The most studied *P21* polymorphism is located at codon 31. However, polymorphisms in untranslated regions have also shown to be important for cellular proliferation, differentiation, tumour suppression and metastasis suppression on several genes.[20–22] There are some reports that studied the *P21* 3′UTR polymorphism, a genetic variation at nt590, and 20 nucleotides downstream of the stop codon in the 3′ end of exon 3, which corresponds to a C → T transition.[21,23,24] Since p53 and its downstream mediator p21 are important factors for cell cycle regulation, it is plausible that polymorphisms in their genes might interfere in the individual susceptibility to cancer. Therefore, the aim of this study was to evaluate the effect of *TP53* codon 72 and *P21* 3′UTR polymorphisms in CC and OC. To the best of our knowledge, this is the first study that correlates these two single nucleotide polymorphisms combined together in association with cervical and ovarian cancer.

## 2. Material and methods

We conducted a case–control study investigating the frequency of the *TP53* codon 72 polymorphism and the *P21* 3′UTR polymorphism in 681 samples from 371 patients with CC, 122 patients with OC and 188 healthy individuals with no evidence of malignant disease. Blood samples were collected at the Portuguese Institute of Oncology, Porto, from Caucasian women from the northern region of Portugal after informed consent, according to the Declaration of Helsinki. Genomic DNA was extracted from the white blood cell fraction of each sample, using a salting-out method.[25]

### 2.1. *TP53 genotype analysis*

The G → C transversion (Arg/Pro) on *TP53* gene was evaluated using the Allele Specific-Polymerase Chain Reaction (AS-PCR) as previously performed by Santos and colleagues.[6] Each reaction was performed separately for each variant, using specific primers for the ARG allele: ARG-(5′-CTGGTGCAGGGGCCACGC-3′) and TP53+ (5′-TCCCCCTTGCCGTCCCAA-3′) amplifying a 141 base-pair (bp) fragment and specific primers for the PRO allele: PRO+ (5′-GCCAGAGGCTGCTCCCCC-3′) and TP53- (5′-CGTGCAAGTCACAGACTT-3′) amplifying a 177 bp fragment. AS-PCR products obtained were analyzed by electrophoresis in a 1.5% (w/v) agarose gel stained with ethidium bromide and visualised under UV light (Fig. 1).

### 2.2. *P21 exon3 genotype analysis*

The C → T transition on *P21* gene was evaluated using a Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) method adapted from a previously described protocol.[23] DNA was amplified in a 50 μL mixture containing primers E3A: 5′-CCCAGGGAAGGGTGTCCTG-3′ and E3B: 5′-GGGCGGCCAGGGTATGTAC-3′, 1× *Taq* Buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.3 μM from each primer and 1 U *Taq* DNA polymerase. Thermocycler parameters were as follows: 94 °C for 5 min; 30 cycles of 94 °C for 60 s, 60 °C for 45 s and 72 °C for 60 s; and a final extension step at 72 °C for 5 min. The PCR products of 300 bp were then digested overnight at 37 °C with 1 U of *Pst*I. The base substitution leads to the destruction of the recognition site for the restriction enzyme, therefore, the C allele when digested, produces two fragments at 174 and 126 bp. The restriction fragments were analysed by electrophoresis in 2% (w/v) agarose gels stained with ethidium bromide and visualised under UV light (Fig. 2).

### 2.3. *Statistical analysis*

Data analysis was performed using the computer software Statistical Package for Social Sciences (SPSS) (version 11.5) for Windows. $\chi^2$ analysis was used to compare categorical variables and a 5% level of significance was used in the analysis. The observed number of each genotype was compared with that expected for a population in Hardy–Weinberg equilibrium using a goodness-of-fit $\chi^2$ test. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measure of the association between *TP53* and *P21* genotypes and cervical and ovarian cancer risk. In addition, OR and its CI were also used to evaluate the influence of the *TP53*

