HORMONAL AND GENETIC REGULATION OF PROSTATE CANCER IN CAUCASIAN AND MALAYSIAN CHINESE ETHNIC GROUPS

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Abstract

Introduction

Prostate Cancer (PC) is the most common cancer in men and the second biggest cause of cancer-related death of men in the Western world. There are significant ethnic differences in the incidence of PC between African Americans, Caucasians and Asians. At the present time, the only method used for PC screening is Prostate Specific Antigen (PSA). Prostate is an androgen regulated gland and it can also affect the concentration of growth hormone by activation of androgen receptor. The aim of this research was to investigate ethnic group-specific relationship between PC incidence, concentration of circulating hormones and polymorphisms in genes that are involved in regulation of androgen metabolism. Specific objectives were: (i) to determine whether circulating concentration of androgens testosterone, dihydrotestosterone (DHT) and a growth hormone insulin-like growth factor-I (IGF-I) differ between Bristol Caucasian (BC) and Malaysian Chinese (MC) men; (ii) to investigate whether variations in concentration of circulating androgens in control and PC cohorts are ethnic group-specific; (iii) to determine whether frequency of DNA polymorphisms in CYP17, SRD5A2 and androgen receptor (AR) genes are ethnic group-specific; (iv) to investigate the relationship between genetic polymorphisms, concentration of circulating androgens and incidence of PC in BC and MC groups.

Material and Method

Blood sample were obtained from newly diagnosed PC patients from Caucasian and MC ethnic groups whose diagnosis was confirmed by biopsy. Control group consisted of patients with confirmed Benign Prostatic Hyperplasia with low risk of PC. The studied populations consisted of 50 Caucasian cancer cases, 50 Caucasian controls, 50 MC cancer cases and 49 MC control participants. Analysis of testosterone, DHT and IGF-I concentrations was performed on serum using ELISA kits. DNA polymorphisms were determined using genomic DNA isolated from whole blood (by PCR amplification) and follow-on DNA sequencing. The obtained DNA sequences were aligned with appropriate DNA sequences of the reference genes. The software “Geneious” was used to identify DNA SNPs and amino acid substitutions in translated protein. Statistical analysis was performed using SPSS version 20.0.

Results

This study established ethnic group-specific differences in circulating androgens between control and PC groups. In particular, testosterone concentration was significantly lower, and DHT/T was significantly higher in the MC cancer patients when compared to Caucasians. IGF-I concentration was significantly higher in the Caucasian cancer and controls than the MC sub-groups. A1/A2 and A2 + A1/A2 alleles of CYP17 gene were associated with a higher risk of PC in both ethnic groups. LL and L/V genotypes of SRD5A2 were prevalent in MC cancer and control group when compared to Bristol Caucasian PC and controls. These genotypes, when present, were associated with increased risk of PC in Caucasians but not in MC group. No relationship was found between a number of CAG-repeats in AR gene and PC in Caucasian or MC populations. No significant relationship was found between SNPs in CYP17, SRD5A2 and AR genes and circulating concentration of androgens and IGF-I in Caucasian and MC populations. The only exception was a positive association between the SRD5A2 polymorphism in the position 261C→G and IGF-I concentration in Caucasian control group.

Conclusions

This study demonstrated positive relationship between the concentration of circulating androgens, namely testosterone, IGF-I growth factor and the severity of the PC in Bristol Caucasians and MC groups. It also established ethnic-group specific polymorphisms in CYP17 and SRD5A2 genes, but not in the AR gene. It was suggested that the CYP17 and SRD5A2 polymorphisms might contribute to the risk of PC development in Caucasians and MC. Further evaluation of the results on a larger cohort and a wider range of ethnic groups are required in order to make definite conclusions about CYP17, SRD5A2 as ethnic-group specific biomarkers for PC.

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I would like to thank my entire supervisory team starting from Prof. Olena Doran for her ideas, guidance and continual support in making this a successful project, Prof. Anthony Rhodes for helping with my application to come here, Mr. David Gillatt for allowing me to work on an honorary basis at the Bristol Urological Institute in order to be able to collect the samples once NHS ethics approval was obtained, Dr. Paul White for all the statistical help and editing of the thesis and last but not the least, Prof. Azad Hassan Razack for helping me to secure this scholarship and to sort out my communication with my collaborators as well as sample collection at the University of Malaya.

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Collaboration

This thesis is a collaborative project between University of the West of England, Bristol, Bristol Urological Institute, Southmead and University of Malaya, Kuala Lumpur, Malaysia. The University of Malaya provided funds for living expenses in Bristol throughout the project and the samples of the Malaysian Chinese participants for this project. Bristol Urological Institute provided access to the Urological outpatient clinic at Southmead hospital to obtain the Caucasian PC and control samples.

Author's Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Academic Regulations and Procedures for research degree programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

Table of contents

List of figures

List of tables

Abbreviations

ANCOVA	Analysis of Covariance
ANOVA	Analysis of variance
AR	Androgen receptor
AUC	Area under curve
BC	Bristol Caucasian
BPH	Benign Prostatic Hyperplasia
BUI	Bristol Urological Institute
CI	Confidence interval
CYP17	Cytochrome P-450 steroid 17α-hydroxylase/17, 20 lyase
DHT	Dihydrotestosterone
DHT/T	Dihydrotestosterone to Testosterone ratio
DNA	Deoxyribonucleic acid
DRE	Digital rectal examination
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
gDNA	Genomic Deoxyribonucleic acid
HSD	Honestly significant difference
IGF-I	Insulin-like Growth Factor-I
LSD	Least significant difference
MBS	Membrane Binding Solution
MC	Malaysian Chinese
MD	Mean difference
MWS	Membrane Wash Solution
N/A	Not applicable
NCBI	National Centre for Biotechnology Information
OR	Odds ratio
PC	Prostate cancer
PCR	Polymerase chain reaction
PIS	Participant Information Sheet
PSA	Prostate Specific Antigen
RAGS	Research and Governance System
RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SNP	Single Nucleotide Polymorphism
SRD5A2	Steroid 5α reductase type II
TAE	Tris-acetate- Ethylenediaminetetraacetic acid
TMPRSS2	Transmembrane protease serine 2
UV	Ultra-violet
UWE	University of the West of England

1 Introduction

Prostate cancer (PC) is the second most common cancer after lung cancer in males. In spite of high incidence of PC, the mortality related to PC is lower when compared to other types of cancer - PC only ranks 6th with regards to cancer-related deaths (Ferlay *et al,* 2010b). There are clear differences in both, incidence and mortality rates between different regions and ethnic groups. Increasing trend in incidence and mortality due to PC has been reported over a 20 year period (1978-1997) in the Asian region among the Japanese, Korean, Singaporean and Thai populations (Sim and Cheng, 2005) as well as in European or Caucasian populations (Baade *et al,* 2009, American Cancer Society, 2010).

PC is a unique and unpredictable disease due to its heterogeneous nature i.e. each individual with the disease differs in stage and grade giving rise to multiple acceptable treatment options for the same stage and grade with very unpredictable response to the chosen mode of treatment. PC can be cured successfully when diagnosed and treated early, but it is lethal when it has progressed to a metastatic stage (Brawley *et al,* 2009). At the same time, a large number of PC cases do not progress to metastatic stage and might not require any treatment as they are very slow to progress to cause any harm to an individual (Albertsen *et al,* 1995). This raises an important question about not only early diagnostics but also about effective and accurate prognosis assessment with regards to cancer progression in order to advocate the best possible treatment option.

There are several risk factors that contribute to unpredictable nature of the disease such as ethnicity, age, family history (genetics) (Bray *et al*., 2010). The literature also suggests that PC development can be influenced by the diet, lifestyle, and sexual behaviour (Damber

and Aus, 2008), although any one or a combination of the above risk factors on PC development have not been fully established.

One of the reasons for the increase in PC incidence in some ethnic groups and regions might be improvement in cancer diagnostics over the last 20-30 years. This is linked to the introduction of PC screening in the more effluent western countries like United Kingdom (UK), United States (US), Scandinavian countries as well as Asian countries like Japan, Singapore and Korea. Prostate Specific Antigen (PSA) test was introduced in 1986 into clinical practice to aid the diagnosis of PC and monitor its progression and response to treatment. However, reliability and specificity of PSA test has been increasingly questioned with regards to over-diagnosis (Etzioni *et al,* 2002), which raises the need for a novel, reliable and more specific test for PC diagnostic and prognosis.

It is known that predisposition to PC varies between ethnic groups (Hsing *et al,* 2000) but the reasons for these variations have not been fully understood. Therefore, there is extreme interest in ethnic-group specific PC screening tests which could be used for diagnostics, prognostics and management of PC. This could ensure reduction of unnecessary expensive and morbid diagnostic procedures such as an invasive biopsy and aggressive treatment. Apart from that, a more ethnic based screening and diagnostic protocol could potentially be developed.

An ideal screening method for a particular disease would be a test that does not require obtaining tissues by performing a biopsy. Ideally, this test would use blood, urine or saliva which can be obtained with minimally invasive methods. In this respect, blood is of particular interest as it contains a large number of proteins which could be used as potential biomarkers. Potential biomarkers for PC might be the circulating androgens such as testosterone and dihydrotestosterone (DHT) as well as the anabolic hormone, Insulin-like

Growth Factor-I (IGF-I). Androgens play an important role in the growth of the prostate gland and have been shown to cause a malignant change in studies on rat models (Noble, 1977). Polymorphisms in genes encoding for enzymes that control testosterone and DHT concentration, might also be potential PC markers. CYP17 gene encodes the enzyme cytochrome P450c17α-hydroxylase and the gene SRD5A2 encodes for the enzyme 5α-reductase type 2 which control the biosynthesis of testosterone and conversion of testosterone to DHT respectively (Picado-Leonard and Miller, 1987, Wilbert *et al,* 1983). Data of the literature on relationship between CYP17 and SRD5A2 polymorphisms and circulating androgen concentration are controversial. This may be the reason for the unpredictable nature of the disease and also explain the low incidence in some ethnic groups especially from the Asian continent. Androgen in the circulation can act on the prostate via regulation of Androgen Receptor (AR) activity. Binding of androgens to the AR and the polymorphism in the AR gene can cause a modification in the function of androgenic response element (Singh *et al,* 2005). One such element that could be affected by the AR gene polymorphism could be the IGF-I metabolic pathway affecting the circulating concentration of IGF-I. Studies have shown positive relationship between circulating IGF-I concentration and PC risk as well as elevated IGF-I level in ethnic groups with higher PC incidence (Rowlands *et al,* 2008, Roddam *et al,* 2008).

The overall aim of this project was to investigate ethnic group-specific relationships between PC incidence, concentration of androgens in circulation and presence/frequency of polymorphisms in the genes that control androgen metabolism. The study was conducted on Bristol Caucasians (BC) and Malaysian Chinese (MC) men. These ethnic groups were chosen because of large difference in incidence of PC, with the PC incidence at least 6 times higher in the BC than MC men (Jemal *et al,* 2011).

The hypotheses for this project are:

1. The concentration of circulating androgens (testosterone and DHT) and the growth hormone Insulin-like Growth Factor-I (IGF-I) are positively related to the incidence of PC in BC and the MC men. The serum concentration of all these hormones are expected to be significantly higher in the BC compared to the MC men because of the higher risk and incidence of PC in the Caucasians compared to Asians.
2. The presence and frequency of selected polymorphisms in the CYP17, SRD5A2 and the AR genes are related positively to the higher incidence of PC in the BC but not in MC men.
3. Polymorphisms in CYP17, SRD5A2 and AR genes are associated with increased concentration of the hormones testosterone, DHT and IGF-I in the circulation.

Specific Objectives

1. To compare the concentration of circulating androgens (testosterone and DHT) and IGF-I in BC and MC ethnic groups and to investigate ethnic-group specific relationship between androgens, IGF-I concentration and PC incidence.
2. To sequence the specific regions in CYP17, SRD5A2 and AR genes from BC and MC ethnic groups in order to identify ethnic-group specific polymorphisms.
3. To investigate the relationship between ethnic group-specific DNA polymorphisms, the circulating androgens and growth hormone concentration and the incidence and risk of PC.

2 Literature Review

This section of the thesis aims to give a review of the literature on the following aspects:

I. Background and important issues in PC management.
II. Role of androgens in PC.
III. An overview of involvement of testosterone, DHT and IGF-I in PC development.
IV. Overview of polymorphisms in CYP17, SRD5A2 and AR genes and their association with PC.
V. Ethnic aspects of hormonal status and genetic polymorphisms in relation to PC risk and incidence.

2.1 Background on prostate cancer

This section gives an overview of PC and discusses the important issues involved in management of PC with regards to the incidence, risk factors, problems with diagnostic and screening.

2.1.1 Anatomy and function of prostate gland

The prostate gland is situated in the male pelvis between the urinary bladder and the penis at the bladder neck region (Figure 2.1). It is traversed by the prostatic part of the urethra which enables the urine from the bladder to pass through. It is the size of a walnut and normally weighs about 18 g. It has anterior, posterior and lateral surfaces with a narrow apex inferiorly and a flat base superiorly (Wein *et al,* 2012). The function of the prostate gland is to secrete fluid into the semen to keep it in alkaline state in order to protect the spermatozoa.

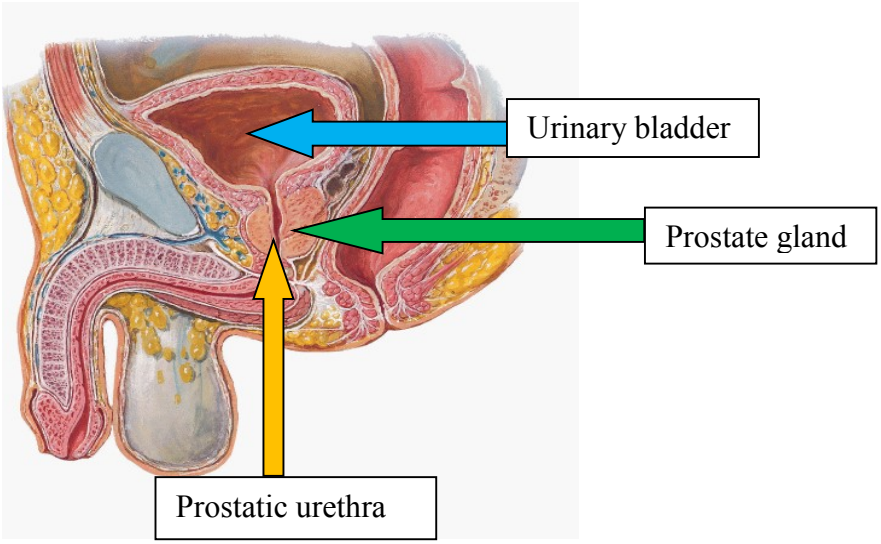

Figure 2.1 Male pelvic anatomy.

Figure shows the position of the prostate gland in relation to the urinary bladder and urethra.

The enlargement of prostate gland with age is a natural occurrence and starts at the age above 40 years which subsequently obstructs the outflow of urine causing lower urinary tract symptoms (Garraway *et al,* 1991) (Figure 2.2). However, the need to initiate treatment is based on the severity of the symptoms because not all individuals will be affected by the symptoms and only 30% of those in their early 70's are symptomatic with 1-2% going into urinary retention (Webber, 2006). As this is a slow progressing condition, treatment is only advocated to those whose quality of life is affected by the symptoms. This condition is called Benign Prostatic Hyperplasia (BPH).

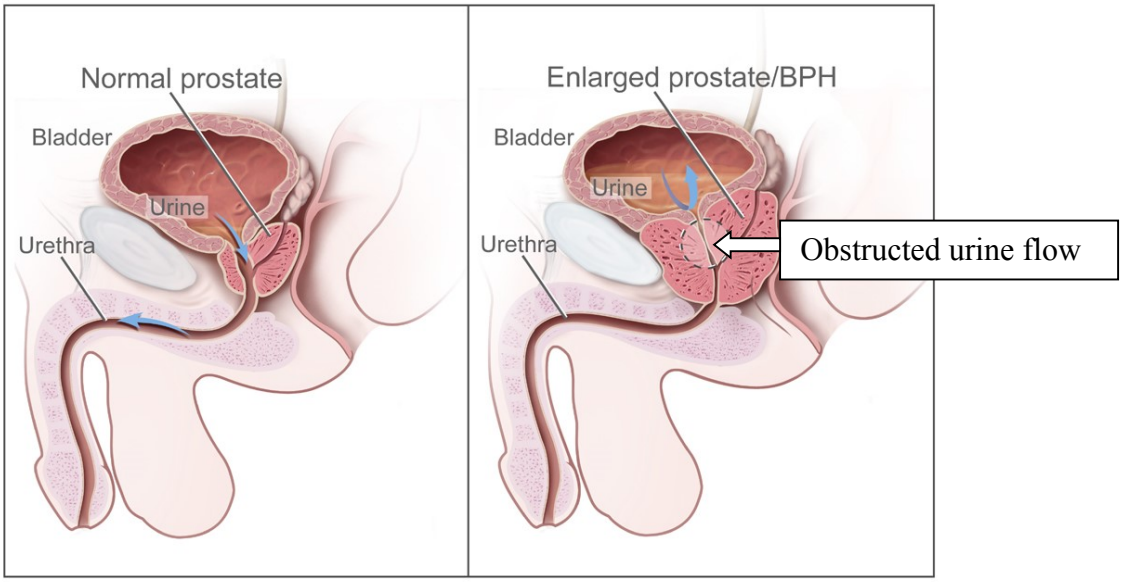

Figure 2.2 Schematic presentation of normal prostate gland and benign prostatic hyperplasia (BPH).

Left- normal prostate gland, right- benign prostatic hyperplasia. Obstructed urinary outflow due to the BPH is shown by blue arrows.
Adapted from http://en.wikipedia.org/wiki/Benign_prostatic_hyperplasia

The prostate gland can undergo a malignant change due to genetic, hormonal, dietary, life-style and environmental factors (Damber and Aus, 2008, Kolonel *et al,* 2004, Parkin *et al,* 1992, Kopper and Tímár, 2005). A multi-step mechanism that takes place during the transformation from normal prostate to cancer is not well understood. Most researchers have agreed that reactive oxygen species (ROS) play an important role in this mechanism via oxidative damage which is described in section 2.1.3 of this thesis.

2.1.2 Epidemiology of prostate cancer

Latest figures from developed countries demonstrate that PC is the most common cancer diagnosed in men (Ferlay *et al,* 2013). In the USA, it has been estimated that 1 in 6 men will be diagnosed with PC during their lifetime (American Cancer Society, 2010).

Estimated new cases of PC diagnosed in 2013 in the USA would exceed that for lung and bronchial cancer by more than 100,000 cases in men (Figure 2.3).

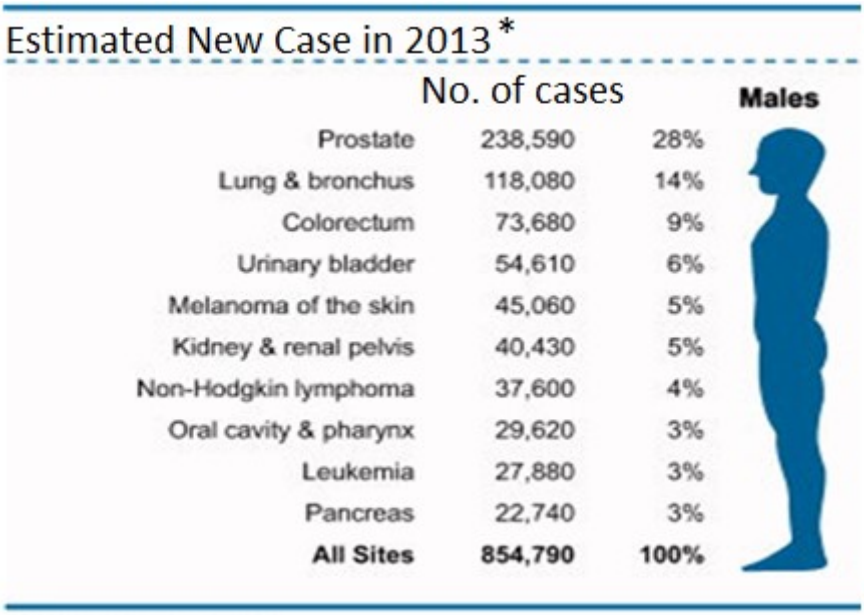

Figure 2.3 List of leading causes of cancer in males for the year 2013 in U.S.
Figure shows the number of cases by organ involved and the percentage (%) of the total number of cancer cases. Adapted with permission from WILEY and accessed via http://onlinelibrary.wiley.com/doi/10.3322/caac.21166/full#fig1. * Excludes cutaneous malignancies and in-situ carcinomas except bladder.

In the UK, PC also leads the way with the highest incidence among males with new case rate of 41,736 cases in the year 2011 (Cancer Research UK, 2011). This incidence rate is only second to breast cancer in females with and incidence of 49,936 cases for the same year. PC is the cause of cancer in 1/4 of males in the UK, ahead of lung and bowel (14% each) and all other cancers like bladder, oesophagus, haematological, kidney, brain and unknown primary sites of the 167,487 new cancer cases in males diagnosed in 2011 (Figure 2.4).

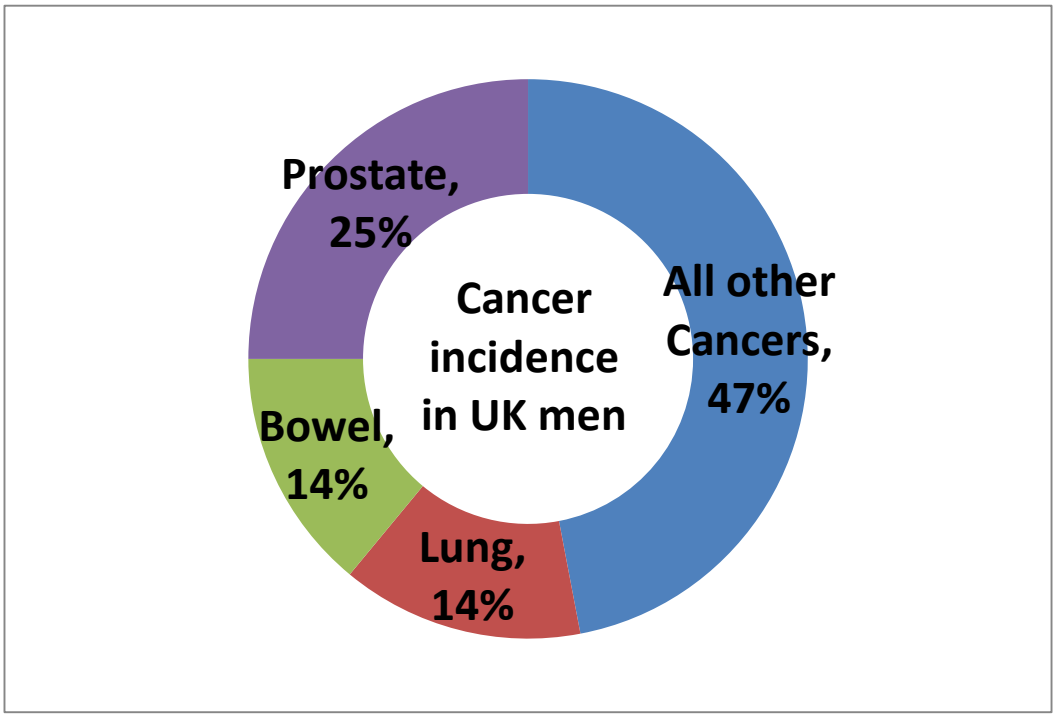

Figure 2.4 Percentage of all cancer cases in males in the UK in 2011.

Adapted from: Cancer Research UK, http://www.cancerresearchuk.org/. Accessed October 2013.

In spite of the high incidence of PC in some developed countries worldwide, PC is only the 4th common cancer after lung, breast and bowel cancers (Cancer Research UK, 2011). This is likely to be due to the incidence of PC being particularly low in Asia which might be related to variation of PC incidence between different ethnic groups in China, India, Taiwan, Malaysia and Japan compared to Western countries. The ethnic aspect of PC will be described in section 2.1.3.

Introduction of PSA testing in 1986 for screening of PC has given rise to a tremendous increase in recording of incidence of PC. For example, the recorded incidence of PC in the UK has significantly increased between 1993 and 2011 (Figure 2.5). In the Asian countries

such as Japan and Singapore there was a 100% increase in recorded PC cases after introduction of PSA testing (Sim and Cheng, 2005). A similar increase in PC cases was noted in the USA in early 1990s but there was some reduction in newly diagnosed PC cases since the late 1990s. One reason for this might be the selective use of PSA in screening, where the clinicians selected the patients appropriately to subject them for this test following clinical guidelines (American Cancer Society, 2010).

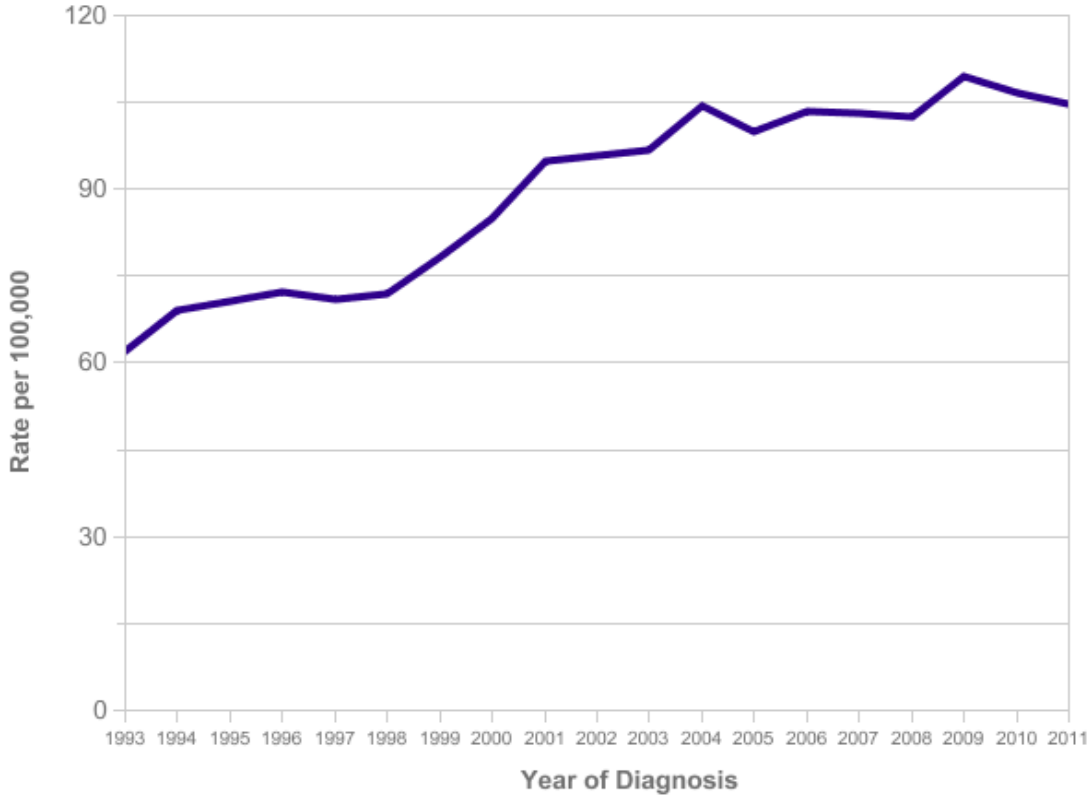

Figure 2.5 Age-standardised PC incidence rates per year in the UK from 1993 to 2011. Y-axis denotes the number of cases diagnosed per 100,000 male population per year. Source: Cancer Research UK, http://www.cancerresearchuk.org/cancer-info/cancerstats/types/prostate/incidence/. Accessed October 2013

One would expect that the steep increase in the number of PC cases over the years would normally lead to increased mortality due to this malignancy. However, PC has a high

incidence to mortality ratio compared to any other cancer (Hsing *et al,* 2000) which indicates that the increase in PC incidence did not have impact on mortality rates in a population. This can be explained by impact of widespread PC screening which led to improved diagnostics (especially early stage cancer) (Albertsen *et al,* 1995). The highest mortality was reported in the Caribbean population (26.3 per 100,000 male) and the lowest was found in the Eastern Asian region (2.5 per 100,000 male population) (Ferlay *et al,* 2010b). Most developed countries report a stable mortality rate or a slight reduction in mortality due to extensive screening with PSA as well as improved treatment modalities developed for the disease (Figure 2.6).

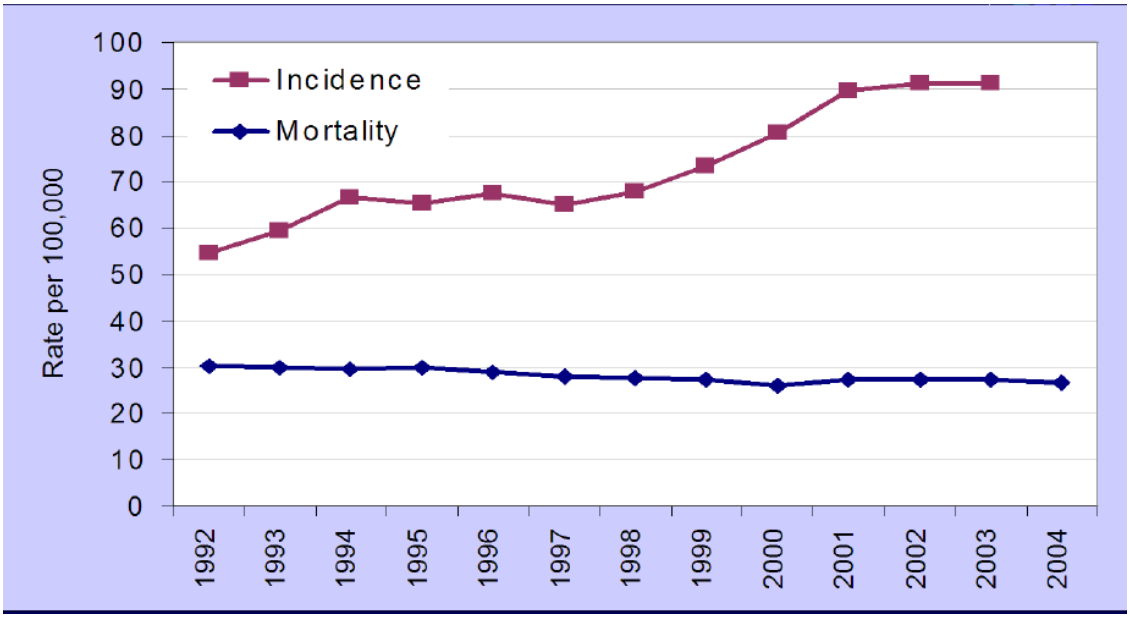

Figure 2.6 Annual incidence and mortality rates due to prostate cancer in the UK.

Y-axis shows the number of cases per 100,000 male population per year. X-axis denotes the year the data was accumulated. Source: Cancer Research UK, http://info.cancerresearchuk.org/cancerstats/faqs/#How. Accessed October 2013.

2.1.3 Risk factor for prostate cancer development

It is very important to establish risk factors of a certain disease in order to be able to predict its possible development and progression to map out the strategies in diagnosis, prevention and treatment.

The normal prostatic cells undergo malignant changes in a multistep process with progression from a normal epithelial cell to a pre-malignant condition called prostatic intraepithelial neoplasia (PIN) (Figure 2.7). This is followed by further progression to a histologically confirmed PC which subsequently breaches the prostatic capsule to spread either locally or to a distant site (Thompson Jr *et al,* 2014).

A combination of either dietary factors like anti-oxidants, androgen imbalances due to aging and associated genetic polymorphism can cause oxidative stress leading to irreversible changes in prostate cells (Nelson and Montgomery, 2003).

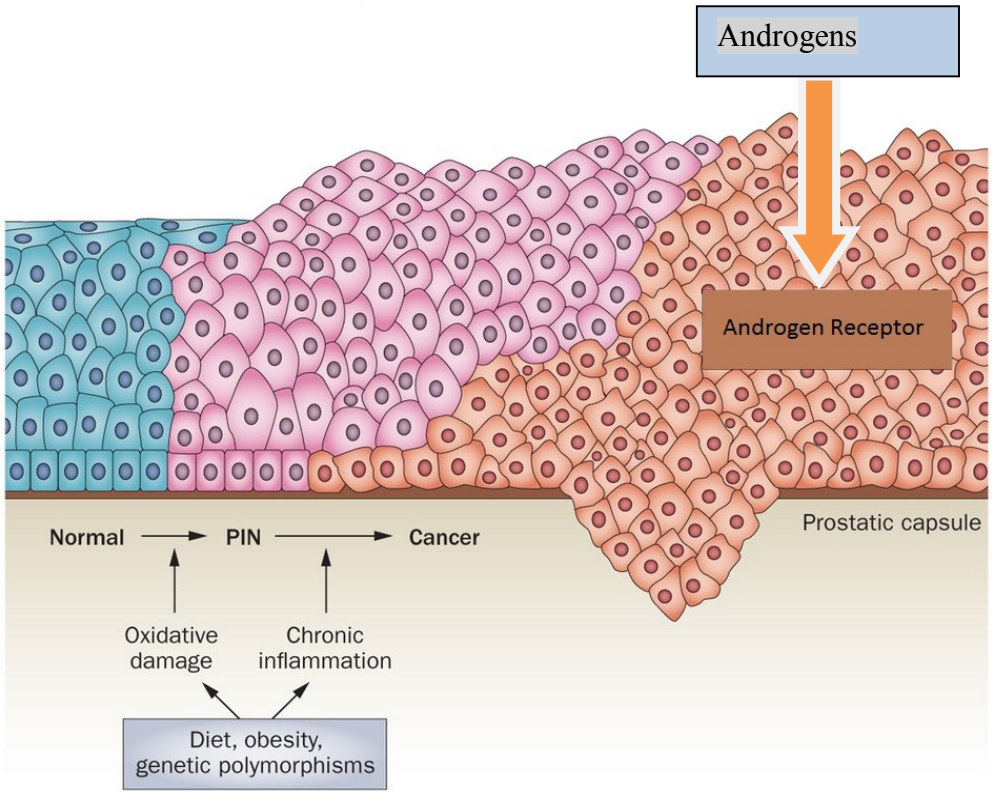

Figure 2.7 Prostate gland cell changes associated with PC progression.

PIN- Prostatic intraepithelial neoplasia. Adapted and modified from Thompson Jr *et al* (2014) with permission from Nature Publishing Group.

The process of oxidative stress leading to PC development has been increasingly gaining importance with the probable role of reactive oxygen species (ROS)-mediated oxidative stress and PC risk remains one of the most important theories in this disease (Figure 2.8) (Khandrika *et al,* 2009). Many factors like those that are intrinsic to the cell (aging, androgen imbalance, anti-oxidant imbalance) and environment factors like inflammatory process in the prostate, dietary fat intake and exposure to radiation can cause increased ROS production in the prostate. This will lead to prostate dysfunction and subsequently increases the ROS levels further. At this point, the anti-oxidant system could act as a defence mechanism to regulate the ROS in prostate and return the function to normal and

hence arrest the progression from normal to a malignant prostate. Impaired anti-oxidant defence mechanism leads to uninhibited oxidative stress. This will lead to further increase in mutation of the DNA within mitochondria, the formation of aggressive phenotypes and further DNA damage causing proliferation of abnormal prostate cells to undergo malignant change (Naka *et al,* 2008, Sauer *et al,* 2001).

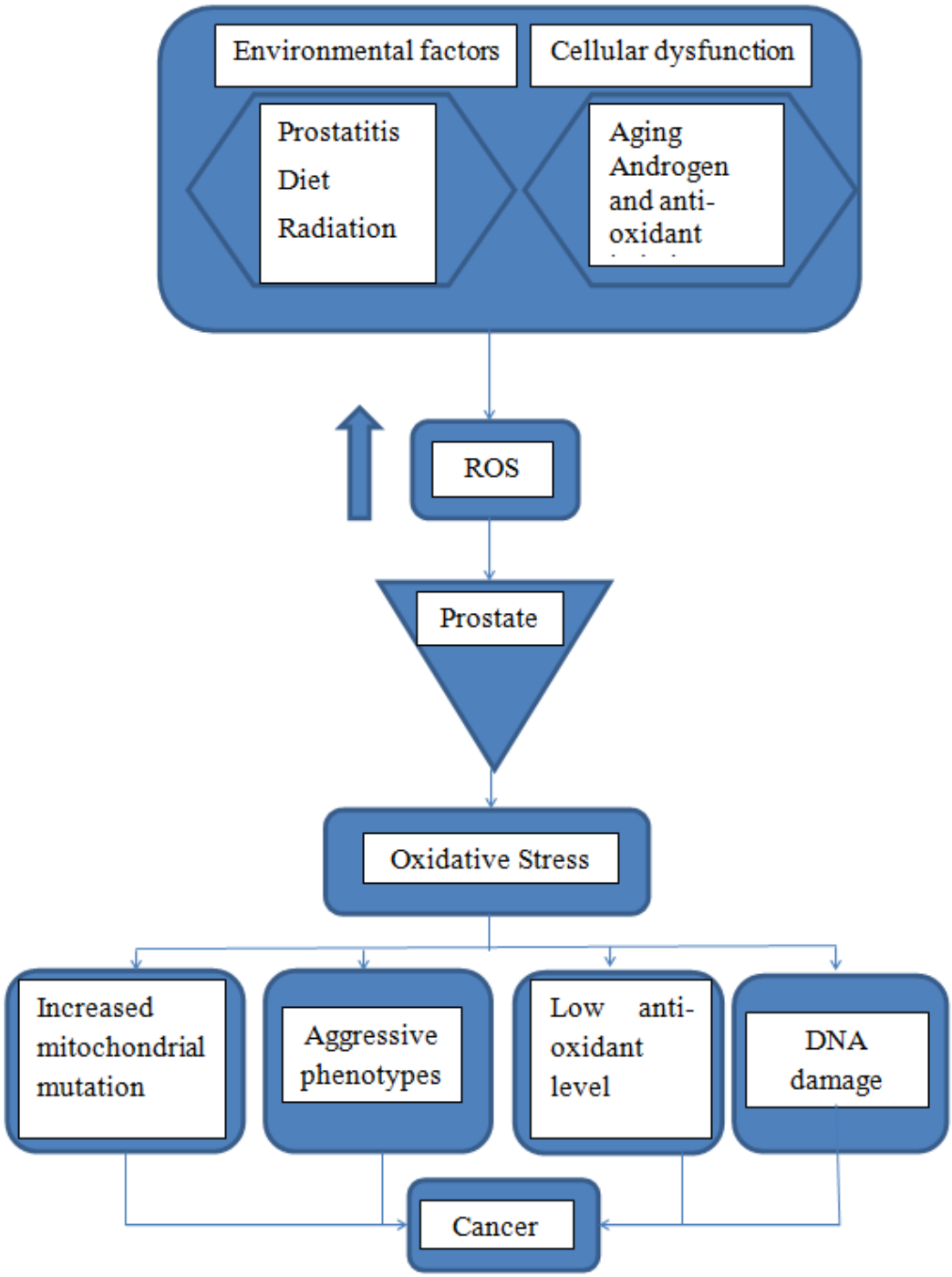

Figure 2.8 Schematic presentation of mechanism of prostate cancer development.

