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Polymorphism of Free Radicals Detoxification Genes GSTM1 and GSTT1 is Associated to Prostate Cancer Risk in men: Case Study in Benin

Firmin H. Sagbo[1,2], M. Mènonvè Atindehou[1,2], Haboubacar Elhadji Yaou Moussa[1,3], Sara Y. Houngue[1,3], Josué Avakoudjo[4], Lucie Ayi-fanou[1,2], Callinice D. Capo-chichi[1,3,*]

[1]Unit of Biochemistry and Molecular Biology/Laboratory of Biochemistry, Molecular Biology and Environment/Faculty of Sciences and Techniques/University of Abomey-Calavi, Cotonou 04 BP 0320, Benin
[2]Laboratory of Biochemistry, Molecular Biology and Environment
[3]Laboratoire des Biomarqueurs Moléculaires en Cancérologie et Nutrition
[4]National University Teaching Hospital (CNHU-HKM), Cotonou Benin
*Corresponding author: callinice.capochichi@gmail.com

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Abstract **Background**: Most diseases including cancers are subsequent to the imbalance between oxidation and reduction in cells. Glutathione-s-transferases (GSTM1 and GSTT1) are cell detoxification enzymes which are involved in the conversion of free radicals derived from reduced oxygen (known to harm DNA) in oxidized molecules which are not harmful for the DNA. Cells with deficient GSTM1 or GSTT1 activity, have impaired cellular detoxification which exposed them to free radicals derived from environment, unhealthy nutrition, or some drugs, all of which can harm DNA and cause genomic instability leading to cancer. Hence, their implication in cancers affecting men in Benin was not investigated and needs to be addressed. The aim of this study is to assess the association between the loss of GSTM1 and GSTT1 and prostate cancer in Benin. **Methods**: GSTM1 and GSTT1 analyses were done with blood samples of prostate cancer patients and pseudo healthy individuals exposed to environmental pollutions. For this pilot study, a questionnaire was used to recruit 53 prostate cancer patients and 53 pseudo-healthy motorized bike drivers as well as matching healthy controls (already published elsewhere). Signed informed consent was collected before peripheral blood withdrawal in a 5 ml EDTA tube. DNA extraction was carried out in the laboratory with the phenol/chloroform method. The Multiplex polymerase chain reaction (PCR) was used to amplify and detect the presence of GSTM1 and GSTT1. **Results**: Our results showed that prostate cancer was more predominant in elderly men 60-80 years than 40-59 years ($p < 0.001$), and the majority of cancer patients were uneducated (62%). We have also noticed that most of them were polygamous (78%) and reside in urban areas. No significant association was observed between the loss of GSTM1 or GSTT1 and prostate cancer. However, the combined losses of GSTM1 and GSTT1 showed a significant association with prostate cancer ($P<0.05$). However, we have noticed more deletion of GSTT1 in prostate cancer. **Conclusion**: Overall, our study determines that the impairment of GSTM1 and GSTT1 is associated with prostate cancer. Thus, the deficiency of GSTM1 and GSTT1 could be used as a predictive biomarker of predisposition to cancers including prostate.

Keywords: *Prostate, Cancer, Glutathione-S-transferases, detoxification, gene, multiplex PCR*

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1. Introduction

The origin of neoplastic diseases is still the subject of several scientific studies, including the analysis of the imbalance between oxidation and reduction [1]. Many risk factors such as smoking, alcohol consumption, poor diet, absence of physical activities, hormonal and immune dysregulation, environmental pollution, epigenetic changes and genetic mutations can increase free radicals while decreasing the activity of antioxidants molecules that contribute to intracellular homeostasis [2]. If the quantity of free radicals is not neutralized by the detoxification enzymes, the organism becomes weak and defenseless to DNA damaging molecules. These damages include DNA mutations and genomic instability all of which leading to carcinogenesis.

