Association of codon 72 polymorphism of p53 with the severity of cervical dysplasia, E6-T350G and HPV16 variant lineages in HPV16-infected women

George D. Moschonas,1† Dimitris Tsakogiannis,1*† Konstantinos A. Lamprou,1 Eirini Mastora,1 Tilemachos G. Dimitriou,1 Zaharoula Kyriakopoulou,1 Christine Kottaridi,2 Petros Karakitsos2 and Panayotis Markoulatos1

Abstract

Purpose. Polymorphic variability in the tumour-suppressor protein p53 at codon 72 has a considerable impact on cervical cancer development. The present study clarified the association between p53 codon 72 genotypes and the risk of cervical disease in Greek patients. We also examined whether the presence of specific p53 genotypes in combination with HPV16 variants or E6 T350G sequence variation can modify an individual’s susceptibility to cervical disease.

Methodology. The analysis of p53 genotypes was performed through PCR-RFLP. Sequence and phylogenetic tree analyses of the HPV16 E6 gene were also performed in order to identify HPV16 variants and T350G sequence variation.

Results/Key findings. The outcomes of the present analysis revealed that women who are homozygous for the arg genotype are at a 4.17-fold higher risk of developing HPV16-associated HSIL+ (OR=4.17, 95% CI:1.48–4.9, P=0.0049). Moreover, p53 arg/arg patients infected by an HPV16 prototype strain were associated with an increased risk of more severe lesions, while a significant relationship between the p53 arg/arg genotype in patients with T350G sequence variation and the risk of high-grade squamous intraepithelial lesions (HSILs) was revealed.

Conclusion. The oncogenic potential of the virus is increased by the presence of the p53 arg/arg genotype in the Greek population in such a way that the specific protein interaction E6 (L83V)–p53 (Arg-72) can modify an individual’s susceptibility to cervical disease.

INTRODUCTION

Human papillomaviruses (HPVs) comprise a heterogeneous group of non-enveloped, capsid-enclosed, double-stranded DNA viruses that infect stratified epithelium, while HPV infections are characterized as mucosal or cutaneous [1, 2]. In general, it is thought that persistent infection with high-risk HPV genotypes is the leading cause for the development of precancerous and cervical cancer lesions, with the HPV16 genotype being detected in more than half of cervical cancer cases worldwide [3–5]. The increased prevalence of HPV16 DNA in cervical cancer triggered extensive investigation of the viral DNA sequence and HPV16 life cycle. In particular, sequence analysis of the long control region (LCR) and the E6 gene of HPV16 DNA revealed single nucleotide polymorphisms that classify the viral genome into four major variant lineages and sub-lineages that are associated with specific geographic and ethnic groups [6–11]. The HPV16 intratypic variant lineages are described as (i) European–Asian (including the sub-lineages European and Asian); (ii) African type I; (iii) African type II; and (iv) Asian–American [11].

In recent decades several studies have investigated the distribution and oncogenic capacity of the nucleotide...
polymorphism T350G of the HPV16 E6 oncogene in diverse geographic populations [9, 12, 13]. The T350G sequence variation is prevalent in both the European and Asian–American variants, while the European variant lineage can be further grouped into isolates that harbour either E6 350T or 350G. The T350G sequence variation leads to the amino acid substitution L83V in the E6 oncoprotein. Several investigations have proposed that L83V is associated with viral persistence and thus it is regarded as a considerable risk factor for the development of more severe dysplasia and cervical cancer [9, 13–17]. The E6 oncoprotein exhibits a fundamental impact on cell proliferation and tumourigenesis. In particular, the viral oncoprotein forms a complex with the E6AP (E3 ubiquitin ligase E6-associated protein) that subsequently targets the p53 pro-apoptotic tumour-suppressor protein, resulting in the recruitment and polyubiquitination of p53 via the ubiquitin-mediated degradation pathway [18]. The degradation of p53 in turn promotes cell cycle entry that leads to prolonged proliferation of infected cells and finally to the accumulation of DNA damage and cancer development [18–20].

