

## An *MGMT* Allelic Variant Can Affect Biochemical Relapse in Prostate Cancer Patients

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**Abstract.** *Background/Aim:* Prostate cancer (PCa) is one of the most frequent neoplasms in men around the world. In recent years, the search for new biomarkers with greater prognostic potential for PCa has intensified. This study aimed to evaluate single nucleotide polymorphisms (SNPs) and a combined panel of these polymorphisms in relation to biochemical recurrence in patients who were through prostatectomy, with an average of 7 years of follow-up. *Materials and Methods:* Patients diagnosed with PCa (n=197) participated in this cohort study. Thirteen SNPs were analyzed: rs2279115 (*BCL-2*), rs26677604 (*CASP3*), rs1052571 (*CASP9*), rs11781886 (*NKX3-1*), rs2735343 (*PTEN*), rs2494750 (*AKT1*), rs2699887 (*PI3KCA*), rs3195676 (*AMACR*), rs17302090 (*AR*), rs2536 (*mTOR*), rs1695 (*GSTP1*), rs2308321 (*MGMT*) and rs1544410 (*VDR*). Variants were combined and four main panels were defined: cell death, cell survival, growth receptors, and metabolism. Genotyping was performed by real-time PCR. *Results:* We did not observe any significant relation between the panels of variants analyzed, apart from the rare allele (G) of rs2308321 (*MGMT*) that was associated with a higher risk of recurrence ( $p=0.036$ ) when compared to the prevalent (A) in the allelic model. *Conclusion:* This *MGMT* variant occurs in an exon, and it could potentially affect DNA repair and, therefore, the biochemical relapse of PCa patients.

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Since the end of the 20<sup>th</sup> century, prostate-specific antigen (PSA) has been the most used prostate cancer marker during follow-up, detecting recurrences in approximately 25% of men after primary treatment with prostatectomy (1, 2). PSA is also used in combination with pathological (TNM) and local staging (D'Amico stratification) as a prognostic marker in the clinic, but PSA has low specificity regarding tumor aggressiveness (3). Besides that, its capacity to differentiate recurrence risk is limited, stimulating research for new prognostic markers capable of complementing the above-mentioned factors in risk stratification and assisting in clinical decision-making (4).

Aly *et al.* (1) stated in their review that biological markers, especially genetic changes associated with prostate tumor aggressiveness, are valuable tools in individualizing treatment. Several alterations in genes have already been associated with the occurrence of PCa where they have been linked to tumor susceptibility (5), prognosis (6) and recurrence (7). These alterations can modify the cell homeostasis, as well as survival and death mechanisms, resulting in genomic instability, with cells often losing their function and entering a state of immortality, characteristic of tumor cells (8).

Single nucleotide polymorphisms (SNPs) have been researched regarding their relation to the carcinogenic process. Individually, the role of SNPs in predicting susceptibility to malignant prostate tumors may not be significant. However, the combination of several variants in a genotypic profile of related pathways can present significant results regarding the understanding of how genes affect the neoplastic process (1, 6, 7).

Thus, the present study aimed to evaluate the role of SNPs in genes that play an essential role in the cellular pathways associated with PCa relapse (cell death, cell survival, growth

receptors, and metabolism) and to propose individual SNPs or a panel of SNPs that may be relevant for future use in the clinical setting.

## Materials and Methods

*Patient follow-up and identification of biochemical recurrence.* This was a longitudinal cohort study, in which 197 men from the region of Londrina, southern Brazil, who were diagnosed with PCa and underwent prostatectomy at the Hospital do Câncer de Londrina (HCL, Paraná, Brazil) from 2006 to 2015, were investigated for tumor recurrence.

The research was approved by the Ethics Committee in Research with Human Beings of the State University of Londrina (CAAE19769913.0.0000.5231). All patients signed an informed consent form and answered a modified questionnaire by Carrano and Natarajan (9). Social, clinical, and laboratory data were obtained through medical records and questionnaires.

The occurrence or non-occurrence of tumor recurrence was determined through periodic consultations of medical records and patients who relapsed within 10 years of prostatectomy were included in the study, with an average follow-up time of seven and a half years.

The criterion used to indicate tumor recurrence was biochemical recurrence through prostate-specific antigen (PSA) measurement, as indicated by the European Association of Urology (EAU) guidelines. A 0.2 ng/ml increase and a second confirmatory 0.2 ng/ml increase (or a single 0.4 ng/ml increase) in serum PSA levels relative to PSA nadir (lowest PSA achieved after primary treatment) were considered biochemical PCa relapse (10, 11). The PSA value was monitored through medical records. The risk of recurrence of D'Amico for each patient was estimated from PSA values, Gleason score, and TNM staging at diagnosis (2). All patients underwent a prostatectomy procedure with curative intent. Thus, the prostate tissue removed from each patient was submitted to histopathological analysis, where the surgical margins were analyzed to evaluate their possible compromise. These data were considered in the statistical analysis in all follow-up analyses. The biochemical relapse data were compared with the relapse risk proposed by D'Amico. For this, the risk of D'Amico was categorized, following the data already established (2), establishing the risk of relapse for patients as low, medium, or high.