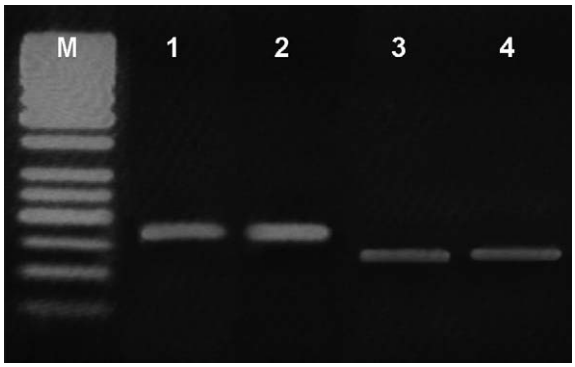


**Fig. 1 – AS-PCR products of the *TP53* codon 72 polymorphism: M – 50 bp ladder; lanes 1 and 2, Arg allele (141 bp); lanes 3 and 4, Pro allele (177 bp).**

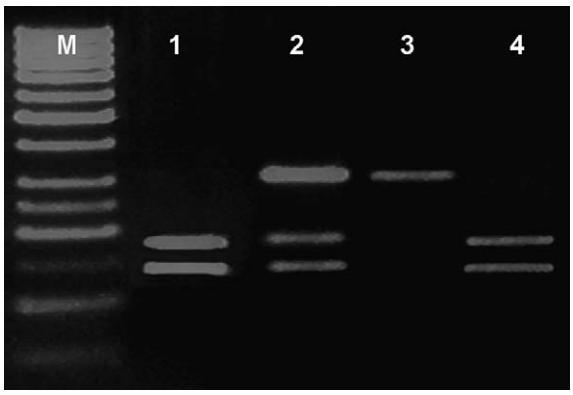


**Fig. 2 – Analysis of the P21 3′UTR genotypes by RFLP: M – 50 bp ladder; lanes 1 and 4, homozygous CC; lane 2, heterozygous CT; lane 3, homozygous TT.**

genotypes in *P21* genotypes among cervical and ovarian cancer women.

Logistic regression analysis was used to calculate the adjusted OR and 95% CI for the influence of the *TP53* and *P21* genotypes in ovarian cancer, with adjustment for age. In addition, we calculated the attributable proportion (AP) using the following formula: $AP = PRF \times (1 - 1/aOR)$, where AP is the fraction of disease attributable to the risk factor; PRF is the percentage of the risk factor in case subjects and aOR is the adjusted odds ratio.

## 3. Results

We analysed our data separately according to the type of neoplasia studied. Table 1 shows the distribution of the three different genotypes of the *TP53* polymorphism among cervical cancer cases and controls. From the 371 CC cases (median age 49 years), we observed that 67.4% were found to be homozygous for the Arg allele, 26.1% were heterozygous and 6.5% homozygous for the Pro allele; whereas for the 188 controls the genotype frequencies were 62.2%, 30,9% and 6.9%, respectively. The genotype distributions for *TP53* polymorphism in the control group were as expected according to the Hardy–Weinberg equilibrium ($P = 0.551$). However, no statistical differences were found when associating Arg carriers and risk for cervical cancer ($P = 0.841$). Similarly, when we stratified the analysis, it was possible to observe that Arg carriers were not associated to increased risk of high-grade squamous intraepithelial lesion (HGSIL), squamous cell carcinoma (SCC) and adenocarcinoma (ADC) ($P > 0.05$). Regarding *P21* polymorphism, the genotype distributions in the control group were as expected according to the Hardy–Weinberg equilibrium ($P = 0.839$). No significant association was observed between the *P21* genotype frequencies in the control group and in women with CC (data not shown). Furthermore, investigating the frequency of the *TP53* genotypes within the *P21* CC genotype (Table 2) we did not find a statistically significant association, even considering HGSIL, SCC or ADC and the control group ($P \geqslant 0.05$).

**Table 1 – Distribution of TP53 codon 72 polymorphism genotypes among cervical cancer cases and controls**

| | *TP53* genotypes | | | | | | Arg carrier | | OR | 95% CI | P[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A/A | | A/P | | P/P | | | | | | |
| | *n* | % | *n* | % | *n* | % | *n* | % | | | |
| Controls (*n* = 188) | 117 | 62.2 | 58 | 30.9 | 13 | 6.9 | 175 | 93.1 | 1.00 | Reference | |
| Cases (*n* = 371) | 250 | 67.4 | 97 | 26.1 | 24 | 6.5 | 347 | 93.5 | 1.07 | 0.53-2.16 | 0.841 |
| HGSIL (*n* = 96) | 60 | 62.5 | 27 | 28.1 | 9 | 9.4 | 87 | 90.6 | 0.72 | 0.30–1.74 | 0.463 |
| SCC (*n* = 243) | 170 | 70.0 | 58 | 23.9 | 15 | 6.2 | 228 | 93.8 | 1.13 | 0.52–2.44 | 0.757 |
| ADC (*n* = 32) | 20 | 62.5 | 12 | 37.5 | 0 | 0.0 | 32 | 100.0 | 1.18 | 1.12–1.25 | 0.125 |