Currently, the drastic increase in the incidence of cancer is a growing problem, representing the leading cause of death worldwide. According to the World Health Organisation (WHO), cancer is the cause of almost 10

million deaths worldwide annually [3,4]. The death rate linked to cancers showed that breast cancer ranks number one with 2.26 million cases; followed by lung cancer with 2.21 million cases; colorectal cancer with 1.93 million cases and in fourth position is prostate cancer with 1.41 million cases [3,4]. In Africa, the incidence of prostate cancer continues to rise each year with more than 1.1 million cancer cases and a mortality rate of up to 700,000 per year have been reported [5]. Cancer report showed that prostate cancer affected 1,931,590 men in 2020 with 375,304 deaths [3]. According to some reports, the European incidence of prostate cancer in 2013 was 12.4/100,000, while the mortality rate linked to that disease in Poland was 12.1/100,000 [6,7]. The frequency of prostate cancer in Benin in 2019 was 4.7 % [8]. More recently, the incidence of prostate cancer increased to 30.5 / 100,000 population [9]. Some prostate cancers can be attributed to genetic predisposition linked to BRCA1 mutations [10].

The search for genes associated with prostate cancer such as BRCA 1 and 2 has been addressed in several studies [10,11,12,13]. Candidate modifier genes for prostate cancer could include those involved in carcinogen metabolism such as Glutathione-S-transferase genes (GSTM1 and GSTT1). These genes code for cytosolic enzymes involved in the detoxification of various exogenous and endogenous reactive species in the body [14]. GSTM1 and GSTT1 function as dimers and catalyze the coupling of mutagenic electrophilic substrates to glutathione. The numerous GST family members are cytosolic, mitochondrial and microsomal proteins [15]. The cytosolic family has eight distinct classes which are: alpha (A), kappa (K), omega (O), pi (P), sigma (S), zeta (Z), mu (M) and theta (T) [16]. Each class is composed of several subunits members called genetic polymorphisms. Thus, allelic variants associated with altered detoxification function leading to potential carcinogens build up in the body, have long been postulated to confer increased susceptibility to cancers [16]. The GSTM1 and GSTT1 genes have functional polymorphisms that are frequently present and were more studied than the others. The GSTM1 gene is located on chromosome 1p13.3 and its homozygous deletion leads to the complete absence of its enzymatic activity [17]. The GSTT1 gene is located on chromosome 22q11.2 and its homozygous deletion is also a source of a defect in enzyme activity [18]. The deficiency of these enzymes leads to a detoxification defect causing the accumulation of free radicals and a genotoxic environment all together underlying several diseases such as cancers including the one of prostate [1,19]. Research has been carried out on the loss of DNA damage repair genes associated to prostate cancer in Africa [2,20]. Nevertheless, the involvement of GSTM1 or GSTT1 deficiency in prostate carcinogenesis was not reported.

The objective of this study is to evaluate the loss of GSTM 1 and GSTT 1 integrity due to mutation, and the association to the development of prostate cancer. It is understandable that deficiency in GSTM1 and GSTT1 may lead to carcinogenesis due to the disruptions of cellular detoxification processes. In this study we verified the association between the loss of GSTM 1 and GSTT 1 and the occurrence of prostate cancer.

2. Material and Methods

Our study populations consisted of prostate cancer patients who came to the health centers for post-diagnosis consultation and professional pseudo-healthy motorized bike drivers exposed to gas pollution environment and at risk to develop prostate cancer. We have already published the matching control data for GSTM1 and GSTT1 in healthy individuals [21]. Before entering the study, each participant gave written informed consent. The inclusion criteria were men over 30 years of age who were seen in consultation for prostate cancer and professional pseudo-healthy motorized bike drivers with at least five (05) years of service. We recruited 53 prostate cancer patients and 53 professional pseudo-healthy motorized bike drivers.

The protocol of this study was evaluated and approved by the Local Ethics Committee for Biomedical Research of the University of Parakou (CLERB-UP) under ref: 0416/ CLERB-UP/P/SP/R/SA before blood collection.

3. Extraction of genomic DNA

Venous blood was collected sterile in 5 ml EDTA tubes. DNA was extracted from white blood cells by the phenol/chloroform method [22]. DNA quantification was performed using nanodrop (Termo Scientific, Germany) and stored at -20 °C until use. DNA is of good quality when its ratio (260/280) is between 1.8 and 2.

Genotyping of GSTM1 and GSTT1 genes by multiplex PCR.

The multiplex PCR technic was carried out to amplify both GSTM1 and GSTT1 genes [23]. Set of two primer pairs were used (forward and reverse). The primer sequences and amplicon sizes are reported in Table 1.