*Sampling and genotypic analysis.* After confirming the diagnosis of PCa using histopathological analysis, the patient's blood was collected by intravenous puncture in tubes with EDTA (ethylenediaminetetraacetic acid). The samples were centrifuged, and the intermediate layer of leukocytes was transferred to 1.5 ml microtubes which were stored at  $-20^{\circ}\text{C}$  until DNA extraction. Intravenous blood collection and genetic material extraction were performed by our research group from 2006 to 2015 and the genetic material was stored at  $-80^{\circ}\text{C}$ .

Genomic DNA was extracted from the leukocyte layer of peripheral blood using the High Pure PCR Template Preparation Kit (Roche®, Indianapolis, IN, USA) according to the supplier's instructions and quantified using Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) at 260/280nm wavelengths; samples with a ratio between 1.8 and 2.0 were considered pure. Each sample was coded, and the individuals' information remained confidential.

From each patient, 13 SNPs were genotyped, each one being validated in public databases (Database of Single Nucleotide Polymorphisms/NCBI, 2021). The evaluated SNPs were: rs2279115 (*BCL-2*), rs26677604 (*CASP3*), rs1052571 (*CASP9*), rs11781886 (*NKX3-1*), rs2735343 (*PTEN*), rs2494750 (*AKT1*), rs2699887 (*PI3KCA*), rs3195676 (*AMACR*), rs17302090 (*AR*), rs2536 (*mTOR*), rs1695 (*GSTP1*), rs2308321 (*MGMT*) and rs1544410 (*VDR*). The technique used for genotyping was Real-Time qPCR with TaqMan® probes (Applied Biosystems, Foster City, CA, USA) through a hydrolysis system, genotyping Master Mix (Applied Biosystems) and 5 ng/ml of genomic DNA, following the recommendations of the manufacturer, using the PrimeQ Real-Time TECHNE® thermal cycler.

Genotyping was performed in the Mutagenesis and Oncogenetics laboratory of the State University of Londrina, Brazil, from 2009 to 2020. SNPs investigated in this study were also previously used in case-control studies carried out in our laboratory (5, 12).

*Statistical analysis.* To determine the association of social variables with biochemical relapse, the Cox regression test was used and considered significant when  $p \leq 0.05$ . Whenever the relation of data with biochemical recurrence was evaluated, data on surgical margin analysis, follow-up, and relapse time were entered to adjust the statistical model.

The association of the D'Amico risk stratification with the occurrence of biochemical recurrence was also evaluated. For this, the D'Amico scores were divided into low ( $\leq 3$ ), intermediary (4-6), and high-risk ( $\geq 7$ ) of recurrence according to each patient's PSA, Gleason score, and local tumor staging. The risk of recurrence was evaluated by the non-parametric Mann-Whitney test. Results were expressed as median and percentiles (25%-75%) and were considered significant when  $p \leq 0.05$ .

The following pathological data were collected from the patient's records at diagnosis: tumor staging, Gleason score, tumor laterality, extracapsular extension, seminal vesicle, and perineural or lymph node invasion, and were tested for their association with biochemical recurrence. To this end, we used Cox regression adjusted by age and follow-up time, where the results were exposed as hazard ratios with a 95% confidence interval. Those with  $p \leq 0.05$  were considered statistically significant.

To determine possible associations between single and multiple SNPs in relation to biochemical recurrence, a Cox Proportional-Hazards Model was applied. To this end, the model was adjusted by the following parameters: patient age, surgical margin compromise, follow-up time, and time to recurrence after primary treatment. The hazard ratio was calculated with a 95% confidence interval and statistically significant differences were considered for  $p \leq 0.05$ . For the combinations, we used the combination of variants most frequently in our sample for each panel as a reference for the analysis. Combinations with a frequency smaller than 1% were disregarded from the combined analyzes due to their rarity and decreased statistical power. All analyzes were performed using the SPSS version 22.0 (IBM, Armonk, NY, USA).

## Results

The social and clinical data collected from the patients and their relationship with biochemical recurrence are presented in Table I. The patients who composed the sample of this study ( $n=197$ ) were aged between 47-84 years, with a mean of  $65 \pm 7$  years at diagnosis. Of these, 92 had a biochemical recurrence in the

follow-up years. Patients' age ( $p=0.162$ ) was not associated with biochemical relapse in the regression analysis. However, the PSA value at diagnosis ( $p=0.022$ ) was statistically significant with biochemical recurrence. Of the patients who had PSA greater than 10 (worst prognosis), 60.47% had a biochemical recurrence in the years of follow-up, while out of the patients who had PSA between 4 and 10 (best prognosis), only 35.64% had a recurrence in the same follow-up time.