OR, odds ratio; CI, confidence interval; A/A, Arg/Arg genotype; A/P, Arg/Pro genotype; P/P, Pro/Pro genotype; HGSIL, high grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; ADC, adenocarcinoma.
a $\chi^2$ test.

**Table 2 – Influence of TP53 codon 72 genotypes in P21 CC genotype among cervical cancer cases and controls**

| | *P21* CC | | | | OR | 95% CI | P[a] |
|---|---|---|---|---|---|---|---|
| | TP53 PP | | TP53 Arg carrier | | | | |
| | *n* | % | *n* | % | | | |
| Controls (*n* = 159) | 9 | 5.7 | 150 | 94.3 | 1.00 | Reference | |
| Cases (*n* = 298) | 20 | 6.7 | 278 | 93.3 | 1.2 | 0.53–2.70 | 0.661 |
| HGSIL (*n* = 76) | 7 | 9.2 | 69 | 90.8 | 0.59 | 0.21–1.65 | 0.312 |
| SCC (*n* = 195) | 13 | 6.7 | 182 | 93.3 | 0.84 | 0.35–2.02 | 0.120 |
| ADC (*n* = 27) | 0 | 0.0 | 27 | 100.0 | 1.18 | 1.11–1.26 | 0.205 |

OR, odds ratio; CI, confidence interval; P/P, Pro/Pro genotype; HGSIL, high grade squamous intraepithelial lesion; SCC, squamous cell carcinoma; ADC, adenocarcinoma.
a $\chi^2$ test.

Regarding the group of ovarian cancer patients (122), the analysis was only possible in 99 cases due to DNA extraction or amplification problems. Table 3 displays the *TP53* codon 72 genotype distribution and clinicopathological features of the OC cases studied (histological type and stage according to the FIGO staging system). It was possible to verify that among the 99 OC cases, 49.5% were Arg/Arg, 40.4% were Arg/Pro and 10.1% were Pro/Pro compared to the frequencies 62.2%, 30.9% and 6.9% among controls, respectively. Statistical significant difference was found when considering the association between Pro carriers and risk of OC development (OR = 1.68; 95%CI 1.03–2.75; $P = 0.038$). This association was more evident when FIGO staging was considered, as there was a twofold increased risk of advanced OC (stages III and IV) for women carriers of the *TP53* P allele (OR = 2.00; 95%CI 1.12–3.58; $P = 0.018$). Regarding the histological types, no statistically significant differences were observed ($P > 0.05$). Furthermore, we did not find a significant association between the frequency of the *P21* genotypes in controls and in OC women ($P > 0.05$, data not shown). However, considering the *TP53* Pro carriers among *P21* CC genotype, we observed a more accentuated risk of OC (OR = 2.09; 95%CI 1.15–3.77; $P = 0.014$) that was more evident among stage III/IV (OR = 3.01; 95%CI 1.43–6.32; $P = 0.003$, Table 4).

Logistic regression analysis adjusted for age (Table 5) confirmed the influence of the *TP53* Pro carrier genotype in OC risk (OR = 1.74; 95%CI 1.03–2.92; $P = 0.038$) and in advanced cancer risk (OR = 2.07; 95%CI 1.12–3.85; $P = 0.020$), as well as its influence among the *P21* CC genotype cases with twofold increase for OC risk (OR = 2.34; 95%CI 1.30–4.21; $P = 0.005$) and a threefold increase for advanced OC risk (OR = 3.05; 95%CI 1.45–6.42; $P = 0.003$).

The proportion of ovarian cancer cases attributed (AP) to *TP53* Pro carrier genotypes was 21.5% in the entire cases group and 32.9% among patients that have the *P21*CC and a *TP53* Pro carrier genotype. Among advanced stage disease, these proportions were higher in *TP53* Pro carriers (28.3%) and in patients with the *P21* CC and a *TP53* Pro carrier genotype (44.2%).

