Research Article

Effect of ageing and Single Nucleotide Polymorphisms associated with risk of aggressive prostate cancer in a New Zealand population

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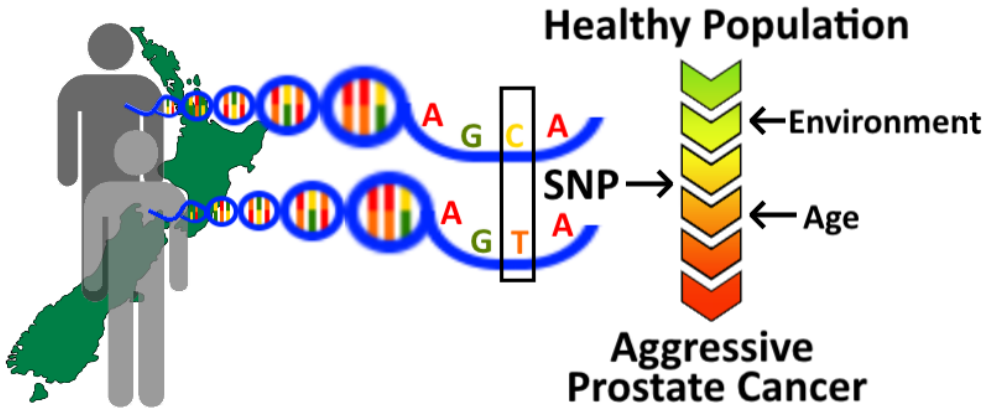
Abstract: Prostate cancer is one of the most significant male health concerns worldwide, various researchers carrying out molecular diagnostics have indicated that genetic interactions with biological and behavioral factors play an important role in the overall risk and prognosis of this disease. Single nucleotide polymorphisms are increasingly becoming strong biomarker candidates to identify susceptibility of prostate cancer. We carried out risk association of different stages of prostate cancer to a number of single nucleotide polymorphisms to identify the susceptible alleles in a New Zealand population and checked the interaction with environmental factors as well. We have identified a number of single nucleotide polymorphisms to have associations specifically to the risk of prostate cancer and aggressiveness of the disease, and also certain single nucleotide polymorphisms to be vulnerable to the reported behavioral factors. We have addressed "special" environmental conditions prevalent in New Zealand, which can be used as a model for a bigger worldwide study.

Pictorial Abstract:



Keywords: prostate cancer; SNP genotyping; ageing; SEQUENOM MassArray technology

1. Introduction

Prostate cancer (PCa) is one of the most significant non-skin cancer male health concerns worldwide [1]. Moreover, it is estimated that at least 1 in 6 PCa patients is at risk of developing aggressive PCa [2]. These are very alarming statistics. The identification of a predictive biomarker and/ or treatment of this disease is therefore of much importance, more so from the New Zealand point of view, because the highest rate of recording of men with PCa, relative to the population of men, is observed in the Oceania region [3,4]. With various biological and behavioral factors established as playing crucial role in the overall risk and prognosis of PCa [1,5-7], SNPs are increasingly appealing biomarker candidates for the identification of PCa susceptibility [8-10].

Although, age, ethnicity, and family history are the three most widely accepted risk factors for PCa [7,11,12], yet nothing much can clinically be done to alter or reverse the effect of these on human health and immunity. Of these three risk factors , age is the most significant risk factor for aggressive PCa [13,14]. In the same line, we believe that healthy ageing, can control the expression of the aggressive form of this disease.

We recently identified gene x environment interaction(s) and the risk of aggressive PCa in a New Zealand population and defined a trend that certain lifestyle habits and effects such as tobacco smoking, and high body mass index (BMI), also have an influence on the aggressiveness of the disease [1]. Even with progressing age, which cannot be curtailed, certain lifestyle habits may stay put. Here, we employed some statistical tools and analysed data generated by genotyping single nucleotide polymorphisms (SNPs) of interest to understand the effect of ageing on external factors and effects such as tobacco smoking, alcohol consumption; and high BMI and risk of aggressive PCa.

Here we present the analysis of the data obtained following the genotyping of 138 SNPs, using SEQUENOM MassArray iPLEX® assay and TaqMan® SNP genotyping procedures in a New Zealand cohort. The cohort includes New Zealand men of self-declared European ethnicity with different clinically diagnosed grades/stages of PCa, and gender matched healthy controls within similar age range. We have identified the association of SNPs as risk for aggressive PCa as well as the influence of external factors including age in risk modification. This, we believe, is the first such study on genetic and environmental risk association with ageing and risk of aggressive PCa in a New Zealand cohort.

2. Materials and Methods

2.1 Study population

A total of 254 patients with different clinical classifications of PCa voluntarily participated in our study after providing informed consent, as mentioned in Vaidyanathan et al., (2017) (Ethics reference NTY05/06/037 by Northern B Ethics Committee, New Zealand, previously, Northern Y Ethics Committee, New Zealand) [1]. Additionally, 369 males from the Auckland region of New Zealand who had no reported clinical diagnosis of PCa were considered as healthy controls for this study (Ethics reference NTY/06/07/AM04 by Northern B Ethics Committee, New Zealand, previously, Northern Y Ethics Committee, New Zealand).

Because of the influence of age in this disease [14], care was taken to invite men between the age categories of 40 to 90 years (at the time of diagnosis for patients with PCa and at the time of recruitment for healthy controls) to participate in this study. We have considered men more than 65 years of age as elderly or older person, as per the norms of World Health Organization (WHO) [15].

2.2 Definition of aggressiveness:

The aggressiveness of PCa, for this study, is based on the classification followed by the American Urological Association [16]. This schema of classification, first proposed by D'Amico *et al*. (1998), defines high-risk or aggressive PCa as clinical T stage ≥cT2c, or Gleason score ≥8, or serum PSA level >20ng/ml [17].