Most of the individuals in the sample ( $n=165$ ) declared themselves to be Caucasian. There was no significant relationship between the presence of biochemical recurrence and the parameters of literacy, smoking, alcoholism, vasectomy, relatives with a history of tumor or relatives who already had a prostate tumor, sexually transmitted infections, and diagnosis of malignant tumors other than prostate.

The prognostic factors for prostate cancer including extracapsular extension ( $p=0.038$ ), seminal vesicle invasion ( $p=0.002$ ), and prostatectomy Gleason score 7 and 8-10 ( $p=0.044$  and  $0.024$ , respectively) were statistically significantly associated with biochemical recurrence. In addition, the biopsy Gleason score showed a tendency ( $p=0.057$ ) to be associated with biochemical PCa recurrence (Table II).

Tumor staging presented a higher risk for recurrence ( $p=0.014$ ) when patients had locally advanced stages (Pt3 and Pt4). Positive surgical margins were associated with a higher occurrence of biochemical recurrence (Table II). Table II also shows the other results of the analysis concerning the association of prognostic parameters with the presence or absence of biochemical recurrence in patients.

The recurrence risk of each patient was estimated from the histopathological parameters, as established by D'Amico *et al.* (2), and was associated with the presence of biochemical recurrence in our sample. Statistical significance was observed between relapse data and the risk of D'Amico ( $p=0.004$ ). Both the group of patients with and without recurrence had an intermediate risk median. However, the median of the group without recurrence was 5 (4–6), while the group with recurrence had a median score of 6 (5–7).

The individual SNP association analysis with the incidence of biochemical recurrence in patients presented the *MGMT* gene variant as statistically significant. The presence of the G allele indicated an increased risk of biochemical relapse in these patients in the allelic model (Table III). However, in the other models, the presence of the G allele was not associated with recurrence.

Figure 1 represents the recurrence over the years of follow-up, represented in months, for rs2308321 (*MGMT* gene). The risk of recurrence in the allelic model ( $p=0.036$ ) throughout the follow-up is expressed in Hazard Ratio in Table III. With approximately 50 months of follow-up, patients with the GG genotype have increased recurrence, while patients carrying the A allele (genotypes AA and AG) maintain similar recurrence.

Table I. Social and clinical data of patients with prostate cancer collected at diagnosis and their relationship with biochemical recurrence.

Social and clinical data	Biochemical recurrence	
	n (Recurrence %)	p-Value
Age at diagnosis		
$\leq 64$	87 (42.5%)	Reference
$\geq 65$	110 (50.0%)	0.162
Ancestry		
Caucasian	165 (46.1%)	-
Non-Caucasian	32 (50.0%)	0.948
Literacy*		
Graduated	29 (48.3%)	-
High School	125 (46.4%)	0.843
Illiterate	41 (43.9%)	0.901
Smoking		
Non-smoker	151 (45.7%)	-
Smoker	46 (50.0%)	0.796
Alcoholism		
Non-alcoholic	103 (52.4%)	-
Alcoholic	94 (40.4%)	0.660
Vasectomy*		
Non-vasectomized	191 (46.6%)	-
Vasectomized	4 (50.0%)	0.702
Relatives with cancer		
No	83 (42.1%)	-
Yes	114 (57.9%)	0.955
Relatives with PCa*		
No	72 (45.8%)	-
Yes	39 (41.0%)	0.074
PSA value at diagnosis*		
Less than 4	9 (44.4%)	-
From 4 to 10	101 (35.6%)	<b>0.022</b>
More than 10	86 (60.5%)	0.728
STIs*		
No	174 (47.1%)	-
Yes	22 (45.4%)	0.244
Other tumors*		
No	127 (50.2%)	-
Yes	11 (48.9%)	0.844

Data is expressed as an absolute number (%). Cox proportional hazards model. \*It was not possible to evaluate the parameters for all patients. PSA, Prostate-specific antigen; STIs, sexually transmitted infections; PCa, prostate cancer. Statistically significant  $p$ -values are shown in bold.

The analysis of multiple SNPs was made according to proteins pathways: (I) Receptors (*VDR* and *AR*), (II) Cell Metabolism (*GSTP1* and *AMACR*), (III) Cell Survival (*PTEN*, *AKT1*, *PI3KCA*, and *mTOR*), and (IV) Cell Death (*CASP3*, *CASP9*, *NKX3-1*, *MGMT*, and *BCL-2*), regarding their association with biochemical recurrence (Table IV). The combination of multiple SNPs did not show a statistically significant association with the presence of biochemical PCa recurrence in any of the analyzed combinations (Table IV).

Table II. Clinical and laboratory prognostic factors of patients with prostate cancer collected at diagnosis and their relationship with biochemical recurrence.