## 4. Discussion

Genetic alterations involved in cancer can activate processes or block negative pathways. Thus, lesions in tumour suppres-

**Table 3 – Distribution of TP53 codon 72 polymorphism genotypes among ovarian cancer cases and controls**

| | TP53 genotypes | | | | | | Pro carrier | | OR | 95% CI | P[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A/A | | A/P | | P/P | | | | | | |
| | *n* | % | *n* | % | *n* | % | *n* | % | | | |
| Controls (*n* = 188) | 117 | 62.2 | 58 | 30.9 | 13 | 6.9 | 71 | 37.8 | 1.00 | Reference | |
| Cases (*n* = 99) | 49 | 49.5 | 40 | 40.4 | 10 | 10.1 | 50 | 50.6 | 1.68 | 1.03–2.75 | 0.038 |
| *Histological type*[b] | | | | | | | | | | | |
| serous (*n* = 52) | 26 | 50.0 | 19 | 36.5 | 7 | 13.5 | 26 | 50.0 | 1.65 | 0.85–3.20 | 0.111 |
| others (*n* = 46) | 23 | 50.0 | 20 | 43.5 | 3 | 6.5 | 23 | 50.0 | 1.65 | 0.82–3.31 | 0.129 |
| *FIGO stage* | | | | | | | | | | | |
| I and II (*n* = 37) | 21 | 56.8 | 15 | 40.5 | 1 | 2.7 | 16 | 43.2 | 1.26 | 0.58–2.71 | 0.531 |
| III and IV (*n* = 62) | 28 | 45.2 | 25 | 40.3 | 9 | 14.5 | 34 | 54.8 | 2.00 | 1.12–3.58 | 0.018 |

OR, odds ratio; CI, confidence interval; A/A, Arg/Arg genotype; A/P, Arg/Pro genotype; P/P, Pro/Pro genotype; FIGO, International Federation of Gynaecology and Obstetrics.
a $\chi^2$ test.
b Data not available in 1 case.

**Table 4 – Influence of TP53 codon 72 genotypes in P21 CC genotype among ovarian cancer cases and controls**

| | P21 CC | | | | OR | 95% CI | P[a] |
|---|---|---|---|---|---|---|---|
| | TP53 AA | | TP53 Pro carrier | | | | |
| | *n* | % | *n* | % | | | |
| Controls (*n* = 159) | 97 | 61.0 | 62 | 39.0 | 1.00 | Reference | |
| Cases (*n* = 63) | 27 | 42.9 | 36 | 57.1 | 2.09 | 1.15–3.77 | 0.014 |
| *Histological type* | | | | | | | |
| serous (*n* = 37) | 16 | 43.2 | 21 | 56.8 | 2.05 | 0.94–4.51 | 0.050 |
| others (*n* = 25) | 11 | 44.0 | 14 | 56.0 | 1.99 | 0.74–5.07 | 0.108 |
| *FIGO stage* | | | | | | | |
| I and II (*n* = 25) | 14 | 56.0 | 11 | 44.0 | 1.25 | 0.49–3.10 | 0.608 |
| III and IV (*n* = 38) | 13 | 34.2 | 25 | 65.8 | 3.01 | 1.43–6.32 | 0.003 |

OR, odds ratio; CI, confidence interval; A/A, Arg/Arg genotype; FIGO, International Federation of Gynaecology and Obstetrics.
a $\chi^2$ test.

**Table 5 – Odds ratio for overall ovarian cancer risk and ovarian advanced cancer risk, using logistic regression analysis adjusted for age**

| | OR | 95% CI | P[a] |
|---|---|---|---|
| *Cancer risk* | | | |
| TP53_Pcarrier | 1.74 | 1.03–2.92 | 0.038 |
| P21_CC | 1.70 | 0.65–4.40 | 0.278 |
| P21_CC/TP53_Pcarrier | 2.34 | 1.30–4.21 | 0.005 |
| *Advanced cancer risk* | | | |
| TP53_Pcarrier | 2.07 | 1.12–3.85 | 0.020 |
| P21_CC | 2.83 | 0.63–12.67 | 0.173 |
| P21_CC/TP53_Pcarrier | 3.05 | 1.45–6.42 | 0.003 |