Although the extensive analysis of the HPV16 E6 oncoprotein has conferred considerable insights regarding HPV16-induced cervical carcinogenesis, in recent years interest in the role of the p53 codon 72 arg/pro polymorphism in cervical cancer risk has grown [21–24]. In particular, a specific sequence variation (G to C) in exon 4 (rs1042522; Arg72-Pro) leads to the amino acid substitution of arginine to proline at position 72 of the p53 protein. In response to amino acid substitution, two structurally different protein forms are generated, which display divergent oncogenic properties [25, 26]. With regard to cervical cancer, Storey et al. [27] first proposed that the p53 Arg-72 form demonstrates augmented vulnerability to E6-mediated degradation compared to the p53 Pro-72 form. Consequently, many studies followed and focused on the association between p53 codon 72 genotypes and HPV-related cervical cancer risk in different geographic populations, but with controversial outcomes.

To this end, the present study focused on the distribution of p53 codon 72 genotypes in HPV16-positive Greek women in order to elucidate the relationship between p53 codon 72 genotypes and the risk of cervical disease in the Greek population. Moreover, we investigated whether the presence of specific p53 genotypes in combination with HPV16 variants or the presence of particular p53 genotypes in association with E6 T350G sequence variation can alter an individual’s susceptibility to more severe dysplasia and consequently be used as valuable biomarkers that could further advance early prognosis.

METHODS

Cervical specimens

A prospective study was conducted that enrolled a total of 80 women who were positive for HPV16 infection and attended the colposcopy clinic of the 3rd Department of Gynecology and Obstetrics in the tertiary care ‘ATTIKON’ University General Hospital between June 2013 and June 2015. The study population did not represent a normally screened population, since most patients attended the outpatient clinics after a referral for abnormal cytology and/or a colposcopy. In particular, 35 samples were diagnosed as low-grade squamous intraepithelial lesions (LSILs), 35 samples were diagnosed as high-grade squamous intraepithelial lesions (HSILs) and 10 cervical samples were diagnosed as cervical cancers (9 paraffin-embedded cervical biopsies and 1 ThinPrep). All patients signed an informed consent form, while the study was approved by the Bioethics committee of the hospital (approval number 5/14-06-2013).

DNA preparation and HPV16 identification

DNA from ThinPrep samples was extracted with the chaotropic agent guanidine thiocyanate (GuSCN) [28], while DNA from formalin-fixed, paraffin-embedded cervical tissues was extracted using the established protocol of proteinase K [29]. The identification of HPV16 DNA was conducted according to a previously described methodology via multiplex PCR [30]. In particular, the assay facilitates the detection and identification of HPV genotypes 16, 18, 45, 35, 66, 33, 51, 58 and 31 using L1 type-specific primer sets in multiplex reactions, while it enables the simultaneous detection of the human b-actin gene as an endogenous control for the assay [30].

Determination of p53 codon 72 genotypes

The p53 codon 72 genotypes were determined by PCR-RFLP [21]. Briefly, a 309 bp fragment of p53 exon 4 was amplified by PCR using the forward primer 5’-TTCACCCA TCTACAGTCC-3’ and the reverse primer 5’-CTCAGGG- CAACTGACCCT-3’, while the amplicons were further subjected to digestion with the BstU1 restriction enzyme.

PCR was performed in a final volume of 50 μl. The PCR mixture consisted of 50 pmol of primer set, 10× PCR buffer (Agilent Technologies, Santa Clara, CA, USA) containing 2 mM MgCl2, 0.25 mM from each dNTP (Invitrogen, Life Technologies, Carlsbad, CA, USA) and 2.5U of thermostable DNA polymerase (Paq5000TM DNA polymerase, Agilent Technologies, Santa Clara, CA, USA). The cycling conditions were 50 cycles of 20 s at 95 °C, 20 s at 52 °C and 30 s at 72 °C. The first cycle was proceeded by a 2 min denaturation step at 95 °C and the last cycle was followed by a 5 min elongation step at 72 °C. Then the amplified fragments were subjected to digestion with 20 units of BstU1 restriction enzyme (New England Biolabs, Ipswich, MA, USA) at 60 °C for 1 h.