Clinical and laboratory data	Recurrence (%)	Odds ratio (95% CI)	p-Value
Biopsy Gleason			
≤7 (3+4)	164 (43.9%)	Reference	1
7 (4+3)-10	32 (62.5%)	1.530 (0.858-2.728)	0.057
Prostatectomy Gleason			
3-6	93 (43.0%)	-	-
7	86 (46.5%)	1.75 (1.013-2.447)	<b>0.044</b>
8-10	17 (70.6%)	2.126 (1.106-4.084)	<b>0.024</b>
Extracapsular extension			
No	106 (37.7%)	-	-
Yes	73 (63.0%)	1.576 (1.026-2.422)	<b>0.038</b>
Seminal vesicle invasion			
No	165 (41.8%)	-	-
Yes	24 (70.8%)	2.352 (1.358-4.074)	<b>0.002</b>
Perineural invasion			
No	161 (42.9%)	-	-
Yes	27 (63.0%)	1.136 (0.665-1.938)	0.641
Compromised surgical margin			
Negative	179 (43.6%)	-	-
Positive	36 (77.8%)	4.532 (2.010-10.219)	<b>0.020</b>
Tumor staging			
Pt1- Pt2a	33 (33.3%)	-	-
Pt2b/Pt2c	77 (31.2%)	1.682 (0.811-3.487)	0.162
Pt3/Pt4	78 (64.1%)	2.274 (1.179-4.385)	<b>0.014</b>

Cox proportional hazards model adjusted by age of patients. Data expressed as absolute number (%), hazard ratio (95% CI), and p-value. CI: Confidence interval. Statistically significant p-values are shown in bold.

## Discussion

Our study aimed to evaluate the prognostic potential of several SNPs related to cell death, survival, and growth as complementary tools to PCa prognosis. Among the SNPs analyzed, the *MGMT* polymorphism was linked to the patients' PCa recurrence. The *MGMT* protein is part of an important DNA repair mechanism, functioning both as a transferase and as an acceptor of alkyl groups; it is one of the few proteins capable of repairing DNA damage caused by alkylating agents (13). The genomic instability generated from the accumulation of damage in the genetic material is the basic condition for the loss of homeostasis and consequent disordered cell growth (14), which may have cancer development as one of the consequences. Genomic instability is also a hallmark of aging (14) and this, in turn, is one of the main risk factors for prostate cancer (15), because this disease presents a slow and silent development until its advanced stage.

The mean age of patients in the present study was 65±7 years, corroborating data from the literature that describe PCa as a disease characteristic of men of advanced age (16, 17). Older men have a higher risk of being diagnosed with advanced PCa (18) and, therefore, have a lower overall

survival rate given the treatment difficulties arising from age (17). This reinforces the relation between advanced age and worse prognosis of patients, although, in the present study, age was not associated with biochemical tumor recurrence.

Ancestry is a factor that has been related to the risk of developing PCa in several studies (5, 16, 17, 19, 20). A relation between the worse prognosis of PCa in white men than in black and brown men was observed by Migowski *et al.* (19). However, three studies from Japanese and American populations showed the opposite, with a higher risk of developing PCa and a worse prognosis for black men (16, 17, 20). Unlike the Japanese and American populations, the one studied by Migowski *et al.* (19) was from Rio de Janeiro (Brazil), characterized by high miscegenation. In our Brazilian sample, also highly miscegenated, ancestry was not associated with biochemical recurrence, and the frequency of men who declared themselves to be non-Caucasian was very low. The divergence with the data from Migowski *et al.* (19) can be explained, in part, by the ethnicities of each location. In the region of Londrina, southern Brazil, where our samples were collected, the high rate of miscegenation occurred mainly among European and Asian immigrants, unlike the region of Rio de Janeiro, where the frequency of African origin is higher (21).

Table III. Association of genetic variants with the presence or absence of biochemical recurrence.