Table 1. Sequences and sizes of GSTM1 and GSTT1 primers

Genes	Forward sequences	Reverse sequences	Size
GSTM1	5'- GAA-CTC-CCT-GAA-AAG-CTA-AAG-C-3'	5'- GTT-GGG-CTC-AAA-TAT-ACG-GTG-G-3'	219 pb
GSTT1	5'- TTC-CTT-ACT-GGT-CCT-CAC-ATC-TC-3'	5'- TCA-CCG-GAT-CAT-GGC-CAG-CA-3'	480 bp

To examine GSTM1 and GSTT1 polymorphisms, we amplified 100 ng of extracted DNA in a 20 µl reaction medium containing a mix master PCR reagent, 100 µM of each primer (Eurogentec, France) and sterile distilled water. PCR was performed using a personal thermal cycler (Mastercycler) with a program setup as follows : one cycle of 94 ° C for 5 minutes; followed by 35 cycles of [94 ◦C for 1 minute; 64 ◦C for 1 minute; 72 ◦C for 70 seconds and a final extension at 72 ◦C for 05 minutes]. The PCR products were migrated on 1.5 % agarose gel electrophoresis at 100V for 25 min and the amplified products stained with ethidium bromide were visualized with an UV trans-illuminator [21].

4. Statistical analysis

Data were processed with Statistical Package for Social Science version 19 (SPSS 19). Genotypic frequencies of GSTT1 and GSTM1 gene polymorphisms in patients were performed by direct counting method. Pearson's Chi-squared test was used to compare categorical variables and to test for an association between polymorphisms and prostate cancer. The strength of the association between GSTT1, GSTM1 polymorphisms and prostate cancer was estimated by calculating the Odds Ratio (OR) and the 95% confidence interval (CI). Pearson correlation was used to investigate the strength of the association between age, marital status, residence and prostate cancer occurrence. A p-value of less than 0.05 ($P < 0.05$) indicates a statistically significant difference.

5. Results

Prostate cancer patients and pseudo healthy motorized bike drivers were surveyed for their socio-demographic status as previously [3,20]. The prostate cancer patients were spread over all age groups, with a clear predominance of 60-80 years and 40-59 years. For the pseudo healthy motorized bike drivers, the age distribution shows that 84% and 68% are respectively in the 30-40 years and 59-60 years age groups, but none are in the other age groups. Prostate cancer was more present in age group of 60-80 years old. The age difference between the two study populations was statistically significant with $P < 0.001$ (Table 2). As for their educational levels, the majority of prostate cancer patients were uneducated (62%) and 35% educated; while in pseudo-healthy motor bike drivers 38% were uneducated and 65% educated. The pseudo-healthy motor bike drivers were significantly more educated than the prostate cancer patients with a $P < 0.005$.

In this study, the marital status of our participants was addressed and showed that 78% of prostate cancer patients were polygamous versus 32% monogamous. In contrast, 68% of the pseudo-healthy motorized bike drivers were monogamous and 22% were polygamous.

Men with prostate cancer reside more in urban areas than in peri-urban and rural areas, as well as pseudo-healthy motorized bike drivers.

The frequencies of homozygous deletion of the GSTM1 in the prostate cancer patients and the pseudo-healthy motorized bike drivers were 49% and 51% respectively. The difference was not statistically significant (*P= 0.68; OR = 1.19; CI: 0.53-2.71*) (Table 3). The frequencies of homozygous deletion of the GSTT1 in prostate cancer patients and pseudo-healthy motorized bike drivers were 52% and 48% respectively. But this difference was not statistically significant (*P = 0.43*; *OR = 0.65*; *CI: 0.22-1.86*). However, we have observed more loss of GSTT 1 in prostate cancer patients than in pseudo-healthy motorized bike drivers.

To definitively point out the association between the loss of GSTM1 and GSTT1 genes and prostate cancer development, we combined the loss of these two genes in prostate cancer patients and compared to their losses in pseudo-healthy motorized bike drivers. The losses of GSTM 1 and GSTT 1 were significantly more pronounced in prostate cancer patients than in motorized bike drivers. We observed that in the majority of prostate cancer patients GSTT 1 was mutated or absent (Figure 2).

The metabolism of xenobiotics often leads to toxic metabolites capable of causing changes in the detoxification enzyme genes. In our study, we investigated the impact of GSTM1 and GSTT1 polymorphisms on the risk of developing prostate cancer in a Beninese population. The genotypes of the GSTM1 and GSTT1 genes were characterized using the multiplex PCR technics (Figure 1). The image shows the agarose gel with different bands of the GSTM1 and GSTT1 genes.