The results of digestion were visualized in a 2% agarose gel containing 1 μg of ethidium bromide per ml in Tris-borate-EDTA buffer using a 100 bp DNA ladder (Invitrogen Life Technologies, Carlsbad, CA, USA and Paisley, UK) as a molecular weight marker. In particular, PCR products with proline/proline yielded a fragment that was 309 bp in size after digestion with BstU1, while amplicons with arginine/arginine were restricted into two fragments that were 175 and 134 bp in size, and amplicons with arginine/proline

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were restricted into three fragments that were 309, 175 and 134 bp in size [21].

Identification of HPV16 variants and E6 T350G sequence variation

The identification of HPV16 intratypic variant lineages and the investigation of E6 T350G sequence variation was performed through amplification of the viral region between the E6 and E7 genes using a PCR assay and the primer set HPV16 41/HPV16 757, as previously described by our group [9].

Cloning and sequence analysis of HPV16 E6 and E7 genes

Each individual amplicon was subjected to cloning using the pGEM T-easy vector system (Promega, Madison, WI, USA). The recombinant plasmid DNA was purified using the Nucleospin plasmid kit (Macherey Nagel, Duren, Germany) and the plasmids were sequenced at Macrogen (South Korea). The cloned sequences were characterized by database alignments at the National Centre for Biotechnology Information (NCBI) website and the MEGA BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The detection of specific intratypic nucleotide variations and the analysis of amino acid substitutions were conducted through multiple sequence alignment among the reference sequence of the HPV16 genome, the E6 cloned sequences that were collected from the present study, the E6 cloned sequences that were presented in a previous study by our group [9] and the representative sequences of HPV16 variant lineages available in the GenBank sequence database under the accession numbers for European variants AF436179 (European–German), AY686580, AY686583 and AY686584, for east Asian variant AF534061, for African type I variants AF472508 and AF366180, for African type II variant AF472509 and for the Asian–American variants AF402678, AY686579 and AY686582. The reference sequence of HPV16 DNA is documented at the HPV sequence database PaVE (http://pave.niaid.nih.gov) and is available from the National Center for Biotechnology Information (NCBI GI no. 333031) at http://www.ncbi.nlm.nih.gov. The multiple sequence alignment was performed using the MUSCLE algorithm in MEGA v.5 software [31, 32].

Phylogenetic tree construction

A maximum-likelihood phylogenetic tree (1000 bootstrap replicates) was built using MEGA v.5 software [31] in order to classify the HPV16 sequences to any of the four major phylogenetic branches. In particular, the phylogenetic tree was designed using the E6 cloned sequences that were previously presented by our group [9], the reference sequence of HPV16R and the representative sequences of the HPV16 variant lineages that were mentioned above. The phylogenetic tree was constructed using the general time reversible substitution model plus gamma (GTR+G) with the online software FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html).

Statistical analysis

The odds ratio (OR) and its 95% confidence intervals (CI) were calculated as a measure of the association between the p53 genotype and the risk of more severe cervical disease. The statistical significance of the OR was determined by the chi square test and Fisher’s exact test (for smaller numbers of samples) through GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Two-sided P-values were considered to be significant at the 0.05 cut-off level.

RESULTS

The findings of the present study indicated differences in the distribution of p53 genotypes among the different stages of cervical disease (Table 1). In particular, the p53 arg/arg genotype was detected in 71.1% (23/45) of HSIL+ and 37.1% (13/35) of LSILs (Table 1). The outcomes of the present analysis indicated that the OR of HSIL+ for women with the arg/arg genotype compared to women with the p53 genotypes arg/pro or pro/pro was 4.17 (OR=4.17, 95% CI:1.48–4.9, P=0.0049) (Table 1).

Sequence and phylogenetic analyses of the E6 gene were conducted in the same cervical samples, in order to evaluate whether the relationship between the p53 codon 72 genotypes and the HPV16 variants influences the risk of more severe dysplasia (Table 2). A maximum-likelihood phylogenetic tree was constructed based on the E6 sequences, while extensive sequence analysis of the E6 gene was also performed in order to identify the specific intratypic nucleotide