Genes	Models	n (Recurrence %)	Hazard ratio (95% CI)	p-Value
<i>VDR</i> rs1544410	Allelic model			
	C	271 (46.5%)	Reference	1
	T	123 (47.1%)	1.040 (0.762-1.419)	0.807
	Genotypic model			
	CC	75 (46.7%)	-	-
	CT	122 (46.7%)	1.030 (0.675-1.569)	0.892
	Dominant model			
	CC	75 (46.7%)	-	-
	CT+TT	122 (46.7%)	1.030 (0.675-1.569)	0.892
<i>AR</i> rs17302090	Allelic model			
	G	73 (55.2%)	-	-
<i>GSTP1</i> rs1695	A	124 (47.6%)	1.135 (0.739-1.744)	0.563
	Allelic model			
	A	172 (46.5%)	-	-
	G	222 (46.8%)	0.983 (0.734-1.316)	0.906
	Genotypic model			
	AA	98 (46.9%)	-	-
	AG	74 (45.9%)	0.971 (0.622-1.516)	0.898
	GG	25 (48.0%)	0.915 (0.480-1.744)	0.788
	Dominant model			
	AA	98 (47.0%)	-	-
AG+GG	99 (46.5%)	1.045 (0.692-1.579)	0.833	
	Recessive model			
	AA+AG	172 (46.5%)	-	-
	GG	25 (48.0%)	0.927 (0.501-1.714)	0.809
<i>AMACR</i> rs3195676	Allelic model			
	G	243 (48.6%)	-	-
	A	151 (43.7%)	0.935 (0.690-1.267)	0.664
	Genotypic model			
	GG	79 (46.8%)	-	-
	GA	86 (51.2%)	1.177 (0.759-1.826)	0.466
	AA	32 (34.4%)	0.770 (0.389-1.522)	0.452
	Dominant model			
	GG	79 (46.8%)	-	-
	GA+AA	118 (46.6%)	1.069 (0.702-1.628)	0.756
	Recessive model			
	GA+GG	165 (49.1%)	-	-
AA	32 (34.4%)	1.705 (0.373-1.332)	0.282	
<i>PTEN</i> rs2735343	Allelic model			
	G	239 (44.0%)	-	-
	C	155 (51.0%)	0.816 (0.608-1.095)	0.175
	Genotypic model			
	GG	76 (43.4%)	-	-
	GC	86 (45.3%)	0.816 (0.464-1.436)	0.481
	CC	35 (57.1%)	0.724 (0.409-1.280)	0.267
	Dominant model			
	CC	35 (57.1%)	-	-
	GC+GG	162 (44.4%)	0.769 (0.459-1.289)	0.319
	Recessive model			
	GC+CC	121 (48.8%)	-	-
	GG	76 (43.4%)	0.832 (0.543-1.276)	0.4
<i>AKT1</i> rs2494750	Allelic model			
	C	347 (47.3%)	-	-
	G	47 (42.5%)	0.953 (0.598-1.521)	0.841
	Genotypic model			
	CC	150 (47.3%)	-	-
CG	47 (44.7%)	0.983 (0.601-1.607)	0.944	

Table III. Continued

Table III. Continued

Genes	Models	n (Recurrence %)	Hazard ratio (95% CI)	p-Value
<i>PI3KCA</i> rs2699887	Dominant model			
	CC	150 (47.3%)	-	-
	CG+GG	47 (44.7%)	0.983 (0.601-1.607)	0.944
	Allelic model			
	G	307 (46.9%)	-	-
	A	87 (46.0%)	1.022 (0.717-1.457)	0.902
	Genotypic model			
	GG	121 (47.1%)	-	-
	GA	67 (46.3%)	0.989 (0.635-1.540)	0.843
	AA	9 (44.4%)	1.401 (0.503-3.897)	0.519
<i>MTOR</i> rs2536	Dominant model			
	GG	121 (47.1%)	-	-
	GA+AA	76 (46.0%)	1.023 (0.667-1.567)	0.918
	Recessive model			
	GA+GG	188 (46.8%)	-	-
	AA	9 (44.4%)	1.406 (0.513-3.858)	0.508
	Allelic model			
	T	355 (46.5%)	-	-
	C	39 (48.7%)	0.974 (0.605-1.569)	0.915
	Genotypic model			
TT	175 (46.3%)	-	-	
TC	5 (60.0%)	1.233 (0.388-3.923)	0.722	
CC	17 (47.1%)	0.941 (0.454-1.951)	0.871	
<i>CASP-3</i> rs4647603	Dominant model			
	TT	175 (46.3%)	-	-
	TC+CC	22 (50.0%)	1.006 (0.534-1.895)	0.985
	Recessive model			
	TC+TT	180 (46.7%)	-	-
	CC	17 (47.1%)	0.935 (0.452-1.935)	0.856
	Allelic model			
	C	341 (45.7%)	-	-
	T	53 (52.8%)	1.122 (0.746-1.690)	0.58
	Genotypic model			
CC	150 (44.0%)	-	1-	
CT	44 (54.5%)	1.231 (0.759-1.994)	0.399	
TT	3 (66.7%)	1.456 (0.351-6.045)	0.605	
<i>CASP-9</i> rs1052571	Dominant model			
	CC	150 (44.0%)	-	-
	CT+TT	47 (55.3%)	1.245 (0.777-1.993)	0.362
	Recessive model			
	CT+CC	194 (46.4%)	-	-
	TT	3 (66.7%)	1.362 (0.331-5.607)	0.669
	Allelic model			
	G	195 (43.1%)	-	-
	A	199 (50.2%)	1.159 (0.866-1.552)	0.32
	Genotypic model			
GG	47 (38.3%)	-	-	
GA	101 (47.5%)	1.203 (0.698-2.072)	0.506	
AA	49 (53.1%)	1.372 (0.748-2.517)	0.307	
<i>NKX3-1</i> rs11781886	Dominant model			
	GG	47 (38.3%)	-	-
	GA+AA	150 (49.3%)	1.256 (0.748-2.109)	0.39
	Recessive model			
	GG+GA	148 (44.6%)	-	-
	AA	49 (53.1%)	1.202 (0.761-1.899)	0.43
Allelic model				
T	269 (46.1%)	-	-	