2.3 Statistical analysis:

SNP genotyping was done for a total of 136 SNPs, but after checking for compliance with Hardy Weinberg Equilibrium (HWE), and in linkage, 97 SNPs were employed for the final analysis [1]. The HWE and linkage analyses were done by employing P-Link software version 1.07 [18].

Compared groups	Pathology	N′			Percentage of men ≥65 years	OR (95% CI)	p-value
		G1 (≤64 years)	G2 (≥65 years)	Total			
Aggressive vs Healthy Control	Aggressive	90	107	197	54.31%	3.070334 (2.1399 – 4.4052)	**7.979E-10**
	Healthy Control	266	103	369	27.91%		

Analysis of the data previously reported for SNPs association with PCa based on aggressiveness and gene x environment interaction [1] was further analysed for the influence of age using P-Link software version 1.07 [18] and reported in tables 2.1 to 2.3. The analysis of the influence of age was not reported prior as it was beyond the scope of the theme focused at that time. In order not to miss any relevance, to the progression of PCa, we carried out the analysis under three broad classifications being between patients with aggressive PCa and healthy controls, between patients with non-aggressive PCa and healthy controls and between patients with aggressive PCa and non-aggressive PCa. Statistical significance for variation was set at $p<0{\cdot}05$. Correction for multiple testing was applied to the analysed data obtained, so as to maintain the linearity of genotype-phenotype relationship [19]. As the tested SNPs are already proven as associated with PCa incidence by other researchers, variations that showed significance before Bonferroni correction were also considered for discussion in our study [1].

3. Results

3.1 Age, Pathology, BMI and lifestyle:

Since the main aim of this article is to identify the role of ageing and statistically adjusting for this parameter in isolation and in combination with various demographic factors such as alcohol consumption, smoking tobacco, and with levels of obesity among the patients recruited for our study, we are presenting the data for variation in age as risk for aggressive PCa in Tables 1.1 to 1.3.

Table1.1: Association between age and aggressive prostate cancer versus healthy controls.

Table1.2: Association between age and aggressive prostate cancer versus non-aggressive prostate cancer.

Table1.3: Association between age and non-aggressive prostate cancer versus healthy controls.

Tables 1.1- 1.3 legend: N'= number; OR= Odds Ratio; 95% CI= 95% confidence interval

3.2 Genetic polymorphism variations and risk of prostate cancer:

The tables show the results of the statistically significant SNPs associated with risk of PCa between patients with aggressive PCa and healthy controls (Table 2.1), between patients with aggressive and non-aggressive PCa (Table 2.2), and patients with non-aggressive PCa and healthy controls (Table 2.3), all assessed before and after the adjustment for various demographic parameters with and without age aspect. Variations in the tested allele between patients recruited for this study with aggressive PCa, non-aggressive PCa and healthy controls for all the SNPs irrespective of statistical significance have been included in Supplementary Tables 1a and 1b and 2. The relevant 95% CI range has also been mentioned in the supplementary table.

Compared groups	Pathology	N'			Percentage of men ≥65 years	OR (95% CI)	p-value
		G1 (≤64 years)	G2 (≥65 years)	Total			
Aggressive vs Non-Aggressive	Aggressive	90	107	197	54.31%	0.642643 (0.3485 – 1.1850)	0.173763
	Non-Aggressive	20	37	57	64.91%		

Compared groups	Pathology	N'			Percentage of men ≥65 years	OR (95% CI)	p-value
		G1 (≤64 years)	G2 (≥65 years)	Total			
Non-Aggressive vs Healthy Control	Non-Aggressive	20	37	57	64.91%	4.778 (2.649 – 8.615)	**9.3852E-8**
	Healthy Control	266	103	369	27.91%		

117 **Table 2.1**: Statistically significant SNP associated with gene x environment effect on risk of aggressive prostate cancer
118 v/s healthy controls after adjusting for each environmental parameter individually and along with age

Sl. No.	1	2	3	4	5	6	7
Gene location	1p22.3	2p23.1	3p22	3p25	6p21.3	7q33	8q24
SNP ID	rs5845	rs632148	rs1799977	17793693	rs130067	rs10244329	rs6983561
Tested allele	C	C	A	A	A	T	A
Gene name	*SEP15*	*SRD5A2*	*MLH1*	*PPARG*	*CCHCR1*	*LEP*	
Before any adjustment – p Value				0.000173	0.03656	0.02344	0.02883
Before any adjustment – OR				4.534	1.383	1.557	1.885
After adjustment for Age – p Value		0.02485			0.03288	0.04731	
After adjustment for Age – OR		1.642			1.5852	1.526	
BMI – After adjustment for BMI – p Value		0.03795			0.04157	0.02679	
BMI – After adjustment for BMI – OR		1.572			1.5405	1.59	
BMI – After adjustment for BMI + Age – p Value		0.02643			0.04695	0.04839	
BMI – After adjustment for BMI + Age – OR		1.637			1.5365	1.528	
Tobacco smoking – After adjustment for Tobacco smoking – p Value					0.0275	0.02088	
Tobacco smoking – After adjustment for Tobacco smoking – OR					1.5964	1.622	
Tobacco smoking – After adjustment for Tobacco smoking + Age – p Value		0.03503			0.03241	0.04413	
Tobacco smoking – After adjustment for Tobacco smoking + Age – OR		1.595			1.6002	1.54	
Alcohol consumption – After adjustment for Alcohol consumption – p Value	0.04139		0.01607			0.03328	
Alcohol consumption – After adjustment for Alcohol consumption – OR	1.6217		1.547			1.624	
Alcohol consumption – After adjustment for Alcohol consumption + Age – p Value			0.03068				
Alcohol consumption – After adjustment for Alcohol consumption + Age – OR			1.498				