Prostate cancer development is mediated by increased ROS and cellular response to elevated ROS in prostate gland. ROS- reactive oxygen species, DNA- deoxyribonucleic acid. Adapted and modified from Khandrika *et al* (2009) with permission from Elsevier.

PC is a disease of aging with the highest incidence of in the age-group above 55 years old and rarely seen below 50 years of age (Ries *et al,* 2008). The role of aging in the development of cancer is thought to be due to accumulation of molecular and DNA damage by free radicals (Harman, 1981). Cellular oxidative stress increases with age due to age-related mitochondrial DNA mutation leading to increase in ROS generation (Khandrika *et al,* 2009).

Prostate gland development and its functions in human depend on androgens and its derivative. The metabolism of androgens and their actions through the AR is described in section 2.2 of this thesis. The theory of increased oxidative damage as a result of ROS when there is abnormal and excessive androgen stimulation is probably the likely scenario for PC development (Pathak *et al,* 2008).

Reduction of ROS level via inclusion of anti-oxidants in the diet has been receiving increasing attention as a tool for prevention of not only PC but also other types of cancer. An important role of dietary anti-oxidants in cancer prevention was first analysed by Lee et al. (2005) who performed a study in the USA to demonstrate the role of vitamin E supplement in reducing incidence of cancer and cancer-related deaths. Data from the above study did not show any benefit of vitamin E supplement to reduce the incidence of cancer (Lee *et al,* 2005). Data of the literature on effect of dietary anti-oxidants on PC development are also controversial. Research by Lippman *et al* (2009) showed that overall risk of PC was not affected by dietary supplementation of vitamin E or selenium. Although anti-oxidant supplement can possibly reduce the ROS production and subsequently may reduce the ROS level, other strategies aiming at reduction of ROS production are likely to be beneficial. In addition to dietary supplementation of vitamin E, other ways to reduce ROS production could be reduction of dietary fat, animal protein and sugar intake. The

ROS reduction strategy also includes reduction of alcohol consumption, smoking and exposure to radiation as well as life-style modification to avoid stress and psychological disturbances (Devasagayam *et al,* 2004).

Chronic bacterial or non-bacterial prostatitis, which is an inflammatory condition of the prostate also can lead to stromal and epithelial cell damage that can result in an increase in the level of free radicals (De Marzo *et al,* 2007) and associated changes in protein structure, function and DNA damage (Olinski *et al,* 2002) , which subsequently increases the risk of malignancy.

Ethnicity remains one of the most important risk factor in PC. This aspect is discussed in detail below. Ethnic-group specific difference in incidence of PC can be due to a number of factors including differences in life-style, diet and environment. These external factors can be modified to reduce oxidative stress and thus, the ROS production. Feaireheller et al. (2011) demonstrated ethnic differences in the level of oxidative stress and the concentration of inflammatory markers between African-Americans and the Caucasians. There was increased plasma superoxide dismutase (SOD) activity in African-Americans when compared to Caucasians (Feairheller *et al,* 2011). However, there were no significant differences between African-Americans and Caucasians in the concentration of nitric oxide, endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunit and interleukin-6 (IL-6). However, the *in vitro* experiment conducted by the same group on the umbilical vein endothelial cells showed increased level of all the above mentioned parameters except for SOD activity in African-Americans compared to the Caucasians. Although, this study was conducted on a very small population, it gives important preliminary data for future research on ethnic difference in mechanisms of oxidative stress development.

Once the normal prostatic epithelial cell has undergone a malignant change, the clinical disease progression monitoring is based on the digital rectal examination (DRE), the Gleason score (determined by histological analysis of prostate biopsy), PSA as well as any radiological assessment (bone scan or magnetic resonance imaging) (Partin *et al,* 2001).

PC is a disease that is quite intriguing in that sense, as the only established risk factors that can be consistently associated with PC are non-modifiable factors such as age, ethnicity and family history/genetic factors (Damber and Aus, 2008, Patel and Klein, 2009, Zangwill, 2011). There has been increasing attention to understand mechanism of action of these factors to develop effective prognostic and diagnostic tools. Modifiable risk factors like nutrition, obesity, vitamin D profile, medications and hormonal factors have also attracted extensive attention but the data regarding their involvement in PC development are controversial.

I. Effect of age on PC development

PC is known as the disease of the elderly and it is very uncommon to be diagnosed with the condition in males of age below 45 years (Figure 2.9). A study assessing the data from five continents for age related incidence of PC have shown uniformity in all ethnic groups studied and concluded that the incidence of PC is very rare in men under 50 (Quinn and Babb, 2002, Jung *et al,* 2014). The incidence increases with age reaching a peak rate at the age of 70-74 years (Cancer Research UK, 2011, Quinn and Babb, 2002, Hemminki *et al,* 2005). Beyond this age, there is a drop in the incidence probably due to the recommended guideline for the use of PSA test only in men who are clinically healthy with a life-expectancy of more than 10 years (Carter *et al,* 2008, Drazer *et al,* 2011, Williams *et al,* 2011).

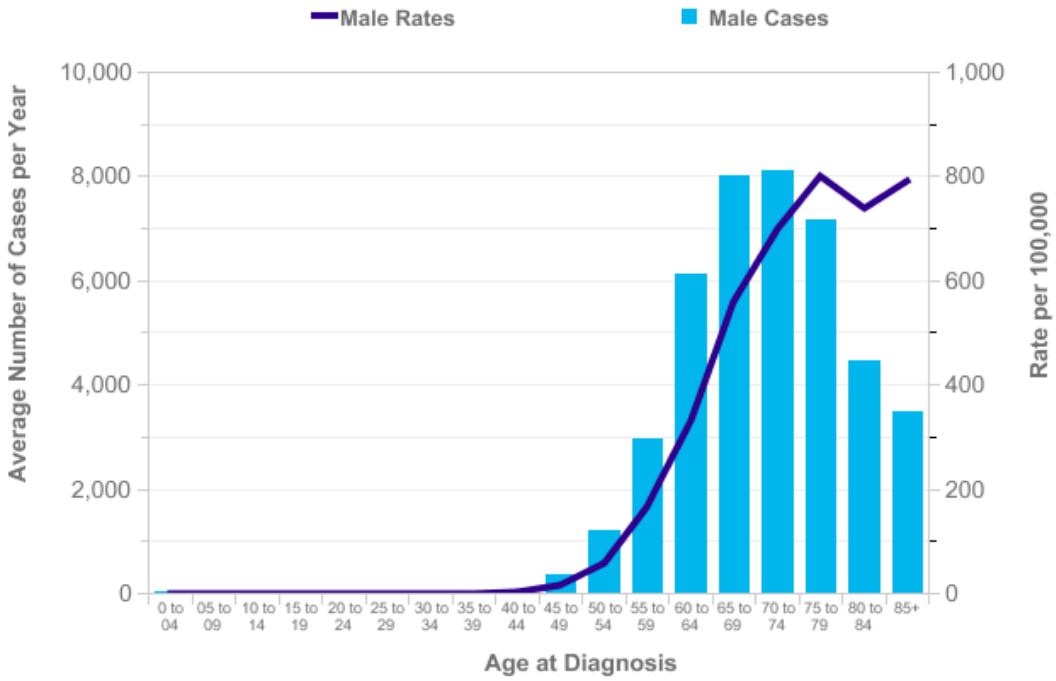

Figure 2.9 Age-specific incidence rates of prostate cancer in the UK from 2009 to 2011.

The blue line indicates the number of PC cases per 100.000 male population in the age group. The blue bar show the average number of new PC cases diagnosed in the particular age group. Source: Cancer Research UK, http://info.cancerresearchuk.org/cancerstats/faqs/#How. Accessed October 2013.

II. Effect of ethnicity on prostate cancer

The ethnic background plays a major role in the risk of PC development. Ethnicity can also be a factor in determining the severity of the disease and its prognosis. PC incidence is higher in developed regions such as Western Europe, Northern Europe, Northern America and Oceania, and in some of the developing regions such as the Caribbean and Southern Africa. The PC incidence rates are particularly low in Asian communities and on the majority of the African continent (Figure 2.10).

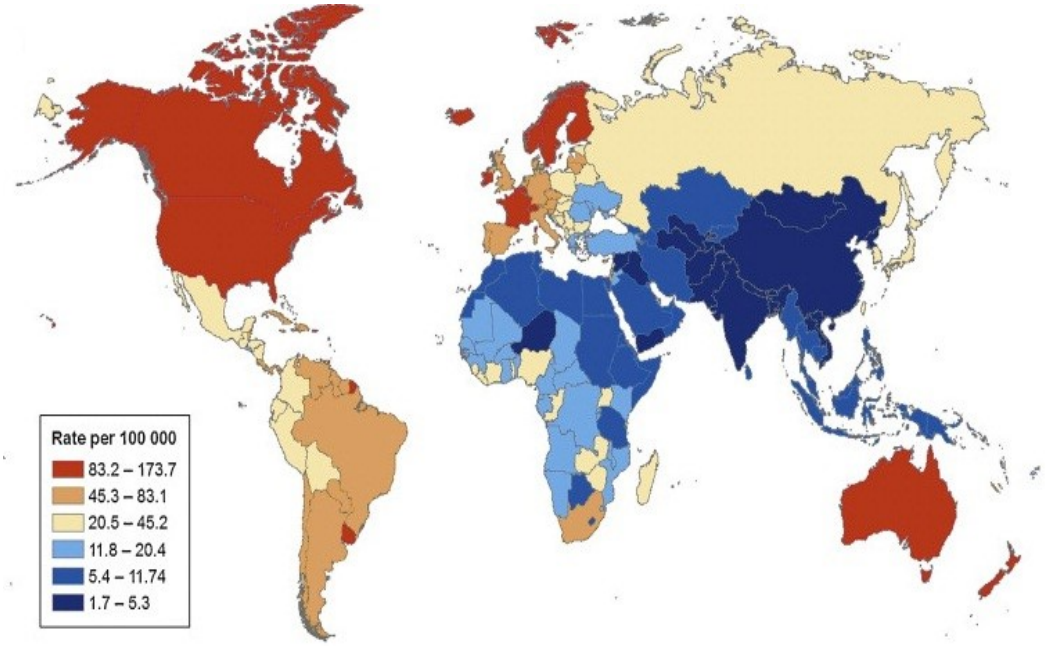

Figure 2.10 Geographical distribution of age-standardised prostate cancer incidence rates. Source: GLOBOCAN 2008 (Ferlay *et al,* 2010a).

Latest figures from GLOBOCAN 2012 have shown that the highest incidence of PC is in the USA and Australia with age-standardised rates of 98.2 and 115.2 cases per 100,000 population respectively (Ferlay *et al,* 2013). Other countries with high PC incidences were Sweden (119.0 per 100,000 population), Trinidad and Tobago (123.9 per 100,000 population), Barbados (123.1 per 100,000 population) and France (127.3 per 100,000 population) (Ferlay *et al,* 2012) .

The above data can be partially explained by the increased PC screening in the developed countries. However, widely used screening methods alone cannot explain the extreme differences in PC incidence in different ethnic groups.

PC incidence is very low in Asia and Africa but there is an increasing trend of these figures. In particular, PC rate per 100,000 population is as follows: 3.9 in India, 6.9 in

China, 8.5 in Korea and 22.3 in Japan (Jemal *et al,* 2010) . Not only PC incidence but also the rate of increase of PC incidence differ between countries with a 3.1% average increase in the Philippines (1985-2002) and Thailand (1988-2002) compared to a 13.8% increase in the Republic of Korea (1999-2007) (Center *et al,* 2012). The incidence of PC has doubled in 1986-1997 in Japanese and Singapore Chinese population from about 7.0 to 14.0 per 100,000 male population (Sim and Cheng, 2005). The latest PC incidence rate for Japan and Singapore showed a further increase to 22.3 and 17.3 per 100,000 respectively (Jemal *et al,* 2010). Malaysia, being a multi-ethnic (Malays, Chinese and Indians) country in Asia, shows an overall PC incidence of 6.2 per 100,000 but when ethnicity is taken into consideration, the Malays show the lowest PC incidence of 4.9 per 100,000 males when compared to the Chinese and Indians (8.7 and 5.8 per 100,000 males respectively) (Zainal Ariffin and Nor Saleha, 2011). PC incidence on African continent is also low (Gunderson *et al,* 2011) but this might be an underestimation due to absence of extensive PC screening programmes (Odedina *et al,* 2006). The age-standardised rates of PC incidence are not uniformly spread within the African continent. For example, some of the Eastern, Western and Southern regions of Africa such as Uganda (39.0), Ivory Coast (31.0) and South Africa (40.0) exhibit a very high rate of PC incidence when compared to other countries on African continent such as Senegal (7.5) and Kenya (16.6) (Delongchamps *et al,* 2007).

Ethnicity has a role in both, the severity and prognosis of PC. Most of the ethnic studies on PC were conducted in the USA due to a high diversity of ethnic groups living in the same environment. About 10% of the American population are African-Americans and the incidence and mortality of PC among the African-Americans according to the National Cancer Institute Surveillance, Epidemiology and End Results Program, are higher when compared to Caucasian-American males (Table 2.1) (Brawley, 2012). Table 2.1 also demonstrates that PC incidence and PC-related mortality are almost doubled in African-

Americans when compared to the Caucasian-Americans. In the UK, men of African and Caribbean descent had higher age-adjusted incidence rate (166.0 per 100,000) than Caucasian men (56.4 per 100,000), although this rate was not as high as the American populations (Ben-Shlomo *et al,* 2008). Ben-Shlomo *et al* (2008) showed no difference in PC incidence between men from African or Caribbean origin in the USA. Several articles have shown that the mortality rate of males of African-descent were definitely higher than that for Caucasian males (Evans *et al,* 2008, Bernstein, 2003, Godley *et al,* 2003). There might be a number of reasons for this high mortality rate.

Table 2.1 Incidence rate and mortality (per 100,000) by race in the USA from 2003 to 2007.

Race/Ethnicity	Incidence	Mortality
All races	156.0	24.7
Caucasian-Americans	149.5	22.8
African-Americans	233.8	54.2
Asian/Pacific Islander	88.3	10.6
American Indian/Alaskan Native	75.3	20.0
Hispanic	107.4	18.8

Adapted from Brawley (2012) with permission from Springer.

One of the reasons might be a lower socio-economic and associated co-morbidity status of the black ethnic group. However, according to Robbins et al (2000), the socio-economic status cannot fully explain the increase mortality in the black population. The other possible reason might be the fact that the white population is more likely to be tested for PC with PSA (Gilligan *et al,* 2004). The black population were also noted to be less likely to undergo aggressive treatment for PC which might be another reason for the higher mortality rates (Shavers and Brown, 2002, Shavers *et al,* 2004). Furthermore, genetic

predisposition to PC should also be taken into account and this pre-disposition can differ between different ethnic groups. This aspect is discussed in the next chapter.

III. Family history and genetics.

Family history of PC increases the risk of PC. Studies have shown that presence of one first-degree relative (brother or father) with PC increases the risk of PC by 2-3 folds (Platz E, 2006, Brandt *et al,* 2010).

Hereditary PC gene 1 (HPCG) is known to increase risk of PC. The Ribonuclease L (RNASEL) gene variants at HPCG locus 1 has been extensively studied in families with history of PC and found to be positively associated with PC risk (Rökman *et al,* 2002). RNASEL gene encodes a component of the interferon-regulated system that functions in the anti-viral and anti-proliferative roles of interferon.

A large breast cancer 2 (BRCA2) gene study, which included screening 1864 men with PC from the UK Genetic Prostate Cancer Study (UKGPCS) cohort, had shown that the frequency of mutations in BRCA2 is positively associated with PC development in patients below 65 years of age. This suggests that the BRCA2 gene mutations might have more impact on PC incidence then family history in the younger age group (Kote-Jarai *et al,* 2011). This gene is a human tumour suppressor gene that help repair damaged DNA or destroy cells if the DNA cannot be repaired. The BRCA2 mutations investigated in the mentioned above study were the protein-truncating mutations within exons 10 and 11, where 3 were in-frame deletions and 69 were insignificant missense variants.

Another gene which attracted attention in relation to PC is NKX3.1 gene that encodes for protein NKX3.1 in human which functions as a prostatic tumour suppressor in an androgen-regulated manner (Abate-Shen *et al,* 2008). It is located on chromosome 8p21.2

that displays loss of heterozygosity in almost 85% of high-grade prostatic intraepithelial neoplasia (PIN) lesion and adenocarcinomas of the prostate (Bethel *et al,* 2006). Earlier studies had shown that there is a complete loss of NKX3.1 expression in advanced cancers (Bowen *et al,* 2000). However, more recent studies with the use of specific antibodies showed a low level of NKX3.1 expression in almost all the PC cases studied (Gurel *et al,* 2010).

It has also been shown that PC patients exhibit a form of fusion between androgen-regulated gene transmembrane protease serine 2 (TMPRSS2) and erythroblastosis virus E26 transcription factor gene (Tomlins *et al,* 2009, Mosquera *et al,* 2009). Mosquera *et al* (2009) and Clark *et al* (2006) have shown that the most common fusion observed in PC patients was TMPRSS2-ERG which was found in 50% of localized PC patients and 15% of high-grade PIN patients. The functional significance of TMPRSS2-ERG fusion in PC is still unknown with theories suggesting that (i) ERG can bind to AR target genes and disrupt the AR signalling which leads to inhibition of prostate epithelial differentiation and (ii) Erythroblast transformation-specific (ETS) gene activation promotes tumour invasive properties of PC (Yu *et al,* 2010, Wang *et al,* 2008).

There has been increasing interests in the role of various DNA single nucleotide polymorphisms (SNPs) in PC risk and progression (Van den Broeck *et al,* 2014). Extensive genome-wide association studies identified multiple SNPs which, when taken individually, have been moderately associated with PC risk. However, this association was higher when a combination of SNPs was analysed (Takata *et al,* 2010, Xu *et al,* 2012). DNA SNPs may play an important role in identifying men who are at high risk of developing PC (Pepe *et al,* 2004). A total of 77 susceptibility loci have been instrumental in explaining about 30%

of familial risk of PC (Eeles *et al,* 2013). The role of specific SNPs in the genes controlling androgen metabolism is described in detail in section 2.3.

IV. Relationship between nutrition and prostate cancer

The role of diet in the development of PC is complicated due to the fact that food is usually consumed whole rather than individual nutrients, which resulted in controversial data of the literature (Wu *et al,* 2006, Hori *et al,* 2011, Masko *et al,* 2013). Low-fat diet with high soy content can contribute to a lower PC incidence in Asian communities compared to the western countries. This effect of soy has been attributed to a high content of isoflavones (phyto-oestrogens) (Ozasa *et al,* 2004, Hedelin *et al,* 2006). The mechanism by which phyto-oestrogen protects against PC development is yet to be fully established, but several theories suggest that the concentration of serum sex hormone-binding globulin (SHBG) can be increased by high intake of tofu which is rich in isoflavones (Habito *et al,* 2000). This subsequently reduces the concentration of the free testosterone due to higher availability of SHBG to bind to testosterone (Nagata *et al,* 2000). This theory favours the association of androgens with PC development.

Another theory is based on laboratory investigations on mice which showed that dietary supplementation of isolated soy protein can inhibit an experimentally induced prostate tumour. This was suggested to be due to reduction of cellular proliferation and angiogenesis together with induction of apoptotic activity (Bylund *et al,* 2000, Zhou *et al,* 2002). The phyto-oestrogens also have anti-oxidant and anti-inflammatory effect that can reduce the DNA damage by reactive oxygen species and thus prevents the transformation of normal cell to malignant cell in the prostate gland (Thomasset *et al,* 2007, Surh, 2003).

Diet high in anti-oxidant compound like lycopene, and micronutrients like selenium and vitamin E might have protective effect against PC development. Lycopene, is the carotenoid compound, that can protect against PC development via a number of mechanisms including acting as a scavenger of free radicals (Bravo *et al,* 2012, van Breemen and Pajkovic, 2008), and inhibition of cancerous cell progression at G_0-G_1 stage of cell cycle. High concentrations of lycopene were demonstrated to inhibit cancer cells growth *in vitro* on human mammary cancer cell line (Karas *et al,* 2000). The scavenging property of lycopene provides protection for lipoproteins and prevents DNA damage (Stahl and Sies, 2003, Hadley *et al,* 2003). However, data of the literature on relationship between lycopene intake and PC risk are controversial. Whilst Wei and Giovannucci, (2012) did not find significant relationship between dietary lycopene concentration and PC, a case-control study in China involving 130 PC patients and 274 controls showed reduction of PC risk with increase of lycopene intake. (Jian *et al,* 2007).

Another pair of nutritional supplement that have been studied in relation to cancer risk are selenium and vitamin E. Mechanisms of their action on PC are not well established. It is hypothesized that selenium and vitamin E can inhibit carcinogen-induced oxidative damage (El-Bayoumy, 2001). However, no evidence for this hypothesis was found in an experiment on dietary selenium and vitamin E intake and PC incidence (Lippman *et al,* 2009). Furthermore, a follow-on study even showed a significant increase in PC occurrence under dietary regime with vitamin E supplementation (Klein *et al,* 2011).

There have been a large number of studies on effect of vitamin D on PC development but the results are controversial. It has been suggested that vitamin D deficiency can increase PC risk due to anti-proliferative effect of vitamin D in the PC cells (Skowronski *et al,* 1993). However, dietary experiments did not produce conclusive evidence that vitamin D

supplementation reduces the risk of PC. A meta-analysis (Gilbert *et al,* 2011) has showed no major protective impact of vitamin D on PC risk or progression. Another study has shown a positive result i.e. increased risk of PC due to high circulating vitamin D concentration (Albanes *et al,* 2011).

V. Relationship between obesity or physical activity and prostate cancer

Obesity is frequently associated with increased oestrogen and decreased testosterone concentration in the circulation (Zumoff, 1987). Sex steroids are known to play critical role in PC development (Bosland, 2006). Androgens, especially testosterone and DHT, are the most important sex steroid hormones involved in PC development. This has been demonstrated in the studies on Nb strain rats, when subcutaneous injection of pellets containing estrone or testosterone 10mg each (every 6 to 8 weeks) into the rats for 6 months or more resulted in increased incidence of PC (Noble, 1977). The mechanism by which androgens regulate PC development will be discussed in section 2.2.1.

Body size and parameters (weight, height, waist circumference and the Body Mass Index (BMI)) was studied in a meta-analysis which revealed that there is no significant relationship between these parameters and PC incidence and death (MacInnis and English, 2006). At the same time Cao and Ma (2011) found a 15-20% increased risk of mortality due to PC and biochemical recurrence for each 5 kg/m^2 increase in BMI in 26 pooled studies from the USA, Netherlands and Japan with more than 1 million participants. However, this study does not take into account the state of participants BMI during the pre-diagnosis period. Therefore, the impact of hormonal imbalances prior to the diagnosis due to obesity cannot be ascertained. The above finding, however, shows that obese males have a higher risk of developing more aggressive disease compared to non-obese person and,

subsequently have a higher mortality rate as a result of the PC (Buschemeyer III and Freedland, 2007).

In addition to body mass, an effect of physical activity on incidence of PC has also been studied. There was a significant inverse relationship between physical activity and PC incidence reported in a meta-analysis of 43 studies involving 88,294 cases (Liu *et al,* 2011). It has also been demonstrated the overall risk reduction with physical activity can be as high as 10-30% (Friedenreich and Orenstein, 2002). Several studies have found no association at all between physical activity and PC risk (Dosemeci *et al,* 1993, Liu *et al,* 2000). Taking together, data of the literature do not provide strong evidence of relationship between physical activity and PC. At the same time, benefit of increased physical activity either in the course of the individual's occupation or for recreation purposes cannot be denied.

2.1.4 Screening, diagnosis and staging in prostate cancer

In this section, the review will focus on the issues related to the contemporary screening test PSA which is used for early detection of PC and to assist treatment decisions. PSA is a glycoprotein enzyme secreted by the epithelial cells of the prostate gland. PSA test detects the amount of PSA in the serum of man in whom there is a suspicion of PC. PSA is not a specific test in detecting PC as it may also be elevated in other benign prostatic disorders like prostatitis and benign prostatic hyperplasia. A brief description of the clinical stage, diagnostic modality and risk stratification will also be discussed.

(i) Screening in prostate cancer

It is widely accepted that PC is a slow developing disease and most patients diagnosed with it may not be affected by the disease in their lifetime (Holman *et al,* 1999). A controversial question is whether screening for PC significantly decreases the mortality rate due to the disease. Evidence from meta-analysis of five randomised control trials (RCT) suggests that screening does not significantly reduce the PC related mortality (Ilic *et al,* 2006).

Digital rectal examination (DRE) is the mandatory examination of a patient who presents to the clinician with prostatic symptoms to look for prostatic nodules and it is unreliable due to evidence suggesting that the DRE alone has a low predictive value in patients with PSA below 3.0 ng/ml of serum and also in a large group of people with PSA between 3.0 to 9.9 ng/ml of serum (Schroder *et al,* 1998). Therefore, relying on DRE alone to proceed for a diagnostic biopsy for PC may lead to a large number of unnecessary procedures.

This brings us to the advent of PSA test in 1986 into clinical practice. PSA is a blood test which identifies patients who are at risk of PC in order to subject the patients to a diagnostic biopsy. Generally, a PSA value less than 4.0 ng/ml is accepted as normal (Catalona *et al,* 1994). A biopsy is generally considered when the value is above 4.0 ng/ml. The PSA test can also be used to monitor PC progression and its response to treatment. However, reliability and specificity of PSA test has been increasingly questioned with regards to over diagnosis and unnecessary biopsy (Etzioni *et al,* 2002, Draisma *et al,* 2003). Although the recommended cut-off for PSA is 4.0 ng/ml, almost 2/3 of patients in this group do not have PC and the biopsy would have been unnecessary. Almost 15% of patients with a PSA level of less than 4.0 ng/ml could have PC (Crawford *et al,* 1999). It is

recommended that the combination of DRE and PSA is used to increase the sensitivity and specificity of PC detection by Crawford *et al* (1999).

Two large randomised trials studied the effect of PSA screening in the reduction of mortality rates due to PC. The USA -based Prostate, Lung, Colorectal and Ovarian (PLCO) found that there was no difference in the mortality rates of the screened participants with PSA compared to those who did not have PSA follow-up (Andriole *et al,* 2009). The European Randomised study of screening for prostate cancer (ERSPC) showed that there is a 20% reduction of mortality rates in the screened population (Schröder *et al,* 2009).

Therefore, there is a strong need for a reliable and more specific diagnostic test for PC. Such a test would help to reduce unnecessary biopsies, patient's anxiety of being diagnosed with PC, and unnecessary treatments of PC cases which are unlikely to progress to advanced stages.

(ii) ***Establishing the diagnosis of prostate cancer***

The definitive diagnosis of PC is made by the Trans-rectal Ultrasound (TRUS) guided biopsy of the prostate gland. The biopsy gives the clinician a histological diagnosis and the patient selected for the biopsy should either have an abnormal DRE or PSA more than 4.0 ng/ml. This is a procedure that is very uncomfortable due to the insertion of a rectal Ultrasound probe to visualise the prostate to take the biopsy. This biopsy has a risk of bleeding in the urine, stool and seminal fluid. Apart from this, there is a risk of infection and pain although it is performed under local anaesthesia (Clements *et al,* 1993).

In view of the morbid and very uncomfortable nature of the procedure, it will be of utmost importance if the biopsy could be reserved only for patients who have a significant risk of PC. Therefore, a more effective test to screen the patients for PC would be very useful.

(iii) ***Grade and stage of PC***

The most common grading system used in the assessment of the biopsy or the operated specimen of the PC is the Gleason grading system, which assesses the architecture of the cells and describes a score from 2 to 10 from well differentiated (least aggressive) to poorly differentiated (most aggressive) (Gleason and Mellinger, 1974) (Figure 2.11). This scoring is the sum of the 2 most common patterns seen from 2 to 5 with the grade < 7 being a low-grade cancer and ≥ 7 being a more aggressive cancer.

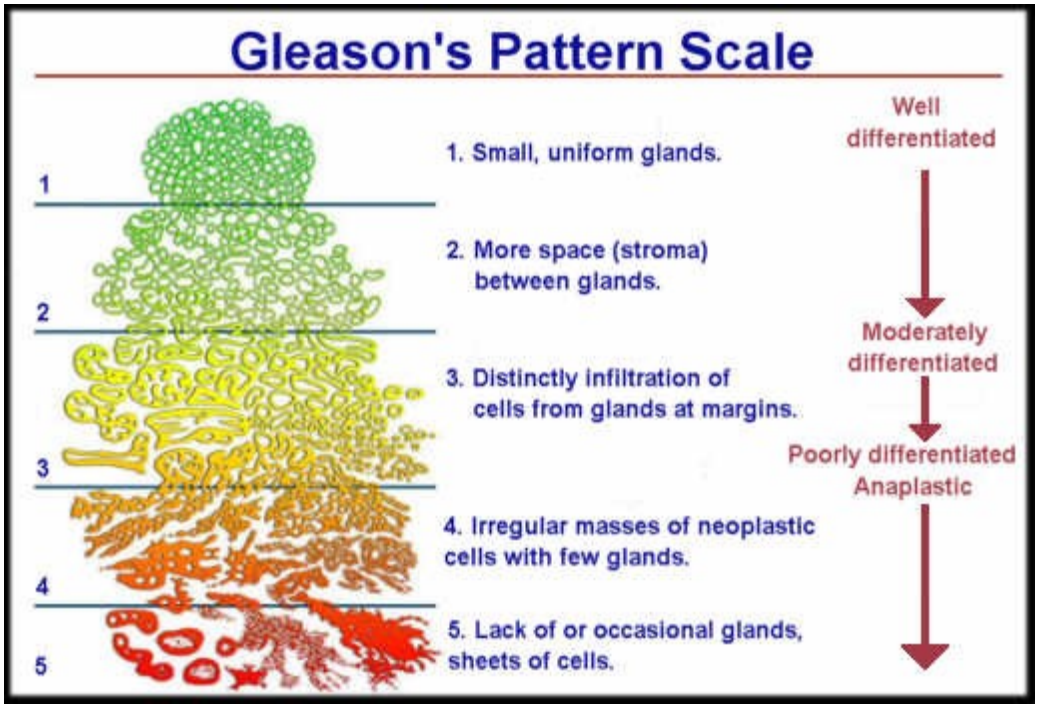

Figure 2.11 Grading of the prostate biopsy/operated specimen.
Figure shows the various grades of prostate cancer from well differentiated to poorly differentiated tumour. Adapted from http://trialx.com/curetalk/2011/07/interpret-your-gleason-grading-score/. Accessed October 2013. Source: Gleason (1977).

A clinical staging for the PC is still done by careful digital examination of the rectum to assess the tumour status (T staging) followed by magnetic resonance imaging (MRI) or computerised tomography (CT) scan to assess the nodal status (N) and the metastatic status of the cancer (Sobin *et al,* 2011). The risk group stratification of the newly diagnosed PC patients based on DRE, PSA and the Gleason score has been described by Damber and Aus (2008). Low risk PC is when the DRE showed no abnormality (T1c) or when a tumour is felt involving less than 50% of a single lobe of prostate (T2a), PSA 10 ng/ml or less and Gleason score of 6 or less. Intermediate risk PC is noted when the DRE finds a tumour involving >50% of a single lobe of prostate (T2b), PSA greater than 10ng/ml but less than 20 ng/ml, and Gleason score of 7. PC is said to be high risk when tumour felt in both lobes of prostate (T2c), PSA of 20 ng/mL or greater, and Gleason score of 8 or greater. Risk stratification is an important element of PC management in order to decide on an appropriate treatment plan which includes active surveillance, radical surgery, radical therapy or androgen deprivation therapy.

2.2 The role of androgens and insulin-like growth factor-I (IGF-I) in prostate cancer

2.2.1 Role of androgen

The prostate is an androgen-dependent organ, requiring androgens (sex steroid hormones) for growth during foetal and pubertal development (Ntais *et al,* 2003b, Ricke *et al,* 2006). There is large body of evidence to suggest that sex steroids, particularly androgens play a major role in the development of PC, but mechanism or their action remains unclear (Morgentaler and Traish, 2009, Hoffman *et al,* 2000, Andersson *et al,* 1993).

Testosterone, the major male androgen in the circulation, and DHT, the principal and potent androgen in tissue, are the two most important androgens in adult males (Welén and Damber, 2011, Henderson *et al,* 1982). Testosterone production is regulated via the hypothalamic-pituitary-gonadal axis (Figure 2.12) where Gonadotropin-releasing hormone which is secreted by hypothalamus, stimulates the pituitary gland to release the luteinizing hormone which acts on the Leydig cells in the testis to induce testosterone production. This contributes up to 90% of the testosterone production with the remaining 10% produced by the adrenal glands under control of adrenocorticotrophic hormone released from the pituitary gland (Ntais *et al,* 2003b, Coffey, 1988).

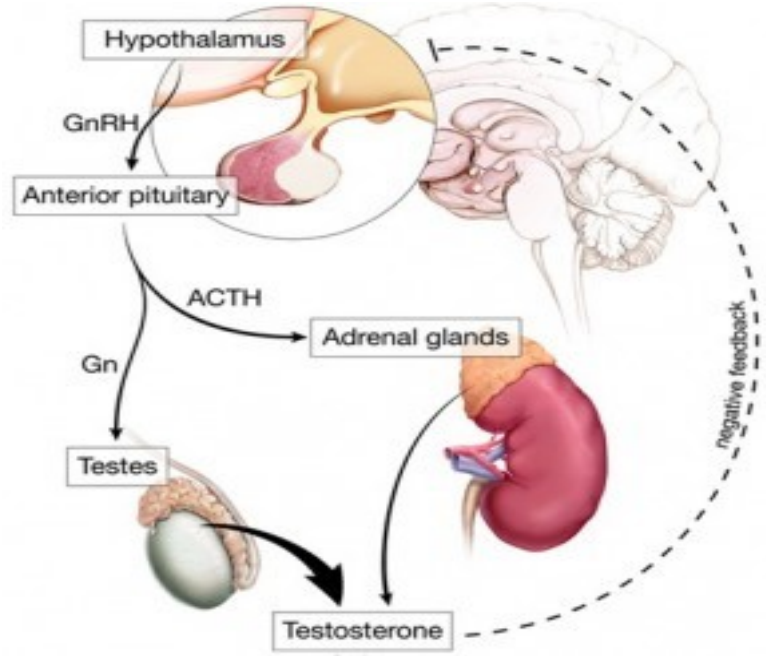

Figure 2.12 Mechanism of testosterone synthesis.

Testosterone synthesis regulated by the hypothalamo-pituitary-gonadal axis. GnRH-Gonadotropin releasing hormone, Gn-Gonadotropin, ACTH- adrenocorticotrophic hormone. Image adapted from Bioscience.org.

DHT in the circulation is produced from testosterone in the prostate gland in a reaction catalysed by 5α-reductase. About 25% of DHT is produced in other peripheral tissues such as skin and scalp (Ntais *et al,* 2003b). The metabolic pathway of androgen production is shown in Figure 2.13 (Li *et al,* 2013a).