Table 2. Socio-demographic characteristics of prostate cancer patients and motorized bike drivers

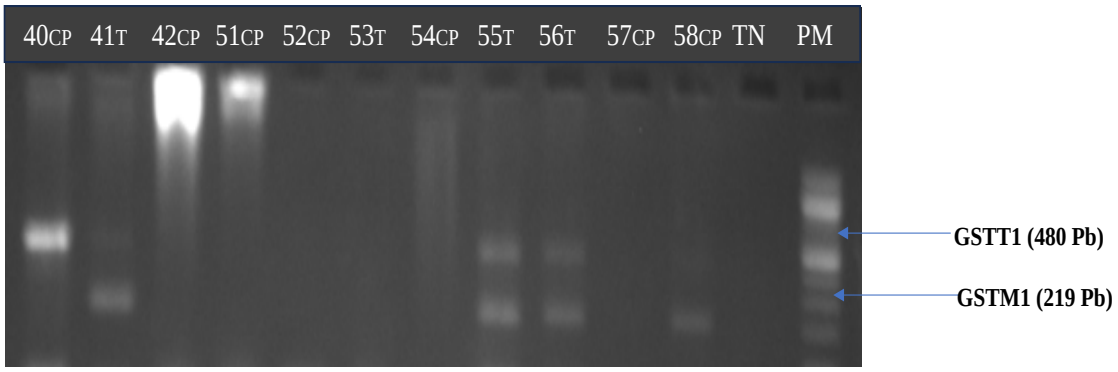
Characteristics		Prostate cancer patients	Motorized bike drivers	P-Value
Age	[20-40]	04 (16 %)	21 (84 %)	<0.001
	[40-60]	15 (32 %)	32 (68 %)	
	[60-80]	33 (100 %)	-	
	[80-100]	01 (100 %)	-	
Educational level	educated	16 (35 %)	30 (65 %)	<0.006
	Uneducated	37 (62 %)	23 (38 %)	
marital statue	Monogamous	21 (32 %)	44 (68 %)	<0.001
	Polygamous	32 (78 %)	09 (22 %)	
Residence	Urban	23 (74 %)	08 (26 %)	<0.005

Table 3. Distribution of GSTT1 and GSTM1 genotypes in the prostate case and motorized bike drivers

Genotypes		Prostate cancer patients	Motorized bike drivers	OR	95%CI	P-value
GSTM 1	GSTM 1 (-)	35 (49 %)	37 (51 %)	1.19	0.53-2.71	0.68
	GSTM 1 (+)	18 (53 %)	16 (47 %)			
GSTT 1	GSTT 1 (-)	46 (52 %)	43 (48 %)	0.65	0.22-1.86	0.43
	GSTT 1 (+)	07 (41 %)	10 (59 %)			

Distribution of GSTT1 and GSTM1 genotypes in the prostate cancer case and pseudo healthy motorized bike drivers groups

In order to investigate the effect of age on the homozygous deletion of GSTM1(-) and GSTT1(-) on prostate cancer incidence, we asked these patients and pseudo-healthy motorized bike drivers about their ages. This allowed us to examine the correlation between age and the absence of GSTM1 and GSTT1 in the occurrence of prostate cancer. The results in the Table 4 show that ages were significantly correlated with the polymorphisms and the loss of the genes.



T : Motorized bike driver, CP : Prostate Case, PM: Molecular weight marker, TN: Negative control

Figure 1. GSTM1 and GSTT1 profile of the prostate case and pseudo healthy motorized bike drivers groups

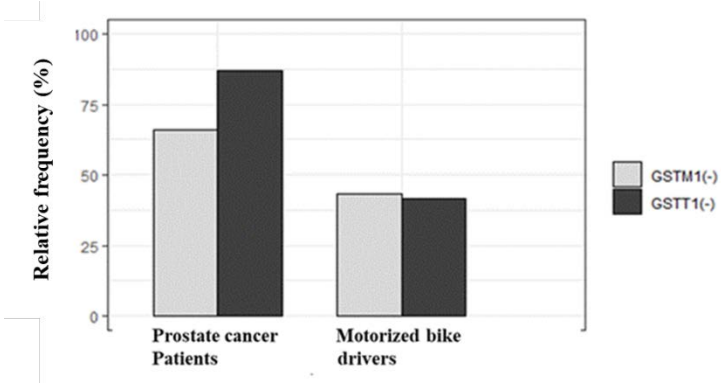


Figure 2. Distribution of absence of polymorphisms according to the groups prostate cancer (case) and pseudo healthy motorized bike driver (témoin)