17	16	15	14	13	12	11	10	9	8
19q13	19q13	17q21	17q25	11q12	11q13			10p15	10q11
rs2659122	rs887391	rs799923	rs6502051	rs2727270	rs11228565	rs10896438	rs7931342	rs12529	rs7920517
A	A	T	C	C	G	T	G	C	A
KLK3	*SLC26A6*	*BRCA1*	*FASN*	*FADS2*	*MYEOV*			*AKR1C3*	*MSMB*
0.04748	0.005094			0.04184	0.02189	0.002322	0.0007423	0.04685	0.01227
1.345	1.594			1.525	1.433	1.4985	1.565	1.294	1.400
0.04928	0.01177		0.04996		0.009652	0.0007877	0.001017		0.006002
1.5163	1.845		1.329		1.9872	1.6231	1.6		1.4729
0.0305	0.01913				0.01887	0.001213	0.000222		0.005373
1.5673	1.744				1.8382	1.5810	1.682		1.4679
0.03905	0.009695		0.04415		0.01579	0.000432	0.000271		0.002065
1.5750	1.892		1.346		1.90439	1.6806	2.2742		1.5615
0.04364	0.02494	0.04582			0.01279	0.001449	0.000637	0.0378	0.01025
1.5151	1.702	1.5384			1.9080	1.5669	1.612	1.3049	1.4196
	0.01295				0.01067	0.0006136	0.000783		0.005115
	1.84				1.9704	1.6463	1.626		1.4861
0.01887	0.04069				0.04425	0.001049	0.001481	0.0258	0.004713
1.6498	1.653				1.7418	1.61134	1.593	1.3424	1.5035
0.02252	0.02386		0.03186		0.03105	0.000411	0.001756		0.00248
1.6556	1.775		1.392		1.8549	1.7070	1.598		1.5710

18		rs17632542	C		0.008268	1.998														
19	20q13	rs3918256	C	*MMP9*							0.04894	1.3116								
20	Xp11	rs5945619	T	*NUDT11*	0.005749	1.694														

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Table 2.2: Statistically significant SNP associated with gene x environment effect on risk of aggressive prostate cancer v/s non-aggressive prostate cancer after adjusting for each environmental parameter individually and along with age

Sl. No.	1	2	3	4	5	6	7
Gene location	2p23.1	2q37.2	7q32	7q33	9q33.1	19q13	19q13.33
SNP ID	rs632148	rs2292884	rs3735035	rs10244329	rs11536889	rs887391	rs17632542
Tested allele	C	A	T	T	G	C	T
Gene name	*SRD5A2*	*MLPH*	*PODXL*	*LEP*	*TLR4*	*SLC26A6*	*KLK3*
Before any adjustment							
p Value	0.01731	0.02614	0.03126	0.03222	0.02251	0.02063	0.04647
OR	1.799	1.801	1.621	2.062	2.303	1.793	3.194
After adjustment for Age							
p Value	0.0124		0.02702	0.03285			
OR	2.121		1.674	2.318			
BMI							
After adjustment for BMI							
p Value	0.01134		0.03816	0.03593			
OR	2.138		1.612	2.288			
After adjustment for BMI + Age							
p Value	0.01165		0.02806	0.03623			
OR	2.14		1.67	2.286			
Tobacco smoking							
After adjustment for Tobacco smoking							
p Value	0.01361		0.03528	0.02697			
OR	2.097		1.625	2.417			
After adjustment for Tobacco smoking + Age							
p Value	0.01396		0.02661	0.02732			
OR	2.097		1.676	2.413			
Alcohol consumption							
After adjustment for Alcohol consumption							
p Value	0.009997		0.0361	0.03179			
OR	2.173		1.62	2.332			
After adjustment for Alcohol consumption + Age							
p Value	0.01083		0.02693	0.03143			
OR	2.165		1.674	2.339			

8	20q13.12	rs3918256	A	*MMP9*	0.04959	1.555	0.02392	1.9260	0.0324	1.8601	0.02871	1.8925	0.02672	1.8989	0.0219	1.9493	0.02991	1.8733	0.02456	1.9215

Table 2.3: Statistically significant SNP associated with gene x environment effect on risk of non-aggressive prostate cancer v/s healthy controls after adjusting for each environmental parameter individually and along with age

Sl. No.	Gene location	SNP ID	Tested allele	Gene name	Before any adjustment		After adjustment for Age		BMI				Tobacco smoking				Alcohol consumption			
									After adjustment for BMI		After adjustment for BMI + Age		After adjustment for Tobacco smoking		After adjustment for Tobacco smoking + Age		After adjustment for Alcohol consumption		After adjustment for Alcohol consumption + Age	
					p Value	OR	p Value	OR	p Value	OR	p Value	OR	p Value	OR	p Value	OR	p Value	OR	p Value	OR
1	2q37.2	rs2292884	G	*MLPH*	0.02375	1.774														
2	7q32	rs3735035	C	*PODXL*	0.03493	1.572														
3	9q33.1	rs11536889	C	*TLR4*	0.02727	2.198														
4	15q26.3	rs4965373	A	*SEPS1*	0.02413	1.801														

Tables 2.1- 2.3 colour legends risk association:

	SNPs statistically significantly associated with risk of aggressive PCa across various classifications both, before and after adjusting for the environmental and age parameters

4. Discussion

It is well-established that there are three major risk factors for PCa, namely, advancing age, ethnicity, and familial history [11]. Recent studies indicate alterations in genetic and epigenetic make-up as the basis for the development of various malignancies [20] and is in line with our findings with regards risk of aggressive PCa [1]. In the current article, the data obtained by SNP genotyping and reported in Vaidyanathan *et* al., (2017) [1] was further analysed to identify risk association with aggressive PCa with the effect of non-genetic or environmental factors after being adjusted statistically with and without the influence of ageing on them.