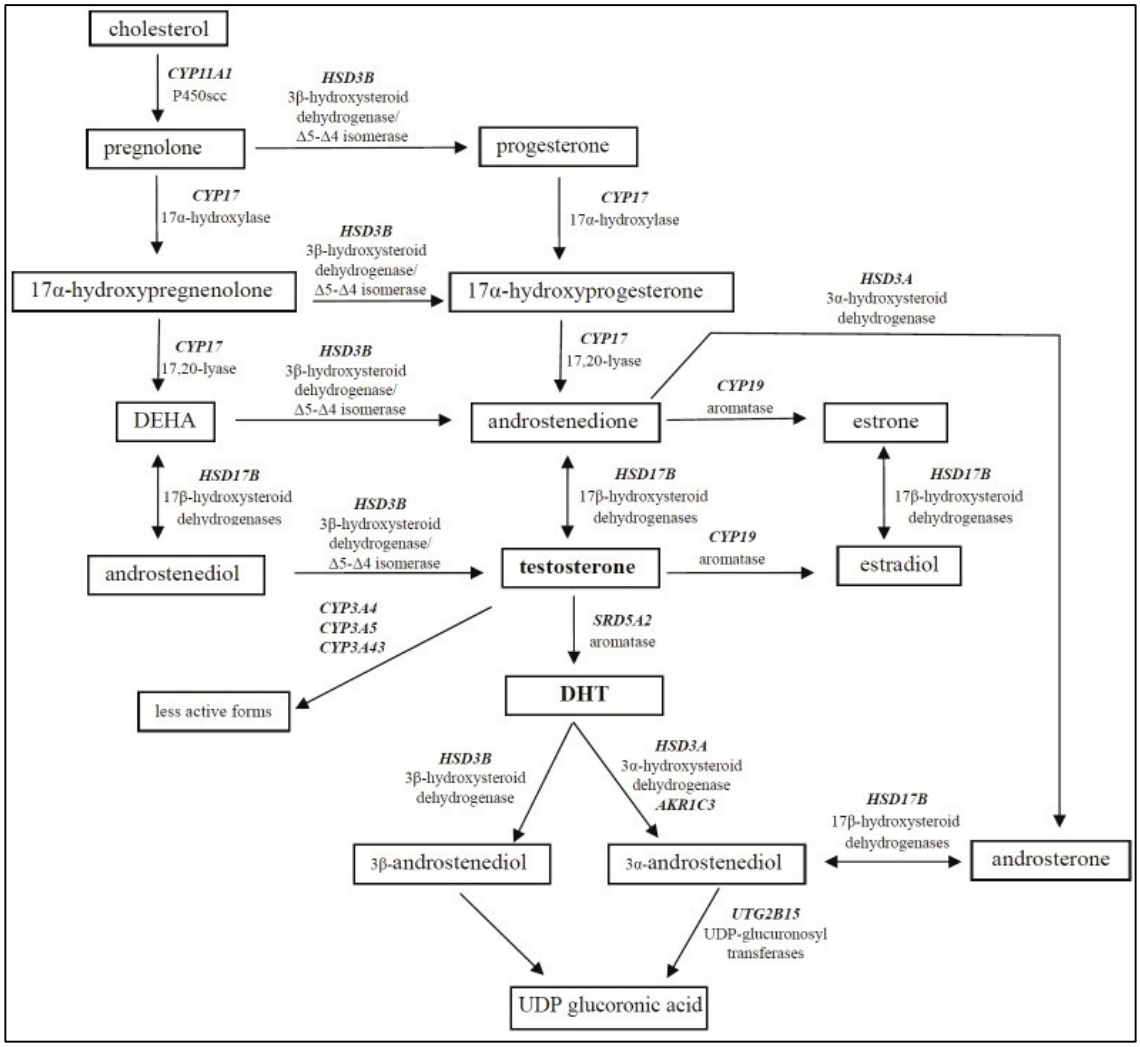

Figure 2.13 The androgen pathway.

Figure shows genes involved in androgen biosynthesis and metabolism. DHEA-dehydroepiandrosterone DHT- dihydrotestosterone. Adapted from Li *et al* (2013) with permission from the author.

The metabolic pathway above shows that the production of testosterone commences with the breakdown of cholesterol in multiple reactions catalysed by various enzymes out of which, the most common and essential ones are the 17α-hydroxylase and the 17, 20-lyase which are encoded by CYP17. (Li *et al,* 2013a, Schleutker, 2011). The enzyme 17α-hydroxylase catalyses the reaction which converts pregnolone and progesterone to 17α-hydroxypregnenolone and 17α-hydroxyprogesterone respectively (Figure 2.13). The enzyme 17, 20-lyase takes part in the reaction that produces dehydroepiandrosterone and androstenedione from 17α-hydroxypregnenolone and 17α-hydroxyprogesterone respectively (Figure 2.13).

The conversion of testosterone to DHT is mediated by 5α-reductase encoded by SRD5A2 gene. This process takes place in the testis and the prostate gland. DHT is the more potent hormone, with five to ten-fold higher binding affinity for androgen receptor (AR) than testosterone (Feldman and Feldman, 2001, Wright *et al,* 1999). Testosterone in circulation can be present in three forms: bound to albumin, bound to sex hormone binding globulin (SHBG) and free testosterone. Free testosterone is converted to DHT within the prostate gland and promotes DNA synthesis and cell replication by binding to the intracellular AR which activates gene transcription and cell proliferation. Increased cell division heightens the potential for mutations, leading to a higher likelihood of PC (Hsing, 2001, Lindström *et al,* 2006). This takes place via the formation of an intracellular DHT-AR complex which is then translocated to the nucleus and binds to the androgen response element of the prostate to induce cellular proliferation (Hsing *et al,* 2002, Heinlein and Chang, 2004) (Figure 2.14).

The overall effect of androgens in cells expressing AR is related to increase of cyclin-dependent kinase activity and stimulation of cells to enter the S phase of the cell cycle, thereby inducing cellular proliferation (Ntais *et al,* 2003b).

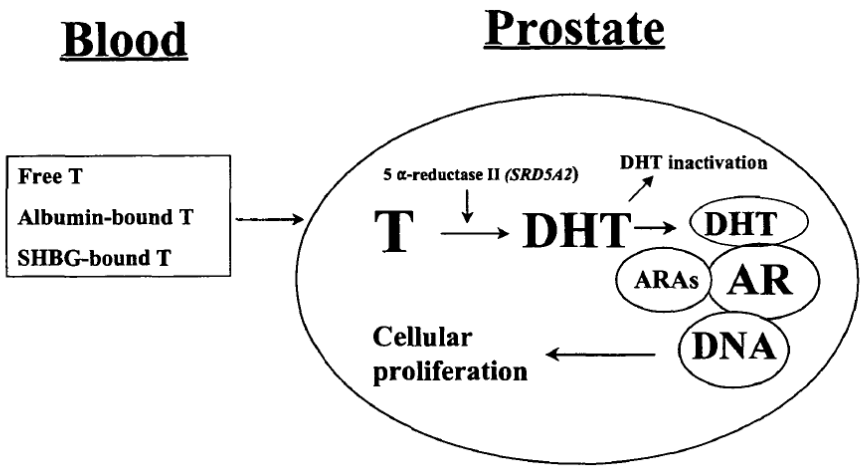

Figure 2.14 Androgenic action in the prostate gland.

T-testosterone, SHBG-sex hormone binding globulin, DHT-dihydrotestosterone, AR-androgen receptor, ARA-androgen receptor-associated proteins, DNA-deoxyribonucleic acid. Adapted from Hsing (2001) with permission from Oxford University Press.

Androgen deprivation therapy has been an important mode of treatment for advanced or metastatic PC for a long time by either chemical (use of anti-androgens) or surgical castration (Bare and Torti, 1998, Damber, 2005). Figure 2.15 shows the various levels of androgen blockade that can be administered in order to reduce androgen production. This includes oestrogen administration to block the stimulation of anterior pituitary, administration of LHRH analogues or antagonists which can block metabolic pathways controlling testosterone production. CYP17 is the main gene encoding for enzymes involved in biosynthesis of testosterone and therefore it could be an ideal candidate for the

blockade therapy. This therapy could also involve the use of anti-androgen drugs inhibiting the binding of DHT to the AR, and the use of 5α-reductase inhibitors and thus, reducing production of DHT (Welén and Damber, 2011).

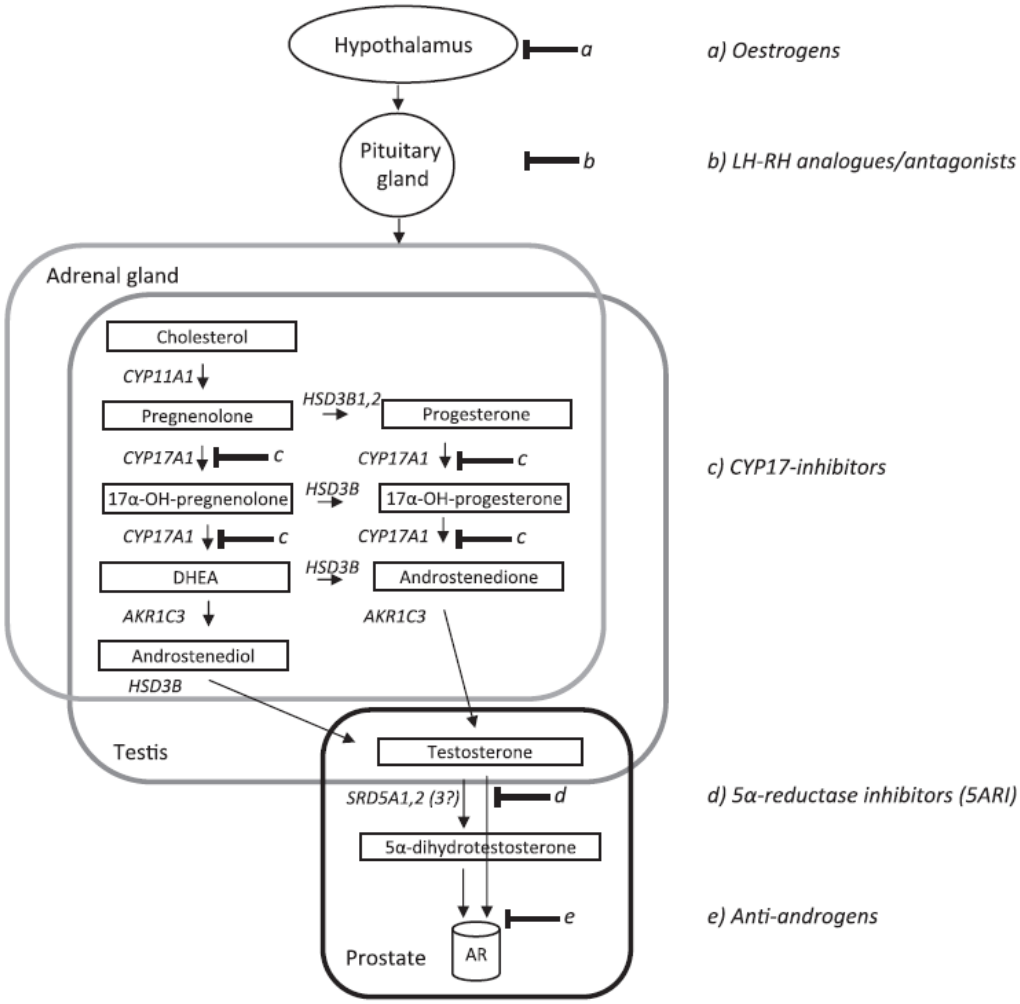

Figure 2.15 Simplified schematic representation of androgen biosynthesis and metabolism.

Figure shows options of androgen deprivation therapy that are available at different stages of androgen pathway. CYP11A1: cholesterol 20– 22 desmolase; CYP17A1: steroid 17-alpha-hydroxylase/17, 20 lyase; AKR1C3: 17-β-hydroxysteroid dehydrogenase; HSD3B: 3-b-hydroxysteroid dehydrogenase; SRD5A: steroid 5α-reductase. Adapted from Welén and Damber (2011) with permission from Elsevier.

Data of the literature on the role of androgens in PC development are controversial and is described below. An increasing number of DNA polymorphisms that can contribute to ethnic differences in PC epidemiology have been identified and noted that allele frequency differences at regulatory polymorphisms also account for some population differences in prevalence of complex diseases (Spielman *et al,* 2007). This chapter will also focus on discussion of polymorphisms in CYP17, SRD5A2 and AR genes which play significant role in the control of testosterone and DHT concentration. It has been described in previous sections of this thesis that the prostate gland is an androgen dependent gland. One of the PC development hypotheses by Gann *et al* (1996), namely a hormonal hypothesis, suggests that the circulating concentration of androgens should be higher in cancer patients compared to the non-cancer participants. Therefore, it is logical to suggest that populations with high PC incidence, such as the Caucasian compared to Asians would have a higher concentration of circulating androgens. The following review intends to look at literature to ascertain the role of androgens in the risk of PC development and ethnic-specific aspects of androgen effects on PC incidence.

Majority of studies in this area were conducted on ethnic groups in the USA (Wu *et al,* 1995b, Litman *et al,* 2006). Population data on hormonal status in European and Asian populations mainly studied single ethnic groups within the same environment. Data of the literature on association between circulating androgens concentration, PC incidence and ethnic variations are inconsistent.

A large population case-control study (N=1127) involving three different PC risk groups (African-Americans, White-Americans and Asian-Americans in USA and Canada) was conducted in order to determine the concentration of testosterone, DHT, the DHT to testosterone ratio and sex hormone-binding globulin (SHBG) (Wu *et al,* 1995a). The risk

of PC was highest in the African-Americans, followed by White-Americans and lowest in the Asian-Americans (either of Japanese or Chinese origin). This study showed that the testosterone concentration in African-Americans was not significantly different from that in White-Americans. However, it was noted that the Japanese-American and the Chinese-American had significantly higher total testosterone concentration than the White-Americans. The African-Americans and the Asian-Americans of Japanese origin showed a significantly higher DHT concentration compared to the White-Americans.

The Boston Area Community Health (BACH) survey involving almost 1900 participants (Black-Americans, White-Americans and Hispanics) investigated the racial/ethnic group differences in androgen concentration and also the DHT to testosterone ratio (Litman *et al,* 2006). This study showed that the concentration of testosterone did not differ significantly between the ethnic groups irrespective of whether the results were adjusted for co-variants (physical status, BMI, smoking, alcohol intake etc.). The DHT concentration and the DHT to testosterone ratio were found to be significantly elevated in the Black-Americans compared to the White-Americans and the Hispanics which might be related to the variations in PC incidence and prognosis between these ethnic groups (Litman *et al,* 2006). Another study comparing the Black-American and the White-American populations found a decrease in testosterone concentration with age in both populations studied. After adjustment for age and BMI, it was noted that the Black-American community had a 3% higher testosterone concentration than the White-Americans (Gapstur *et al,* 2002). No differences in androgen concentration were found between Kuwaiti Arabs and Chinese groups but a significant 20-50% reduction in the concentration of androgens was observed in Kuwaiti Arabs compared to the Black and White Americans, Chinese, Germans and Nigerians (Kehinde *et al,* 2006). It should be noted that the study by Kehinde *et al* (2006) was not performed in a single lab and therefore the results should be interpreted with

caution. A study by Kubricht III *et al* (1999) compared testosterone concentration in White-American (N=264) and Black-American (N=189) men who underwent a prostatic biopsy. The study did not find significant differences between the mean concentration of testosterone in the PC and control groups or between the two ethnic groups (White-American and Black-American) (Kubricht III *et al,* 1999).

In order to consider androgens (testosterone and/or DHT in particular) as potential biomarkers for PC, it is important to have clear understanding of relationship between the circulating androgen concentration and occurrence of PC. Data from the literature on this issue is controversial. There might be a number of reasons for inconsistency between data of the literature including the use of different assay for androgens analyses, different blood collection time and difference due to ethnicity.

The nested case-control study by Nomura *et al* (1996) in Japanese-Americans in Honolulu showed that the testosterone and DHT concentration do not differ significantly between the PC and control participants. In this study the androgen concentrations were analysed by radioimmunoassay (RIA) technique and the time of day of blood collection was not stated. The blood collection time is very important for androgen analysis because of the diurnal variation observed in the concentration with peak concentration being in the morning from 8.00 am to 12.00 noon (Rose *et al,* 1972, Brambilla *et al,* 2009). There are other studies on different ethnic group which concur with findings of Nomura *et al* (1996). Dorgan *et al* (1998) did not find significant relationship between testosterone, DHT and the PC risk in a Finnish population. The blood collection time in this case was specified to be in the morning and RIA was used as the technique for assessment of hormones concentrations. The above finding is also consistent with reports of Chen *et al* (2003) on Caucasian-Americans, Sawada *et al* (2010) on Japanese men, Heikkila *et al* (1999) on Finnish cohort

and Mohr *et al* (2001) on Caucasian-Americans. Mohr *et al* (2001) also used RIA technique and blood collected in the morning for androgen detection. Sawada *et al* (2010) used electro chemiluminescence immunoassay (ECLIA) for testosterone assay and the blood drawn could not be ascertained whether it was performed in the morning. Gann *et al* (1996) demonstrated that testosterone (but not DHT) concentration was significantly higher in PC cases in USA Caucasian ethnic group. Gann *et al* (1996) performed the hormonal assays by RIA technique but the time of blood collection was not exclusively in the morning. Similarly, Hsing *et al* (1993) found elevated DHT to testosterone ratio but the time of blood taking in the report was not indicated. de Jong *et al* (1991) demonstrated that there was no significant association between PC and androgen concentration in the Dutch and Japanese population but they showed a non-significant lower testosterone and DHT to testosterone ratio in the Japanese men.

To summarise, data of the literature do not provide a consistent view and strong evidence regarding the relationship between androgens concentration in PC incidence and development. Furthermore, there have been a limited number of studies on ethnic-group specific aspects of circulating androgen concentration and its correlation with PC, although population study suggests a higher androgen concentration in the Black-American population when compared to White-Americans and Asian-Americans. The above highlights the need for detailed investigation into ethnic-group specific androgen concentrations, especially from the Asian region using a well-designed case-control study. The Asian region has a very low incidence of PC and data from this region has not been widely studied in relation to the role of androgens in PC. Majority of the studies published so far, are based on nested case-control study which used blood samples drawn at the time of patients recruitment, which were stored and analysed only when PC had been

diagnosed. Another important issue appears to be the different methods used in the androgen assay and the time at which the samples collected were not consistent.

2.2.2 Role of insulin-like growth factor-I

IGF-I hormone is mainly produced in the liver under the regulation of the complex mechanism controlled by the hormones and nutritional factors (Livingstone, 2013). A schematic presentation of this process is given in Figure 2.16. The rate of production of growth hormone (GH) in the pituitary gland plays a key role in this process. GH is transported to the liver where it binds to the growth hormone receptor to produce IGF-I which is secreted into the circulation (Livingstone, 2013). Almost 99% of IGF-I is bound to the IGF binding proteins (IGFBP). Six proteins of IGFBP family have been identified and they include IGFBP3 which has the highest affinity to IGF-I and subsequently determines the concentration and half-life of IGF-I (Livingstone, 2013, Firth and Baxter, 2002, Pollak *et al,* 2004, Lima *et al,* 2009). The small proportion of unbound IGF-I in circulation known as the free IGF-I, is the active component and it can bring out the mitogenic activity. The mitogenic activity is initiated by binding of the free IGF-I to the IGF-I receptor on target tissues (Livingstone, 2013). Apart from GH that controls the IGF-I concentration, nutrition also plays a role in the maintenance of IGF-I concentration in circulation. There are evidences showing that IGF-I concentration drops under malnutrition and increases again upon feeding, supporting the idea that IGF-I can be used as a potential nutritional biomarker (Livingstone, 2013, Counts *et al,* 1992). The hepatic IGF-I production can also be stimulated by the circulating insulin produced by the pancreas in relation to the carbohydrate content in the body as well as the nutritional status of the individual (Hanaire-Broutin *et al,* 1996). This explains the reason for the increase and decrease IGF-I concentration as described above due to malnutrition and feeding.

IGF-I has various functions in the human body including short-term effects such as stimulation of amino acid uptake into skeletal muscle and stimulation of peripheral glucose uptake (Jacob *et al,* 1989, Bilan *et al,* 1992) which is an important function that mimics action of insulin. IGF-I also has long-term effect on cellular proliferation, differentiation and anti-apoptotic effect which can all lead to the progression of normal cell to a malignant cell (Yu and Rohan, 2000, Jones and Clemmons, 1995) .

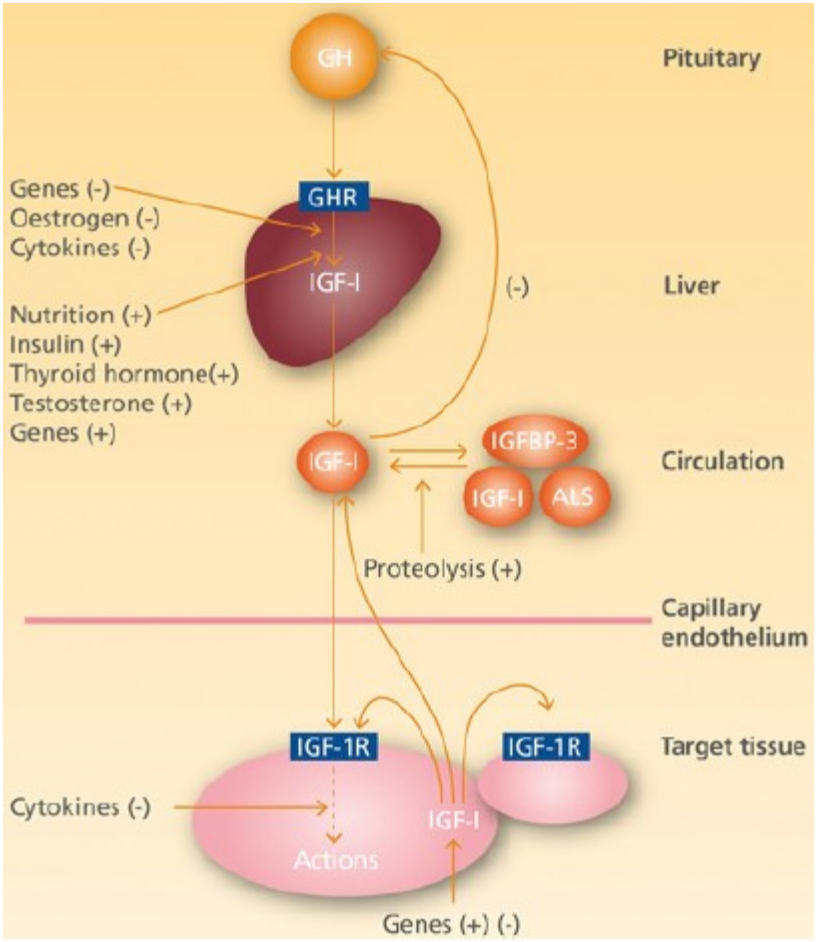

Figure 2.16 Regulation of circulating and tissue concentration of Insulin-like growth factors. Positive and negative regulatory effects are shown at "+" and "-" respectively. GHR-growth hormone receptor, GH-growth hormone, IGF-I-Insulin-like growth factor-I, IGFBP-Insulin-like growth factor binding protein, IGF-IR- Insulin-like growth factor-I receptor, ALS- acid-labile unit. Source: Livingstone (2013).

Although prostate gland is an androgen-regulated tissue, (testosterone and DHT- regulated in particular), it cannot be denied that PC can also develop in individuals with low testosterone concentration (Morgentaler *et al,* 1996, Morgentaler and Rhoden, 2006). This indicates the involvement of other factors, such as the insulin-like growth factors in PC development.

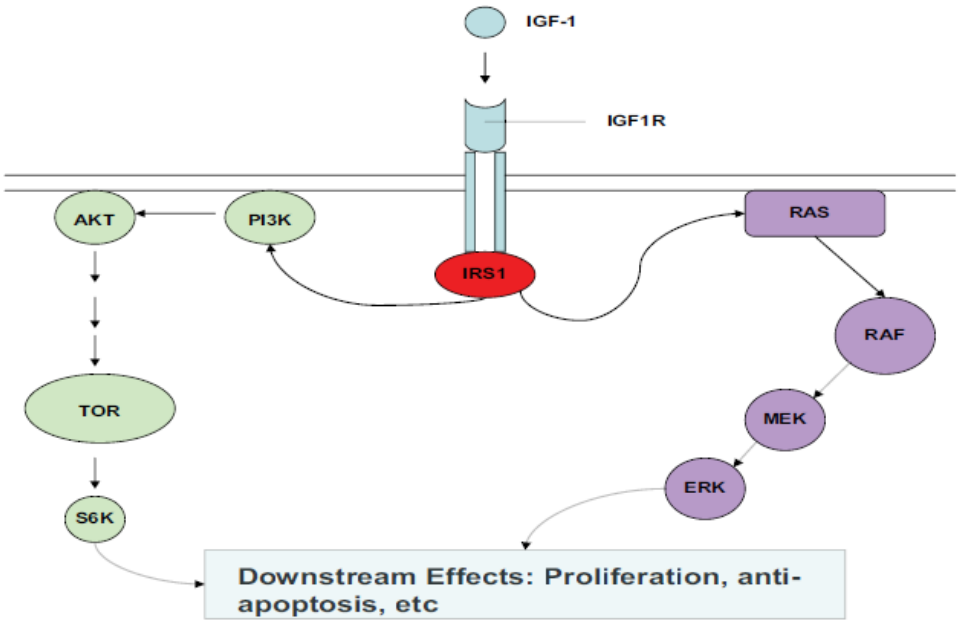

Figure 2.17 Illustration of an overview of downstream signalling from IGF-I receptor.

IGF-I- Insulin-like growth factor-I, IGF1R- Insulin-like growth factor-I receptor, IRS1- Insulin receptor substrate 1, PI3K- phosphatidylinositol 3-kinase, TOR- target of Rapamycin, S6K- S6 kinase, MEK- mitogen activated protein kinase, ERK-extracellular signal-related kinase. Adapted from Pollak *et al* (2004) and Aggarwal *et al* (2011) with permission from Nature Publishing Group.

IGF-I acts by binding to the IGF-I receptor found on the cell membrane and causes the proliferative effect and anti-apoptotic function by activating the tyrosine kinase pathways via 2 routes, namely the PI3K and RAS/RAF protein pathways which activates signal transduction molecules as shown in Figure 2.17 (Lima *et al,* 2009, Aggarwal *et al,* 2011).

Function of tyrosine kinase is to catalyse the phosphorylation of tyrosine residues in proteins that can lead to functional changes of the protein (Nelson *et al,* 2008). This protein can be transcriptional factors which control the transfer of genetic information from DNA to messenger RNA which can be affected by the abnormal tyrosine kinase activity in mitogenesis (Radha *et al,* 1996). This is the reason for unhindered tyrosine kinase activity leading to malignant change in the prostate. Insulin also has proliferative as well as anti-apoptotic effect as the insulin receptors are structurally similar to IGF-I receptor (Mynarcik *et al,* 1997) and the signalling pathway is the same as for IGF-I once insulin binds to the IGF-I receptor. There is a correlation between IGF-I and insulin concentration in the circulation with the occurrence of PC. (Cox *et al,* 2009).

It is known that there is relationship between the androgen biosynthesis pathway and the growth factor metabolism pathway which may contribute to initiation and progression of PC (Hsing, 2001). A review article by Zhu and Kyprianou (2008) reported evidence of functional interaction between AR and various growth factors such as epidermal growth factor, fibroblast growth factor, IGF-I, vascular endothelial growth factor and transforming growth factor-β. The endogenous AR expression and its transcriptional activity that control cellular proliferation and apoptosis, are regulated by insulin and IGF-I pathways similar to that described in Figure 2.17 (Manin *et al,* 2000, Manin *et al,* 2002).

Various epidemiological studies have been performed to investigate the relationship between the circulating IGF-I concentration in the development of PC. The discussion below focuses on IGF-I as a potential ethnic group-specific biomarker for PC. IGF-I serum concentration is age-dependent; it increases slowly from birth to puberty and thereafter declines with age (Moschos and Mantzoros, 2002, Ceda *et al,* 2005). Increased IGF-I concentration in older patients may be a sign of human cancer as a whole (Samani *et al,*

2007). There have been various studies over the years assessing circulating concentration IGF-I in relation to PC risk.

A number of authors linked elevated IGF-I concentration in the circulation with increased risk of developing PC. Stattin *et al* (2004) found a 4-fold increase in the risk of PC in a Swedish cohort with high IGF-I concentration using the immunoradiometric (IRMA) assay. Chokkalingam *et al* (2001) demonstrated by ELISA technique a 2.6-fold increase of PC risk in patients with higher IGF-I in case-control study in Shanghai. Chan *et al* (2002) and Platz *et al* (2005) also described positive relationship between PC risk and IGF-I concentration using the ELISA technique in a mixed American population (all ethnic groups). Wolk *et al* (1998) concurred with the above authors in a Swedish case-control study but they used an IRMA technique for IGF-I assay. Kehinde *et al* (2005) have shown that Arab men have lower IGF-I concentration than Caucasians which may explain a low incidence of PC in the Arabs. Positive associations between circulating IGF-I concentration and PC development and severity was found in several meta-analysis. Renehan *et al* (2004) demonstrated in a meta-analysis which included 21 eligible studies with a case to control ratio of 1 : 2 that high concentrations of IGF-I were associated with an increased risk of PC compared to a lower concentration. Another meta-analysis also showed that high circulating IGF-I concentration can give a 2-fold increase in PC risk (Shaneyfelt *et al,* 2000). An analysis of individual patient data from 12 prospective studies also concluded that the higher IGF-I circulating concentration increases the risk of PC (Roddam *et al,* 2008).

At the same time data of other authors do not agree with the mentioned above studies. Using the IRMA technique for IGF-I analysis, Chen *et al* (2004) found that IGF-I concentration was not associated positively with risk of PC in a mixed USA population. In

the UK, Rowlands *et al* (2012), using the RIA technique also found no association between IGF-I and PC risk. Several studies using ELISA techniques by Borugian *et al* (2008), Allen *et al* (2007) and Severi *et al* (2006) was also in agreement that IGF-I concentration does not affect the PC risk in mixed USA, multiple European and Australian ethnic groups.

There may be several reasons for the inconsistency of results between studies done previously in different ethnic groups. Firstly, most of the studies done previously were mainly based on nested case-control study design. These studies are retrospective and the samples are collected before the diagnosis is made and are usually a part of a different large population study. Once the follow-up of the participants have confirmed that they have developed PC, then the analysis of all such patients are performed for the required parameters. The selection of controls is age-matched among those in the same cohort. Although this type of studies are cost-effective as samples are already available but the selection of controls may be biased due to the possibility of unknown occult PC. The samples obtained are also pre-diagnostic samples which have been stored for a long time since recruitment which can be up to 20 years in some study. This may impact the results as it does not reflect the actual state of the participant in the preceding years before the diagnosis of PC due to the effect of factors like diet and lifestyle on the hormone concentration.

Generally, there has been a lack of well-designed prospective case-control and ethnic-group based studies comparing multiple ethnic groups from different environment. There is also a lack of data from various other low PC incidence cohorts like Asians and Africans. It should also be noted that previously conducted studies used different techniques for IGF-I detection which makes it difficult to interpret results.

2.3 DNA polymorphisms in genes controlling androgen pathways and their link to prostate cancer

The importance of androgens in PC development has been described in section 2.2.1. The genes involved in the biosynthesis and metabolism of androgens have been extensively studied and several key genes, such as SRD5A2, CYP17 and AR have been identified as potential candidate genes for PC (Figure 2.13). Specific polymorphisms in these genes in relation to PC will be reviewed in the following section of the thesis.

2.3.1 Polymorphisms in CYP17 gene and their role in prostate cancer

Sex steroid hormone synthesis follows the conversion of cholesterol to pregnenolone. CYP17 enzyme catalyses two key reactions involved in the production of sex steroids from cholesterol, which occur sequentially as follows: 17α-hydroxylase converts pregnenolone to 17α-hydroxypregnenolone and progesterone to 17α-hydroxyprogesterone, while C17,20-lyase converts 17α-hydroxypregnenolone to DHEA and 17α-hydroxyprogesterone to androstenedione (Vasaitis *et al,* 2011, Denisov *et al,* 2005) (Figure 2.18). The precursor DHEA and androstenedione are subsequently converted to testosterone by HSD3B2 and HSD17B3. There has been another pathway described in the production of DHT that by-passes testosterone and is catalysed by enzymes encoded by CYP17 gene (Figure 2.18) (Auchus, 2004). Extensive involvement of CYP17-encoded enzymes in the androgen metabolism indicates importance of this gene as potential therapeutic targets for PC. CYP17-encoded enzymes belong to oxygen-reacting hemeproteins cytochrome P450 group which has 40 types of proteins in humans with a diverse list of activities, one of which is the biosynthesis of sex steroids (Denisov *et al,* 2005, Hasler *et al,* 1999).

CYP17 gene is located on chromosome 10 and encodes the cytochrome P450c17α enzymes. There has been a lot of interest in studying CYP17 polymorphisms and their effects on the CYP17 expression and enzyme activity in relation to the rate of testosterone production. The use of abiraterone acetate, a selective CYP17 inhibitor, demonstrated improvement in PC patients as evidenced by PSA test and bone scan (Reid et al., 2010). These results are in a line with a report on a randomised phase III trial on metastatic PC patients which demonstrated benefit of abiraterone acetate-based drug for overall patient survival (Ryan *et al,* 2013).

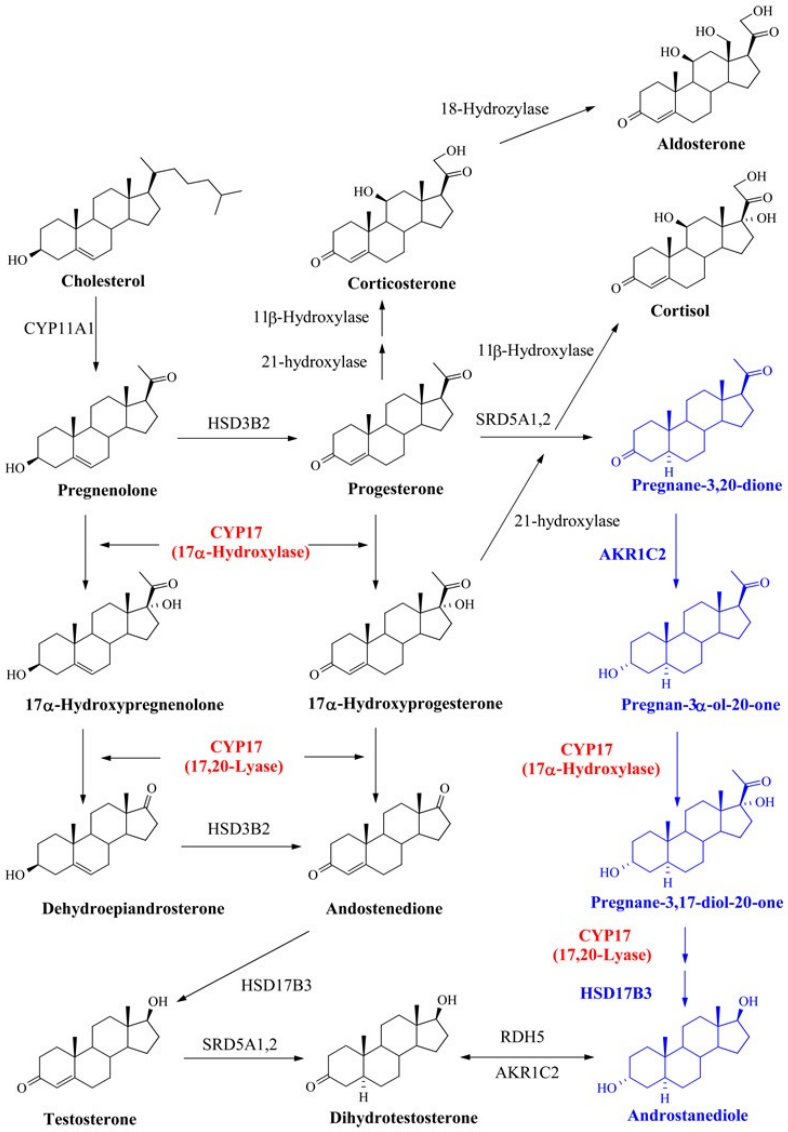

Figure 2.18 Series of reactions involved in the formation of androgens from cholesterol.

Red- Enzymes encoded by CYP17 gene, Blue- second pathway for DHT production catalysed by CYP17 encoded enzymes. Adapted from Vasaitis *et al* (2011) with permission from Elsevier.

The role of CYP17 gene in biosynthesis of testosterone was first described in a study which identified a new single base change (T to C substitution) in 5' promoter region of the CYP17 gene (Carey *et al,* 1994). This SNP creates an additional SP1-type (CCACC box) promoter site, presence of which may positively be related to testosterone production. The variant C allele (designated as A2) may result in increased transcriptional activity and

hence increase testosterone biosynthesis (Carey *et al,* 1994). CYP17 involvement in PC development is supported by finding that CYP17 mRNA expression is up-regulated in tumour compared to normal tissues (Stigliano *et al,* 2007). The same authors also demonstrated increase in CYP17 mRNA expression with the PC grade. Another important evidence in support of links between CYP17, testosterone concentration and PC is a 17-fold increase in CYP17 mRNA in castrate-resistant PC patients (i.e. in those whose disease was progressing despite complete androgen blockade achieved as treatment of PC) compared to the patients who had not been treated for their primary tumours (Montgomery *et al,* 2008).