| Table 1. Distribution of p53 Codon 72 genotypes between patients diagnosed as LSILs and HSILs and cervical cancer |
|---|---|---|---|---|---|---|
| p53 genotypes | LSILs [n (%)] | HSILs [n (%)] | Cervical cancer | OR HSIL+ vs LSILs (95% CI) | P-value | OR HSIL+ vs LSILs (95% CI) | P-value |
| pro/pro | 5 (14.3) | 3 (8.6) | 0 (0) | 1 | 1 |
| arg/pro | 17 (48.6) | 6 (17.1) | 4 (40) | 0.98 (0.15–7.69) | 0.98 |
| arg/arg | 13 (37.1) | 26 (74.3) | 6 (60) | 4.1 (0.66–29.46) | 0.14 | 4.17 (1.48–4.9) | 0.0049 |
| Samples (n) | 35 | 35 | 10 | 1 | 1 |
polymorphisms that facilitate the classification of HPV16 DNA into the four major phylogenetic branches (Fig. 1). Our findings demonstrated that the HPV16 prototype sequence was present in 31.4% (11/35) of LSIL and in 22.9% (8/35) of HSIL samples, while the European variant (T350G) was detected in 65.7% (23/35) of LSILs and 71.4% (25/35) of HSILs (Table 2 and Fig. 1). Three samples were characterized as non-European variants (Fig. 1). In particular, the LSIL cervical sample A17 C1 was clustered into the African type II variant lineage, while the two HSIL samples A17 C1 (European variant) and A3 C1 (Asian variant) were grouped into the Asian–American type II variant lineage (Fig. 1). Merging results from HPV16 variant analysis and p53 codon 72 polymorphism genotype investigation revealed that in this sub-class of samples the OR of HSILs for women with the p53 arg/arg genotype was reported in 31.4% (11/35) of LSIL and in European and Asian American variants) encountered the p53 arg/arg genotype (Table 2 and Fig. 1). Merging results from HPV16 variant analysis and p53 codon 72 polymorphism genotype investigation revealed that in this sub-class of samples the OR of HSILs for women with the p53 arg/arg genotype was reported in 31.4% (11/35) of LSIL and in 22.9% (8/35) of HSIL samples. The European variant (T350G) was detected in 65.7% (23/35) of LSILs and 71.4% (25/35) of HSILs, while the European variant (T350G) was 2.76 (OR=2.76, 95% CI: 0.8–8.9). When the results from HPV16 analysis and p53 codon 72 genotyping were considered together it was revealed that the OR of HSILs for women with the p53 arg/arg genotype and the T350G sequence variation compared to women with the p53 arg/pro or pro/pro genotype and T350G was 3.09 (OR=3.09, 95% CI: 1–9.9, P=0.05) (Table 4).

**DISCUSSION**

The p53 tumour-suppressor protein confers effective protection against tumour growth, since it coordinates different cellular mechanisms, including apoptosis, cell cycle arrest and DNA repair in response to stressful stimuli [33]. The distribution of diverse p53 codon 72 genotypes and their association with cervical cancer risk varies among different populations and ethnicities. In particular, it has been proposed that the arg allele is more prevalent in cervical cancer cases in Caucasian compared to Asian or African populations [34–36]. Research studies that have been performed in European population groups reinforced the initial assumption of Storey et al. [27], suggesting that homozygous arg/arg p53 exhibits an increased risk for the development of cervical disease [21, 35, 37, 38]. Interestingly, a considerable association between the p53 codon 72 arg/arg genotype and cervical cancer risk has also been described in population groups from India, Sudan and China [39–41]. On the other hand, a recent meta-analysis in the Chinese population revealed that pro/pro carriers are at increased risk for cervical cancer growth [21], while other investigations suggested that the p53 pro/pro genotype also contributes to cervical cancer development in Indian and Korean women [42, 43]. Moreover, no relationship between p53 codon 72 polymorphisms and cervical cancer risk was documented in Table 3. Our findings revealed that the presence of T350G increases with the severity of cervical disease (Table 3). Moreover, the OR of HSILs for individuals with 350G compared to individuals that harbouried 350T was 1.76 (OR=1.76, 95% CI: 0.61–5.05, P=0.29) (Table 3). When the results from T350G analysis and p53 codon 72 genotyping were considered together it was revealed that the OR of HSILs for women with the p53 arg/arg genotype and the T350G sequence variation compared to women with the p53 arg/pro or pro/pro genotype and T350G was 3.09 (OR=3.09, 95% CI: 1–9.9, P=0.05) (Table 4).
described in patients derived from central and southern Africa, Morocco, Thailand and east Asia [23, 24, 44–46]. One possible explanation for the diverse association of p53 genotypes with cervical cancer risk in various geographic locations is that different population groups around the world have different environmental exposures as well as divergent genetic backgrounds [22, 47]. Hence, the present study investigated whether specific p53 codon 72 genotypes affect susceptibility to more severe dysplasia and cervical cancer in HPV16-positive Greek women and examined the impact of variant-specific synergy between HPV16 and p53 on the progression of cervical disease.