Table III. Continued

Table III. *Continued*

Genes	Models	n (Recurrence %)	Hazard ratio (95% CI)	p-Value
<i>MGMT</i> rs2308321	C	125 (48.0%)	1.038 (0.762-1.413)	0.815
	Genotypic model			
	TT	96 (43.7%)	-	-
	TC	78 (51.3%)	1.195 (0.771-1.852)	0.425
	CC	23 (43.5%)	1.031 (0.517-2.059)	0.93
	Dominant model			
	TT	96 (43.7%)	-	-
	TC+CC	101 (46.5%)	0.966 (0.641-1.455)	0.867
	Recessive model			
	TT+TC	174 (47.1%)	-	-
	CC	23 (43.5%)	0.952 (0.492-1.843)	0.885
	Allelic model			
	A	355 (45.3%)	-	-
	G	39 (59.0%)	1.601 (1.031-2.486)	<b>0.036</b>
	Genotypic model			
	AA	164 (44.5%)	-	-
AG	27 (55.6%)	1.446 (0.823-2.540)	0.200	
GG	6 (66.7%)	2.267 (0.821-6.258)	0.114	
Dominant model				
AA	164 (44.5%)	-	-	
AG+GG	33 (57.6%)	1.567 (0.939-2.615)	0.086	
Recessive model				
AA+AG	191 (46.1%)	-	-	
GG	6 (66.7%)	2.144 (0.781-5.888)	0.139	
<i>BCL-2</i> rs2279115	Allelic model			
	C	210 (44.5%)	-	-
	A	184 (49.5%)	1.123 (0.840-1.501)	0.433
	Genotypic model			
	CC	55 (38.2%)	-	-
	CA	101 (51.5%)	1.412 (0.851-2.345)	0.182
	AA	41 (46.3%)	1.273 (0.684-2.369)	0.447
	Dominant model			
	CC	55 (38.2%)	-	-
	CA+AA	142 (50.0%)	1.372 (0.843-2.234)	0.203
	Recessive model			
	CC+CA	156 (46.8%)	-	-
AA	41 (46.3%)	1.008 (0.608-1.670)	0.976	

Cox proportional hazards model. Adjusted by age of patients, surgical margin of prostatectomy, and follow-up time. Data expressed as absolute number (%), hazard ratio (95%CI) and p-value. CI: Confidence interval. Statistically significant p-values are shown in bold.

Migowski *et al.* (19) also identified that the lower level of literacy was related to a higher risk of recurrence and worse prognosis of PCa, and these patients also had a later diagnosis. A previous study by our research group also showed that men with less literacy had a higher risk of developing PCa (5). However, in the present study literacy was not associated with biochemical recurrence. This parameter is directly related to the greater demand for health services and the greater financial capacity of patients.

The increase in PCa-specific recurrence and mortality was associated with tobacco and alcohol consumption (17); however, the authors highlighted that these data may have been influenced by multiple unknown external factors. In the

present study, drinking and smoking habits were not related to the occurrence of biochemical relapse.

The presence of familial cancer is established as a risk factor in several types of cancer, including PCa. Patients with siblings or parents who had PCa have an increased risk of about 50% for the development of this malignant neoplasm (5, 16). It is estimated that about 25% of familial cases of PCa result from SNP-like alterations, which are inherited by descendants, predisposing them to a greater risk of developing this pathology (1). However, little is known about the influence of heredity on the recurrence of prostate tumors. In our study, we did not observe a relation between the presence of general cancer or PCa in the family and the

occurrence of biochemical recurrence. This could indicate that the familial inheritance for PCa may be related to the development of the disease, but not to its recurrence.

Murata *et al.* (20) demonstrated the efficiency of PSA as a diagnostic and prognostic marker for PCa, especially when it was associated with other clinical parameters, such as Gleason score and TNM staging.

The Gleason score and digital rectal exam in combination with PSA showed important prognostic power in the study by Migowski *et al.* (19), where they were used in pre-treatment risk stratification. This parameter associated with other clinical factors has a prognostic value already determined and used in clinical practice (10).

As proposed by D'Amico *et al.* (2), the use of PSA, Gleason score, and TNM staging has great prognostic value for PCa. Our results reiterate the prognostic capacity that the combination of these markers offers: PSA ( $p=0.022$ ), Gleason score ( $p=0.044/0.024$ ) and TNM staging ( $p=0.014$ ). They were able to predict the occurrence of biochemical recurrence, corroborating data from studies by Murata and Pond *et al.* (20, 22) and reinforcing the value of these parameters as good prognostic markers for PCa.