It is important to highlight that although there has been an increasing number of communications about polymorphism in CYP17 promoter the relevance of these polymorphisms to PC development is not clear and data of the literature are controversial. In particular, this refers to the polymorphism in 5'-untranslated region of the CYP17 gene containing a polymorphic T-to-C substitution (rs743572) at position -34T→C that gives rise to A1 (T) and A2 (C) alleles (Carey *et al,* 1994). There are several other polymorphisms that have been identified in CYP17 gene in relation to PC risk. The reported CYP17 polymorphisms are in rs284851, rs619824, rs10883782, rs1004467, rs17115144, rs2486758 but no significant association was found between these polymorphisms and PC but it should be taken into account that information about these polymorphisms are limited. (Beuten *et al,* 2009). The only SNP that has been consistently studied in different ethnic groups is the rs743572, although outcomes of these studies are inconsistent (Karimpur-Zahmatkesh *et al,* 2013, Severi *et al,* 2008). This SNP will be one of the focuses of this thesis. One of the reasons for controversial data of the literature might be the use of different ethnic groups and a possibility of ethnic group-specific variations in the mechanisms controlling PC development. The studies reviewed reported

frequency of the above polymorphism and the relationship between different CYP17 alleles (A1, A2 and A1/A2) and the risk of PC.

Lunn *et al* (1999) reported the same frequency of A2 allele in Caucasian-Americans and Black-Americans. In Caucasians (but not Black-Americans) this allele was associated with increased risk of PC. Gsur *et al* (2000), Yamada *et al* (2001) and Sarma *et al* (2008) observed a higher frequency of A2 allele in PC patients compared to the controls in the Austrian, Japanese and African-American ethnic groups. Yamada *et al* (2001) also described increased risk of PC in patients with a high frequency of A2 allele compared to A1 or A1/A2 alleles. The A2 allele was noted to increase risk of PC in North Indian, Iranian and Tunisian men (Sobti *et al* 2009, Karimpur-Zahmatkesh *et al* (2013), Souiden et al., 2011).

At the same time, a number of authors disagreed on contribution of CYP17 polymorphisms at the position -34T→C to PC development. These studies were conducted on Chinese, Australian-Caucasian, Brazilian and mixed American men (multiple USA ethnic groups) by Madigan *et al* (2003), Severi *et al* (2008), dos Santos *et al* (2002) and Chang *et al* (2001) respectively. The most likely explanation for controversial data of the literature is the different ethnic groups studied. CYP17 polymorphism may be more common and associated with PC risk in some ethnic groups but not in others. To confirm this hypothesis, a large population study with the use of different ethnic groups under the same experimental conditions is required. It is also important to ensure that such a study will have a proper control group. The selection of control participants has been different in different papers with some using BPH and some using healthy controls. This is an important limitation of all the research and could be one of the reasons for the inconsistency of the results.

No association between CYP 17 SNP (rs743572) and PC was found in meta-analysis on 2,404 PC patients and 2,755 controls (Ntais *et al,* 2003a). However, it should be noted that when the data were analysed by ethnic groups, the presence of A2 allele was associated with increased susceptibility to PC in subjects of African descent. There was no effect of A2 allele on PC risk seen in subjects of European origin or Asian origin. As the number of cases and studies involving African origin were low, further research is needed to confirm these data (Ntais *et al,* 2003a). This indicates the need to undertake a well-designed case-control study in an unreported multi-ethnic society like in Malaysia consisting of Malays, Chinese and Indians in direct comparison to the Caucasian group.

Another important aspect of the CYP17 polymorphism at position -34T→C is its impact on the concentration of circulating testosterone. The first published study on this topic showed no significant association between the CYP17 polymorphism and total testosterone concentration in UK white men (Allen *et al,* 2001). Another nested case-control study within the Physicians' Health Study cohort in the USA involving 590 PC cases and 782 matched controls evaluated the association between CYP17 polymorphism at position -34T→C and circulating steroid hormone concentration in the control group. Among the controls, the carriers of the A2 allele of CYP17 gene were found to have no difference in the circulating steroid hormone concentration compared to the non-carriers (Haiman *et al,* 2001). Research involving 523 participants in the Study of Osteoporotic Risk in Men (STORM) established a significantly higher concentration of free testosterone in White-American men with the A2 allele compared to A1 genotype, with heterozygous (A1/A2) men having intermediate hormone concentration (Zmuda *et al,* 2001). There is not much information on the status of Asian population with regards to CYP17 polymorphism at position -34T→C in relation to the circulating sex hormone concentration. Therefore, one

of the focuses of the present PhD project is to investigate the relationship between CYP17 polymorphism at the position -34T→C and circulating sex hormone concentration.

2.3.2 Polymorphisms in SRD5A2 gene and their role in prostate cancer

SRD5A2 gene encodes 5α-reductase enzyme that catalyses the conversion of testosterone into a more powerful prostatic androgen, dihydrotestosterone (DHT) (Coffey, 1993), which is responsible for prostate growth and may be considered a potential marker for predicting clinical response to the anti-androgen therapy in PC patients (Wilbert *et al,* 1983, Geller *et al,* 1984).

There are two types of 5α-reductase enzyme reported with SRD5A1 gene encoding for the type I enzyme and SRD5A2 gene encoding the type II enzyme. The type 2 enzyme is the key enzyme involved in prostate development and growth (Reichardt *et al,* 1995). The type I enzyme is usually found in the skin and liver and is more important in sexual differentiation (Reichardt *et al,* 1995).

The conversion of testosterone to the more active intracellular metabolite, DHT, by 5α-reductase with NADPH as the cofactor takes place within the prostatic epithelial cell (Figure 2.19). DHT binds to the androgen receptor (AR), and the DHT-AR complex activates a number of genes containing AR-responsive elements (Figure 2.14) (Coffey, 1993).

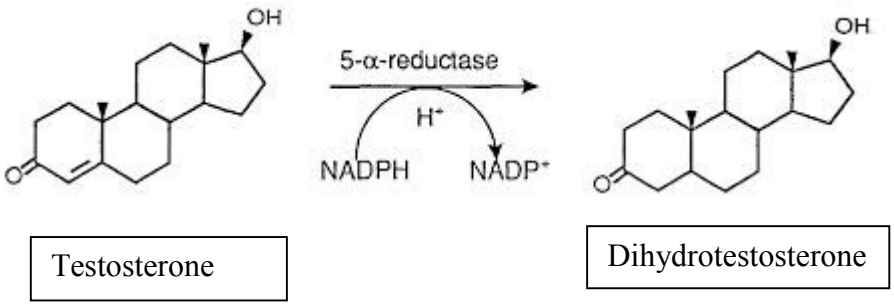

Figure 2.19 Conversion of testosterone to DHT facilitated by 5α-reductase with NADPH as a cofactor.

Adapted and modified from Andrews et al (1996) with permission from author and Bentham Science Publishers.

The human SRD5A2 gene is located on chromosome 2 (2p23) and it spans about 40 kb of genomic DNA, with 5 exons and 4 introns and known to be a highly polymorphic gene (http://www.ncbi.nlm.nih.gov/SNP) (Reichardt *et al,* 1995). There have been 10 missense substitutions reported in SRD5A2 gene (Makridakis *et al,* 2000). Most of these polymorphisms are extremely rare with an incident rate of less than 2%. The two most important polymorphisms in SRD5A2 as far as PC is concerned, are C>G substitution, rs523349, which results in amino acid substitution, Val to Leu at codon 89 (V89L), and G>A substitution, rs9282858 which results in Ala to Thr change at codon 49 (A49T) (Makridakis *et al,* 2000, Makridakis *et al,* 1997, Li *et al,* 2013b). It has been hypothesised that the activity of 5α-reductase enzyme can be controlled by the two polymorphism mentioned above.

The V89L amino acid substitution reduces activity of the enzyme 5α-reductase *in vivo* and *in vitro*, which might lead to reduction in the circulating DHT. This effect could potentially protect against PC development (Makridakis *et al,* 2000). In contrast, the A49T substitution increases 5α-reductase activity *in vitro* and might lead to increased production of DHT from testosterone (Makridakis *et al,* 1997). Zeigler-Johnson *et al,* (2002) investigated frequency of V89L and A49T in African-Americans, Caucasian-Americans,

Ghanaians and Senegalese populations. It was noted that frequency of L genotype at codon 89 was the highest in Caucasian-Americans followed by the African-Americans. The Africans were least likely to carry this polymorphism (Zeigler-Johnson *et al,* 2002). The same study showed that the frequency of the T genotype at codon 49 was highest among White-Americans followed by African-Americans and Africans. An interesting observation was that the frequency of V89L and A49T in the Asians were reported as 54.8% and 0% respectively among the cancer-free participants (Li *et al,* 2010). This reiterates the fact that there is a potential in studying ethnic differences of these variants in cancer patients. Data from epidemiologic study in various populations and the risk associated with PC is described below. The genotypes that are produced in the event of the above C>G polymorphism are denoted based on the amino acid change. It can be VV for Val (no change), LL (for Leu change) and L/V for a heterozygote type of substitution under the V89L variant. Margiotti *et al* (2000) had shown that the presence of the LL genotype reduced the risk of PC non-significantly in Italian men. This was also noted in a study by Hsing *et al* (2001) in a Chinese group but similarly the reduction was not statistically significant. Yamada *et al* (2001) demonstrated that there was a non-significant 5% increase in the frequency of the LL genotype in the Japanese PC cases than in controls. These Asian data may indicate the protective nature of increased frequency of the LL genotype that may explain the low incidence of PC in these Asian communities but the above study did not assess the risk and therefore was unable to comment on the risk of PC. However, larger studies in more Asian communities are required to confirm this finding. Torkko *et al* (2008) in a study among non-Hispanics and Hispanic-whites found that Hispanic-white men with the VV genotype had reduced PC risk in the USA. Giwercman *et al* (2005) did not assess the risk of PC in the Swedish cohort studied but found that the frequency of the LL genotype was significantly higher in the PC group than the control

group. This data was somewhat contradicting the theory that LL genotype frequency should be higher in Asians as shown by Torkko *et al* (2008). Studies by Salam *et al* (2005) and Cussenot *et al* (2007) in mixed American men and Caucasian French men respectively demonstrated that the homozygous LL genotype increase the risk of PC significantly in both the ethnic groups studied compared to the VV or LV genotype. Cussenot *et al* (2007) also reported that the LL genotype is associated with the most aggressive PC. This is in keeping with positive impact of this LL variant in the SRD5A2 gene that may play an important role in increasing the risk of PC in Western men compared to the Asians although data from Asia is limited.

Several authors did not concur with the findings of the previous research above and have reported no association between the V89L variant of the SRD5A2 gene in the frequency or risk associated with PC in the presence of VV, LL or L/V genotypes. These studies with negative association with this polymorphism were reported by Onen *et al* (2007) in Turkish men, Hayes *et al* in the Australians and Rajender *et al* (2009) in South Indian men. These findings above confirm the possibility of ethnic difference in the frequency and risk of PC incidence and warrants larger studies in other ethnic groups. The heterogeneity in the selection of the PC cases and the control groups again may play an important role in the inconsistency of the results. This is because the selection of patients was all not of the same disease stage and grade and therefore led to small numbers in each stage. As in the selection of control group in the polymorphism in the CYP17 gene, there may be a difference in the genetics of healthy controls, BPH and population control groups as described in section 2.3.1.

Most authors in our review of literature have reported that the A49T variant was not found in any of the cases or control patients in their study. Yamada *et al* (2001), Hsing *et al*

(2001) and Rajender *et al* (2009) reported the absence of polymorphism that causes the A49T variant in the PC cases and controls studied in Asians of Japanese, Chinese and South Indian origin respectively. Onen *et al* (2007) was also in agreement with the above finding in the Asian group and reported the absence of A49T variant in any of their samples in Turkish men. Contrasting results were found in some of the Western studies as far as frequency and risk associated with PC incidence is concerned. Margiotti *et al* (2000) reported that there was a non-significant increase in PC risk with the TT genotype but none of the variant was found in any of the controls who were participants with low risk of PC development. Torkko *et al* (2008) reported that the TT homozygote was absent in the Hispanic and non-Hispanic cohort studied in the USA but the AT heterozygote variant found did not increase the risk of PC. Hayes *et al* (2007) reported the frequency of the G>A polymorphism at position 140 from the start codon in SRD5A2 gene in an Australian cohort study (amino acid change was not reported). They found that the frequency of A nucleotide was about 2% higher in PC cases than controls and the G/A heterogeneous nucleotide was also about 3% higher in PC cases than controls. The overall risk of PC in the study by Hayes *et al* (2007) was a significant 60% higher with the presence of either A or G/A nucleotide. Result from the study by Hayes *et al* (2007) was in agreement with an earlier study by Makridakis *et al* (1999) in African-Americans and Hispanic-Americans that showed a significant 7.2 and 3.6-fold increase in PC risk in the two ethnic groups respectively. Based on this review, it is noted that an ethnic variation exists in the prevalence of this A49T variant of SRD5A2 gene. It appears that the variant is absent in Asian population compared to other ethnic groups. The possibility of this variant being one of the reasons for ethnic difference in the incidence should be studied further in various other ethnic groups in Asia in comparison to the Caucasians and African-origin groups.

Information about the described above genetic polymorphisms and their relationship with PC risk might be useful for the development of intervention strategies for PC treatment. There has been increasing interest in developing drugs for chemoprevention (i.e. Finasteride) which act as 5α-reductase inhibitors (Lucia *et al,* 2007, Thompson *et al,* 2003). These drugs are used to treat Benign Prostatic Hyperplasia by shrinking the prostate and have been tried as a chemo preventive agent for PC. In a study comparing Finasteride and placebo in PC prevention, a preventive effect of Finasteride was demonstrated for low grade PC (Lucia *et al,* 2007). Therefore, the use of this drug is beneficial in the prevention of PC and the study of polymorphism in the SRD5A2 gene that encodes for the enzyme 5α-reductase may be correlated with the benefit of using this drug to reduce the incidence of PC. The identification of genetic polymorphism of genes that encode for the enzyme 5α-reductase could provide an explanation for the success or failure of any chemo preventive strategy used.

In view of these contrasting evidence, the need for data from new groups and an overall assessment of the androgen pathway can still establish important information to explain the heterogeneity of the PC disease.

2.3.3 Polymorphism in AR gene and its role in prostate cancer

The biological function of androgens is exerted via activation of the transcriptional activity of androgen receptor (AR) (Huang and Tindall, 2002). The AR is capable of binding both, testosterone and DHT. DHT has a five-fold higher affinity for the AR than testosterone (Heemers *et al,* 2009).

The androgen-induced transcriptional activation of AR is modulated by the interaction of AR with co-regulators and by phosphorylation of AR and AR co-regulators in response to growth factors (Figure 2.20) (Heinlein and Chang, 2004, Richter *et al,* 2007).

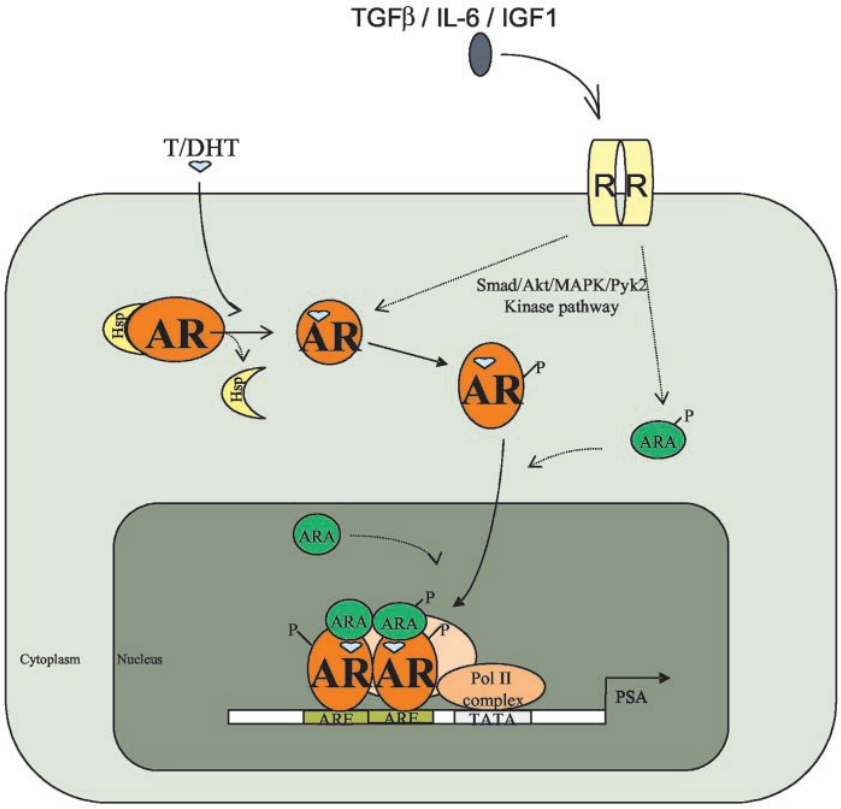

Figure 2.20 Interaction between androgen and AR within the prostate gland.
T- testosterone, DHT- dihydrotestosterone, AR- androgen receptor, ARAs- AR co regulators, AREs- androgen response elements, HSP- heat shock protein, R- membrane receptor, P- protein Phosphorylation. Adapted from Heinlein and Chang (2004) with permission from the author.

Testosterone and DHT bind to the AR in the prostate and promote the association of AR co-regulators (ARAs). AR then translocates to the nucleus and binds to androgen response elements (AREs) in the promoter regions of target genes to induce cell proliferation and

apoptosis. An example of the target gene is the PSA gene which is up regulated to increase the production in case of malignant change (Agoulnik and Weigel, 2009) . Other signal transduction pathways, such as those involving TGFβ, IL-6, and IGF-I, can also enhance AR activity via phosphorylation of AR and/or ARAs (Heinlein and Chang, 2004, Culig *et al,* 2002).

AR integrates signals from multiple regulatory pathways described above and disruptions in any of the pathway may lead to the loss of AR functional integrity. Increases in cell growth-promoting functions and the apparent loss of differentiation properties of AR may play important role in the transformation from a benign prostate to a malignant one (Figure 2.21). This transformation can also be due to mutations in the AR gene which alter ligand interaction or increase transcriptional activity leading to abnormal cellular proliferation and loss of anti-apoptotic signal (Heinlein and Chang, 2004, Richter *et al,* 2007).

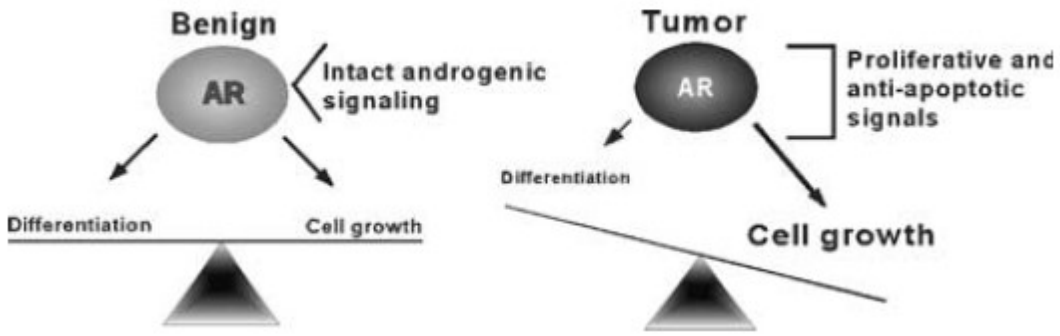

Figure 2.21 The Androgen receptor (AR) balance.

Figure shows overwhelming cell growth and reduced differentiation due to abnormal AR signalling. Adapted from Richter *et al* (2007) with permission from Nature Publishing Group.

The AR gene, located on the short arm of X-chromosome (Xq11-12), is known to be polymorphic, having a highly variable trinucleotide microsatellite of CAG-repeats and GGC-repeats in exon 1 (Kral *et al,* 2011, Ekman *et al,* 1999). The numbers of CAG-repeats can vary in different population groups, with normal numbers ranging from 8–31, with an average of about 20 repeats (Ekman *et al,* 1999, Edwards *et al,* 1992). The number of GGC repeats can vary from 10–30 but the impact of abnormal repeat count on PC is still a matter of research in contrast to the more definitive findings in studies related to the CAG-repeats (Kral *et al,* 2011). The NH2-terminal transactivation domain of AR contains two trinucleotide repeat regions (CAG and GGC) both of which are polymorphic in length and are separated by 248 amino acids of non-polymorphic sequence (Heinlein and Chang, 2004, Kral *et al,* 2011). The CAG trinucleotide encodes for a sequence of glutamine (polyglutamine) while the GGC trinucleotide encodes for a sequence of glycine (polyglycine) and these amino acid sequence length may also vary based on the length of polymorphic CAG and GGC repeat length.

Studies have shown, although not consistently, that the longer AR variants (due to the increased number of CAG-repeats and to a certain extent GGC-repeats) decrease transactivation activity and affinity to bind androgen which may lead to a protective effect on the prostate. The short variants may increase the risk of PC due to increase AR activity (Ekman *et al,* 1999, Chamberlain *et al,* 1994). PC patients appear to have shorter CAG-repeats compared to controls (Irvine *et al,* 1995, Ingles *et al,* 1997). African-Americans, who have the highest risk of PC, generally have shorter CAG-repeats compared to White-Americans and Asian-Americans (Kral *et al,* 2011, Coetzee and Ross, 1994). This PhD project will focus on relationship between the number of CAG-repeats and PC incidence.

The ethnic differences in PC incidence tend to be inversely related to CAG-repeat number in the AR gene. Asian men have the lowest PC incidence and the longest AR gene CAG-repeats, whereas African Americans (AA) have the highest PC incidence and shortest CAG-repeat length (Li *et al,* 2013a, Kral *et al,* 2011). However, there are several studies that found no evidence that the CAG repeats are linked to PC in Taiwanese, non-Hispanic white population, Japanese and Chinese Singaporeans (Edwards *et al,* 1999, Price *et al,* 2010, Huang *et al,* 2003, Chang *et al,* 2002). In contrast, studies conducted on the Americans (Giovannucci *et al,* 1997), Nigerians (Akinloye *et al,* 2011) , Iranians (Ashtiani *et al,* 2011), and South Indians (Krishnaswamy *et al,* 2006) have shown positive links between a number of the repeats and PC. In particular, Giovannucci *et al* (1997) showed that the risk of PC increased in those with CAG-repeat length of less than 18. Akinloye *et al* (2011) used the cut-off length of ≤ 21 and reported that PC risk is increased in shorter CAG-repeats in Nigerians. Ashtiani *et al* (2011) demonstrated significant shorter mean CAG-repeat length in PC compared to healthy controls and in PC compared to BPH. The South Indian study by Krishnaswamy *et al* (2006) found that the risk of PC increased significantly in men with ≤ 19 CAG repeats compared to those with > 19 CAG repeats where the controls were a mixture of healthy and BPH participants. In studies conducted in Chinese men, Zhai *et al* (2014) found that the risk of PC and more aggressive disease was higher in CAG-repeat length of < 21, but in an earlier study by Hsing *et al* (2000), it was noted that there was 65% increase risk of PC in CAG-repeat length of < 23. A large study in multiple ethnic groups within the same environment in a country like Malaysia could possibly establish an ethnic based cut-off for CAG-repeat length to determine risk of PC tailored to specific ethnic-groups.

A meta-analysis confirms association between ethnic differences in predisposition to PC and ethnic-group specific length of CAG-repeats. It has been noted that longer repeat (≥ 20

repeats) were associated with 11% decreased risk of PC in USA population, 53% decreased risk in Europe, and 20% decreased risk in Asia (Gu *et al,* 2012). There are also studies which assessed the circulating concentration of testosterone in relation to the AR polymorphism. The data are controversial with some papers reporting elevated testosterone concentration in case of longer CAG-repeats (Krithivas *et al,* 1999, Lindström *et al,* 2010) with other communications not being able to establish any correlation between testosterone and CAG-repeats length (Zitzmann *et al,* 2001, Van Pottelbergh *et al,* 2001). An important issue in majority of the study in different ethnic groups reviewed above showed a striking difference on cut-off for CAG-repeat length in each community to assess PC risk. This indicates a strong need in determining polymorphic length of CAG in specific ethnic groups.

In this research project, the relationship between the number of GGC repeat and PC was not studied due to lack of evidence in the literature regarding the role of this polymorphism in PC development.

2.4 Summary of literature review

2.4.1 Background on prostate cancer

a) Size of prostate gland increases with age and may cause urinary obstruction symptoms called benign prostatic hyperplasia.
b) Malignant change can take place in the prostate. Mechanism that causes the change is unknown but the most likely theory is the increased production of reactive oxygen species in the prostate caused by risk factors like diet, lifestyle and environmental changes.

c) Worldwide incidence of PC is rising and it is the most common cancer in men in western countries. Despite increase in PC incidence, mortality due to PC has remained quite stable due to diagnosis of clinically insignificant cases that is unlikely to lead to mortality in the life–time of the patient.

d) The only established PC risk factors are the non-modifiable ones such as age, ethnicity, family history and genetics

e) Ethnicity plays a major role in PC incidence. The risk of PC is the highest in Africa-Americans followed by Caucasians. Asian ethnic groups have the lowest risk of PC.

f) Modifiable PC risk factors have been extensively studied but the results are inconsistent so far. These risk factors include nutritional factors (vitamin E, vitamin D, Selenium, and Lycopene), obesity, physical activity, and hormonal factors (androgens and growth factor).

g) PSA is the only established screening test widely used for PC screening. There is an issue with PC over diagnosis due to low sensitivity and specificity of the test. This lead to unnecessary diagnostic procedure which are morbid.

2.4.2 Role of androgen in prostate cancer

a) Prostate gland is an androgen regulated organ. Testosterone and DHT are the major sex hormones that play important role in PC development. The mechanism of androgens involvement in PC development is not fully understood.

b) It is suggested that the conversion of testosterone to a more potent DHT takes place in the prostate. DHT binds to AR and activates gene transcription and cellular proliferation. Uninhibited proliferation can lead to malignant change in the prostate gland.

c) Data of the literature are inconsistent with regards to relationship between androgen concentration and PC risk in different ethnic groups.

2.4.3 Role of insulin-like growth factor-I in prostate cancer

a) IGF-I is produced in the liver. The production is regulated by growth hormone and nutrition.
b) IGF-I binds to IGF-I receptor to bring about metabolic and anabolic activity. Its long-term effect includes cellular proliferation, cell differentiation and anti-apoptosis effect via the tyrosine kinase pathway.
c) Changes in AR function due to binding of DHT to the receptor can cause functional changes in the activity of growth factors such as IGF-I.
d) Data of the literature is inconsistent with regards to effect of IGF-I on risk of PC, It is suggested that higher concentration of circulating IGF-I increases the risk of PC.

2.4.4 Review of genetic polymorphisms

a) CYP17 and SRD5A2 play an important role in control of androgen metabolism. AR gene controls the binding capability of AR to DHT.
b) CYP17 gene:
    i. Polymorphism at -34T→C is the most studied in relation to PC risk and incidence.
    ii. CYP17 gene encodes P450c17α enzyme that catalyses two key reactions involved in the production of sex steroids from cholesterol. The two reactions are (i) conversion of pregnenolone to 17α-hydroxypregnenolone

and progesterone to 17α-hydroxyprogesterone, and (ii) conversion of 17α-hydroxypregnenolone to DHEA and 17α hydroxyprogesterone to androstenedione.

iii. The polymorphism mentioned above is suggested to increase production of testosterone via androgen metabolism pathway.

c) Data of literature on CYP17 polymorphisms are controversial with multiple studies from different ethnic groups reporting varying PC risk associated with A1, A2 and A1/A2 alleles.

d) SRD5A2 gene:

i. SRD5A2 gene encodes for 5α-reductase, an enzyme that catalyses the conversion of testosterone into the more powerful prostatic androgen, DHT.

ii. A49T and V89L are the most extensively studied variants with regards to PC. Nucleotide substitution at the position 261C→G causes the amino acid change from Val to Leu at codon 89 (V89L) and nucleotide substitution at the position 140G→A results in amino acid change from Ala to Thr at codon 49 (A49T) in this gene.

iii. V89L variant reduces activity of 5α-reductase thus reducing the conversion of testosterone to DHT. A49T variant increases activity of the above enzyme and therefore increases the production of DHT.

iv. Data of the literature are not conclusive as for association between these two polymorphisms and PC risk.

e) AR gene:

i. Mutation in AR gene can alter the binding capacity of the receptor with DHT or increase transcriptional activity leading to abnormal cellular proliferation and loss of anti-apoptotic signal.

ii. AR gene is known to be polymorphic, with a highly variable trinucleotide microsatellite of CAG-repeats and GGC-repeats in exon 1. Number of CAG repeats has been widely studied in relation to PC.

iii. It is thought that a lower number of CAG-repeat is associated with the higher the risk of PC. However, the data differ depending on ethnic groups studied.

The hypotheses for this project are:

1. The concentration of circulating androgens (testosterone and DHT) and the growth hormone Insulin-like Growth Factor-I (IGF-I) are positively related to the incidence of PC in BC and the MC men. The serum concentration of all these hormones are expected to be significantly higher in the BC compared to the MC men because of the higher risk and incidence of PC in the Caucasians compared to Asians.
2. The presence and frequency of selected polymorphisms in the CYP17, SRD5A2 and the AR genes are related positively to the higher incidence of PC in the BC but not in MC men.
3. Polymorphisms in CYP17, SRD5A2 and AR genes are associated with increased concentration of the hormones testosterone, DHT and IGF-I in the circulation.

3 Materials and methods

This study was a coordinated research between University of the West of England (UWE), Bristol Urological Institute (BUI) and University of Malaya, Kuala Lumpur (UM), aiming to investigate ethnic-group specific mechanisms of PC risk and development.

This study was conducted on 200 samples of whole blood and serum collected from the two ethnic groups, Bristol Caucasian (BC) and Malaysian Chinese (MC) men (100 samples from each ethnic group). The samples from each ethnic group comprised of control samples and PC patients (50 samples per group).

The samples from the BC men were collected at the Urology outpatient's clinic at Southmead Hospital, Bristol, UK. Samples from the MC group were obtained at the Urology outpatient clinic of the University Malaya Medical Centre Kuala Lumpur (UMMC). Some control samples in the Malaysian group were also obtained during Prostate Awareness Week. All the sample collection was done in accordance to Ethical approvals and following strict participant selection criteria described in Table 3.1. The same protocol was used for the sample collection in Malaysia and the UK.

This study also includes a pilot experiment which was conducted in order to optimize laboratory techniques and protocols, particularly in relation to molecular biology and genetic experiments. This experiment was conducted on the whole blood samples from five healthy volunteers recruited at the University of the West of England. Ethical approval for the recruitment of the volunteers was obtained from the Faculty Ethical Committee of the University of the West of England which permits the sample to be stored for 5 days at -80°C before being processed as in section 3.1.3. All volunteer samples were discarded after 5 days.

All the collected blood samples from the participants were immediately processed as described in section 3.1.3 and stored at -80°C (up to 3 months) until analysed.

Schematic presentation of the experimental design is shown in Figure 3.1.

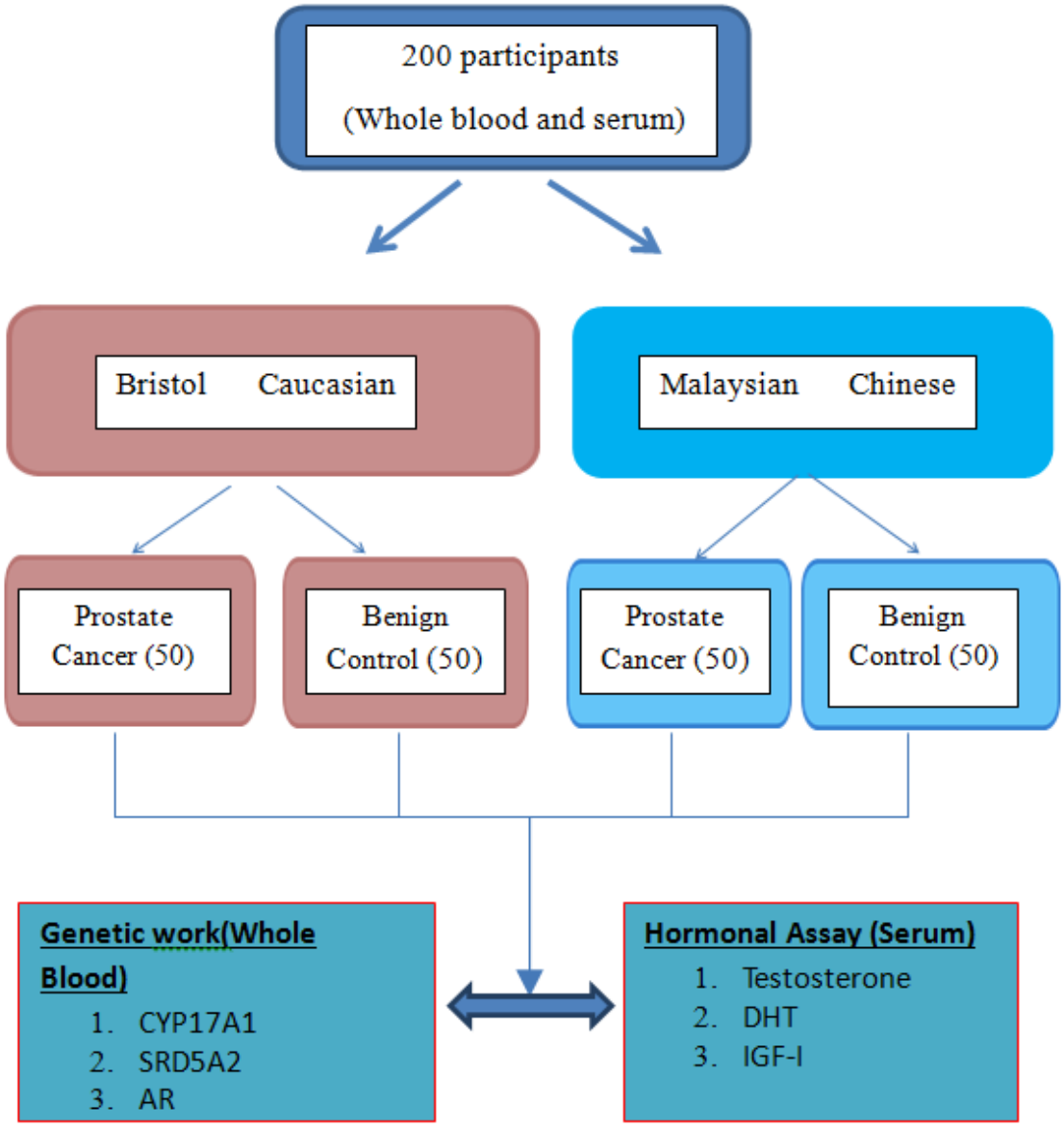

Figure 3.1 Schematic presentation of experimental design

CYP17A1- cytochrome P-450 steroid 17α-hydroxylase/17,20 lyase, SRD5A2- steroid 5α reductase type II, AR- Androgen Receptor, DHT- Dihydrotestosterone, IGF-I- Insulin-like growth factor-I.

3.1 Study Design

3.1.1 Ethical Approval

This research was based on work with human tissue (whole blood and serum) and therefore a National Health Service (NHS) ethical approval was required prior to sample collection and experimental work. The PC patients and control group recruitment was conducted in accordance with UK and local Ethical Regulations (South-West Committee). The protocol for PC patients and control group recruitment was approved via the Research and Governance System (RAGS) of the University of the West of England for both the main study and the pilot study with involvement of volunteers. The South-West ethical approval was obtained on 14th May 2012.

Ethical approval was also obtained from the Medical Ethics committee of the University Malaya Medical Centre (UMMC), Kuala Lumpur, for the patient recruitment and samples collection at the hospital in Malaysia.

3.1.2 Patients and Controls Recruitment

This study recruited (i) patients who were newly diagnosed with PC and (ii) a control group with Benign Prostatic Hyperplasia (BPH).

The inclusion and exclusion criteria for recruitment were the same for both ethnic groups investigated and are presented in Table 3.1. All the participants in this study were more than 40 years old. The PC group consisted of newly diagnosed PC cases. The diagnosis was established based on prostate biopsy. Indications for prostate biopsy were either abnormality felt on digital rectal examination or an elevated PSA level (above 4.0 ng/ml). Participants in the control group were of similar age with low risk of PC.

Table 3.1 Inclusion and exclusion criteria for prostate cancer control groups in BC and MC participants.

Parameters	Inclusion	Exclusion
Prostate Cancer Group	All newly diagnosed prostate cancer patients. Diagnosis was based on Transrectal Ultrasound guided biopsy of prostate. Age above 40 years old. Bristol Caucasian and Malaysian Chinese ethnic groups.	Men of ethnic origin other than Bristol Caucasian and Malaysian Chinese. Age less than 40 years old.
Control Group	Patients who have been diagnosed with Benign Prostatic Hyperplasia (BPH). Normal digital rectal examination (DRE) findings. Prostate specific antigen level less than 4.0 ng/ml. No family history of prostate cancer.	Prostate Specific Antigen level more than 4.0 ng/ml Positive family history of prostate cancer. Abnormal digital rectal examination finding. Men of ethnic origin other than Bristol Caucasian and Malaysian Chinese.

BC- Bristol Caucasians, MC- Malaysian Chinese.

The Bristol Caucasian (BC) participants with PC were approached regarding the recruitment for this study during the counselling visit after the treatment decision has been made by the clinicians at Southmead Hospital. This was the second visit where the patient had been informed about the diagnosis during the first visit. The patients received a Patient Information Sheet prior to the second visit. This approach was adopted so that the anxiety of being diagnosed with cancer can be reduced. The Malaysian participants were also recruited during (i) outpatient appointments after the treatment decisions for PC have been

made and (ii) during outpatient follow-up appointments for consultation and investigation, or during awareness campaign for the Benign Prostatic Hyperplasia cases.