The results that emerged from the present study demonstrated that women who are homozygous for the arg genotype are at a 4.17-fold higher risk of developing HPV16-associated HSIL+ (OR=4.17, 95 % CI : 1.48–4.9, \( P =0.0049 \)). Our data confirmed the initial assumption of Storey et al. [27] and several previous analyses which proposed that p53 arg/arg homozygosity increases a patient’s susceptibility to cervical disease [21, 35, 37, 38].

Another aspect of the current analysis was to investigate whether the p53 arg/arg genotype in association with the HPV16 prototype strain or specific HPV16 variants can influence an individual’s susceptibility to more severe dysplasia. It is noteworthy that previous analyses in British and Dutch populations observed a considerable overrepresentation of the p53 arg/arg genotype in women with the HPV16 prototype sequence in cervical cancer cases, while this overrepresentation was not detected in HSIL cases [37, 38]. Moreover, a more recent analysis in Italian women revealed that there is no association between the p53 arg/arg genotype in women infected by the HPV16 prototype strain and the risk for the development of more severe dysplasia [21].

In contrast to these findings, our study revealed that the p53 arg/arg genotype in patients with the HPV16 prototype is associated with a 18.6-fold higher risk for the development of HSILs (OR=4.17, 95 % CI : 1.5–222, \( P =0.02 \)). Sequence analysis of the E6 and E7 genes of the HPV16 prototype strains revealed no sequence variations that could be further associated with the p53 arg/arg genotype and the risk of cervical disease. As a result, additional biological or biochemical factors of viral life cycle must be investigated in combination with the p53 codon 72 genotypes, regardless of the HPV16 variant lineages. These factors might comprise the integration of viral DNA into the host chromosome, the methylation status of HPV16 DNA or mutations into viral genes [48]. These aspects could further explain not only the relationship between p53 genotypes and the risk of cervical disease, but also the association between the p53 arg/arg
genotype and the reference strains of HPV16 that are circulating among different populations. At present, no significant conclusions have been drawn regarding the relationship between HPV16 DNA’s physical status and the p53 genotypes [49], while no investigations have focused on the methylation status of viral DNA in relation to the p53 codon 72 genotypes.

Moreover, our results revealed that the p53 arg/arg genotype in patients infected by HPV16 European variant have a 2.76-fold higher risk for the development of more severe cervical disease, but this association did not reach the limits of statistical significance (P=0.087) (Table 2). These results highlight previous findings in the Italian population that supported the contention that the oncogenic effect of HPV16 intratypic variant lineages may not be influenced by p53 genotypes [21]. Interestingly, in the present study the two cervical samples that were found to be infected by the HPV16 Asian–American variant only encountered the p53 arg/arg genotype. It is widely accepted that the HPV16 Asian–American variant lineage confers the highest risk for cervical cancer development compared to European, African type I and African type II variant lineages [8–11]. Considering the overrepresentation of the p53 arg/arg genotype in more severe dysplasia and cervical cancer, as well as the strong implication of HPV16 Asian–American variant in cervical cancer risk worldwide, we suggest that further analyses are required in order to evaluate the prospect of specific interactions between p53 (arg/arg) and the HPV16 Asian–American variant. These results will clarify whether p53 arg/arg women who are infected by the HPV16 Asian–American variant have a higher risk for cervical disease development.