Out of the 97 SNPs studied by us, only 5 SNPs were identified to be significantly associated with risk of aggressive PCa when compared with healthy control across all combinations before and after adjustment, 4 SNPs were significantly associated with risk of aggressive PCa when compared with non-aggressive PCa across all combinations before and after adjustment, and no SNPs were identified to be significantly associated with risk of non-aggressive PCa compared to healthy controls across all combinations before and after adjustment.

Although the genome-wide association studies (GWAS) are used for the identification of the direct role SNP association plays as for aggressive PCa, yet we believe that SNP interactions with demographic and lifestyle factors could also add to the allelic effect producing a modified risk of a disease. These SNPs identified herewith to have come up significant could be indicating a unique situation for New Zealand men with PCa, and can be used as a model for other chronic diseases.

4.1 Age at diagnosis and age at recruitment (prostate cancer patients and healthy controls respectively) and risk of prostate cancer:

Age is a major risk factor for PCa, as reported [14,21]. However, in the data presented in our present study we did not consider the role of ageing, as we wanted to see the effect of gene and environment aspects in the expression and progression of PCa. Age, being irreversible, but other environmental factors being more under one's control we focused on those aspects to identify any link and define the means by which high-risk PCa can be controlled.

We found correlation of age to aggressive PCa when compared to healthy controls. It is often suggested that older men (≥65 years of age) are more likely to develop the aggressive form of PCa, if they develop PCa, and are also more likely to die of the same as compared to younger men (≤64 years of age) [22]. This is in line with the findings in our cohort as well (Table 1.1). Consistent with the findings of other groups, we found that age of an individual is associated with risk of non-aggressive PCa when compared with healthy controls (Table 1.3), but has no significant correlation with aggressive PCa when compared with non-aggressive PCa (Table 1.2), as is understandable. Diseases such as PCa often have an onset with progressing age [23], but the aggressiveness may not be solely age-dependent [1].

4.2 BMI, smoking tobacco, and alcohol consumption (external factors) at recruitment and risk of prostate cancer:

In our previous approach, we combined the effect of the three external factors to extract as much from the prevalent factors common among New Zealand men and risk of PCa and not miss any SNP of interest. However, in this current analysis, we split the three parameters, and analysed the effect they have individually and with age as well as risk for PCa with statistical adjustments.

The data for the demographic analyses related to high BMI, tobacco smoking, and alcohol consumption has previously been reported [1].

4.3 Gene x environment interaction and risk of prostate cancer and effect of adjustment for age:

Knowledge of gene x environment interaction is important for risk prediction and the identification of certain high-risk populations to inform public health strategies for targeted prevention [24]. We associated the environmental factors with the genotypes of the men in our study to identify the risk alleles for specific kind of external factors such as BMI, smoking tobacco and alcohol consumption. Since these factors play an important role in the risk association of PCa and yet can be controlled by individuals, it is therefore of importance to understand and limit this disease.

4.3.1 SNP genotyping, the effect of environmental factors, and of age as a risk of aggressive prostate cancer vs healthy controls:

We had previously identified 14 SNPs when we analysed the data for gene x environment interactions without any adjustments (Table 2.1) [1]. This gave us a good idea of the influence of environmental factors on various SNPs in and near certain genes, and the prevalent environmental conditions in New Zealand. Of the 14 SNPs, three were found near the gene *MYEOV* (*Myeloma Overexpressed*)- rs7931342, rs10896438, rs11228565; two near the gene *KLK3* (*Kallikrein-3*)- rs2659122, rs17632542; and one each near the genes *MSMB* (*Microseminoprotein Beta*)- rs7920517, *FADS2* (*Fatty acid desaturase 2*)- rs2727270, *LEP* (*Leptin*)- rs10244329, *PPAR-γ* (*Peroxisome Proliferator-Activated Receptor Gamma*)- rs17793693, *CCHCR1* (*Coiled-Coil alpha-Helical Rod protein1*)- rs130067, *AKR1C3* (*Aldo-Keto Reductase family 1 member C3*)- rs12529, *SLC26A6* (*Solute carrier family 26 member 6*)- rs887391, and *NUDT11* (*Nucleoside Diphosphate-linked Moiety X Motif 11*)- rs5945619; and in the region 8q24- rs6983561.

These results were partly expected and partly novel to New Zealand conditions and the risk of aggressive PCa. *MYEOV* is a putative oncogene [25], and it made absolute sense that the highest number of SNPs were recorded in this gene with regards aggressive PCa in our population [1]. The genes *KLK3*, and *MSMB* are both involved in the PSA metabolism pathway were understandably identified as statistically significant in our study, due to their proven risk association to PCa, and same with the SNP in *AKR1C3* [1,4,7,26] and the SNP in *CCHCR1*, which has been previously reported in rheumatoid arthritis- a possible side-effect of androgen deprivation therapy for PCa [20]. The gene *SLC26A6* is a fusion gene and plays a vital role in the development and progression of a number of cancers and is interestingly just 10Mb centromeric to the gene *KLK3*, which we have already identified as an important gene of interest with regards studies on PCa [27]. *NUDT11* is a paralogous human gene, and is predominantly expressed in the testes, and assumed to be playing a major role in signal transduction [28,29]. Various GWAS and case control studies have also indicated about the susceptibility locus at *NUDT11* being involved with the risk of PCa [30-32]. The presence of a SNP as risk for PCa in the gene desert region of 8q24 has also been observed in a number of cancers including the prostate [33].