Participant information sheets were given out during appointments for Benign Prostatic Hyperplasia consultation/investigation clinics or PC clinics. The participant information sheets made it clear that the study was voluntary and that choosing to refuse to participate would not in any way affect the patients' treatment plan. It was also made clear to the participants that the samples would be made anonymous, and the nature of the experiments to be conducted on the samples was explained. The document also explained the benefit of this study to the future of prostate cancer management. A written consent was taken from each participant who agreed to participate in the study after at least 24 h going through the participant information sheet. Blood samples were then taken from consenting participants by a qualified nurse or a doctor at the clinic.

The Malaysian collaborating team followed the same recruitment protocol, but also included some participants who were diagnosed with Benign Prostatic Hyperplasia and PC during the Prostate Awareness Programmes. Recruitment through this additional programme was related to a very low incidence of PC in a single hospital.

3.1.3 Blood collection and storage

The protocol for the collection and storage of blood samples was the same for BC and MC participants. The blood sample from all consenting participants was obtained in the mornings. This was due to the known fact of diurnal variation in the concentration of circulating testosterone with the peak concentration known to be in the mornings (Brambilla *et al,* 2009).

The blood sample (12 ml) were drawn by venepuncture by either a phlebotomy trained nurse or a clinician at the Urology out-patient clinics at Southmead Hospital, Bristol and University Malaya Medical Centre, Kuala Lumpur. The blood samples were then divided in three tubes labelled with unique identity number. 3ml of blood each was placed in two tubes containing anti-coagulant Ethylenediaminetetraacetic acid (EDTA) and another 6ml of blood was placed into a plain tube not containing preservatives or anti-coagulant.

The sample in the EDTA-tube was transferred immediately to a -80°C freezer at Bristol Urological Institute and at University Malaya Medical Centre, Kuala Lumpur facility. These samples were used for genetic analysis. It has been previously reported that storage of blood samples at -80°C does not compromise the quality and integrity of the DNA in blood or serum used for hormonal assay (Peakman and Elliott, 2008). For hormonal assays, blood samples were left in plain tubes and were allowed to clot for one hour and subsequently subjected to centrifugation at 1500 x g for 15 min at room temperature to separate the serum from the cellular components (http://www.scienceboard.net/resources/protocols). Equal volumes of serum were carefully pipetted out into three 1.5ml Eppendorf tubes, one tube per assay of each hormone. The tubes were stored at -80°C until analysed (the duration of storage varied from 1 to 6 months). Aliquoting in three tubes was necessary to ensure that the samples were not subjected to repeated freeze-thaw cycles that can contribute to the potential damage to the quality of the serum (Mitchell *et al,* 2005).

3.1.4 Sample transfer

The BC samples were transferred from Bristol Urological Institute to the Centre for Research in Biosciences at the University of the West of England in a biohazard labelled cold-storage box containing ice on the day of the analysis.

The Malaysian samples (isolated DNA from whole blood and the serum) were transported to the University of the West of England by courier (World Courier (Malaysia) Pvt. Ltd. Petaling Jaya) at -80°C. DNA isolation method was agreed by both parties before hand and is described in Section 3.3.1 below. Anonymity of the samples was ensured at all times with accurate labelling with unique identity number.

3.1.5 Clinical and demographic data collection

All clinical and demographic data were collected during interview with the patients or from medical records and were entered into a standard proforma. The data collected were: age, diagnosis, PSA level, the Gleason score (in cancer cases) and digital rectal examination (DRE) findings. The information from the proforma was entered into a password secure computer for later use in statistical analysis. A unique identity number was generated for each participant and this number cannot be traced back to the patient to ensure anonymity of each sample. The Malaysian samples and data were collected with the help of the Malaysian collaborators from University of Malaya, Kuala Lumpur following the uniform protocol and subsequently transferred to the University of the West of England for analysis.

3.2 Analysis of serum hormone concentration

The concentration of circulating testosterone, DHT and IGF-I were analysed in the serum samples which were collected, stored and transferred to the University of the West of England as described in Sections 3.1.3 and 3.1.4.

The samples were analysed in batches of 40 for each hormone. Each analysis was conducted in duplicates. Intra-assay coefficient of variability (CV) was calculated for samples within the same plate to ensure the repeatability of the results and precision in the techniques. For the intra-assay CV, the mean of the mean concentration of hormone for each sample and the standard deviation (SD) was calculated for each plate. The SD was then divided by the mean and multiplied by 100 i.e. SD/ mean of mean concentration X 100 to give the percentage CV. Acceptable CV percentage is < 10%. The percentage CV was calculated for each kit and the average for each test in our study was noted to be 8.7%, 6.1% and 10.7% for testosterone, DHT and IGF-I respectively. Each batch included equal number of samples from Caucasian cancer, Caucasian control, MC cancer and MC control groups (10 samples per batch).

The laboratory analysis of testosterone, DHT and IGF-I concentration were performed using commercially available Enzyme-Linked Immunosorbent Assay Testosterone ELISA kit (Immuno Biological Laboratories International, Hamburg, Germany), 5alpha-dihydrotestosterone ELISA (Immuno Biological Laboratories International, Hamburg, Germany) and Quantikine® ELISA Human IGF-I Immunoassay (R&D System Europe Ltd. Abingdon, UK) respectively following the recommended protocol. Fig. 3.2 shows the work-flow in the analysis of the stored serum obtained from the participants.

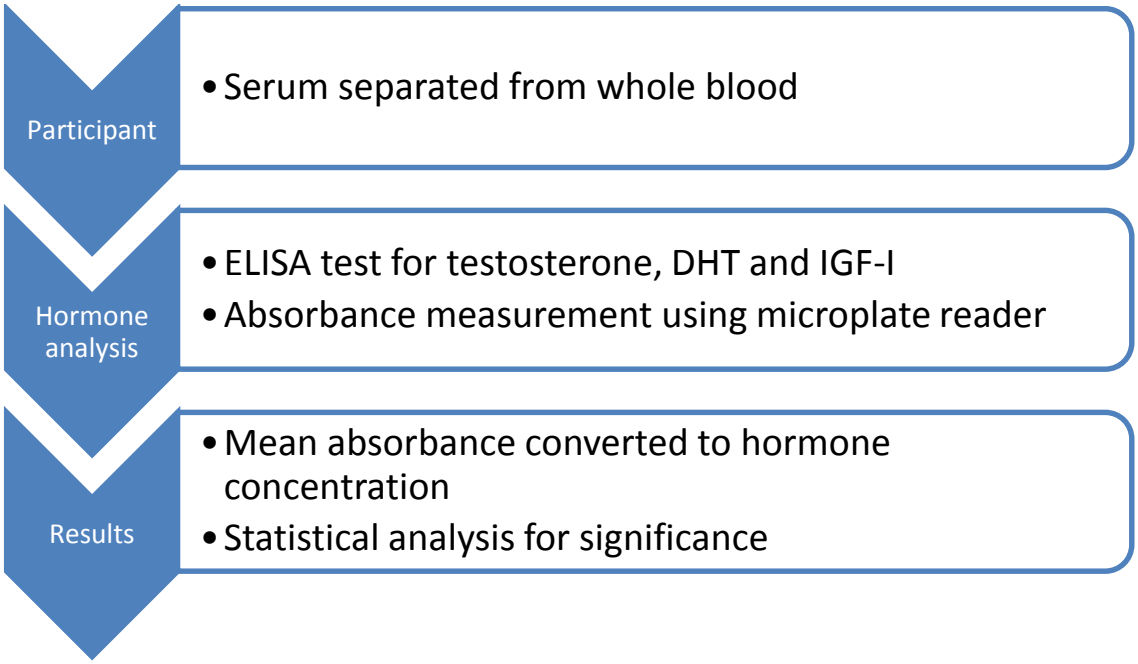

Figure 3.2 Schematic presentation of the work-flow for hormonal analysis. ELISA-Enzyme-linked immunosorbent assay, DHT-Dihydrotestosterone, IGF-I-Insulin-like growth factor-I.

3.2.1 Analysis of serum testosterone

The concentration of circulating testosterone in the serum was determined using the Testosterone ELISA kit (Immuno Biological Laboratories International, Hamburg, Germany).

All the reagents and serum samples were brought to room temperature. All standard solutions, control and cancer samples were run in duplicates concurrently to ensure same laboratory conditions and minimise the variations. 25µl of standard solution and 25µl of samples were dispensed into marked wells coated with mouse monoclonal anti-testosterone antibodies. 200µl of enzyme conjugate (testosterone conjugated to horseradish peroxidase) was added to each well. The content of the well was thoroughly mixed and incubated at room temperature for 60 min, then briskly shaken out and rinsed with 400µl of wash solution (provided in the kit). The washing step was repeated 3 times. A substrate solution

(Tetramethylbenzidine, 200µl) was added to each well and incubated again at room temperature for 15 min. The enzymatic reaction was stopped by adding 100µl of Stop Solution from the kit (0.5M H_2SO_4) to each well and the optical density was measured using a microtitre plate reader (Bio-Tek Synergy 2, Winooski, U.S) at 450nm within 10 min of adding the Stop Solution. The mean absorbance was determined by calculating the average of the duplicates for each sample. Figure 3.3 shows the flow of the experiment for testosterone concentration assessment.

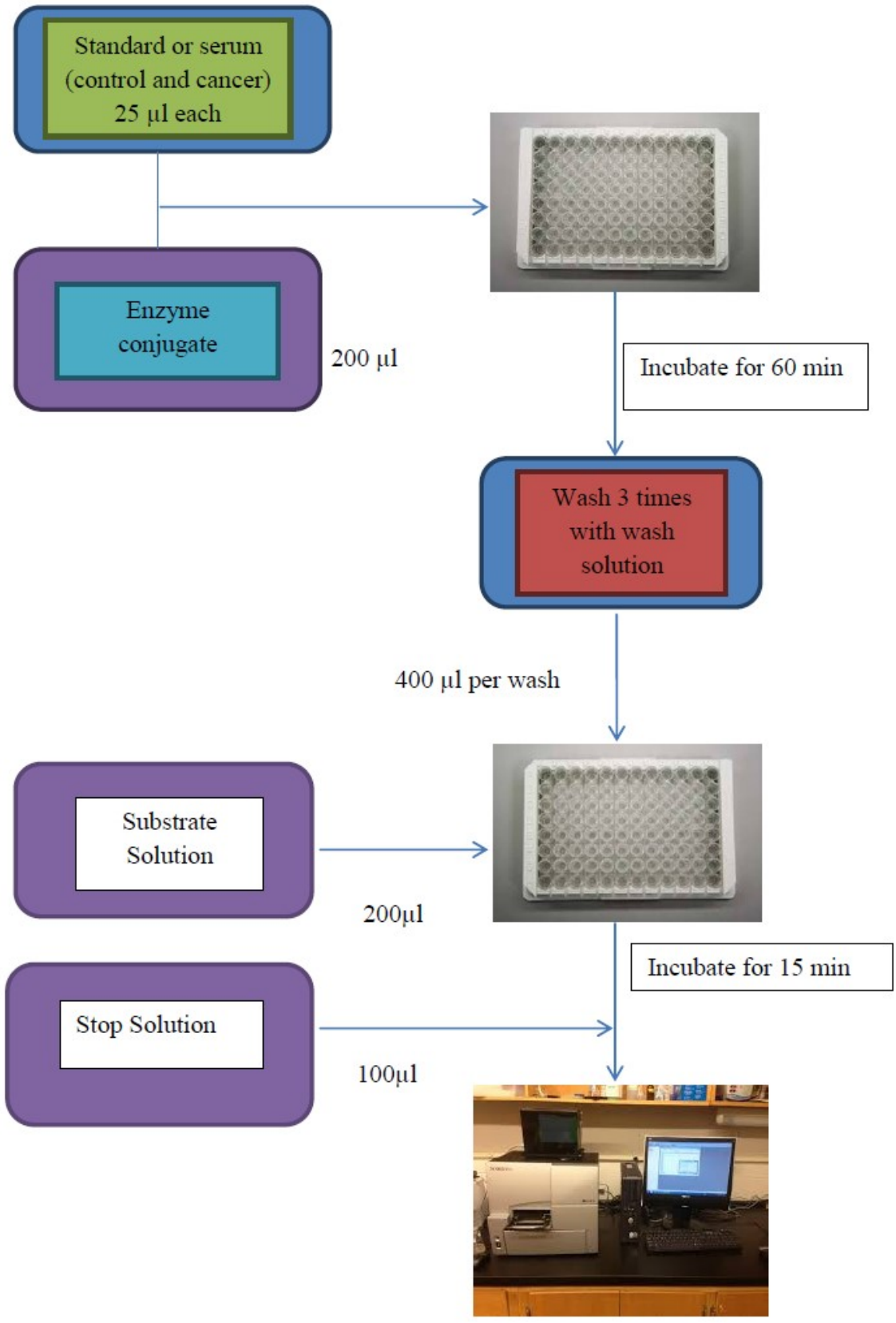

Figure 3.3 Schematic presentation of the steps in analysis of testosterone concentration.

A standard curve (Fig. 3.4) was constructed using the value of the mean absorbance of each of the standard (provided in the kit) plotted against its given concentration. A new standard curve was constructed for each kit.

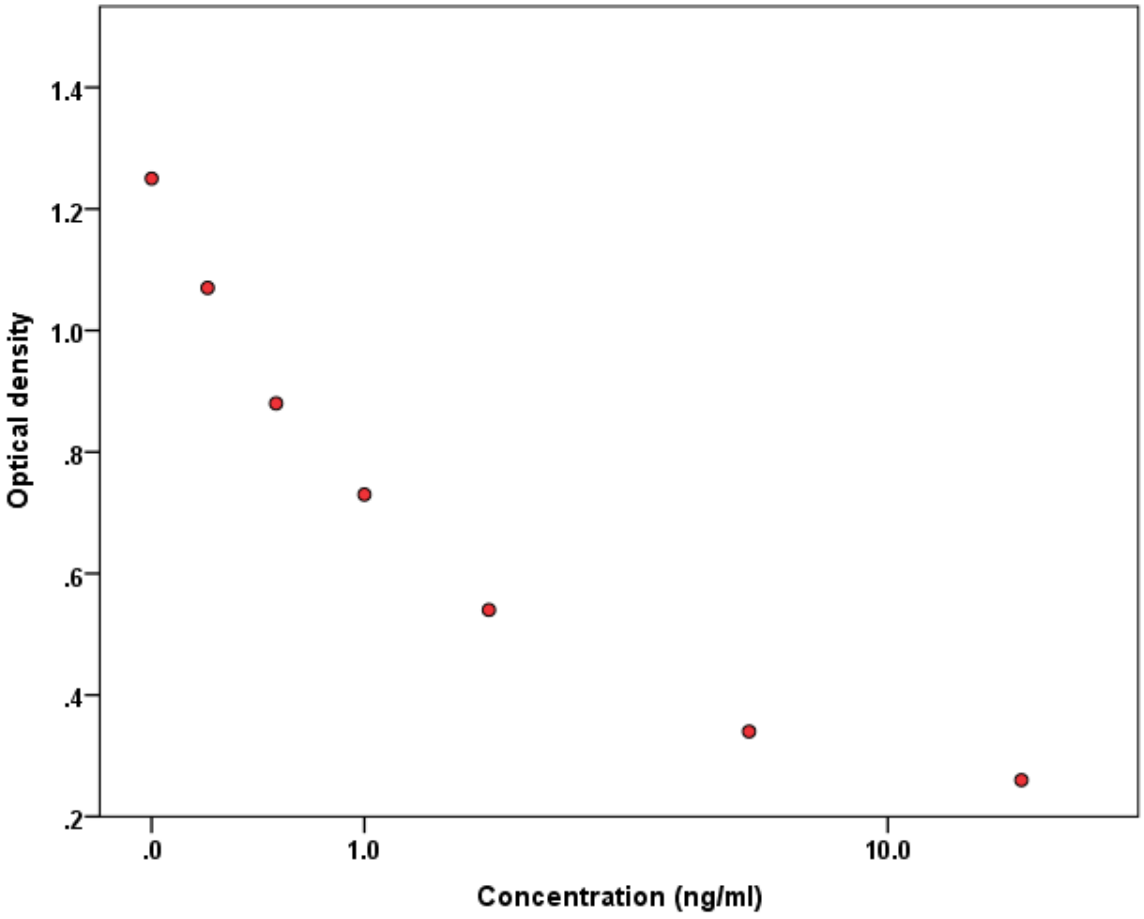

Figure 3.4 An example of a standard curve for testosterone.
Each point represents the mean optical density of 2 measurements for Standard Solution of given concentration.

Concentration of the testosterone in control and experimental serum samples was calculated using the standard curve. Regression modelling was used as described by the manufacturer to derive the formula for calculation of testosterone concentrations. The calibration curve produced a good fit for the observed sample data with an R^2=0.983. This was simplified using the formula derived statistically as below:

Testosterone concentration = Anti-log of [1.616- (2.202 x Mean Optical Density)]

This formula was used for all the samples for testosterone concentration and tabulated for statistical analysis later.

3.2.2 Analysis of serum dihydrotestosterone (DHT)

The concentration of circulating DHT in the serum was determined using the 5alpha-Dihydrotestosterone ELISA kit (Immuno Biological Laboratories International, Hamburg, Germany).

All the reagents and serum samples were brought to room temperature. The standard solutions, controls and cancer samples were run in duplicates concurrently to ensure same laboratory conditions and minimise the variations. A working solution of DHT-Horseradish Peroxidase conjugate was prepared by diluting the stock solution 1:100 with the assay buffer (protein-based buffer with non-mercury preservative). 50 µl standard, control and cancer samples were pipetted into marked wells coated with rabbit anti-DHT antibody. 100µl of conjugate working solution was dispensed into each well. The plate was then incubated on a plate shaker (~200 rpm) at room temperature for 1 h. The wells were washed three times with the diluted wash buffer (300µl each time) and tapped against an absorbance paper to ensure that the wells are dry. The substrate solution (Tetramethylbenzidine 150 µl) was added to each well and incubated at room temperature on a plate shaker for 10-15 min. Stop solution (1M H_2SO_4 50 µl) was added into each well after the incubation. The absorbance was then read using a microtitre plate reader (Bio-Tek Synergy 2, Winooski, U.S) at the recommended wavelength by the manufacturer which was 450nm within 20 min of adding the Stop Solution. The mean absorbance was

determined from the optical density by calculating the average of the duplicates for each sample. Figure 3.5 shows the flow of the experiment for DHT concentration assessment.

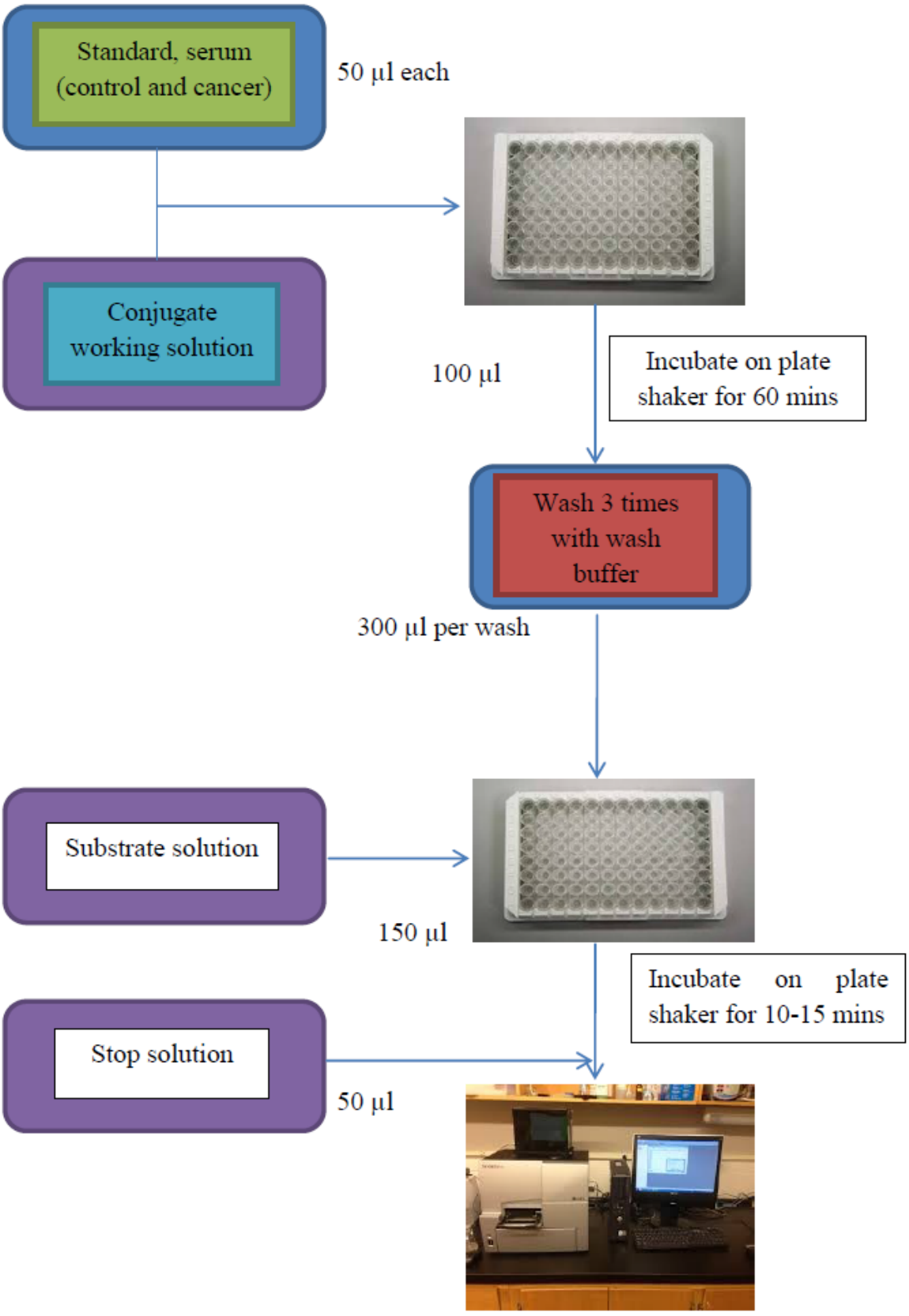

Figure 3.5 Schematic presentation of the steps in analysis of DHT concentration.

A standard curve (Figure 3.6) was constructed using the value of the mean absorbance of each of the standard (provided in the kit) against standard concentration. A new standard curve was constructed for each kit.

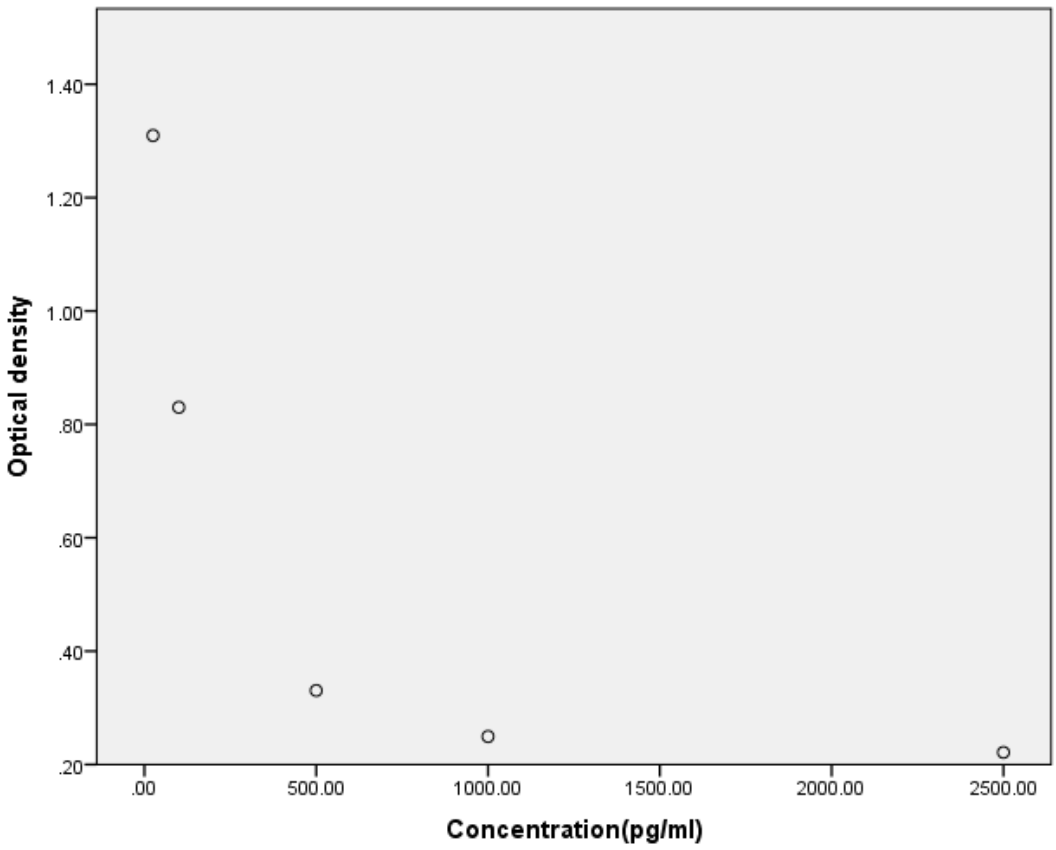

Figure 3.6 An example of a standard curve constructed with a 5alpha Dihydrotestosterone ELISA kit for the calculation of DHT concentration.

DHT concentration was determined for each control and experimental (cancer) sample using the standard curve. Regression modelling was used to derive the formula for calculation as described by the manufacturer. The calibration curve produced a good fit for the observed sample data with a R^2=0.938. This was simplified using the formula derived statistically as below:

DHT concentration = Anti-log of [3.462- (1.638 X Mean Optical Density)]

This formula was used for all the samples for DHT concentration and tabulated for statistical analysis later. The unit of the calculated DHT concentration is then converted to ng/ml for standardisation purpose by dividing with 1000.

The DHT/T was then calculated by dividing the obtained concentration for the corresponding patient.

3.2.3 Analysis of serum Insulin-like Growth Factor-I (IGF-I)

IGF-I concentration was measured using the Quantikine® ELISA Human IGF-I Immunoassay (R&D System Europe Ltd. Abingdon, UK).

I. Samples and Reagents Pre-treatment

The samples were pre-treated with the pre-treatment reagent A (acidic dissociation solution) and B (buffered protein with blue dye and preservative) from the kit to release the IGF-I from the binding proteins. 20µl of serum sample was added to 380 µl of the pre-treatment A in a polypropylene tube, mixed by vortex and incubated for 10 min at room temperature. 50 µl of the mixture was pipetted into 200 µl of the pre-treatment reagent B, mixed and analysed immediately. The substrate solution was prepared by mixing colour reagents A and B in equal volumes within 15 min of use. The substrate solution was kept protected from light. The standard IGF-I (lyophilised) solution was reconstituted with 1.0 ml distilled water to produce a stock solution of 60 ng/ml IGF-I. The standard solution was allowed to sit for 15 min before use. Calibrator Diluent RD5-22 (360 µl) was pipetted into the first tube and 200 µl into subsequent tubes. 40 µl of IGF-I stock solution was added to the first Calibrator Diluent tube and after mixing, 200 µl from each tube was transferred to the remaining tubes to obtain a serial dilution of the standard (Figure 3.7).

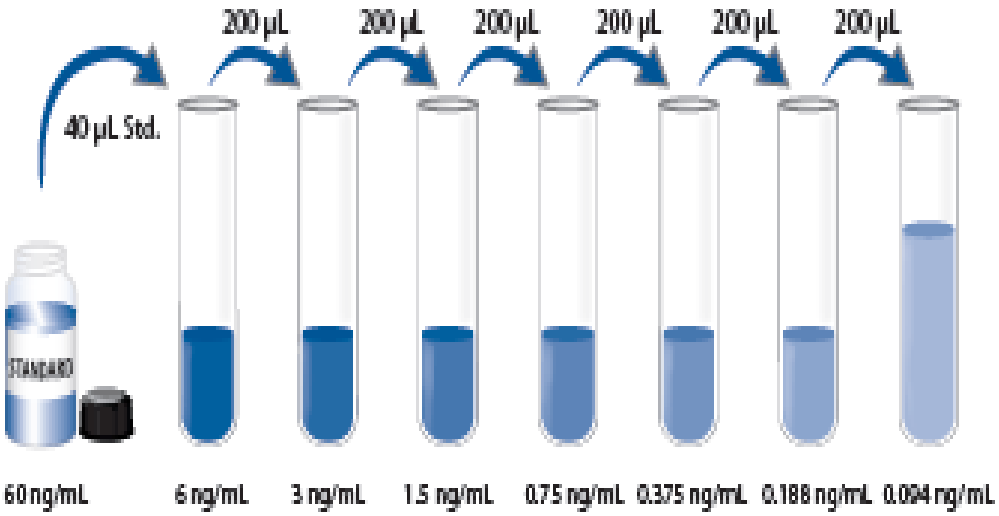

Figure 3.7 Serial dilution of the IGF-I standard solution using the Calibrator Diluent RD5-22. Adapted with permission from manufacturer insert provided by R&D System Europe Ltd. Abingdon, UK.

II. Assay procedure

Assay diluent RD1-53 (150 µl) was added to each well of microtitre plate coated with mouse monoclonal anti-IGF-I antibody. Another 50 µl of the pre-treated standard, serum from control and cancer participants were added to each well and covered with an adhesive strip provided. This covered unit was then incubated at 2-8^{o}C for 2 h. Thorough washing was performed with 400 µl of wash buffer and repeated 4 times. Complete removal of liquid ensured by tapping on absorbent paper. 200 µl of cold IGF-I conjugate (polyclonal antibody against IGF-I conjugated to Horseradish Peroxidase) was added to each well and covered with a new adhesive strip before incubating again at 2-8^{o}C for 1 h. The washing was repeated another 4 times as described above before adding 200 µl of Substrate Solution to each well. The mixture was incubated at room temperature for 30 min

protected from light. After this, Stop Solution was added (50 µl per well) and the colour change from blue to yellow was noted (Figure 3.8).

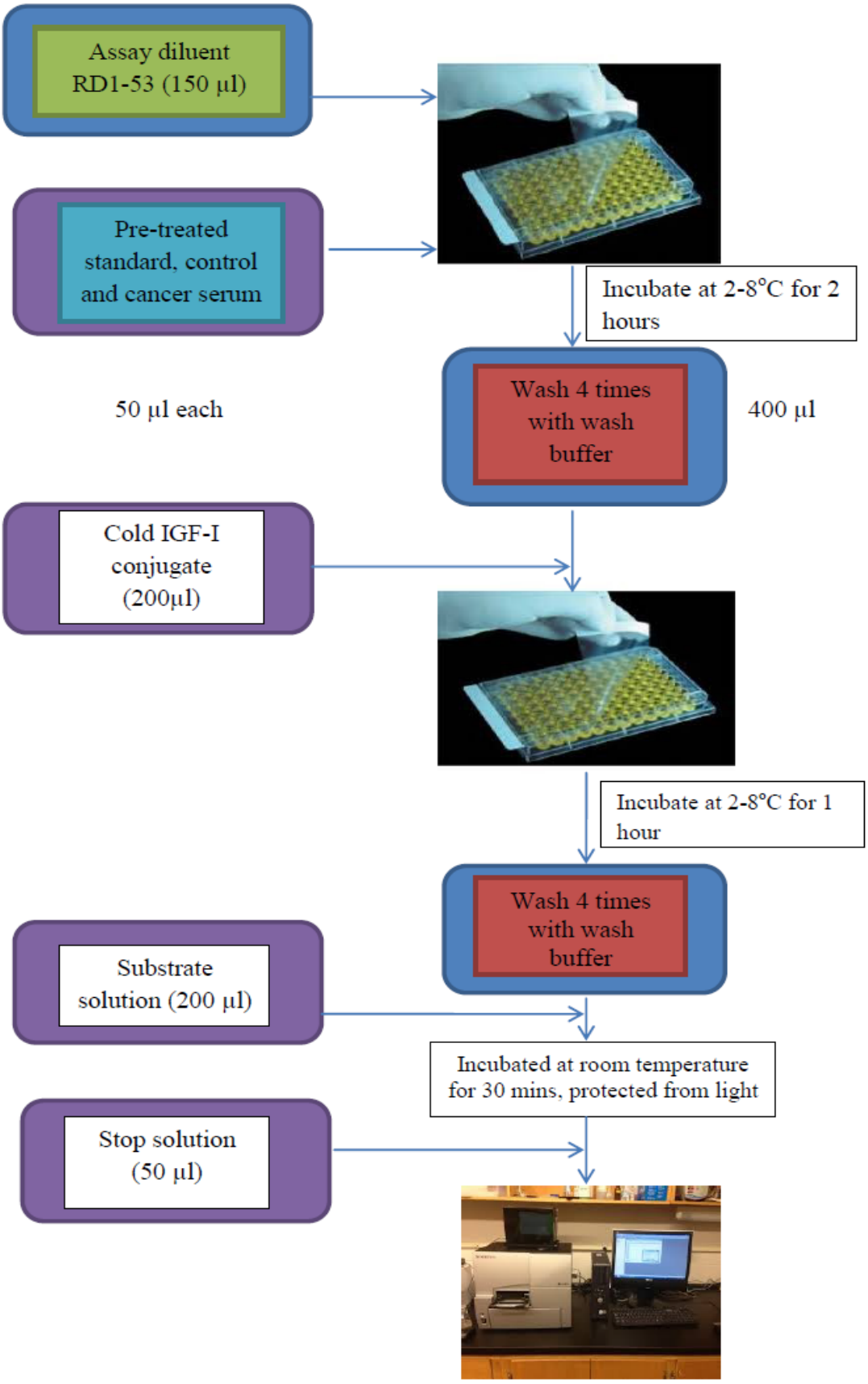

Figure 3.8 Schematic presentation of steps in the analysis of serum IGF-I concentration.

Optical density of the samples was determined in duplicates using a microtitre plate reader (Bio-Tek Synergy 2, Winooski, U.S) at the wavelength recommended by the manufacture within 30 min of adding the Stop Solution. In this case, the recommended reading was conducted by subtracting the absorbance at 450nm from that obtained at 570nm. The mean absorbance value was calculated for duplicates for each sample.

A standard curve (Fig. 3.9) was constructed by plotting value of the mean absorbance of each of the standard (provided in the kit) against its given concentration. A new standard curve was constructed for each kit.

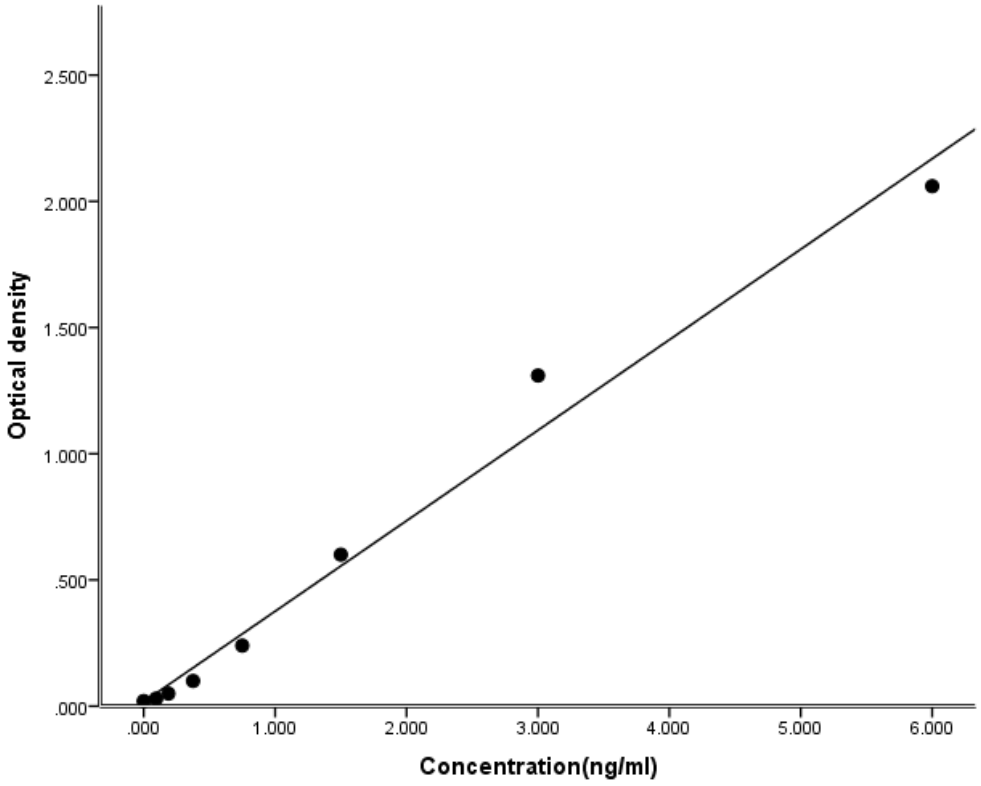

Figure 3.9 An example of a standard curve constructed with an IGF-I ELISA kit for the calculation of the IGF-I concentration.

Corresponding concentration of IGF-I for each sample was determined using the standard curve. Regression modelling, as described by the manufacturer, was used to derive the formula for calculation. The calibration curve produced a good fit for the observed sample data with a R^2=0.981. This was simplified using the formula derived statistically as below:

IGF-I concentration = Anti-log of [0.477 + (log10 Mean Optical Density x 0.746)]

This formula was used for all the samples for IGF-I concentration and tabulated for statistical analysis later.