Moul *et al.* (23), evaluated tissue samples obtained from a biopsy of patients with PCa and found a relation between the overexpression of BCL-2 protein in prostate tissue with greater tumor recurrence and lower survival for patients who had the homozygous CC genotype. In other studies that evaluated the same SNP in patients with PCa, the AA and CA genotypes were associated with a higher risk of recurrence and a worse prognosis (24, 25). Our work did not corroborate such data from the literature, as the presence of the allelic variant in the *BCL-2* gene did not have a significant influence on the occurrence of biochemical recurrence.

Holeckova *et al.* (2020) used next-generation sequencing to analyze 14 DNA repair genes in patients with metastatic castration-resistant prostate cancer (mCRPC) and identified three variants [*BRCA2* (rs80359306), *RAD50* (rs786201531), and *ATM* (rs1555099760)] that are likely to affect DNA repair efficiency and eleven other variants with pathogenic potential (26).

The MGMT protein is one of the few proteins able to transfer CH<sub>3</sub> radicals removed from O<sup>6</sup>-methylguanine to a cysteine site within it; this transfer restores the methylated base to its original form, repairing possible pairing errors, but prevents MGMT from returning to its functional conformation, making it a single-acting protein (27). A previous study suggested that the *MGMT* gene has multiple polymorphic sites and that some could be related to the occurrence of PCa (28). In a review study, Zhang *et al.* (29) evaluated the influence of several *MGMT* gene polymorphisms on PCa and showed that in 11 of the case-control studies reviewed, rs2308321 was not associated with PCa susceptibility. However, the authors highlighted the heterogeneity between the articles reviewed as

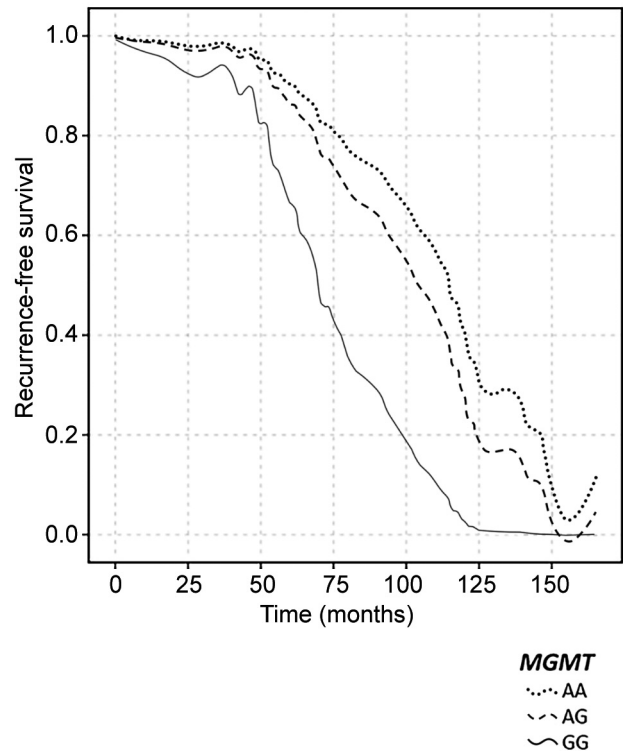


Figure 1. Kaplan-Meier survival analysis. Follow-up of patients diagnosed and treated for prostate cancer (PCa), distinguished by their genotype of rs2308321 (*MGMT*).

a limiting factor to the exposed results (29). In the present study, when rs2308321 was analyzed in the allelic model, the presence of the G allele significantly increased the risk of recurrence in PCa patients. The presence of the G variant causes an amino acid change in the final protein, which has the potential to affect the gene's functionality as a transferase and acceptor of alkyl groups, and in turn, allow further DNA damage to accumulate in the cell.

H3K27 histone in its trimethylated state (H3K27me3) inactivates several gene promoters and has been referred to as an epigenetic marker, as well as associated with PCa progression (30). Sanchez *et al.* (2020) found that the demethylation of H3K27 histone was dependent on the Jumonji domain-containing protein 3 (JMJD3) demethylase and showed the link between JMJD3 and the regulation of MGMT gene in tumor progression in PCa cell lines. The authors emphasized that it would be interesting to see the effects of JMJD3 inhibition on MGMT *in vivo*, as a potential therapeutic target for PCa (31).

Thus, although the literature data did not report an association between rs2308321 of the *MGMT* gene and susceptibility to PCa, in our study it was associated with an increased risk for biochemical tumor recurrence after surgical

Table IV. Analysis of the association of multiple single nucleotide polymorphisms (SNPs) related to receptors, cell metabolism, survival and cell death with biochemical recurrence.