Correlation of GSTM1(-) and GSTT1(-) according to the age of the participants

Table 4. Correlation between age, marital status, residence and deletion of GSTM1 and GSTT1

	Prostate cancer cases	Motorized bike drivers	P-Value
GSTM 1 (-)			
[20-40[	02 (12 %)	14 (88 %)	0.001
[40-60[	10(30 %)	23 (70 %)	
[60-80[	22 (100 %)	-	
[80-100[	01 (01 %	-	
GSTT 1 (-)			
[20-40[	04 (21 %)	15 (79 %)	0.001
[40-60[	13 (32 %)	28 (68 %)	
[60-80[	28 (100 %)	-	
[80-100[	01 (100 %)	-	
Monogame	14 (33 %)	29 (67 %)	0.001
Polygames	21 (72 %)	08 (28 %)	
GSTT 1 (-)			
Monogame	18 (33 %)	37 (67 %)	0.001
Polygames	28 (82 %)	06 (18 %)	
GSTM1 (-) et GSTT1 (-)			
Urbane	16 (76 %)	05 (24 %)	0,034
Peri-urbane	10 (40 %)	15 (60 %)	
Rural	09 (45 %)	11 (55 ¨)	

The correlation between the marital status and the loss of GSTM 1 and GSTT 1 among our two study populations and their association to prostate cancer risk are summarized in Table 3. Losses of GSTM1 and GSTT1 genes were more common in prostate cancer cases compared to pseudo-healthy motorized bike drivers and showed a statistically significant difference *P= 0.001*.

We have also correlated the area of residence with the deletion of GSTM1 and GSTT1 (Table 3). Our results showed that the residence areas were more associated with the deletion of GSTM1 and GSTT1 genes. Comparison of the double null deletion of both genes with prostate cancer has also showed significant associations (*P=0.034*).

6. Discussion

Prostate cancer is a multifactorial disease with a complex etiology in which genetic and environmental factors combined, contribute to the pathogenesis of the disease. Among the genetic factors, the role of GSTs has received particular attention as a potential risk factor for prostate cancer [24,25]. Understanding the contribution of GST polymorphisms and their interactions with other relevant factors can improve some pre-diagnosis tests for prostate cancer risk. Thus, in this study, we evaluated the potential association of GSTM1 and GSTT1 deletion with prostate cancer in the Beninese population. The present study comparing GSTM1 and GSTT1 profile among prostate cancer patient and motorized bike drivers showed that prostate cancer is predominantly found in the age group of 60 - 80 years with a statistically significant difference (*P= 0.001*). Our results showed that this age correlates with the WHO age of prostate cancer development. These results are in line with works done in United States of America (USA) and China in which it was shown that the mean age of prostate cancer patients was 65 years [26,27]. However, our results were in contrast to a study reported by Huang who found an

increase in the incidence of prostate cancer in younger men under the age of 50 [9,28]. The educational level of prostate cancer patients and motorized bike drivers were also addressed in this study. Our results revealed that prostate cancer patients' group has more uneducated individuals (62%) than the one of motorized bike drivers with a significance of *P= 0.006*. This increase could be explained by the lack of awareness and information about prostate cancer. In other studies, prostate cancer patients have a good level of education which contrasts with the results of our study [29,30,31]. Marital status was also a risk factor for prostate cancer. Indeed, both polygamous and monogamous individuals were included in this study and our results showed that the disease affects more polygamous (78%) than monogamous (32%) compared to pseudo-healthy motorized bike drivers (*P= 0.001*). Previous studies have shown that prostate cancer patients were less polygamous than other statuses but this is not associated with prostate cancer and widowed men suffer more from this disease than others [32,33,34]. In Benin, marital status may be an independent predictor of prostate cancer and overall mortality in men developing that disease.

Geographical considerations show that majority of men with prostate cancer, lived in the urban area (74%), compared to rural area (46%) and peri-urban area (37%) with *P=0.005*. This could be explained by the presence of pollutants that they inhaled through briefing air causing then many pathologies in Beninese population [21] Overall, considering the living geographical location, the incidence of prostate cancer risk did not differ among the two study groups.