3.3 Genomic DNA extraction and analysis

Genomic DNA (gDNA) from Bristol Caucasian group was extracted from blood sample stored in EDTA at -80^{o}C.

gDNA from Malaysian samples was extracted at University of Malaya, Kuala Lumpur and transferred to the University of the West of England. The gDNA extraction protocol is described in Section 3.3.1 and it was standardised between the 2 collaborating teams to ensure uniformity.

Figure 3.10 represents a flow-chart of experiments involving the whole blood analysis for genetic polymorphism.

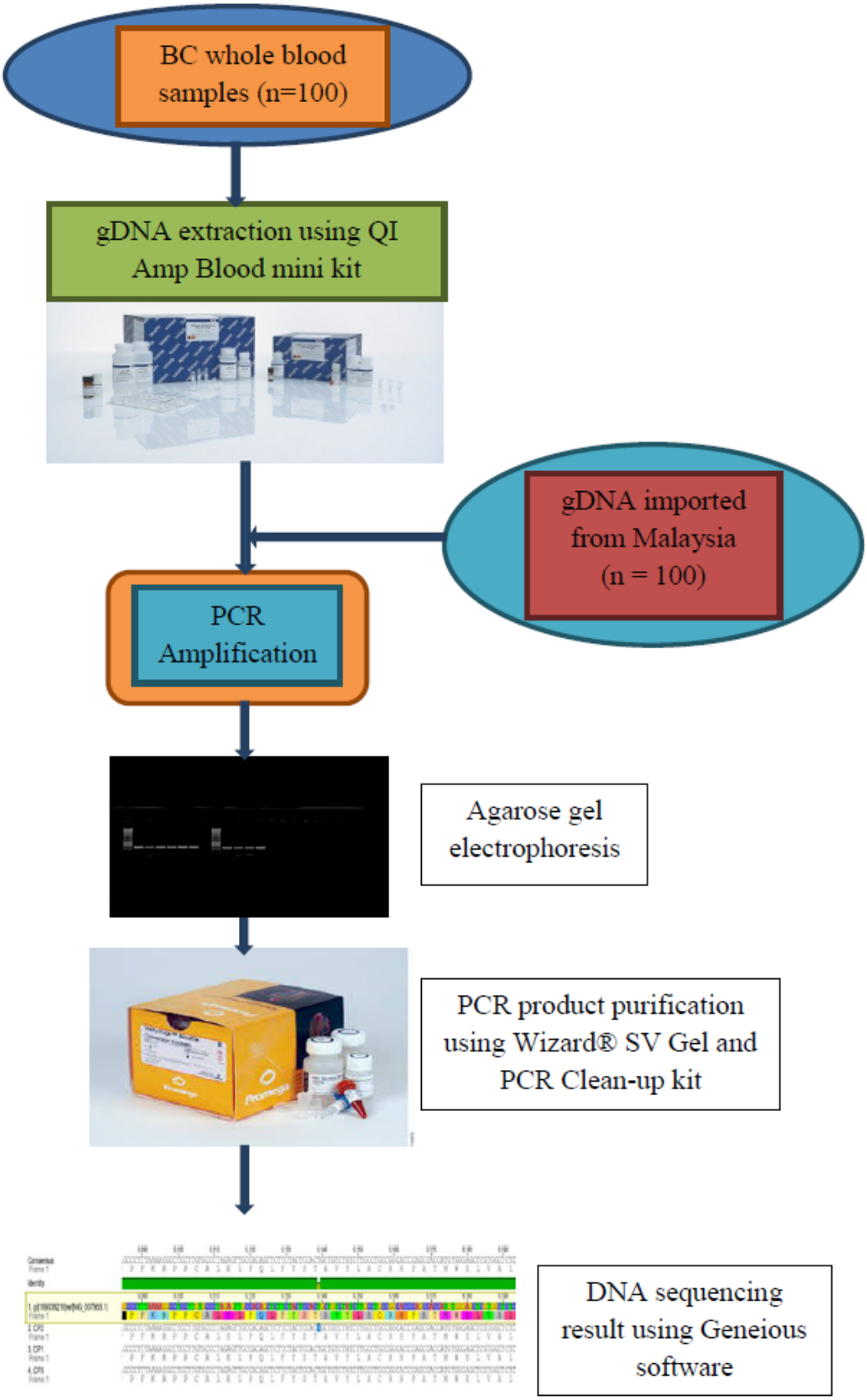

Figure 3.10 Schematic presentation of the work-flow in the analysis of genetic polymorphism in the Bristol Caucasians and Malaysian Chinese groups.

3.3.1 Genomic DNA (gDNA) extraction.

The QI Amp Blood mini kit (Qiagen Ltd, Crawley, UK) was used for extraction of gDNA from human whole blood. The DNA extraction in the Malaysian samples were performed using the same kit and protocol by trained technical staff at University of Malaya, Kuala Lumpur, under direct my direct supervision.

The blood samples were defrosted on ice. 20µl of Qiagen Protease (or proteinase K) was pipetted into 1.5ml Eppendorf tubes. A volume of 200µl of the whole blood sample and the same volume of the buffer AL (which causes cell lysis to expose DNA) was added into the same tube, and mixed by pulse-vortexing for 15 sec. The mixture was then incubated in a water bath at 56°C for 10 min. The tubes were briefly centrifuged and 200µl of ethanol (96–100%) was added to the samples followed by pulse-vortexing for 15 sec and another brief centrifugation to remove the drops from the mixture from inside the rim. This mixture was then placed carefully in a QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x g for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube and the filtrate was discarded. The QIAamp Mini spin column was opened and centrifuged twice, once with 500µl Buffer AW1 (prepared by adding 96-100% ethanol to the concentrate containing Guanidium chloride- denatures the protein and flows through the column) at 6000 x g for 1 min and the second time with 500µl Buffer AW2 (contains mainly ethanol to remove salts from the column) at full speed (20,000 x g) for 3 min. The filtrate was discarded. The QIAamp Mini spin column was then placed in a new 2 ml collection tube and centrifuged at full speed to eliminate the chance of possible buffer AW2 contamination. After discarding the filtrate, the QIAamp Mini spin column was placed in a clean 1.5 ml Eppendorf tube. Buffer AE (contains Tris-Cl and EDTA- elutes the DNA from the membrane and enables long-term storage) was added (75µl) and

incubated at room temperature for 5 min before centrifuging at 6000 x g for 1 min. Resulting gDNA was stored at –20°C for later use.

This gDNA extraction method was optimised and tested on blood samples from volunteers. Quantity and quality of the gDNA isolated from blood of volunteers was checked as described in Section 3.3.2 and was demonstrated to be appropriate for the purpose of this study.

3.3.2 Genomic DNA Quantification

Quality and quantity of extracted gDNA was assessed using the NanoDrop 1000 (Thermo Scientific, Basingstoke, UK). The gDNA concentration was expressed as ng/µl and the absorbance ratio at 260/280 (indicates the amount of protein contamination) was measured to evaluate quality of gDNA. The optimal 260/280 ratio of 1.8 or higher is generally accepted as an indicator of minimal protein contamination of the gDNA samples. Isolated gDNA was stored at –20°C until used for PCR amplification.

3.3.3 Primer Design for genetic polymorphism and tandem repeat analysis

PCR primers were designed for amplification of the regions of interest in CYP17, SRD5A2 and the AR genes. The human sequence for each gene of interest derived from the National Centre for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/):

- CYP17: ref|NG_007955.1| Homo sapiens cytochrome P450, family 17, subfamily A, polypeptide 1.
- SRD5A2: ref|NG_008365.1| Homo sapiens steroid-5-alpha-reductase, alpha polypeptide 2 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 2).
- AR: ref|NG_009014.2| Homo sapiens androgen receptor.

The areas of interest in CYP17, SRD5A2 and the AR genes were identified based on the data of literature review. The areas selected contained polymorphisms which are likely to have an impact on PC development.

- CYP17: position -34T→C (SNP reference rs743572) (Carey *et al,* 1994).
    - T to C polymorphism at the 34th base preceding the translation initiation point.
- SRD5A2: A49T and V89L variants (SNP rs523349 and rs9282858) (Makridakis *et al,* 2000).
    - Amino acid Ala to Thr change at codon 49 in Exon 1 due to polymorphism at position 140G→A.
    - Amino acid Valine to Leucine change at codon 89 in Exon 1 due to polymorphism at position 261C→G.
- AR: Number of polymorphic CAG repeat in Exon 1 (Ekman *et al,* 1999).

The forward and reverse primers were designed to include 400-600 base pairs of the specific regions of the genes where polymorphisms of interests were reported. Primer designing was done based on the available software Primer 3 (http://frodo.wi.mit.edu/primer3/) and was checked using the Primer-BLAST to ensure the specificity of the amplified region. The primers and their characteristics are as shown in Table 3.2. The positions of the primers in DNA sequence are demonstrated in Figure 3.11-3.13.

Table 3.2 Forward and reverse primers with their characteristics.

Gene	Type of Primer	Primer (no. of bp) (5' to 3')	Annealing Temp. (^{o}C)	GC content (%)
CYP17 (NG_007955.1)	Forward	GAGCCCAGATACCATTCGCA (20)	59.4	55.0
	Reverse	GCCCCATCTATTCGGTTCGT (20)	59.4	55.0
SRD5A2 (NG_008365.1)	Forward	GCGCTCCATAAAGGGGTTGC (20)	61.4	60.0
	Reverse	GGACGCTAGAGAGTTGACAAGCG (23)	64.2	56.5
AR (NG_009014.2)	Forward	TCCCGCAAGTTTCCTTCTCT (20)	57.3	50.0
	Reverse	CCCACTTTCCCCGGCTTAA (19)	58.8	57.9

bp- base pairs, Temp.-Temperature, NG- accession number.

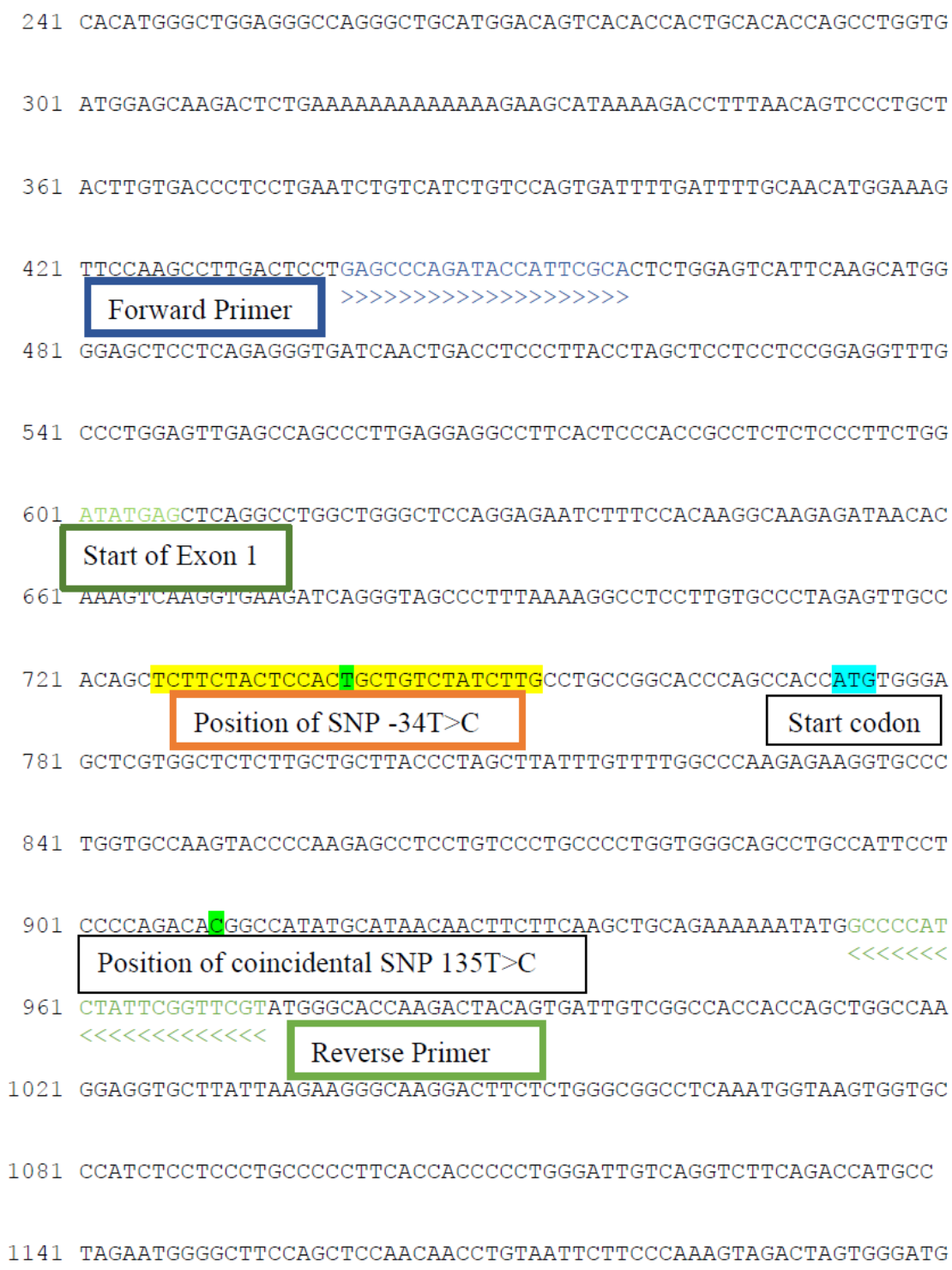

Figure 3.11 CYP17 gene primers on gDNA sequence (ref NG_007955.1) for SNP rs743572 (SNP -34T>C).

Product size: 535bp. Numbering on the left indicates the reference point from origin of sequence of gDNA used for primer design.

Figure 3.12 SRD5A2 gene primers on gDNA sequence (ref NG_008365.1) for SNP rs523349 (261C→G) and rs9282858 (140G→A).

Product size: 550bp. Numbering on the left indicates the reference point from origin of sequence of gDNA used for primer design.

Figure 3.13 AR gene primers on gDNA sequence (ref NG_009014.2) for CAG repeats at Exon 1.

Product size: 671bp. Numbering on the left indicates the reference point from origin of sequence of gDNA used for primer design.

3.3.4 Polymerase chain reaction (PCR)

PCR reactions were performed for all the 200 gDNA samples from the Caucasian and Malaysian cancer and control groups. The reactions were conducted for identification of SNPs of interests in the CYP17, SRD5A2 and AR genes.

PCR reactions were performed using a GeneAmp High Fidelity PCR System (Applied Biosystems, Cheshire, UK). PCR primers were first diluted with molecular grade water in the volume recommended by the manufacturer to obtain a concentration of 100pmol/µl. This was further diluted to 1:10 and stored at -20°C before use. A dNTP mix containing dATP, dCTP, dGTP and dTTP each at a final concentration of 10mM was purchased from Fermentas (Sankt Leon-Rot, Germany). This was also diluted 1:10 with molecular grade water and stored at -20°C. On the day of experiment, diluted primers, diluted dNTPs, and 10 x PCR buffer with $MgCl_2$ (provided in the kit) as well as gDNA (produced in section 3.3.1) were defrosted on ice. At all times, the batch of gDNA to be amplified, always consisted of equal number of samples across all the studied groups. For each PCR, 100ng of gDNA and was mixed with molecular grade water to make up a volume of 15µl in a 0.5 ml PCR tube. A Master mix was made by combining 2µl each of the forward and reverse primer, 10µl dNTPs per sample, 5µl 10 x PCR buffer with $MgCl_2$ and 0.5 µl High Fidelity Enzyme mix (from kit, taken straight from -20°C freezer to minimise enzyme degradation) per sample in a 0.5ml Eppendorf tube. A volume of 35µl was pipetted out from the Master mix and added to the above 0.5 ml PCR tube and pipette-mixed. The PCR tubes containing 50µl of PCR mix each were then placed in an MJ Research PTC-200 thermo cycler (Bio-Rad, Hertfordshire, UK) and the program was run as follows:

- Initialisation: 94°C for 3 minutes.
- Denaturation: 94°C for 30 seconds.
- Annealing: 59°C for 30 seconds.
- Extension: 72°C for 1 minute.

} Repeated for 35 cycles.

- Finalisation: 72°C for 7 minutes.
- Hold: 4°C until removed from machine.

PCR experiments for CYP17 gene were conducted with the annealing temperature of 59°C. Annealing temperature of 62°C and 58°C was used for SRD5A2 and AR gene respectively. The annealing temperature for each pair of primers was identified after a series of pilot experiments which tested a range of temperature recommended by the primer designing software.

3.3.5 Agarose Gel Electrophoresis

An agarose gel was prepared by dissolving 1.0g of powdered agarose in 100ml of Tris-acetate-EDTA (TAE) buffer by heating in a microwave. The molten agarose was left to cool for 5 min before adding 1µl of ethidium bromide from the stock solution 10 mg/ml and gently mixed. The agarose was poured into a gel mould, a comb was placed and the gel was left to set at room temperature for 30-40 min. Once the gel has set, 3µl of loading dye (Bioline, London, UK) was added to 10µl of the PCR products from the Section 3.3.4. The mixture was loaded on the gel. 10µl HyperLadder I (Bioline, London, UK) was loaded into a spare well. The gel was run for 40 min at 100 V with TAE buffer. The gel was then placed on a UV Transilluminator and strong 500 bp bands were identified (Figs 3.14 to 3.16). The size of the bands was an expected size of PCR products which confirmed that the PCR reaction was successful.

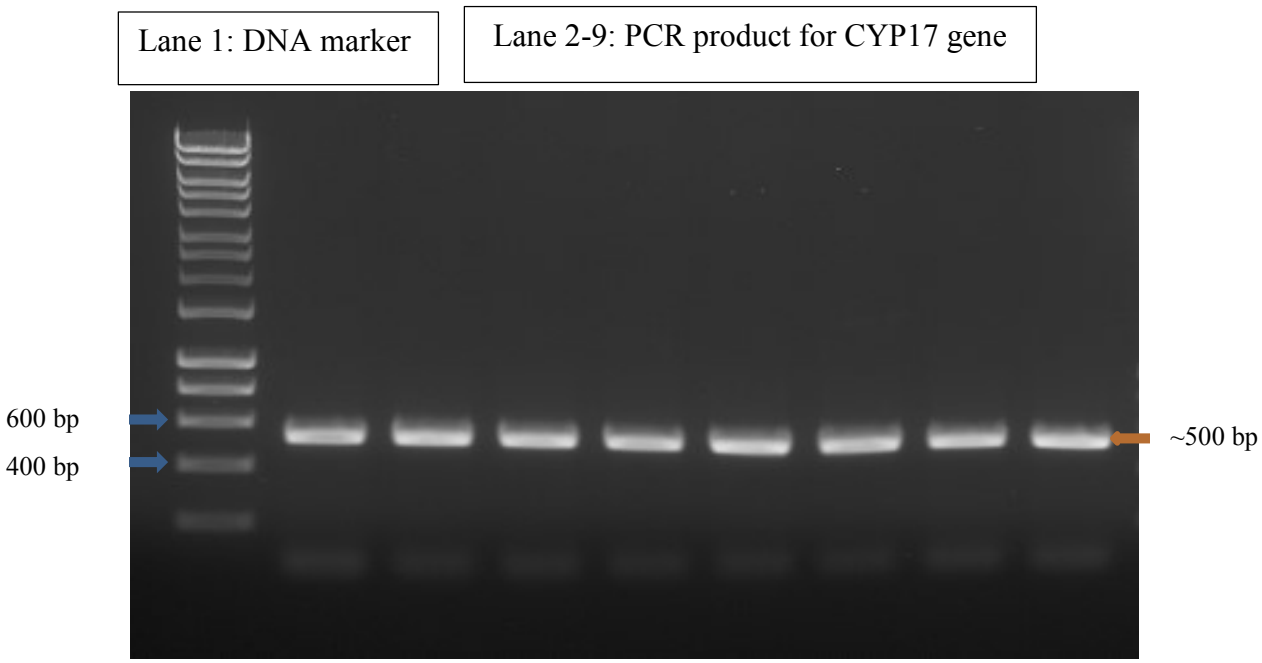

Figure 3.14 UV transilluminator image showing successful PCR amplification for CYP17 gene.

Blue arrow indicates the size of the DNA markers and the brown arrow indicates PCR amplification product of about 500bp size.

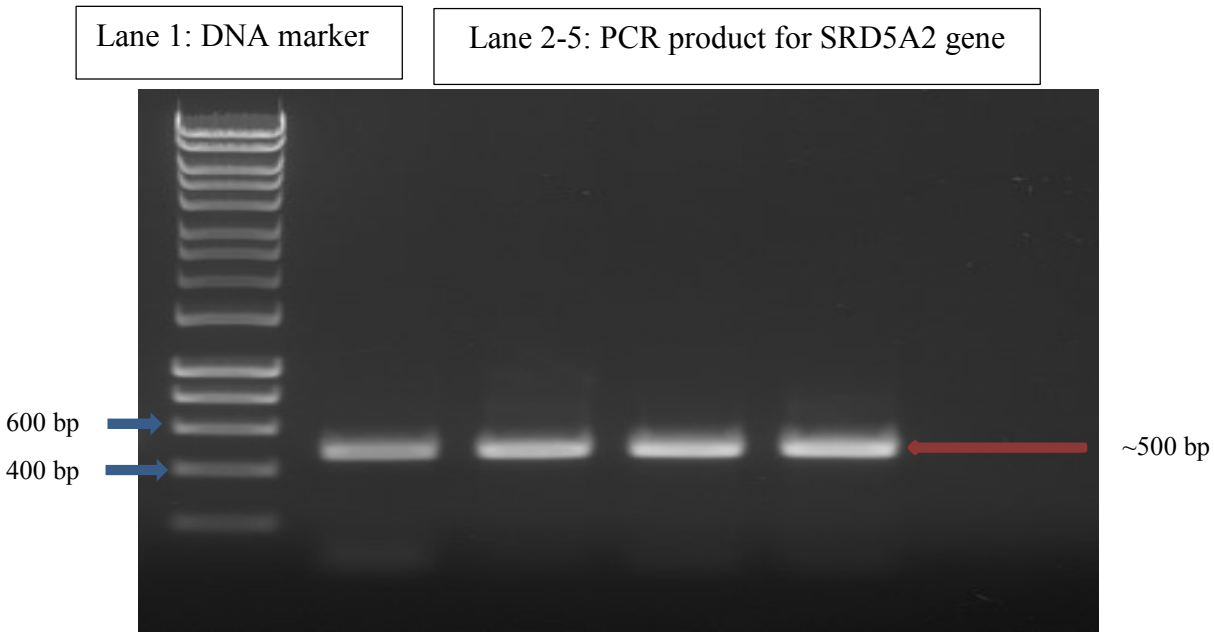

Figure 3.15 UV transilluminator image showing successful PCR amplification for SRD5A2 gene.

Blue arrow indicates the size of the DNA markers and the brown arrow indicates PCR amplification product of about 500bp size.

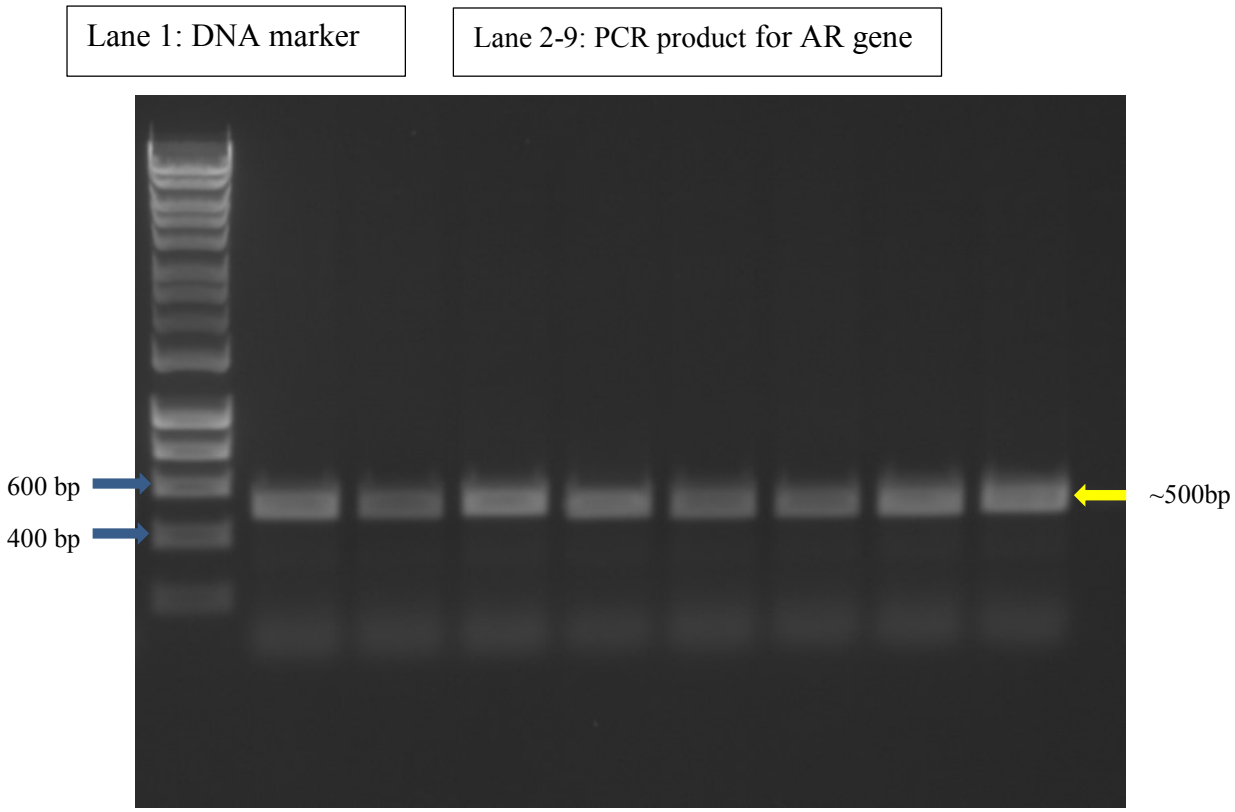

Figure 3.16 UV transilluminator image showing successful PCR amplification for AR gene. Blue arrow indicates the size of the DNA marker and the horizontal yellow arrow indicates PCR amplification product of about 500bp size for AR gene.

3.3.6 Polymerase chain reaction product purification

PCR product obtained in the Section 3.3.4 was purified before sending for sequencing to Eurofins MWG Operon (Ebersberg, Germany). The purification process was done using the Wizard® SV Gel and PCR Clean-up kit (Promega, Southampton, UK).

Equal volume of Membrane Binding Solution (MBS) (provided in the kit) was added to the PCR amplification product (38µl). The DNA binding process was undertaken by transferring the PCR preparation above into an SV minicolumn in a collection tube. This mixture was incubated at room temperature for 1 min, centrifuged at 16,000 x g for 1 min. The flow-through was discarded. The membrane was washed twice with 700µl and 500µl

of Membrane Wash Solution (MWS) (ethanol added) and follow-on centrifugation at 16,000 x g for 1 and 5 min respectively. The collection tube was then emptied and the assembly re-centrifuged at full speed for 1 min with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol. The minicolumn was transferred to a new 1.5ml Eppendorf tube. 50µl of Nuclease-Free water was added to the minicolumn and incubated at room temperature for 1 min before centrifuging at 16,000 x g for 1 min. The minicolumn was then discarded and the purified gDNA obtained was stored at -20^{0}C.

3.3.7 DNA sequencing and analysis

The purified PCR products were sent for sequencing to Eurofins MWG Operon (Ebersberg, Germany) using the on-line Value Read Tube service. Purified PCR products were split into two tubes so as to perform a forward and reverse read for each sample using the same forward and reverse primers in the PCR reactions for each gene in this study. Sequencing results were downloaded from the Eurofins MWG Operon website and analysed using licensed Geneious Pro 5.6.4 software for Microsoft Windows 7 (Fig. 3.16).

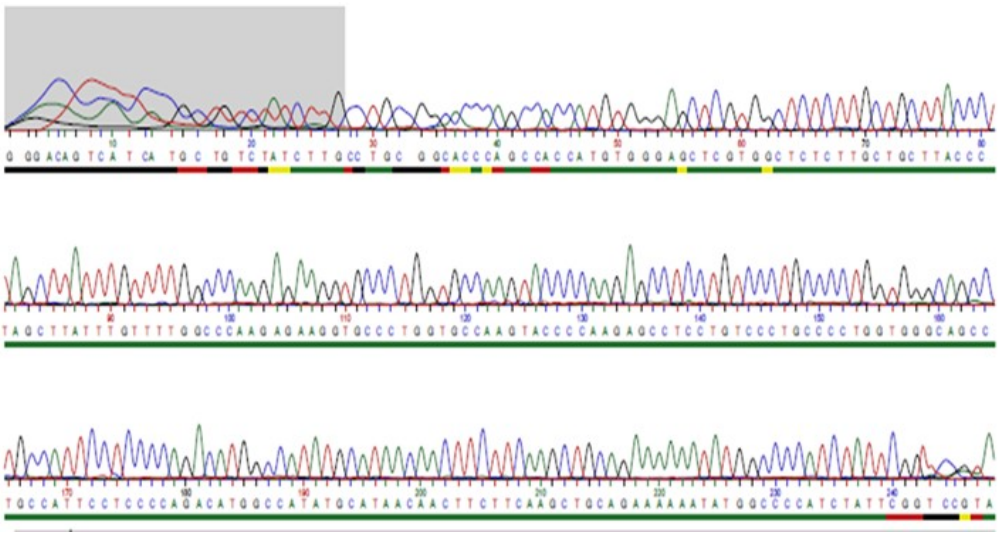

Figure 3.17 Example of DNA sequence presenting the region of CYP17 gene.
Sequence data is colour-coded from green to black indicating quality of data from excellent to poor. Only segment in continuous green segment used for alignment.

The downloaded sequence for each sample was aligned with the NCBI database sequences in the region of the studied SNPs and any polymorphism identified were noted (Fig. 3.17).

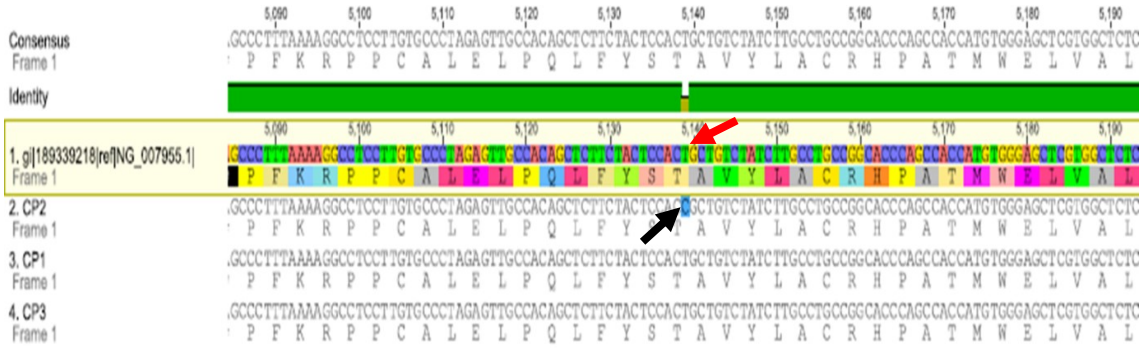

Figure 3.18 An example of alignment of CYP17 experimental sequence with database sequence.
Alignment shows polymorphism from nucleotide T (red arrow) to nucleotide C (black arrow).

3.4 Statistical method

The sample size was determined using power calculations and to ensure feasibility and practicality, this research consisted of 100 samples from Malaysian Chinese (N = 50 Prostate Cancer, N = 50 Benign Prostate Hyperplasia) and 100 samples from Bristol Caucasian (N = 50 Prostate Cancer, N = 50 Benign Prostate Hyperplasia). The primary outcome measures were androgen concentration (testosterone, dihydrotestosterone) which are inherently continuous measures. For the proposed sample sizes the appropriate primary omnibus analysis had in excess of 95% power for detecting medium sized effects between the prostate cancer group and the benign prostate hyperplasia group. The detection of hormonal effects between those with and without prostate cancer within each ethnic group was of concern. For these a priori reasoned contrasts, the study retained in excess of 85% power to detect a medium sized effect (Cohen's d = 0.5) for an analysis using the independent samples t-test.

Quantitative data analysis was undertaken using standard recognised statistical techniques.

The wider proposed study took the form of a 2 by 2 fully between-subjects factorial balanced design with N = 50 replicates (N = 200 in total) with multiple non-commensurate dependent variables. The design had Disease Status (Prostate Cancer, Benign Prostate Hyperplasia) as a two level independent factor and ethnicity as a second two level independent factor (UK Caucasian, Malaysian Chinese). The dependent variables were androgen concentration (testosterone, dihydrotestosterone) and IGF-I concentration and polymorphisms in the CYP17, SRD5A2 and AR genes.

For each hormone, the primary omnibus analysis was an analysis of variance for a 2 by 2 fully between subjects design. The main effect of Disease Status on androgen concentration was of primary concern. Such an analysis made reference to the F-

distribution on 1 and 196 degrees of freedom and had in excess of 95% power for detecting medium sized effects across Ethnic groups. However, the detection of hormonal effects between those with and without prostate cancer within each ethnic group was of concern and the study needed to be powered for this subgroup analysis. For the subgroup analysis, the independent samples t-test was used (N = 50 per group, degrees of freedom = 98). Elevated mean hormone concentrations were anticipated in the prostate cancer groups and assuming a medium effect size (Cohen's d = 0.5) the study retained a power in excess of 85%.

The efficacy of the hormone data in developing a predictive or diagnostic tool for prostate cancer was evaluated using a ROC analysis, and confidence intervals for sensitivity and specificity for the optimal use of hormone concentration was derived.

The presence and absence in the CYP17 and SRD5A2 genes was cross-tabulated against disease status and association assessed using the chi-square test of association. The significance of the number of CAG repeats was analysed as done in the hormonal analysis using the analysis of variance for the 2 X 2 design. The strength of association was determined using the odds ratio. Good markers for the disease were sought. The odds ratio for good markers was expected to have a value in excess of 1 (i.e. the presence or absence of a marker doubles the likelihood of correct disease classification). On this expectation a sample of N = 200 (N = 100 for prostate cancer, N = 100 for benign prostate hyperplasia) should provide approximately 80% power or greater over a wide range of proportions. All statistical analysis was performed using IBM SPSS Statistics package version 20.0.

4 Results

This chapter consists of three sub-chapters which describe results of the following sets of experiments:

i. ***The participants' demography***: This sub-chapter summarises information about the participants' ethnic group, age and other aspects relevant to this study.
ii. ***Evaluation of hormones*** concentration: This section reports results of analysis of the circulating testosterone, DHT and IGF-I in the participants of different ethnic groups and with or without prostate cancer.
iii. ***Investigation of genetic polymorphisms***: This part of results (a) reports ethnic-groups specific DNA polymorphisms in CYP17, SRD5A2 and AR genes in relation to their role in androgen metabolism and (b) investigates whether the polymorphisms are related to the incidence of prostate cancer.
iv. ***Correlation study:*** This sub-chapter presents analysis of relationship between circulating concentration of testosterone, DHT and IGF-I hormones and DNA polymorphisms in the genes encoding for these hormones in control and prostate cancer patients.

4.1 Participants demography

This study recruited a total of ***200 participants*** from the Bristol Caucasian group from Southmead Hospital, Bristol, UK and the Malaysian Chinese (MC) participants from the Urology and community clinics in UMMC, KL.

Table 4.1 shows demographic distribution of the participants. The MC participants (N = 100) had a mean age higher than the Caucasians (N = 100) by 1.9 years across control and cancer groups. The cancer patients (combined MC and Caucasian ethnic groups, N = 100) were also found to have a significantly higher mean age when compared to the combined control group (N = 100) by 5.1 years (by about 7%) ($P < 0.05$). The participants were divided into 4 sub-groups: MC cancer, MC control, Caucasian cancer and Caucasian control. Each of the sub-groups comprised of 50 participants. It was found that the MC cancer participants were older with a mean age of 71.5 years when compared with the Caucasian cancer group by 5.5 years (by about 8%) respectively. This difference was statistically significant ($P < 0.05$).

Table 4.1 Demographic data of Caucasian and Malaysian Chinese participants.

Parameters		Age		PSA		Gleason Score	
		Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Caucasian	**Control**	64.5	10.35	2.16	0.98	N/A	
	Cancer	66	7.39	27.47	65.84	6.84	0.766
Malaysian Chinese	**Control**	62.8	8.86	1.71	1.03	N/A	
	Cancer	71.5*	8.05	201.42	623.32	7.40	1.278
Caucasian + MC	**Control**	63.7	9.62	N/A		N/A	N/A
	Cancer	68.8*	8.18	N/A		N/A	N/A

SD- Standard Deviation, PSA- Prostate Specific Antigen, N/A-not applicable.

*** Significant at P < 0.05.**

Figure 4.1 shows the age of individual participants in Caucasian control (A) and Caucasian cancer (B) sub-groups. The age of participants in both groups varied from 41 to 85 years. The mean age of the Caucasian control and cancer patients were 64.5 and 66.0 years respectively.