With no direct connection yet established between obesity and risk of PCa, it was interesting to find SNPs associated with risk of PCa in our population in 3 genes. The genes *FADS2*, *LEP*, *PPAR-γ* are associated with obesity and diabetes mellitus which is a major risk of PCa [1,34,35]. This is interesting because New Zealand has the third highest adult obesity rate among Organisation for Economic Co-operation and Development countries [36], and is a major external factor in the potential risk for aggressive PCa [37].

When we, next, adjusted the SNP genotyping data for age of the cohort and continued to analyse the data, we found certain SNPs to have lost their power of statistical significance on risk of aggressive PCa, and certain SNPs were identified statistically significant which were not identified without the adjustment. SNPs rs632148 and rs6502051 in genes *SRD5A2* (*Steroid 5α-reductase type 2*) and *FASN* (*Fatty Acid Synthase*) respectively were identified as statistically significant to the risk of aggressive PCa when compared to healthy controls. The gene *SRD5A2* has previously been reported by groups working on various aspects related to and causing PCa in Caucasian populations and not restricted only to studies discussing its role in the quality of sperms [38]. It is well established that with progressing age, there is a drop in testicular function, and thus certain genes pertaining to virility, including *SRD5A2*, may be functioning differentially [39]. The SNP in a gene pertaining to obesity [40,41]- *FASN* also identified as a risk for aggressive PCa is also in line with the theory that ageing may cause certain physiological alterations leading to major effects such as , and not limited to, PCa [14]. Since obesity is classically considered to be proportional to progressing age [42], we feel that our findings are further strengthening the theory of age as a risk factor for PCa [14], especially aggressive PCa. The other SNPs that were identified to be statistically associated as risk for aggressive PCa, even after the adjustment for age, were rs7931342, rs10896438, and rs11228565 near the gene *MYEOV*; rs7920517 near the gene *MSMB*, rs2659122 near the gene *KLK3*; rs10244329 near the gene *LEP*; rs130067 *CCHCR1*; and rs887391 *SLC26A6*.

Next, we adjusted the data for BMI, and identified that apart from the SNP rs6502051 near the gene *FASN*, the other SNPs that were identified to have statistical significant association as risk for aggressive PCa when compared to healthy controls after adjusting for age remained significant. This helps us define the role of BMI as risk for aggressive PCa with ageing [42].

We then adjusted the data for BMI and age. Interestingly, instead of getting a lesser number of SNPs associated with the risk of aggressive PCa, we identified three more SNPs. Since the data was adjusted for BMI and age, this, statistically, implies the effect of alcohol consumption and tobacco smoking on our health. The additional SNPs identified as significantly associated with the risk of aggressive PCa were rs3918256, rs5945619, and rs6502051 present near the genes *MMP9* (*Matrix metallopeptidase 9*), *NUDT11*, and *FASN* respectively. The SNPs in gene *FASN* has previously been discussed with regards its role as risk for aggressive PCa, but the SNP in the gene *MMP9*- an inflammation marker [43] was not previously identified when seeing the role gene x environment interaction plays. Both, tobacco smoking and alcohol consumption have been studied in the recent past to be altering the levels of expression of MMP9 protein [44,45].

Next we adjusted the data for tobacco smoking only, in order to identify the risk age, BMI, and alcohol consumption have as a risk of aggressive PCa when compared to healthy controls. We identified two new SNPs, compared to the result generated by adjusting the data for age, being rs12529, in the gene *AKR1C3* and rs799923 near the gene *BRCA1*. The crosstalk between tobacco smoking and the SNP rs12529 in the gene *AKR1C3* has previously been explored by our group [46]. Interestingly, the identification of the SNP rs799923 near the gene *BRCA1*, a tumour suppressor [47], indicates that with progressing age, certain genes may function differently in the presence of external stresses such as alcohol consumption .[48]

We got further evidential proof with regards the effect of age on the expression and effect of tumour suppressor genes such as *BRCA1* on diseases such as aggressive PCa, when we analysed the data after adjusting for tobacco smoking and age and found that the gene was no longer significantly associated as a risk for the disease. Interestingly the significant association of risk of aggressive PCa was lost in the SNPs in the genes *AKR1C3* and *KLK3* too. The result pertaining to the SNP in the gene *AKR1C3* is interesting. As aforementioned, we have found some interesting correlations between the gene *AKR1C3*, tobacco smoking and the risk of PCa [46] and when we adjusted for age, the role of the SNP as a potential risk for aggressive PCa, compared to healthy controls, was not found to be statistically significant. We believe age-long smoking tobacco has a more potent effect on the risk of aggressive PCa rather than not. Consistent with the effect of adjusting the data for BMI and age, we identified SNP 632148 in the gene *SRD5A2* to be significantly associated with the risk of aggressive PCa. This, we believe, helps understand the nexus between ageing and the effect of certain genes and the influence of external factors leading to oxidative stress in a body.

In the final set of adjustments of our data to analyse the effect of SNPs as risk of aggressive PCa, we considered alcohol consumption and the combination of alcohol consumption and age. Interestingly, the SNP rs1799977 present in the gene *MLH1* (*MutL homolog 1*), which plays a major role in DNA (deoxyribonucleic acid) mismatch repair [49], and more so because rs1799977 is an exonic SNP [1,50]. DNA mismatch repair mechanism is an important fight-back against major diseases such as cancer [51]. SNPs in the genes *SEP15* and *FASN* are found significantly associated with risk of aggressive PCa when compared with healthy controls with adjustments for just alcohol and combination of alcohol and age respectively. The effects of smoking and BMI have always been a matter of controversy, but according to Kaufman *et al*., (2012), tobacco smoking can have a wide range of effects including limited physical activities, and it itself being a "gateway" habit, the effect on increasing BMI and obesity should be accepted [52].