- Cellular dysfunction resulting from polymorphisms and post-translational modifications such as gene promoter methylation contributes to the risk of prostate cancer. Changes in GST gene expression are a risk factor involved in the development many affictions including prostate cancer [25]. This dysfunction may initiate the accumulation of reactive oxygen species (ROS) responsible for DNA damages and mutations in cells. These damages include homozygous double deletion (GSTM1-/GSTT1-) which could exacerbate prostate cancer pathogenesis. The present study in Benin is the first to assess the GSTM1 and GSTT1 polymorphism in prostate cancer patients and compares it to pseudo-healthy motorized bike drivers in order to highlight the risk of prostate cancer to which the latter are exposed. The results of this study show that there is more loss of GSTM 1 in pseudo-healthy motorized bike drivers (51) than in prostate cancer patients (49%). We do not find a significant association between the loss of the GSTM1 gene and prostate cancer incidence (p = 0.68; OR = 1.19; CI: 0.53-2.71). The increased loss of the GSTM1 gene may be due to repetitive exposures to toxic metabolites present in inhaled polluents. Pseudo-healthy motorized bike drivers have briefed more of these environmental toxic polluents and they are at risk of developing pathologies among which we have prostate cancer. Regarding GSTM1, our data were in agreement with previously published results that revealed a higher loss of GSTM1 in pseudo-healthy motorized bike drivers with a frequence of 55% compared to healthy controls with a frequence of 47% [24,37,38]. The loss of GSTM1 in prostate cancer patients has a non-significant association [24,37,38]. Our results are in contrast to other studies that have reported a significant association of GSTM1 deletion with prostate cancer cell proliferation [24]. In our study we have also investigated the association of GSTT1 polymorphism with the risk of developing prostate cancer. The results showed that there is more loss of GSTT 1 in our study prostate cancer patients (52%) compared to pseudo-healthy motorized bike drivers (48%). This statistical analysis revealed a non-significant difference (p = 0.43 ; OR = 0.65; CI: 0.22-1.86). Our

Considering the combination of the absence of amplified PCR products for both GSTM1 and GSTT1 genes (fig. 2), our results showed that the loss of GSTM1 and GSTT1 genes is higher in prostate cancer patients than in motorized bike drivers. This difference was statistically significant (P< 0.05). Additionally, the loss of GSTT1 is higher than the one of GSTM1 in prostate cancer patients. Our results confirm those previously reported after investigating the combined deletion of both genes and showed a significant increase in prostate cancer risk in men with the GSTM1/GSTT1 double deletion [39]. As age is one of the parameters highlighted in the genesis of prostate cancer, we correlated the deletion of both GSTM1/GSTT1 genes with ages. Our statistical analyses showed a significant correlation between the deletion of both GSTM1/GSTT1 genes and the ages of the two study groups (p = 0.001). These results highlight that as age increases, men are at greater risk of developing prostate cancer. Our results are consistent with those of Malik et al. who showed that the loss of GST and acquired polymorphisms changes with age [40].

The correlation of the deletion of both genes with the places of residence was addressed in this study. The results showed a significant correlation between the loss of GSTM 1/GSTT 1 and the place of residence of the study population (p= 0.034). This difference could be explained by the presence of mutagenic and carcinogenic compounds such as polycyclic aromatic hydrocarbon particles responsible for the generation of free radicals [41]. Free radicals binding to DNA initiate mutations or act as secondary messengers modifying the regulation in the cell leading to higher the risks of developing prostate cancer. All of these pointed out the importance of healthy nutrition with food containing anti-oxidants molecules to neutralize free radicals while reducing genomic instability [10,21].

In conclusion, we have observed an association between the deletion of both GSTM1 and GSTT1 genes and prostate cancer. Therefore, deletion of these two genes increases the susceptibility of pseudo-healthy motorized bike drivers to develop prostate cancer. Furthermore, in the group of pseudo-healthy motorized bike drivers, the loss of GSTT1 is more likely to be a risk factor for prostate cancer when associated to GSTM1 deletion. Our results suggest that the GSTT1 genes may be a predictive biomarker of prostate cancer susceptibility.

Hence, the pre-screening of prostate cancer risk is possible with the assessment of GSTM1/GSTT1 profile in the general population and could also be prevented with healthy anti-oxidants containing nutrition.

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Conflict of interest

The author declares no conflict of interest

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