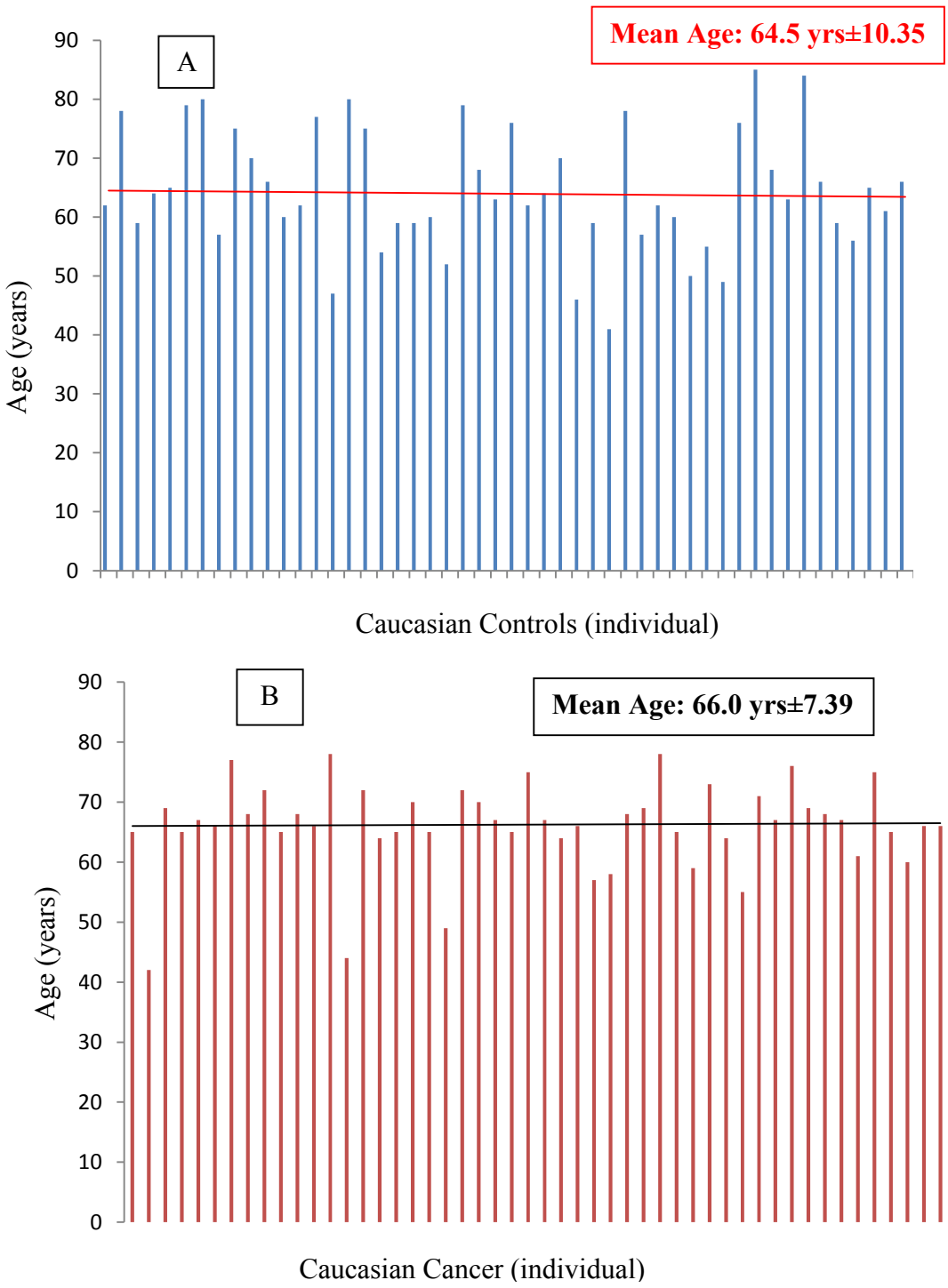

Figure 4.1 Age of individual participants in Caucasian control (A) and Caucasian cancer (B) group.

Each bar represents and individual participant. The solid line on the bar shows the average age for a given group ± standard deviation.

Figure 4.2 shows the age of individual participants in the MC control (C) and MC cancer (D) sub-groups. The age of participants in both groups ranged from 45 to 88 years. The mean age of the MC control and cancer patients were 62.8 and 71.5 years respectively.

Table 4.1 also includes data on PSA level and Gleason score. PSA is currently the most commonly used test to screen a patient for PC or to identify high risk of PC patients and together with Gleason score can be used to make treatment decisions (Brawley *et al,* 2009). MC cancer patients had a mean PSA significantly higher (by 9.5 times) when compared to Caucasian cancer group. This might be related to the fact that patients in Malaysia usually visit doctors when the symptoms or disease are in more advanced state. In contrast to the cancer group, PSA values did not differ significantly between MC and Caucasian control groups, where the mean PSA values were 2.16 and 1.71 respectively. This was expected as the control group participant recruitment was performed with a predefined PSA level (< 4.0 ng/ml) as one of the recruitment criteria. It was noted in this study, that the mean Gleason score for all cancer patients in MC and Caucasian groups were almost the same (Table 4.1). The Gleason score for the control groups were not available as these participants were not subjected for a biopsy of the prostate.

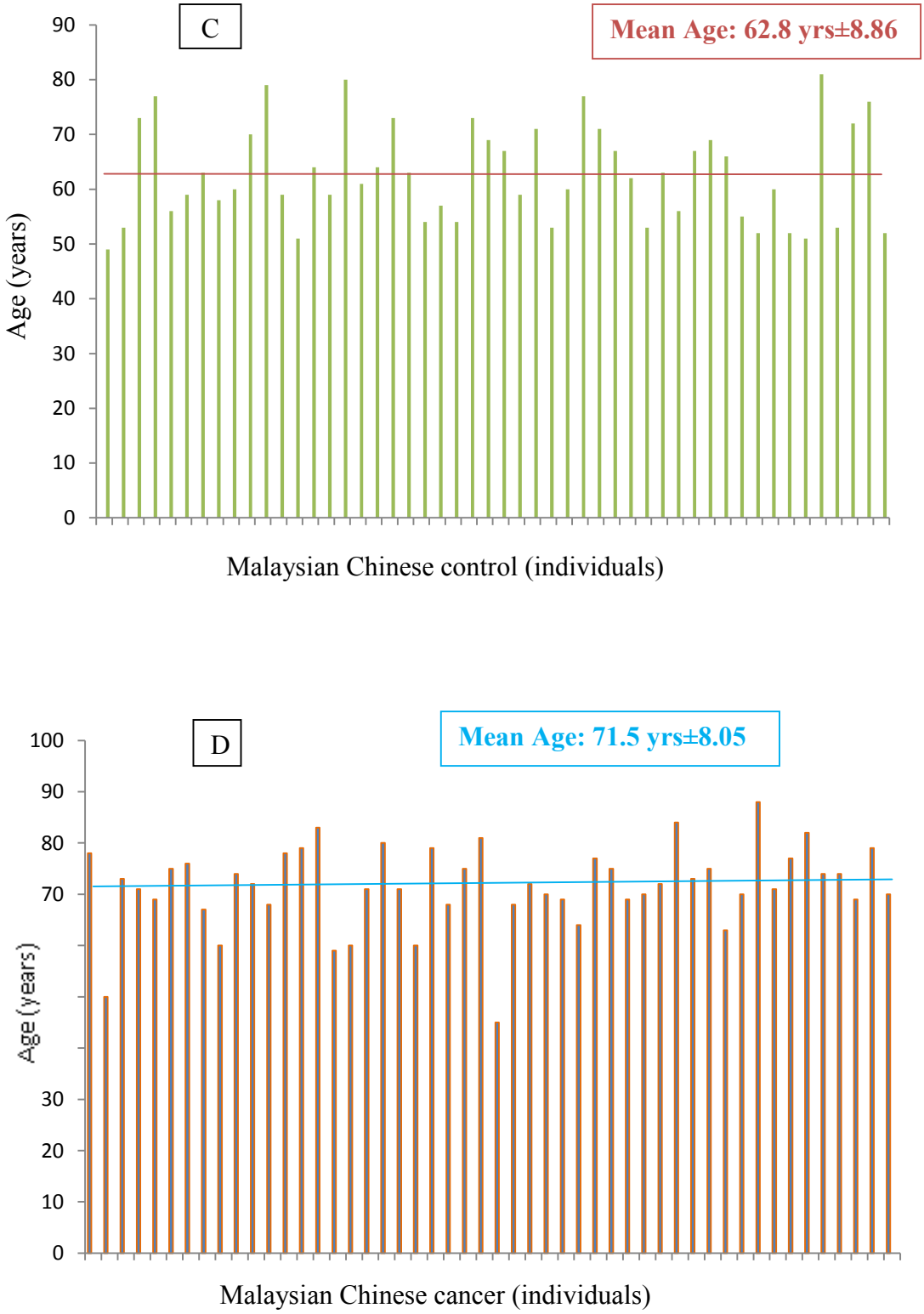

Figure 4.2 Age of individual participants in Malaysian Chinese control (C) and Malaysian Chinese cancer (D) group.

Each bar represents and individual participant. The solid line on the bar shows the average age for a given group ± standard deviation.

4.2 Evaluation of the concentration of androgens in Caucasian and Malaysian Chinese groups

This section presents results of experiments on evaluation of concentration of male sex hormones testosterone, DHT and the growth hormone IGF-I in the circulation system using serum separated from the whole blood of the control and experimental groups. The measurements were conducted using commercially available ELISA kits (see Material and Methods for details).

4.2.1 Analysis of serum testosterone concentration

Testosterone is the male sex hormone, also known as androgen, which is produced in the testis and adrenal glands in human. Testosterone is the most important circulating male sex hormone and plays critical role in the development of prostate and has been suggested to be involved in the development of PC (So *et al,* 2003).

Table 4.2 Circulating testosterone concentration (ng/ml) in different ethnic groups, control and cancer patients and interaction between these parameters.

	Group	N	Mean	SD	P value**
Ethnicity	**Caucasian**	100	3.73	1.45	0.810
	MC	100	3.67	2.38	
Diagnosis	**Control**	100	4.34	1.67	< 0.001*
	Cancer	100	3.07	2.05	
Interaction	**Ethnicity vs. Cancer &Control**	200	3.70	1.97	0.008*

N-number of samples, SD- standard deviation, MC-Malaysian Chinese.* Significant at P-value < 0.05. ** ANOVA Test

This study showed that the mean testosterone concentration of the Caucasians was slightly higher (by 1.6%) than that in MC men (Table 4.2). This difference was, however not significant (P = 0.810). The comparison of mean testosterone concentration between the control and the cancer groups, which contained combined data from both ethnic groups, showed that the control group had a statistically significant higher mean testosterone value (by 34.3% P < 0.001). There was also a significant effect in the interaction between the combined ethnic groups and the diagnosis (cancer and control taken as one data set) on the concentration of circulating testosterone (P = 0.008) (Table 4.2).

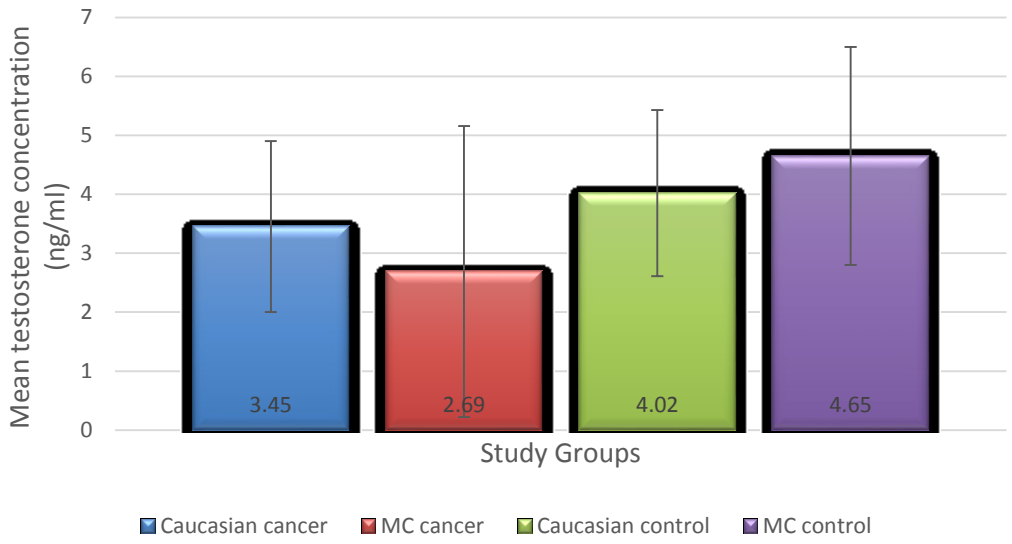

Figure 4.3 Mean testosterone concentration for control and cancer patients in Caucasian and MC ethnic groups.

The data are presented as Mean +/- SEM. Each study group consisted of 50 participants.

To gain further insight into ethnic-group specific relationship between cancer and circulating testosterone, serum testosterone concentrations were compared between cancer and control patients within Caucasian and MC ethnic groups. Figure 4.3 demonstrates that the difference in the mean values for testosterone between control and cancer groups within MC population was bigger than the difference in the mean values between control and cancer groups within the Caucasian population ($P < 0.001$ and $P = 0.125$ respectively).

It is known that the concentration of male sex hormones declines with age (Gray *et al,* 1991). Taking into consideration that there was a significant difference in the age of the participants between ethnic groups investigated, with the MC cancer group having significantly higher mean age when compared to Caucasians, it was necessary to conduct statistical analysis of testosterone concentration taking into account the age factor. This was achieved by using analysis of covariance (ANCOVA). Table 4.3 demonstrates that age itself has significant effect on testosterone concentration when the data for both ethnic groups were pooled together ($P = 0.016$). However, when effect of age was analysed in relation to ethnicity, the testosterone concentration did not depend on ethnicity (Table 4.3). There was a highly significant difference in the mean values ($P < 0.001$) between the control and the cancer groups when adjusted for age between the two ethnic groups in this study. There was significant interaction between the ethnicity and diagnosis, even after taking the age factor into consideration ($P = 0.033$) (Table 4.3).

Table 4.3 Analysis of covariance for mean testosterone concentration with age.

Parameter	Mean square	F	P value
Age	19.47	5.86	0.016*
Ethnicity	0.002	0.001	0.980
Disease (Control x PC)	54.0	16.25	<0.001*
Ethnicity x Disease	15.32	4.61	0.033*

***Significant when P < 0.05 (ANCOVA). PC- Prostate cancer**

F- Indicates the ratio of the variance due to the effect of the parameters to the variance in the error. The ratio is significant when they are high and less significant when close to zero. Age- mean age of all the participants in the study according to their disease status and ethnicity. Ethnicity- Caucasian and Malaysian Chinese. Disease- indicates either control or PC group, Ethnicity x Disease- interaction between ethnicity and disease status.

The study has also demonstrated that Caucasian cancer patients had a significantly higher mean testosterone concentration than MC cancer patients (P = 0.041). The mean testosterone concentrations were, however, significantly higher in the MC control group when compared to MC cancer (P < 0.001). There were no significant difference between the Caucasian control and cancer groups and MC control vs. Caucasian control groups (Table 4.4).

Table 4.4 Comparison of serum testosterone concentration in control and cancer patients of Caucasian and Malaysian Chinese ethnic groups.

Dataset 1	Dataset 2	MD(1-2)	SE	P value LSD
Caucasian cancer	**MC cancer**	0.759	0.369	0.041*
MC cancer	**MC control**	-1.962	0.369	<0.001*
Caucasian control	**Caucasian cancer**	0.569	0.369	0.125
MC control	**Caucasian control**	0.633	0.369	0.088

MD- mean difference, SE- standard error, LSD-least significant difference, MC-Malaysian Chinese. * Significant when P < 0.05 using post-hoc analysis. Caucasian cancer (n=50), MC cancer (n=50), Caucasian control (n=50), MC control (n=50)

To summarise, the presented results demonstrate that the cancer patients have a lower testosterone concentration compared to the control patients overall. There was no difference in the testosterone concentrations between the ethnic groups when control and cancer data were pooled together. Age factor did not have significant impact on the testosterone concentration. However, there was a significant interaction between age, ethnicity and diagnosis. MC cancer patients had lower testosterone concentration than the Caucasian cancer group.

4.2.2 Analysis of serum dihydrotestosterone concentration

Dihydrotestosterone (DHT), also known as 5α-dihydrotestosterone is derived from testosterone in the prostate. It is 10-times more potent in its effect on the prostatic growth compared to testosterone (Deslypere *et al,* 1992).

Table 4.5 demonstrates that the Caucasian men had a significantly higher mean serum DHT concentration compared to the MC men (by 15.4%) when the cancer and control groups were analysed as one dataset (P = 0.027). It was also found that there was a significant difference in the mean DHT concentration between the control cancer groups when ethnicity was analysed as one dataset (P = 0.005). The control group had the mean DHT concentration of 18.8% higher than the cancer group. Despite the significant effects of ethnicity and diagnosis on DHT concentration when analysed as individual dataset, this significance was lost when the analysis was performed to assess the interaction between the ethnicity (Caucasian or MC) and the disease (control or cancer) (P = 0.118) .

Table 4.5 Circulating DHT concentration (ng/ml) in different ethnic groups, control and cancer patients and interaction between these parameters.

	Group	N	Mean	SD	P value**
Ethnicity	**Caucasian**	100	0.63	0.26	0.027*
	MC	100	0.54	0.29	
Diagnosis	**Control**	100	0.64	0.25	0.005*
	Cancer	100	0.53	0.29	
Interaction	**Ethnicity vs. Cancer &Control**	200	0.59	0.28	0.118

N-number of samples, SD- standard deviation, MC-Malaysian Chinese, DHT-dihydrotestosterone. * Significant at P-value < 0.05. ** ANOVA Test

The comparison of mean values of circulating DHT concentration between the 4 participating groups (MC control, MC cancer, Caucasian control and Caucasian cancer) showed that the difference in DHT concentration between the control and cancer group is bigger within MC than the Caucasian group (Figure 4.4).

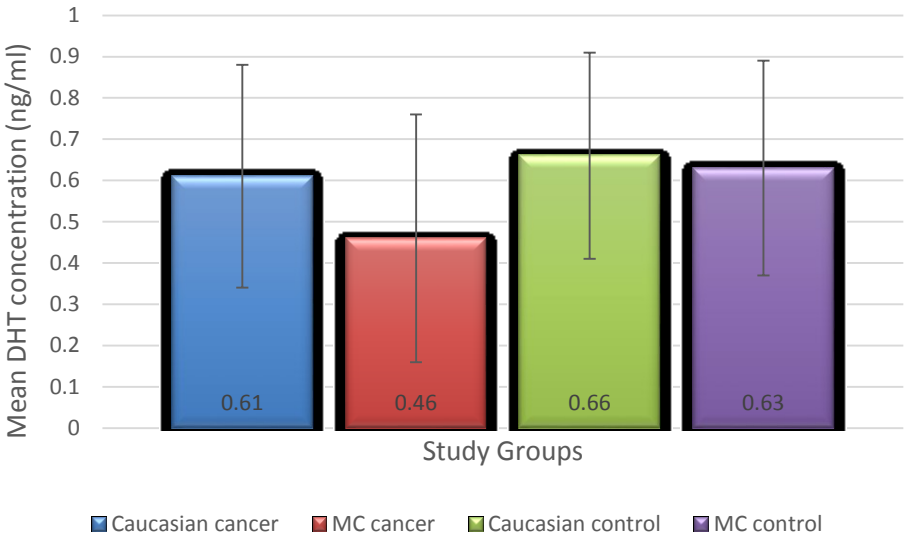

Figure 4.4 Mean DHT concentration for control and cancer groups in Caucasian and MC ethnic groups.

DHT- Dihydrotestosterone. The data are presented as Mean +/- SEM. Each study group consisted of 50 participants.

Similarly to the study on testosterone, an effect of age on DHT concentration was analysed (Gray *et al,* 1991). When age was taken into account, it cancelled the differences in DHT concentration between the Caucasian and MC ethnic groups (P = 0.064) and between control and cancer groups (combining Caucasian and MC participants) (P = 0.079).There was no significant (P = 0.420) interaction between the ethnicity, diagnosis (cancer or control) and DHT concentration when the age factor was taken into account (Table 4.6).

Table 4.6 Analysis of covariance for mean DHT concentration with age.

Parameter	Mean square	F	P value
Age	0.990	14.46	<0.001*
Ethnicity	0.238	3.47	0.064
Disease (Control x PC)	0.213	3.11	0.079
Ethnicity x Disease	0.045	0.652	0.420

***Significant when P < 0.05 (ANCOVA), PC- Prostate cancer**

F- Indicates the ratio of the variance due to the effect of the parameters to the variance in the error. The ratio is significant when they are high and less significant when close to zero. Age- mean age of all the participants in the study according to their disease status and ethnicity. Ethnicity- Caucasian and Malaysian Chinese. Disease- indicates control or PC group, Ethnicity x Disease- interaction between ethnicity and disease status.

To summarise, result presented in this sub-chapter showed that there was a significant difference in the concentration of DHT between the MC and the Caucasian groups (when control and cancer were analysed as one dataset) as well as difference between the control and the cancer groups (when Caucasians and MC were analysed as one dataset). However, when age of the participants was taken into account, no statistical significance in DHT concentration was observed. Therefore, in contrast to study on testosterone concentration (section 4.2.1), where age of participants did not affect the results, in the study on DHT, the participants age was an important confounding factor, which, when included, had strong impact on the findings.

4.2.3 Analysis of dihydrotestosterone to testosterone ratio

Testosterone and DHT (which is produced from testosterone) are two male sex hormones which play critical role in the development of prostate gland (Deslypere *et al,* 1992). It is important not only to know the concentration of both hormones but also DHT/Testosterone (DHT/T) ratio which gives information about the fraction of DHT produced from the testosterone in the circulation.

Table 4.7 shows that there was a significant (P = 0.001) ethnic group effect on the mean value of the DHT/T with the MC group showing a higher ratio than the Caucasian group. It was also established that the cancer group had a significantly higher DHT/T (P < 0.001) than the control group (when ethnicity was analysed as one data pool). The interaction between the ethnicity and the diagnosis was also significant (P < 0.001) (Table 4.7).

Table 4.7 Comparison of mean DHT/T in different ethnic groups, control and cancer patients and interaction between these parameters.

	Group	N	Mean	SD	P-value**
Ethnicity	**Caucasian**	100	0.18	0.07	0.001*
	MC	100	0.38	0.64	
Diagnosis	**Control**	100	0.16	0.07	<0.001*
	Cancer	100	0.40	0.63	
Interaction	**Ethnicity vs. Cancer &Control**	200	0.28	0.46	<0.001*

N-number of samples, SD- standard deviation, MC-Malaysian Chinese. * Significant at P-value < 0.05. ** ANOVA Test

This study demonstrated that the difference in DHT/T between MC cancer and MC control groups were bigger (by 122%) when compared to the difference between the Caucasian

cancer and Caucasian control groups (Figure 4.5). In fact, there was no significant difference in DHT/T ratio between the Caucasian cancer and control group (P = 0.914).

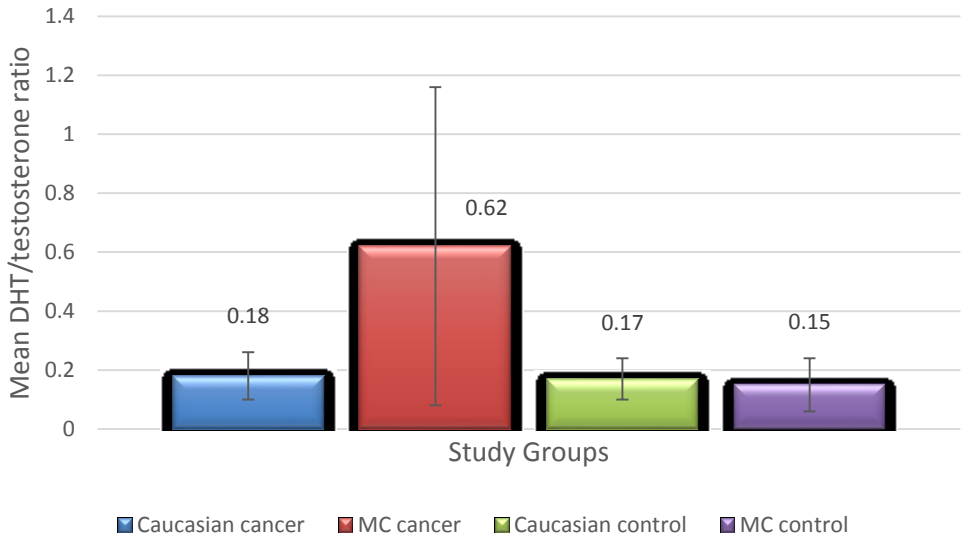

Figure 4.5 Mean DHT/T ratio for control and cancer patients in Caucasians and Malaysian Chinese ethnic groups.

DHT/T- Dihydrotestosterone toTestosterone ratio. The data are presented as Mean +/- SEM. Each study group consisted of 50 participants.

As discussed in sections 4.2.1 and 4.2.2, the analysis for the DHT/T was also performed taking into account the age of participants as a confounding factor that may affect this parameter. The study showed that there was no significant effect of age on DHT/T (P = 0.297) (Table 4.8). The DHT/T was significantly higher in the MC ethnic group when compared to the Caucasians (for cancer and control groups pooled together) (P = 0.001). The DHT/T was found to be significantly higher in the cancer group compared to the control group (when MC and Caucasian ethnic groups were pooled together) (P < 0.001). When age was factored into the analyses, the interaction between ethnicity and the

diagnosis was still highly significant (P < 0.001). These results suggest that DHT/T might be potentially used as a predictor of prostate cancer in different ethnic groups.

Table 4.8 Analysis of covariance for mean DHT/T ratio with age.

Parameter	Mean square	F	P value
Age	0.197	1.09	0.297
Ethnicity	1.921	10.68	0.001*
Disease (Control x PC)	2.291	12.73	<0.001*
Ethnicity x Disease	2.30	12.77	<0.001*

***Significant when P < 0.05 (ANCOVA). PC- Prostate cancer**

F- Indicates the ratio of the variance due to the effect of the parameters to the variance in the error. The ratio is significant when they are high and less significant when close to zero. Age- mean age of all the participants in the study according to their disease status and ethnicity. Ethnicity- Caucasian and Malaysian Chinese. Disease- indicates control or PC group, Ethnicity x Disease- interaction between ethnicity and disease status.

Analysis of effect of ethnicity on DHT/T demonstrated that MC cancer patients had a significantly (P < 0.001) higher DHT/T ratio than the Caucasian cancer patients. There was no significant difference in DHT/T between Caucasian control and MC control groups (P = 0.742) (Table 4.9).

Table 4.9 Comparison of DHT/T ratio in control and cancer patients of Caucasian and Malaysian Chinese ethnic groups

Dataset 1	Dataset 2	MD(1-2)	SE	P value LSD
Caucasian cancer	**MC cancer**	-0.436	0.085	<0.001*
MC cancer	**MC control**	0.474	0.085	<0.001*
Caucasian control	**Caucasian cancer**	-0.009	0.085	0.914
MC control	**Caucasian control**	-0.028	0.085	0.742

DHT/T- dihydrotestosterone to testosterone ratio, MD- mean difference, SE- standard error, LSD-least significant difference, MC-Malaysian Chinese. * Significant when P < 0.05 using post-hoc analysis. Caucasian cancer (n=50), MC cancer (n=50), Caucasian control (n=50), MC control (n=50)

One of the most likely factors which might have influenced the DHT/T ratio could be the severity of disease. PC severity is the factor which enables the clinician to predict the disease progression, to assist in making decisions regarding treatment options, and predict responses of patients to chosen treatment protocols. The commonly used indicators of PC severity are the PSA level and the Gleason score which are used in combination (Partin *et al,* 1997).

As the mean DHT/T values in the MC cancer patients were significantly different from the MC control participants, the distribution of these values for each participant in both ethnic groups for both, cancer and controls were plotted (Figure 4.6). DHT/T ratio were all clumped together in the Caucasian groups for both, cancer and the control groups, and the difference in the mean DHT/T was insignificant. The severity of the disease in the

Caucasians did not alter the DHT/T as much as the MC cancer group. It was noted that the MC cancer group had multiple participants with a DHT/T ratio lying outside of normal range of distribution compared to the control group (Figure 4.6).

This is to ascertain whether there is an element of severity of the disease based on the PSA value and Gleason score that may give rise to a significant increase in the mean of DHT/T ratio in the MC cancer groups.

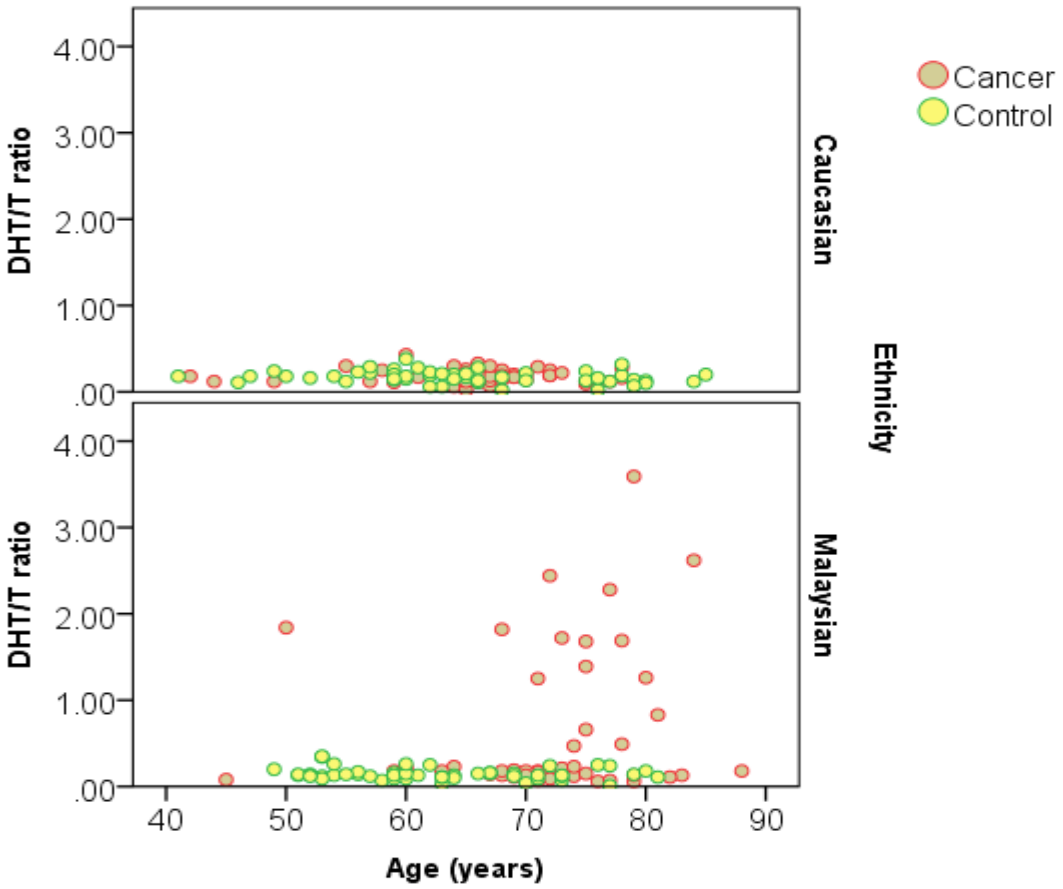

Figure 4.6 Distribution of DHT/T ratio in Caucasian and Malaysian Chinese cancer and control groups.

DHT/T- Dihydrotestosterone to Testosterone.

The individual clinical data of the MC cancer patients (data of 4 individuals were omitted due to extremely high PSA of > 900 ng/ml) was reviewed as far as their PSA values (Figure 4.7) and Gleason scores (Figure 4.8) were concerned. This was to compare the severity of the disease in relation to DHT/T ratio between those having higher and lower ratio than the mean DHT/T in MC cancer group.

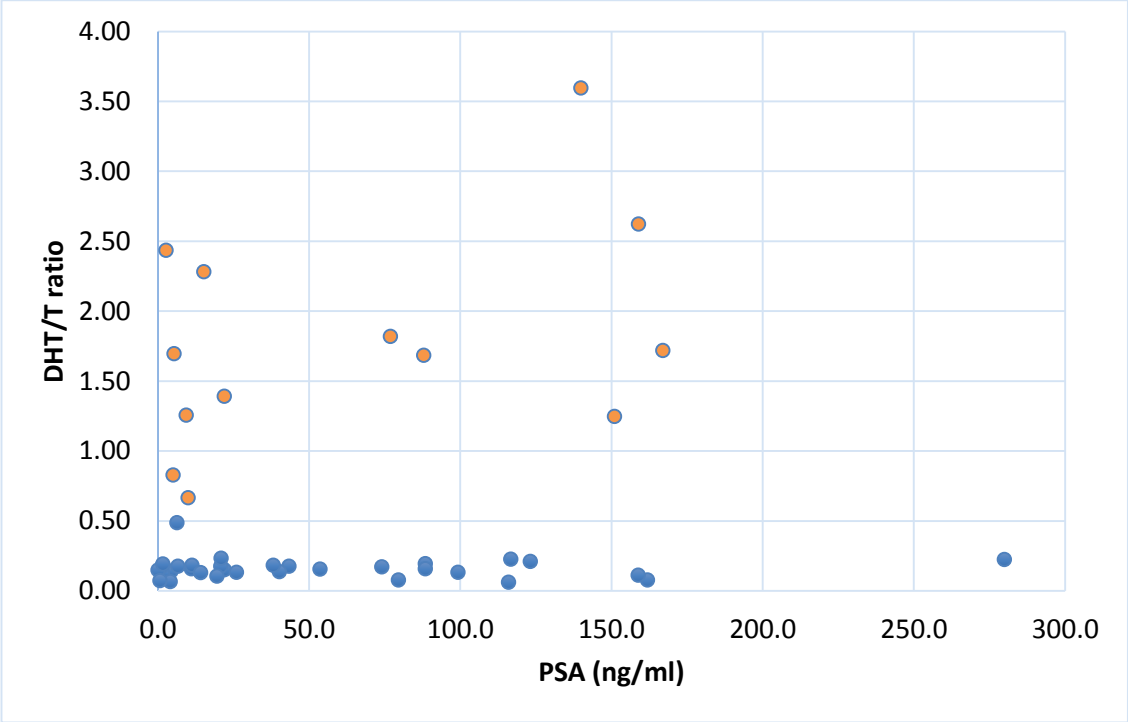

Figure 4.7 Distribution of DHT/T ratio in relation to PSA in MC cancer patients. DHT/T- Dihydrotestosterone/Testosterone, PSA-Prostate Specific Antigen. Individuals with DHT/T above the average DHT/T ratio indicated in amber and below the average is indicated in blue.

It was not clearly established in this study that the higher DHT/T ratio is due to increased disease severity as indicated by high PSA and Gleason score. The trend indicates the possible ethnic variability in the distribution of individual DHT/T ratio comparing the Caucasians to the MC cancer patients. However, this finding needs to be investigated

further with larger cohort due to an almost equal number of patients with the same severity of disease having lower than average DHT/T ratio in this study.

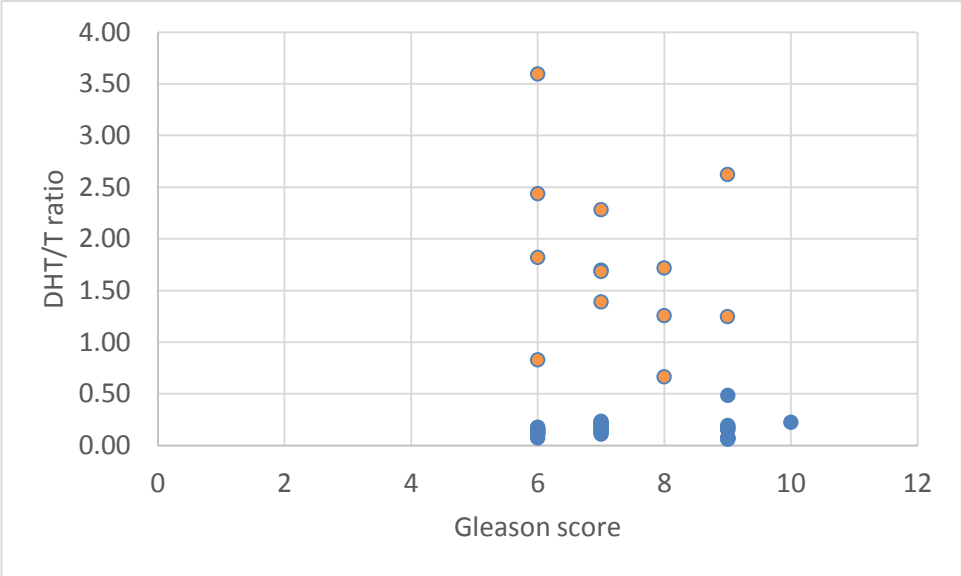

Figure 4.8 Distribution of DHT/T ratio in relation to Gleason score in MC cancer patients. DHT/T- Dihydrotestosterone/Testosterone, PSA-Prostate Specific Antigen. Individuals with DHT/T above the average DHT/T ratio indicated in amber and below the average is indicated in blue.

To conclude, analysis of DHT/T ratio looks promising as a tool for aiding the diagnosis of PC. In this study, the MC cancer group had a higher DHT/T ratio than Caucasian cancer and MC control groups. However, it is important to highlight that these differences may be due to ethnic group differences in severity of the disease. MC cancer patients were in more advanced stage of PC (according to PSA and Gleason score data) when compared to Caucasian cancer. Therefore, the observed differences in DHT/T between MC and Caucasian cancer might not be the "true" ethnic differences. In order to elucidate further

the ethnic differences, it is critical to ensure that all the cancer patients recruited are of the same stages of PC.