The use of such combinations to adjust the data and extract the fine points of a case-control study is quite an unique approach on its own, however, the SNPs in the various genes that we have identified as a risk of aggressive PCa when compared to healthy controls is quite interesting. With as many as five SNPs across three genes- *MYEOV*, *MSMB*, and *SLC26A6* that remained significantly associated as risk for aggressive PCa, it is beyond doubt that these are the most important genes of interest with regards to similar studies. Having said this, it is worthy of bringing to notice that studies in larger populations need to be done to validate these results, though (Figure 1).

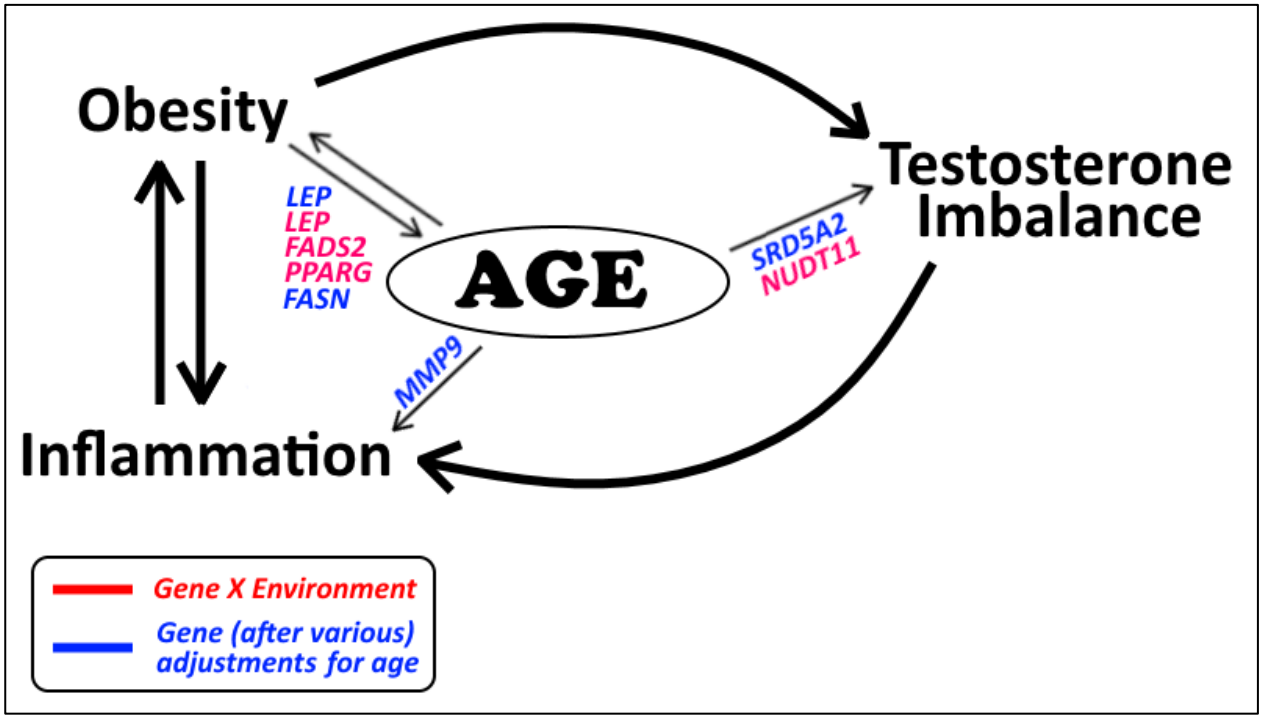

Figure 1: Various pathways and the genes identified to be significantly associated with a risk of aggressive prostate cancer (compared to healthy controls)

4.3.2 SNP genotyping, the effect of environmental factors, and of age as a risk of aggressive prostate cancer vs non-aggressive prostate cancer:

A similar approach was employed to determine the SNPs in genes of interest with regards the risk of aggressive PCa when compared to non-aggressive PCa. If the logic of progression of PCa holds true, non-aggressive PCa is the most crucial stage, as due to cell division with accumulation of cancer cells, and a prolonged weakening of immune cells, non-aggressive PCa could progress to aggressive PCa [1,14]. We believe that this is one of the most important sets of data that we have analysed thus far, as knowledge of these SNPs and corresponding genes is important to arrest non-aggressive PCa from progressing to aggressive PCa.

We first analysed the data without adjustment for any of the four afore mentioned factors, for the gene x environment effect as a risk of aggressive PCa compared to non-aggressive PCa and has been explained in details in one of our recent publications [1]. One SNP each in the genes *SRD5A2*- rs632148, *MLPH* (*Melanophilin*)- rs2292884, *PODXL* (*Podocalyxin-like*)- rs3735035, *LEP* (*Leptin*)- rs10244329, *TLR4* (*Toll-like receptor 4*)- rs11536889, *SLC26A6*- rs887391, *KLK3*- rs17632542, and *MMP9*- rs3918256 were identified as statistically significant risk of aggressive PCa (compared to non-aggressive PCa). As expected, we identified that there is a general trend of a typical textbook-like analysis of progression of any cancer. We identified SNPs in a fusion gene- *SLC26A6* which is well established to aid the development of human cancers [1,27]; *MMP9* and *TLR4*- genes involved in the inflammation pathway [1,53]; *PODXL*- a gene encoding for the cell-adhesion glycoprotein which has previously been reported to be associated with aggressive tumour phenotype and poor prognosis in various cancers [1,54,55]; along with genes pertaining to steroid levels- *SRD5A2*, and overexpressed in the estrogen receptor - *MLPH* [56]; along with a gene pertaining to obesity- an import external risk factor for aggressive PCa [1] and *KLK3*- involved in the PSA metabolism pathway [1]. The data is indicative of a strong gene x environment interaction leading to the progression of the disease.