OR, odds ratio; CI, confidence interval.
a logistic regression analysis adjusted for age.

sor genes can inactivate the inhibition of cell growth. *TP53* gene is one of the major tumour suppressor genes and encodes the p53 protein whose primary role is to maintain the genetic integrity of a cell. Therefore, when DNA damage occurs, the G1 cell cycle arrest allows DNA to be repaired. If this protein or its gene is altered due to some genetic variation, such as polymorphisms, it may not be able to induce cell cycle arrest, leading to a cell cycle progression without control.[6]

Our results demonstrate an approximately twofold increased risk for ovarian cancer in women carriers of the *TP53* P allele (OR = 1.68; 95%CI 1.03–2.75; *P* = 0.038). These results are consistent with a previous report suggesting that the Pro allele induces apoptosis with lower kinetics compared to the Arg allele.[26] Furthermore, the Pro allele was associated to a poorer prognosis and survival in response to chemotherapy treatment in OC.[17,18] Besides, Buller and colleagues suggested that OC tumours that retained a Pro allele were more prone to mutation.[27]

In our study, we have also compared the association of the *P21* polymorphism at nt590 and the susceptibility to ovarian cancer. Additionally, we have studied the influence of this polymorphism in association with *TP53* genotypes and cancer risk. In fact, the increased risk observed among *TP53* Pro carriers was more evident when we analysed the combined effect with the *P21* CC genotype (OR = 2.09; 95%CI 1.15–3.77; *P* = 0.014). These results are in agreement towards what we were expecting, because p53 is responsible for the G1 cell cycle arrest in response to genomic errors and p21 plays a direct role in p53 induced G1 arrest.

The *P21* nt590 polymorphism is located at the UTR (untranslated region) which has been shown to be an important region in cellular proliferation, differentiation, tumour suppression and metastasis suppression, as it is also often implicated in mRNA stability and degradation.[20–22] Therefore, although the nt590 polymorphism is not located at a coding region, it can play an important role in p21 stability, altering the protein and cell cycle normal function; and increasing cell susceptibility to certain types of cancer.[28] Our results are consistent as p21 is a downstream target of p53 and cells with the more aggressive *TP53* genotype would be expected to have an altered p53-mediated p21 induction among the wild type allele (*P21* CC). Thus, in response to DNA damage, it leads to increased susceptibility for cancer development. As a result, *TP53* Pro carrier genotypes (Arg/Pro; Pro/Pro) may contribute to the loss of function on cell cycle arrest and, as a consequence, to cell proliferation without control.

When we stratified the analysis according to the status of disease and FIGO staging system, we observed a twofold increased risk (OR = 2.00; 95% CI 1.12–3.58; *P* = 0.018) for advanced OC (stage III/IV) in *TP53* Pro carriers. No statistically significant differences were found between the *P21* genotypes and risk or OC development, as corroborated by other authors.[24,29] However, when we associated the combined effect of *TP53* and *P21* polymorphisms, the susceptibility was augmented (OR = 3.01; 95%CI 1.43–6.32; *P* = 0.003). Overall, our results suggest that these two polymorphisms may act synergistically increasing susceptibility for OC development, particularly in an advanced clinical stage of the disease.

The association between the *TP53* codon 72 polymorphism and CC has been largely studied due to the interaction between high-risk HPV-E6 and p53, but results remain controversial.[6,13–16,30] We have analysed the influence of *P21* polymorphism in the susceptibility to CC among *TP53* Arg carriers, as this allele has been associated to increased CC risk. Our results do not demonstrate any association between the *TP53* Arg carriers alone or considering the influence of *P21* genotypes in the risk to CC. This is consistent with previous results from several authors who studied the influence of *TP53* Arg genotypes in CC.[6,13,16] Furthermore, no statistically significant differences were found between the *P21* genotypes and risk or CC development

To sum up, as *TP53* alterations are the most frequent genetic event described in various types of cancer, we have analysed the polymorphism on codon 72 in cervical and ovarian cancer patients under the influence of the *P21* polymorphism. We demonstrate that women carrying the *TP53* Pro allele are at higher risk for the development of OC and this was reinforced among the *P21* wild type genotype. Hence, the *TP53* genotype could be considered as a molecular marker, contributing to the genetic profile in OC women.

## Conflict of interest statement

None declared.

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