Genes	Frequency (%)	Hazard ratio (95% CI)	p-Value
<i>VDR + AR</i>			
rs1544410/rs17302090			
TC + A	41.1%	Reference	1
C + G	16.2%	0.72 (0.37-1.43)	0.35
T + A	21.8%	1.26 (0.75-2.12)	0.38
T + G	20.8%	1.15 (0.67-1.96)	0.61
<i>GSTP1 + AMACR</i>			
rs1695/rs3195676			
AA + GG	43.9%	-	-
A + A	26.5%	0.71 (0.33-1.54)	0.39
G + G	19.03%	1.00 (0.42-2.40)	0.99
G + A	10.57%	2.41 (0.69-8.37)	0.17
<i>PTEN + AKT1 + PI3KCA + MTOR</i>			
rs2735343/rs2494750/rs2699887/rs2536			
GG + CC + GG + TT	37.56%	-	-
C + C + G + T	25.98%	0.83 (0.37-1.85)	0.65
G + C + A + T	9.54%	0.41 (0.11-1.49)	0.18
C + C + A + T	6.94%	0.69 (0.20-2.40)	0.56
G + G + G + T	5.86%	0.25 (0.06-1.14)	0.07
G + C + G + C	4.95%	0.82 (0.23-2.90)	0.75
C + G + G + T	1.65%	4.69 (0.15-149.99)	0.38
C + C + G + C	1.37%	1.38 (0.12-15.73)	0.81
C + G + A + T	1.13%	0.08 (0.15-46.55)	0.44
<i>CASP3 + CASP9 + NKX3-1 + MGMT + BCL-2</i>			
rs26677604/rs1052571/rs11781886/rs2308321/rs2279115			
CC + GA + TT + AA + CC	17.67%	-	-
C + G + T + A + A	15.57%	1.07 (0.28-4.10)	0.92
C + A + T + A + A	13.36%	0.38 (0.06-2.29)	0.29
C + A + C + A + A	8.27%	0.33 (0.07-1.51)	0.16
C + A + C + A + C	3.71%	7.15 (0.23-225.64)	0.27
C + G + C + A + A	2.72%	0.17 (0.01-2.69)	0.21
T + G + T + A + C	1.83%	0.07 (0.01-2.76)	0.16

Cox Proportional Hazards Model. Combinations with less than 1% frequency were excluded from the analysis. CI: Confidence interval.

intervention, indicating that risk for PCa recurrence likely involves expression of genes and cellular pathways distinct from those that act in the development of the disease. However, the frequency of the G allele in our sample was low (only 17.88% of our patients carried this allele), which may have been a limiting factor in the statistical analysis. Further studies with larger sample sizes that evaluate the role of this variant in the recurrence of PCa are very important since it is necessary to analyze a greater frequency of this allele in the population so that the data we obtained is confirmed.

Among the combinations of SNPs analyzed regarding receptors (rs1544410 *VDR*; rs17302090 *AR*), cell metabolism (rs1695 *GSTP1*; rs3195676 *AMACR*), cell survival (rs2735343 *PTEN*; rs2494750 *AKT1*; rs2699887 *PI3KCA*; rs2536 *MTOR*) and cell death (rs26677604 *CASP3*; rs1052571 *CASP9*;

rs11781886 *NKX3-1*; rs2308321 *MGMT*; rs2279115 *BCL-2*), no statistical significance was observed in relation to the biochemical recurrence of PCa. Although the panels of multiple SNPs offer a greater insight into their role in tumorigenesis (1, 6, 7), the combination of a panel also reduced the statistical analysis capability, and thus, limited the results of this study. Nonetheless, the association of panels analyzed in the present study is new in the literature and we hope that it may serve as a base for future studies.

## Conclusion

Our work sought to elucidate the role of polymorphisms present in genes of the main pathways related to tumorigenesis and tumor progression in the recurrence of prostate cancer. In

the individual analysis of SNPs, we observed that carriers of the G allele of the *MGMT* gene had an increased risk of biochemical recurrence compared to carriers of the A allele, but further studies are needed on the *MGMT* variant and its effects on DNA repair and PCa recurrence to confirm our findings. This was one of the first studies using combinations of multiple polymorphisms with PCa follow-up, but we did not observe statistically significant associations when using these combinations. However, our results may contribute to future studies on how multiple SNPs combination can affect PCa occurrence, development, and prognosis. We believe that a panel composed of genetic markers related to PCa could aid in clinical decision-making in the future. Therefore, the results obtained in this study serve as a basis for upcoming research on the action of single or multiple polymorphisms as PCa markers. In the future, polymorphisms, including *MGMT* variants, could help clinicians and provide a more complete diagnosis and prognosis, benefiting patients.

### Conflicts of Interest

The Authors have no relevant conflicts of interest to declare.

### Authors' Contributions

Study conception and design: Cólus, IMS; Furini, HH and Fuganti, PE; Data acquisition: Furini, HH; Fukushima, KSSQ; Nóbrega, M.; Souza, MF; Rodrigues, MRS; Mattos, BB. Statistical analysis: Simão, ANC; Flauzino, T.; Furini, HH; Fuganti, PE. Data analysis and interpretation: Furini, HH; Cólus, IMS. Manuscript preparation: Furini, HH; Losi-Guembarovski, R; Cólus, IMS. Critical revision of manuscript: Losi-Guembarovski, R.; Cólus, IMS; Fuganti, PE; Supervision and project administration: Cólus, IMS.

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