We then adjusted the data for age to identify the genes which may be influenced by progressing age [14]. Interestingly, only four of the aforementioned eight SNPs remained significantly associated with the risk of aggressive PCa when compared to non-aggressive PCa. These were identified as the SNPs in the genes *SRD5A2*, *PODXL*, *LEP* and *MMP9*. Incidentally, only these four SNPs remained significantly associated as risk for aggressive PCa when compared with non-aggressive PCa across all statistical adjustments.

The role between inflammation and the development of cancer is a very well established nexus [57,58]. With the progression of cancer, the tissue(s) may change drastically, which may trigger certain homeostatic processes of tissue repair, and the recruitment of inflammatory leukocytes [58] and affect innate immunity as well [57]. Not only *MMP9*, but other members of this family of enzymes with their role in the evolution of the immune system are well known to regulate certain inflammatory and repair processes and hence may be used for predictory analysis for various cancers [59]. The fact that a SNP in this gene was identified as significantly associated as risk of aggressive PCa is understandable.

PODXL is cell-adhesion glycoprotein which is also associated with a number of aggressive tumour outcomes [60]. This transmembrane glycoprotein is expressed in a number of cancers including ovarian [61], epithelium [62] and prostate [1]. PODXL causes an increase in cell migration as well as invasion, leading to an increase in the MMP expression [60], which has an established role in inflammation [58] and innate immunity [57].

One of the other important genes that upregulates the function of some members of the MMP family [63], and is significantly associated with obesity and the risk of a number of cancers is *LEP* [64]. There have been a number of studies to define the role of obesity in carcinogenesis [65], but it is usually poorly understood [64]. With an increase in the world population's BMI, it is vital to identify means to understand the progression of various diseases, including aggressive PCa owing to the SNPs and thereby altered expression of obesity-related genes such as *LEP*.

As expected, the SNP rs632148 present near the gene *SRD5A2* was identified to be significantly associated with the risk of aggressive PCa when compared with non-aggressive PCa, just as was when compared to the healthy controls. The enzyme produced by the gene *SRD5A2* is important for the development and growth of the prostate gland [66]; and assists in the conversion of the male sex hormone, testosterone into the more effective androgen dihydrotestosterone [67]. With testosterone-levels being a matter of debate amongst urologists with regards the risk of PCa [68], it is interesting to find *SRD5A2* as significantly associated with risk of aggressive PCa in our population, because New Zealand is predominantly an overweight population [69], and increase in BMI reduces testosterone levels [70]. This reduction in testosterone levels with increased BMI is interesting, as we feel, an increase in BMI, may increase the dilution factor due to an increase in the overall size of the body, but further work needs to be done to prove this.

The New Zealand story (gene x environment interactions and risk of aggressive PCa) gets firmly knit when we put the results in this section together (Figure 2). It is well established that obesity has a major contribution in the inflammatory pathway [71], which in turn leads to the progression of cancers into advance stages [57,58]. Moreover, age and obesity have a role leading to alterations in testosterone levels, as previously discussed [14], and this hormonal imbalance, in turn, is a risk for aggressive PCa [7,68]. Thus, the effect of age on and with obesity may be playing a major role in our population with regards the total number of cases with aggressive PCa. This, we believe, is a very unique finding.

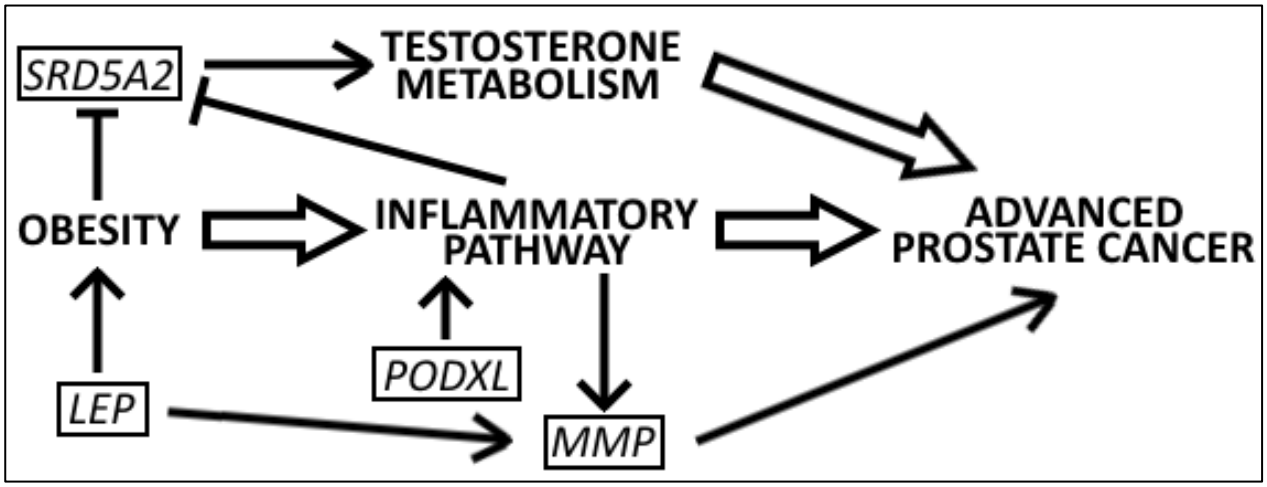

Figure 2: Various pathways and the genes identified to be significantly associated with a risk of aggressive prostate cancer (compared to non-aggressive prostate cancer)

4.3.3 SNP genotyping, the effect of environmental factors, and of age as a risk of non-aggressive prostate cancer vs healthy controls:

Finally, we analysed the data with and without various statistical adjustments to understand the initiation of PCa in our population and effect of age by comparing non-aggressive PCa with healthy controls. We identified only four genes with one SNP in and/or near it that was identified as statistically significant with the risk of non-aggressive PCa. They being rs2292884 in the gene *MLPH*, rs3735035 in the gene *PODXL*, rs11536889 in the gene *TLR4*, and rs4965373 near the gene *SEPS1 (Selenoprotein 1*). With 3 out of 8 genes identified to be common with the risk of aggressive PCa without any statistical adjustments, it indicates that there is a continuation with regards the alteration of certain gene functions with the schematic progression of the disease. Interestingly, however, none of the SNPs were identified to bear any significant association with the risk of non-aggressive PCa after various statistical adjustments including for age were performed. This implies that perhaps the gene x environment interactions, rather the genes on their own play the most important role in the initiation of diseases such as PCa.

The fact that a single gene involved with selenium metabolism- *SEPS1* was also significantly associated with the risk of non-aggressive PCa cannot be ignored, as yet another selenoprotein- *SEP15* was associated with risk of aggressive PCa (compared to healthy controls) when statistically adjusted for certain demographic parameters, as discussed above. The deficiency of trace elements such as selenium in the New Zealand soil is a well-established fact [72], and in the absence of the same, certain people take dietary supplements. However, a direct correlation between the role played by these dietary supplements and risk of PCa was recently identified [6,21,46]. Two of the other three genes involved are pertaining to the inflammatory pathway- *TLR4* and *PODXL*, which again can be due to the side-effect of the prevalence of high number of tobacco smokers in New Zealand [69], and the third one is overexpressed in the estrogen receptor- *MLPH*, which may be influenced by the low levels of Vitamin D among our cohort because of the lesser exposure to sunlight due to ageing [73,74] (Table 3).

Table 3: "New Zealand factors" and risk of non-aggressive prostate cancer

New Zealand factor(s)	Reference	Gene involved	SNP
Low Selenium levels in soil (leading to lower dietary intake)	[72]	*SEPS1*	rs4965373
Low sun exposure (leading to low Vitamin D levels)	[73]	*MLPH*	rs2292884
High tobacco smoking (leading to inflammation)	[69]	*PODXL*	rs3735035
		TLR4	rs11536889

Therefore, it does seem that the inflammatory pathway is one of the most important pathways for the initiation of PCa, along with the local factors such as life-long consumption of food low in selenium, and exposure to low levels of Vitamin D due to various factors with progressing age, and with the effect of hormones pertaining to specific organ of interest that eventually may be critical. The gene x environment interaction with the adjustment for age has brought a completely new way of looking at and understanding the risk for aggressive PCa based on the data generated from our cohort.

5. Conclusions

SNPs, being the most commonly observed variations in the genome, are ideal candidates for identification of biomarkers for various diseases [1]. Genotyping SNPs and observing the gene x environment

interactions is a very useful tool to identify the various local factors and their effect on genes leading on to a bottle-neck population with a particular condition- in this aggressive PCa.

We have identified a number of important individual lifestyle factors and their effect (either due to lifestyle exposure or due to ageing) as risk factors for PCa and aggressive PCa. We propose that the inflammatory pathway is one of the most important pathways responsible for initiating the disease, and certain local demographic factors such as obesity and tobacco smoking play crucial roles in driving non-aggressive PCa to the aggressive stage. SNPs in a putative oncogene (*MYEOV*) play a very influential role as risk for aggressive PCa. These findings are crucial for planning larger scale studies, because, although we recruited men of European ethnicity in our study, and genotyped SNPs that were identified as significantly associated as risk for PCa in various European populations, we could define a clear dependence of age in the progression of the disease based on gene x environment aspects. We propose that further studies based on our case- control analyses should be carried out to define specific biomarkers on a regional-basis, as this will help develop better diagnostic and treatment methods which will be tailor-made.

Supplementary Materials: Table S1a: Case-control association test. Table S1b: Case-control interaction with age test. Table S2: Adjustment for multiple testing Bonferroni_Sidak_FDR_Holm.

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Author Contributions: V.V. and V.N. did the data cleaning and statistical analysis. V. V. did data interpretation and wrote the manuscript. V.V. and V.N. conceived the idea for the results section. C.H.-J.K. did the graphical representations. V.V., V.N., N.K., R.P., A.J., G.M., P.K., and L.R.F. helped conceive the idea of the discussion chapter and proof-read the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AKR1C3: *Aldo-keto reductase family 1 member C3*
BMI: body mass index
CCHCR1: *coiled-coil alpha-helical rod protein1*
DNA: deoxyribonucleic acid
FADS2: *Fatty acid desaturase 2*
FASN: *Fatty Acid Synthase*
GWAS: Genome-wide association studies
HWE: Hardy Weinberg Equilibrium
KLK3: *Kallikrein-3*
LD: linkage disequilibrium
LEP: *Leptin*
MLH1: *MutL homolog 1*
MLPH: *Melanophilin*
MMP9: *Matrix metallopeptidase 9*
mRNA: messenger-ribonucleic acid
MSMB: *Microseminoprotein Beta*
MYEOV: *Myeloma Overexpressed*
NUDT11: Nucleoside Diphosphate-linked Moiety X Motif 11
PCa: prostate cancer
PODXL: *Podocalyxin-like*
PSA: prostate-specific antigen
SNP: single nucleotide polymorphism
SEP15: *Seleoproten 15kDa*

SEPS1: Selenoprotein S
SLC26A6: *Solute carrier family 26 member 6*
SRD5A2: *Steroid 5α-reductase type 2*
TLR4: *Toll-like receptor 4*

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