In addition, in the present analysis it was observed that the p53 arg/arg genotype in patients with the E6 T350G sequence variation are at a 3.09-fold higher risk of developing more severe dysplasia, while the simultaneous detection of T350G and the p53 arg/arg genotype can further stratify the risk by selecting women who are at the highest risk for the development of more severe dysplasia, while the simultaneous detection of T350G and the p53 codon arg/arg genotype can further stratify the risk by selecting women who are at the highest risk for HSILs or worse. As a result, analysis of the polymorphic variability of the p53 and E6 genes could further predict cervical cancer risk, providing essential information regarding the viral tumourigenic capacity and consequently patients’ risk for cancer development.

In conclusion, the present study supports the claim that patients with the p53 arg/arg genotype present an increased risk of developing HSILs or worse, while individuals with the T350G E6 sequence variation and the p53 arg/arg genotype are at considerable risk of developing more severe lesions. These results show that the oncogenic potential of the virus is increased by the presence of the p53 arg/arg genotype, and that the specific protein interaction E6 codon 72 form is more susceptible to be targeted and degraded by the HPV16 E6 oncoprotein with L83V amino acid substitution (T350G), and that this positive synergy in turn increases patients’ vulnerability to cervical disease. Taking these facts into account, it was concluded that the specific interaction between p53 genotypes and E6 variants might influence the carcinogenic potential of HPV16, and consequently it could explain the diverse impact of particular p53 genotypes on cervical cancer susceptibility in different geographic populations. However, further analyses in various population groups are required in order to confirm this assumption.

Finally, our results revealed that the prevalence of T350G increases with the severity of cervical disease [9, 12, 13]. Considering the role of T350G E6 sequence variation, as well as the impact of the p53 arg/arg genotype on cervical cancer growth, it is postulated that their identification could further stratify the risk of disease progression. Notably, detection of the T350G sequence variation enables the stratification of risk by identifying women who have higher risk for the development of more severe dysplasia, while the simultaneous detection of T350G and the p53 codon arg/arg genotype can further stratify the risk by selecting women who are at the highest risk for HSILs or worse. As a result, analysis of the polymorphic variability of the p53 and E6 genes could further predict cervical cancer risk, providing essential information regarding the viral tumourigenic capacity and consequently patients’ risk for cancer development.

In conclusion, the present study supports the claim that patients with the p53 arg/arg genotype present an increased risk of developing HSILs or worse, while individuals with the T350G E6 sequence variation and the p53 arg/arg genotype are at considerable risk of developing more severe lesions. These results show that the oncogenic potential of the virus is increased by the presence of the p53 arg/arg genotype, and that the specific protein interaction E6

### Table 3. Prevalence of E6 T350G in HPV16-positive patients who had been diagnosed as LSIL or HSIL

<table>
<thead>
<tr>
<th>E6 T350</th>
<th>LSILs [n (%)]</th>
<th>HSILs [n (%)]</th>
<th>OR HSILs vs LSILs (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>300T</td>
<td>12 (34.3)</td>
<td>8 (22.9)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>350G</td>
<td>23 (65.7)</td>
<td>27 (77.1)</td>
<td>1.76 (0.61–5.09)</td>
<td>0.29</td>
</tr>
<tr>
<td>Samples</td>
<td>35</td>
<td>35</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4. Distribution of p53 codon 72 genotypes in LSILs and HSILs that harbour the E6 T350G sequence variation

<table>
<thead>
<tr>
<th>p53 genotypes</th>
<th>LSILs [n (%)]</th>
<th>HSILs [n (%)]</th>
<th>OR HSILs vs LSILs (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>arg/pro or pro/pro</td>
<td>13 (56.5)</td>
<td>8 (29.6)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>arg/arg</td>
<td>10 (43.5)</td>
<td>19 (70.4)</td>
<td>3.09 (1–9.9)</td>
<td>0.05</td>
</tr>
<tr>
<td>Samples</td>
<td>23</td>
<td>27</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(L83V)–p53 (Arg-72) can alter an individual’s susceptibility to cervical disease.

Funding information
This work was supported by IKY fellowships of excellence for postgraduate studies in Greece – Siemens programme and by research grants from the Postgraduate Programme ‘Toxology’ code 5050, of the University of Thessaly, School of Health Sciences, Department of Biochemistry and Biotechnology.

Acknowledgements
The authors would like also to thank Dr G. Amoutzias for critical reading of the manuscript and for helpful scientific discussions.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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