4.2.4 Analysis of serum insulin-like growth factor-I

The endocrine hormone IGF-I has been suggested to play a key role in the development of PC, and raised circulating IGF-I concentration has been demonstrated to be positively associated with PC risk (Rowlands *et al,* 2009). Insulin and IGF-I play an important role in cellular proliferation and apoptosis and can be related to the dietary habits of individuals (Pollak *et al,* 2004).

This study showed that the Caucasian participants had a higher mean IGF-I concentration in the circulation compared to the MC participants (by 20%) when cancer and control groups were combined. ($P = 0.007$). This study also demonstrated that the control group (MC and Caucasian data combined) had a significantly higher mean IGF-I concentration compared to the cancer group (MC and Caucasian data combined) by 17.7%. ($P = 0.017$). There was no significant interaction between IGF-I concentration and ethnicity or diagnosis ($P = 0.146$) (Table 4.10).

Table 4.10 Comparison of mean IGF-I (ng/ml) in different ethnic groups, control and cancer patients and interaction between these parameters.

	Group	N	Mean	SD	P-value**
Ethnicity	**Caucasian**	100	93.73	52.02	0.007*
	MC	100	76.68	36.62	
Diagnosis	**Control**	100	92.74	36.97	0.017*
	Cancer	100	77.68	52.09	
Interaction	**Ethnicity vs. Cancer &Control**	200	85.21	45.68	0.146

N-number of samples, SD- standard deviation, MC-Malaysian Chinese, IGF-I- Insulin-like growth factor-I. * Significant at P-value < 0.05. ** ANOVA Test

Difference in circulating IGF-I concentration between control and cancer groups were bigger in the MC group when compared to the Caucasians. Comparison of IGF-I concentration between the ethnic groups demonstrated MC cancer patients had lower IGF-I when compared to Caucasian cancer by 28%, and the MC control group had lower IGF-I when compared to the Caucasian control by 8% (Figure 4.9).

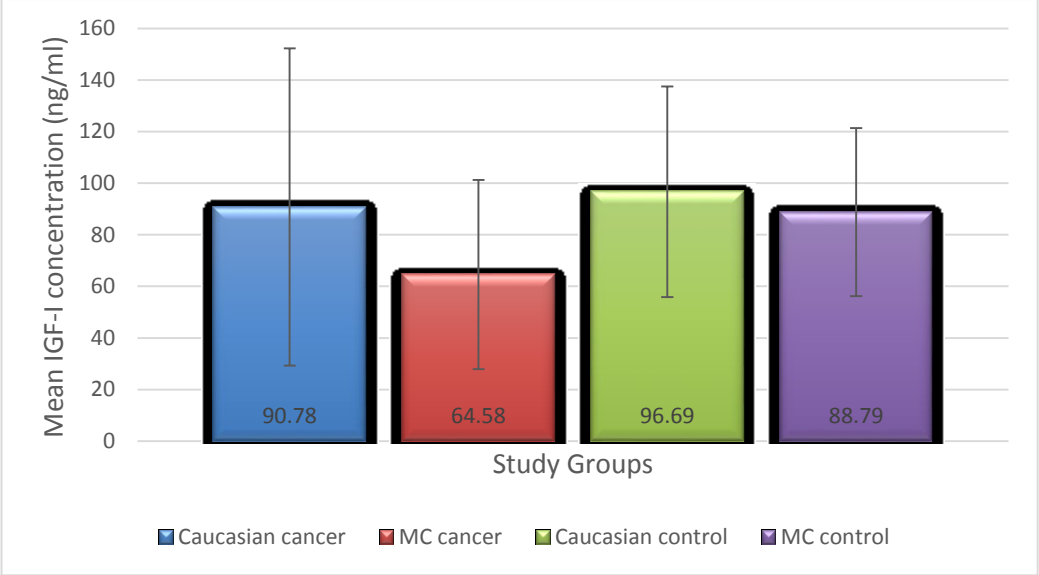

Figure 4.9 Mean IGF-I concentration for control and cancer patients in Caucasians and Malaysian Chinese ethnic groups.

IGF-I- Insulin-like growth factor-I. The data are presented as Mean +/- SEM. Each study group consisted of 50 participants.

IGF-I concentration is known to decline with age (Yamamoto *et al,* 1991) and therefore effect of age on circulating IGF-I concentration was analysed. The results showed that age was a significant confounding factor in the decline of the IGF-I concentration in the study ($P < 0.001$). A significant ethnic difference in IGF-I ($P = 0.018$) was still present after adjusting the data statistically for age between the Caucasians and the MC patients (data for the cancer and control were pooled together). As for the cancer and control groups (combining the MC and the Caucasians for both disease state), there were no significant differences in circulating IGF-I concentration ($P = 0.174$). There was no significant ($P = 0.463$) interaction between ethnicity (MC and Caucasian) and diagnosis (cancer or control) after factoring in the age of participants as far as the circulating IGF-I concentrations are concerned (Table 4.11).

Table 4.11 Analysis of covariance for mean IGF-I concentration with age.

Parameter	Mean square	F	P value
Age	23972.76	12.94	<0.001*
Ethnicity	10561.08	5.70	0.018*
Disease (Control x PC)	3448.27	1.86	0.174
Ethnicity x Disease	1000.30	0.54	0.463

***Significant when P < 0.05 (ANCOVA). PC- Prostate cancer**

F- Indicates the ratio of the variance due to the effect of the parameters to the variance in the error. The ratio is significant when they are high and less significant when close to zero. Age- mean age of all the participants in the study according to their disease status and ethnicity. Ethnicity- Caucasian and Malaysian Chinese. Disease- indicates control or PC group.

Difference in the mean IGF-1 concentration between Caucasians and MC participants and cancer and control groups were assessed using the post-hoc analysis. It was found that IGF-I concentration was significantly higher (P = 0.003) in the Caucasian cancer when compared to MC cancer cases (Table 4.12). The MC control group had a significantly (P = 0.007) higher mean IGF-I concentration than the MC cancer group. Differences in IGF-I between the Caucasian control and cancer group as well as the MC control group with the Caucasian control were found to be not significant (P = 0.506 and 0.374 respectively).

Table 4.12 Comparison of IGF-I concentration in control and cancer patients of Caucasian and Malaysian Chinese ethnic groups

Dataset 1	Dataset 2	MD(1-2)	SE	P value LSD
Caucasian cancer	**MC cancer**	26.21	8.87	0.003*
MC cancer	**MC control**	-24.21	8.87	0.007*
Caucasian control	**Caucasian cancer**	5.91	8.87	0.506
MC control	**Caucasian control**	-7.90	8.87	0.374

IGF-I- Insulin-like growth factor-I, MD- mean difference, SE- standard error, LSD-least significant difference, MC-Malaysian Chinese. * Significant when P-value < 0.05 using post-hoc analysis. Caucasian cancer (n=50), MC cancer (n=50), Caucasian control (n=50), MC control (n=50)

Taken together, results for IGF-I concentration demonstrate that age had an effect on IGF-I in the circulation. Nevertheless, IGF-I concentration still significantly differed between ethnic groups and it was significantly higher in Caucasian cancer patients when compared to MC cancer. Furthermore, IGF-I concentration also allowed to differentiate between MC control and cancer cases even after adjusting for age.

4.2.5 Relationship between hormone concentration and prostate cancer

Currently PSA analysis is the most commonly and widely used test for PC screening. However, there are a number of issues associated with this test including its low sensitivity and specificity (Holmstrom *et al,* 2009). This is associated with the issue of over-diagnosis

of PC and unnecessary biopsy. As a result, there has been increasing interest in new biomarkers for PC, which could form the basis for developing a new effective PC diagnostic test which could be used either in conjunction with PSA or replace PSA. This search for new PC biomarkers has been developing in two directions: identification of biomarkers in circulation (i.e. hormones) and identification of genetic polymorphisms. Research conducted in this study addressed both aspects.

In the sections 4.2.1, 4.2.2, 4.2.3 and 4.2.4, the circulating concentration of testosterone, DHT DHT/T and IGF-I were investigated in control and cancer patients of the 2 ethnic groups, Caucasian and MC. In this chapter, a relationship between the hormonal status and disease as well as impact of ethnicity of this relationship was further investigated.

To ascertain whether detection of these hormones can be used to predict the state of disease, a Receiver Operating Characteristic (ROC) curve was plotted for each of the hormone individually and also by combining two or more hormone marker to assess the Area Under the Curve (AUC) for each parameter. A higher AUC in a ROC curve indicates a better predictability of the test to differentiate between cancer and non-cancer. Furthermore, sensitivity and specificity of hormonal assay was also determined using calculation from the ROC curve so as to analyse the possibility of establishing a specific cut-off value for the assay in the different ethnic groups and the disease state. High sensitivity and specificity of a test will enable its usage for diagnostic purposes.

This study was not able to achieve significant results in the AUC, sensitivity and specificity for testosterone, DHT and IGF-I as individual tests. However, analysis of IGF-I in combination with testosterone and the DHT/T ratio alone has shown high levels of sensitivity and specificity in this study which has been described below.

The use of a combination of testosterone and IGF-I analysis as a diagnostic marker for PC was found to be significantly different for the Caucasian and MC groups, based on the results of the ROC curve. The ROC curve for the Caucasian men in this study (Figure 4.10) showed a significantly (P = 0.009) larger AUC.

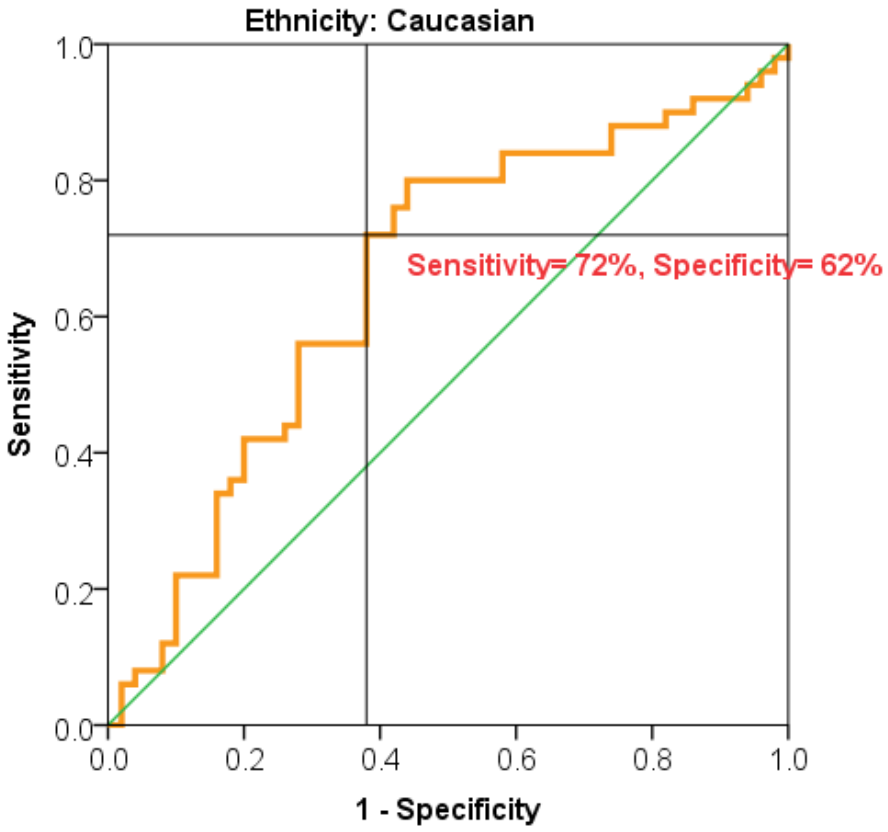

Figure 4.10 Receiver Operating Characteristic (ROC) curve for Caucasian participants using a combination of testosterone and IGF-I

ROC for assessment of diagnostic capability of Prostate cancer. 1-specificity indicates the false positive rate which is given in the X-axis and the specificity is calculated by subtracting this value form 1. IGF-I- insulin-like growth factor-I.

The AUC for the MC men was found to be significantly ($P < 0.001$) larger than the AUC for the Caucasian men when data for both cancer and non-cancer participants were taken into consideration (Figure 4.11). Although both ethnic groups had significant findings but the AUC, sensitivity and specificity for the test is much higher in the MC group compared to the Caucasians (Table 4.13).

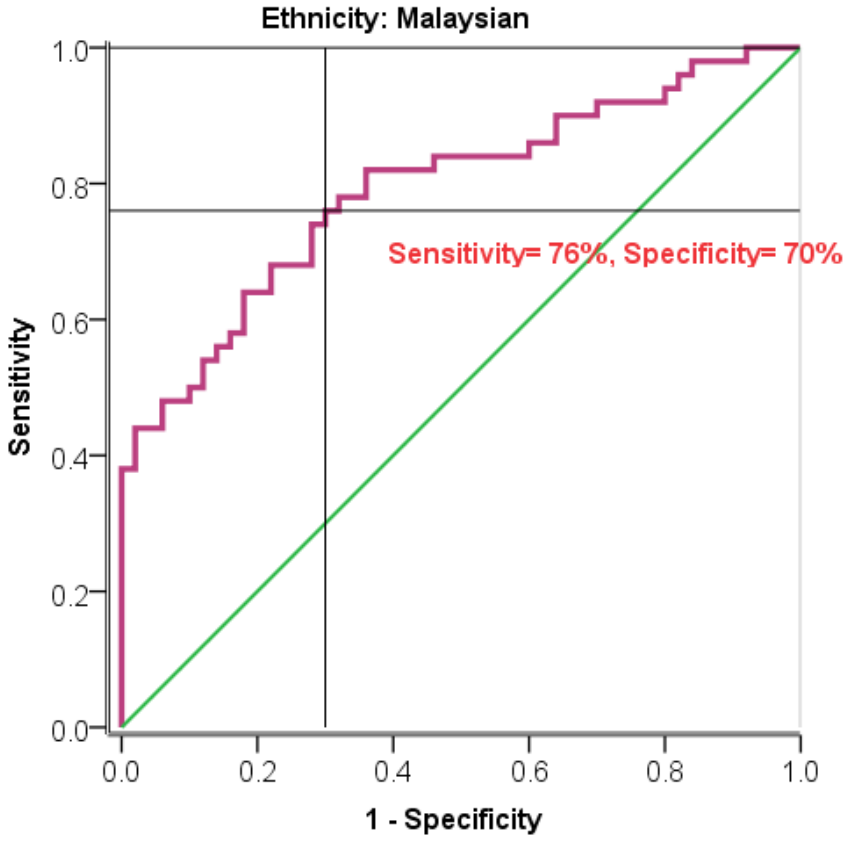

Figure 4.11 Receiver Operating Characteristic (ROC) Curve for Malaysian Chinese participants using a combination of testosterone and IGF-I.

ROC for assessment of diagnostic capability of prostate cancer. 1-specificity indicates the false positive rate which is given in the x-axis and the specificity is calculated by subtracting this value form 1. IGF-I- insulin-like growth factor-I.

Calculation of sensitivity and specificity from ROC curve for both, the Caucasians and the MC groups showed that combination of testosterone and IGF-I might be an effective predictor of PC (when the entire data for cancer and control groups were analysed together (Table 4.13).

Table 4.13 Comparison of area under the Receiver Operating Characteristic (ROC) curve for Caucasian and Malaysian Chinese groups using testosterone and IGF-I combination.

Ethnic group ǂ	AUC (%)	SE	P value	Asymptomatic 95% Confidence Interval		Sensitivity (%)	Specificity (%)
				Lower Bound	Upper Bound		
Caucasian	65.2	0.056	0.009	0.542	0.762	72	62
MC	79.2	0.045	<0.001	0.704	0.880	76	70

SE- standard error, MC-Malaysian Chinese, AUC- area under the ROC curve. *Significant when P < 0.05. ǂ- both cancer and control groups.

In this study we observed difference in DHT/T between Caucasian and MC groups as described in section 4.2.3 above. The ROC curve for the Caucasian group for DHT/T was just about 50% and this indicates that the sensitivity and specificity of this test was not significant (P = 0.741) statistically in PC diagnostics (Figure 4.12).

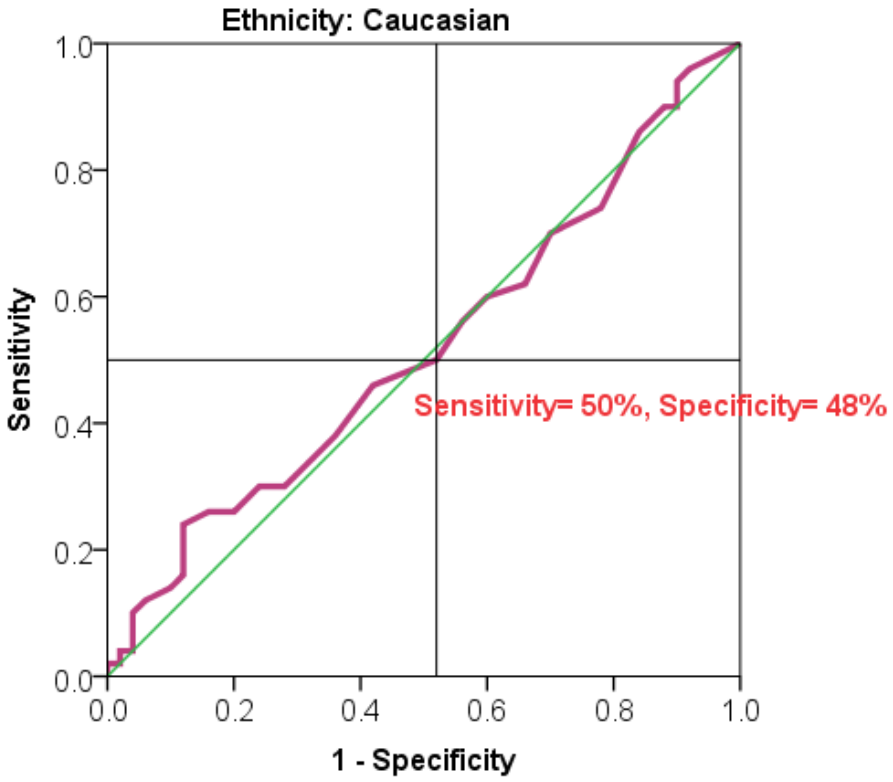

Figure 4.12 Receiver Operating Characteristic Curve (ROC) for Caucasian participants using DHT/T.

ROC for assessment of diagnostic capability of prostate cancer. 1-specificity indicates the false positive rate which is given in the x-axis and the specificity is calculated by subtracting this value form 1. DHT/T- dihydrotestosterone to testosterone ratio.

It was found that MC group had a much larger AUC (by 17.4%, Figure 4.13) compared to AUC of Caucasian group.

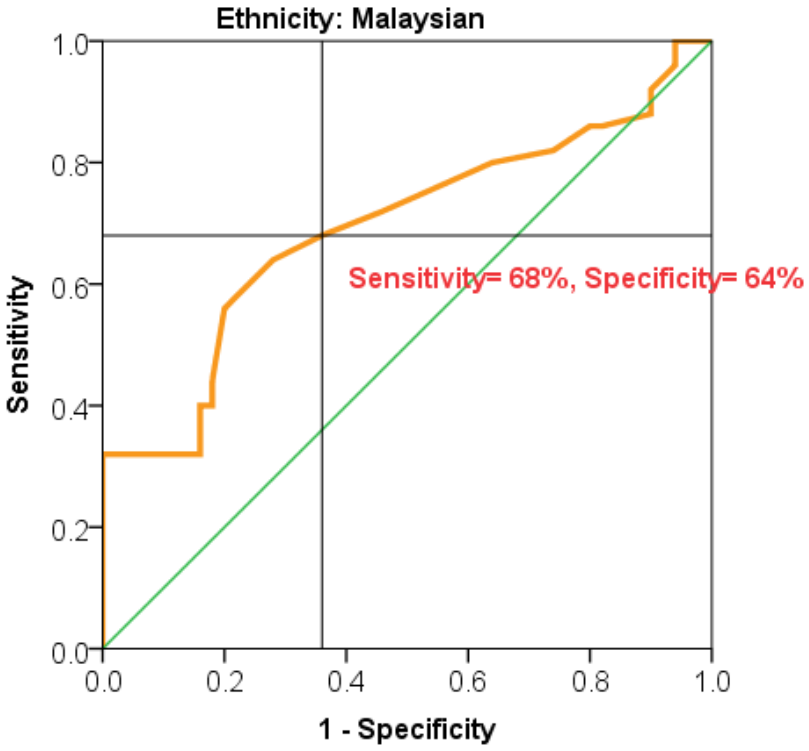

Figure 4.13 Receiver Operating Characteristic Curve (ROC) for Malaysian Chinese participants using a DHT/T.

ROC for assessment of diagnostic capability of prostate cancer. 1-specificity indicates the false positive rate which is given in the x-axis and the specificity is calculated by subtracting this value form 1. DHT/T- dihydrotestosterone to testosterone ratio.

Sensitivity and specificity of DHT/T ratio in predicting PC was calculated from the ROC curve above for both, Caucasians and MC groups. As expected, due to a larger AUC in the MC group, the sensitivity and specificity of DHT/T test in the MC was higher when compared to Caucasians in differentiating PC from the controls (Table 4.14).

Table 4.14 Comparison of area under the Receiver Operating Characteristic (ROC) curve for Caucasian and Malaysian Chinese groups for DHT/T

Ethnic group ǂ	AUC (%)	SE	P-value	Asymptomatic 95% Confidence Interval		Sensitivity (%)	Specificity (%)
				Lower Bound	Upper Bound		
Caucasian	51.9	0.058	0.741	0.405	0.633	50	48
MC	69.3	0.054	0.001	0.587	0.798	68	64

SE- standard error, MC-Malaysian Chinese, AUC- area under the ROC curve. *Significant when P < 0.05. ǂ- both cancer and control groups.

This study has shown that it might be possible to use testosterone in combination with IGF-I or the DHT/T for improving diagnosis of PC even in this small study group. Such a test would be particularly important for MC group where the incidence of PC is lower.

4.3 Investigation of DNA polymorphisms

This section presents results of experiments on (i) identification of ethnic group-specific polymorphisms in CYP17 (rs743572), SRD5A2 (rs523349 and rs9282858) and AR genes and (ii) relationship between these polymorphisms and PC development. These genes were chosen because they encode for enzymes and receptors that are important in androgen metabolism described in section 2.3. The study was conducted on genomic DNA (gDNA) isolated from the whole blood of participants recruited for this study from Caucasian and MC ethnic groups.

This research consisted of (i) a pilot study and (ii) ethnic groups study. The pilot study aimed to optimise a protocol for isolation of gDNA from blood and optimise DNA

amplification, and it was conducted on blood from 4 volunteers (see section 4.3.1. for details). The ethnic group study was conducted on 100 cancer and 100 control patients of Caucasians and MC populations (see section 4.3.2 for details).

4.3.1 Genomic DNA isolation from whole blood samples of volunteers

gDNA isolation was optimised using samples of whole blood (10 ml) from 4 volunteers. The blood from each volunteer was aliquoted into several tubes and frozen at -80º C. Optimisation of gDNA isolation was performed as describe below:

i. Isolation of gDNA following the manufacture protocol using the QI Amp Blood mini kit (Qiagen Ltd, Crawley, UK). DNA quality was assessed using NanoDrop 1000 (Thermo Scientific, Basingstoke, UK) as described below.
ii. If the gDNA quality was found to be unsatisfactory (if the absorbance 260/280 ratio of < 1.8), the gDNA isolation conditions were modified (the elution buffer was replaced with water).
iii. If the gDNA concentration was low (below < 50 ng/µl), the volume of elution buffer was reduced in order to concentrate gDNA.

Table 4.15 presents results of evaluation of gDNA concentrations and quality (measured as 260/280 ratio). The results demonstrate that in a pilot study, gDNA concentration was generally low (< 50 ng/µl) but the 260/280 absorbance ratio was within acceptable limits of > 1.8 with exception of 3 sub-samples of the split volunteer samples.

Table 4.15 Concentration and quality of gDNA isolated from blood of volunteers.

Sample‡	DNA identity [a]	gDNA concentration (ng/µl) (Average of the 2 sub-samples)	260/280
Volunteer 1	V1A1	17.9	2.06
	V1A2		1.79
Volunteer 2	V2A1	27.05	2.01
	V2A2		1.88
Volunteer 3	V3A1	42.75	1.91
	V3A2		1.66
Volunteer 4	V4A1	42.1	1.84
	V4A2		1.67

[a] DNA identity: anonymised sample identification number, gDNA: genomic Deoxyribonucleic acid, 260/280: Absorbance ratio. Single measurement for each sample performed. ‡ Each volunteer blood sample were divided into two tubes for two independent gDNA isolation and measurements.

To increase gDNA yield and improve 260/280 ratio, DNA isolation procedure was modified. In particular, water was used instead of the elution buffer for the final elution of purified gDNA. This was recommended by the manufacturer in the protocol attached with the kit. However, this modification did not improve gDNA quality and, in fact, the absorbance ratio and the gDNA concentration were not significantly different when either buffer or water was used for elution (P = 0.14 and 0.07 respectively) (Table 4.16).

Table 4.16 gDNA concentration and 260/280 ratio

Sample ǂ	Elution	gDNA concentration (ng/µl)	Mean gDNA ± SEM	P value	260/280	Mean 260/280 ± SEM	P value
Volunteer 1	Buffer	8.2	18.0 ±6.46	0.14*	1.74	1.70 ±0.72	0.07*
Volunteer 2		15.6			1.56		
Volunteer 4		30.2			1.80		
Volunteer 1	Water	19.3	18.9 ±2.03		1.60	1.63 ±0.17	
Volunteer 2		22.2			1.66		
Volunteer 4		15.2			1.63		

gDNA concentration obtained with a modified protocol (substituting the buffer with water for elution). gDNA: genomic Deoxyribonucleic acid, 260/280: Absorbance ratio. ǂ Each volunteer blood sample was divided into two tubes for gDNA isolation and two independent measurements was done. Sample from volunteer 3 was not used due to insufficient volume. * denotes independent T-test and significant when P < 0.05.

The other approached used to improve gDNA yield and quality was changing the elution buffer volume in the first elution step (from 200 µl to 25, 50 and 100 µl). This modification allowed production of gDNA of best quality and acceptable concentration (Table 4.17). Based on these findings, a decision was made to use 100 µl and 50 µl of elution buffer for the first and second elution respectively in future experiments.

Table 4.17 DNA concentration and 260/280 ratio in experiments using various volume of elution buffers.

Sample ǂ	DNA identity [a]	Remarks	DNA concentration (ng/µl)	260/280
1a	V1C1	Elute with buffer 25µl	79.2	2.06
1b	V1C1	2nd elution with buffer 50µl	40.4	2.17
1c	V1C2	Elute with buffer 50µl	43.5	1.99
1d	V1C2	2nd elution with buffer 50µl	45.8	2.09
1e	V1C3	Elute with buffer 100µl	39.4	2.13
1f	V1C3	2nd elution with buffer 50µl	39.3	2.12
4a	V4C1	Elute with buffer 25µl	135.1	1.98
4b	V4C1	2nd elution with buffer 50µl	65.1	2.04
4c	V4C2	Elute with buffer 50µl	100.6	2.01
4d	V4C2	2nd elution with buffer 50µl	25.9	2.24
4e	V4C3	Elute with buffer 100µl	63.8	1.87
4f	V4C3	2nd elution with buffer 50µl	80.0	2.03

[a] DNA identity: anonymised sample identification number, gDNA: genomic Deoxyribonucleic acid, 260/280: Absorbance ratio. ǂ Each volunteer blood sample was divided into two tubes for gDNA isolation and two independent measurements was done. Sample from volunteer 2 and 3 were not used due to insufficient volume.

4.3.2 Analysis of quantity and quality of genomic DNA from blood of control and cancer patients of Caucasian and Malaysian Chinese groups.

The optimised protocol (section 4.3.1) was used uniformly for isolation of gDNA from the blood of Caucasian and MC groups.

Table 4.19 presents mean values for 260/280 ratio and gDNA concentration. For all the groups investigated, 260/280 values were above 1.8. The mean concentration of gDNA obtained in the MC group was lower than the Caucasian group by almost 50% (Table 4.18). However, the lower concentration of isolated gDNA in MC ethnic group was not an issue for PCR amplification and subsequent DNA sequencing as the concentration of gDNA used as a template for PCR was standardise to a concentration of 100 ng/µl in all the cases.

Table 4.18 gDNA concentration and 260/280 ratio in blood samples from Caucasian and Malaysian Chinese groups

Parameters		**N**	**Mean gDNA concentration (ng/µl)**	**Mean 260/280 ratio**
Caucasian	**Control**	50	66.1	1.85
	Cancer	50	63.9	1.87
Malaysian Chinese	**Control**	49	35.2	1.90
	Cancer	50	25.5	1.82

N- number of participant in each group. One sample in the Malaysian Chinese control group omitted due to absent gDNA. gDNA- genomic Deoxyribonucleic acid.

Some of the patients in the cancer and control groups from both, Caucasians and MC groups had absorbance ratio below the 1.8 (but not by more than 10%). However, the number of these individuals was only 3 in Caucasian cancer, 8 in Caucasian control, 10 in MC cancer and 4 in MC control group. In this study, PCR amplifications were conducted on the samples with both, high and lower 260/280 ratio to check whether it is possible to obtain a sufficient quality product from samples with lower ratio. The quality of all PCR products was satisfactory (evidenced by high quality PCR band of the correct size, absence of non-specific bands and correct DNA sequences).

4.3.3 Identification of CYP17 polymorphism

Prostate is an androgen-regulated organ. It has been suggested that polymorphisms in genes encoding for enzymes controlling androgen biosynthesis and metabolism or androgen receptor functions may be related to the risk of prostate cancer (Henderson and Feigelson, 2000). One such enzyme is 17α-hydroxylase/17, 20 lyase which is encoded by CYP17 gene. This enzyme acts at multiple points in the androgen metabolism pathway that lead to the formation of testosterone (see Figure 2.14 in section 2.2.1). It has been reported in the literature that a single base pair (bp) change in the 5'-transcribed but untranslated region of CYP17 (rs743572) (position: -34T→C) is positively associated with excessive androgen production (Carey *et al,* 1994).

Research presented in this chapter investigated the presence or frequency of the above CYP17 polymorphism in control and cancer patients in the Caucasian and MC ethnic groups. Furthermore, this study investigated not only CYP17 polymorphism reported in the literature but also any coincidental SNPs present in the DNA region amplified during PCR.

DNA regions of interest were amplified and the size of PCR products was checked using agarose gel electrophoresis. The UV transilluminator image showed strong bands of 500bp. Each gel contained samples from the Caucasian cancer, MC cancer, Caucasian control and MC control groups (Figure 4.14).

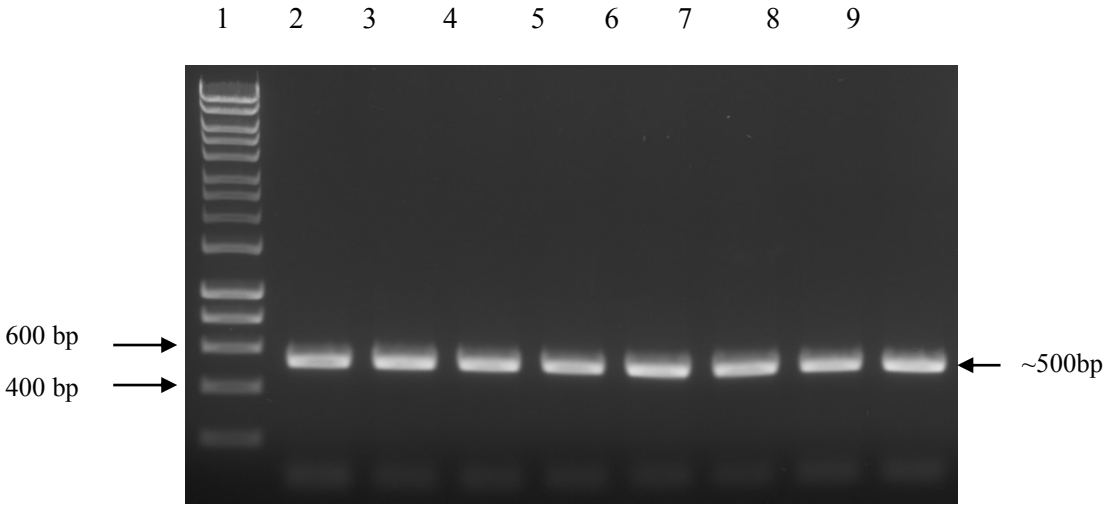

Figure 4.14 Horizontal agarose gel electrophoresis output for PCR product of CYP17 gene fragment.

Lane 1 DNA markers, Lane 2 & 6: Caucasian cancer, Lane 3 & 7: Malaysian Chinese cancer, Lane 4 & 8: Caucasian control, Lane 5 & 9: Malaysian Chinese control. bp- base pair.

The position of SNP -34T→C (rs743572) in CYP17 is shown in Figure 4.15. This figure also presents a position of a coincidental polymorphism that was found in these experiments.

Figure 4.15 Position of SNP -34T→C and 135C→T (coincidental) in CYP17 gene gDNA sequence (ref NG_007955.1).

SNPs -34T→C (rs743572) and 135C→T (coincidental) is located 34 bases preceeding and 135 base from the start codon in Exon 1. Numbering on the left indicates the reference point from origin of sequence of gDNA used for primer design. ATG indicates the position of the start codon.

4.3.3.1 Single nucleotide polymorphism at position -34T→C of CYP17 5' untranslated region.

The -34T→C SNP in the CYP17 gene was identified by DNA sequencing. This SNP is denoted as A1 which represents a homozygote individual where both alleles contain a T nucleotide whilst A2 represents a homozygous for C nucleotide at both alleles whils T/C heterozygote is represented by A1/A2.

Table 4.19 Frequency of CYP17 gene polymorphism among Caucasians and Malaysian Chinese (MC) groups

Ethnic Group	N	Type of polymorphism (Allele)			P-value
		A1	A2	A1/A2	
		n	n	n	
Caucasian	100	59 (59%)	18 (18%)	23 (23%)	0.333*
MC	99	65 (65.7%)	22 (22.2%)	12 (12.1%)	0.381**

A1-T nucleotide (homozygote), A2- C nucleotide (homozygote), A1/A2- T or C nucleotide (heterozygote). N- number of participant in the ethnic group. n- number of participant with polymorphism. Ethnic group- combination of cancer and control cases. *Pearson Chi-Square test **Fisher's Exact test.

It was found that frequency of A1 and A1/A2 allele was by 6.7% and 10.9% higher in Caucasian group, whilst frequency of A2 allele was 4.3% higher in MC group. However, Pearson Chi-Square and Fisher's exact tests showed that the difference was not statistically significant in the frequency profile of the different alleles in the different ethnic groups (P = 0.333 and 0.381) (Table 4.19).

This -34T→C SNP in CYP17 was present in cancer and control cases of both ethnic groups in this study. Frequency of polymorphisms (A2 + A1/A2) was 45% (n=45) in the

cancer group and 30.3% (n=30) in the control group when men from Caucasians and MC ethnic groups were considered together. Comparison between the control and cancer cases overall showed that there was a statistically significant increase (by 14.7%) of polymorphism incidence in the cancer group compared to control group when data for Caucasian and MC participants were analysed as one dataset (P = 0.032 and 0.041 using Pearson Chi-Square test and Fisher's Exact test) (Table 4.20). Frequency of A2 allele was no different between the cancer and control groups but there was a 15% increase in the prevalence of A1/A2 allele in the cancer group. This higher frequency was statistically significant (P = 0.029).

Table 4.20 Frequency of different alleles of CYP17 gene at position -34T→C in cancer and control groups.

Group	**N**	**Type of polymorphism (Allele)**				**P-value**
		A1	**A2**	**A1/A2**	**A2 + A1/A2**	
		n (%)	n (%)	n (%)	n (%)	
Control	99	69 (69.7)	20 (20.2)	10 (10.1)	30 (30.3)	0.032*
Cancer	100	55 (55)	20 (20)	25 (25)	45 (45)	0.041**

A1-T nucleotide (homozygote), A2- C nucleotide (homozygote), A1/A2- T or C nucleotide (heterozygote). N- number of participant in the ethnic group. n- number of participant with polymorphism. Group- All men combined (Caucasian + Malaysian Chinese). *Pearson Chi-Square test **Fisher's Exact test.

Risk of PC development was assessed by calculating the odds ratio (OR) with 95% confidence interval (95% CI). The OR was calculated for A2, A1/A2 and a combination of A1 + A1/A2 compared to the baseline OR for A1 which was considered as a reference point. OR of > 1.0 was considered to have higher risk of PC but the significance was assessed with the range of 95% CI. 95% CI was considered significant if the lower limit was > 1.0 due to the fact that OR of the reference in case of A1 was considered to be 1.0.

Table 4.21 Association of SNP -34T→C in CYP17 gene with the risk of prostate cancer.

Type of polymorphism	All ethnic groups			Caucasian			MC		
	Control	Cancer	OR (95% CI)	Control	Cancer	OR (95% CI)	Control	Cancer	OR (95% CI)
A1	69	55	1.0 (reference)	33	26	1.0 (reference)	36	29	1.0 (reference)
A1/A2	10	25	3.14 (1.39-7.08) *	6	17	3.6 (1.24-10.41) *	4	8	2.48 (0.68-9.07)
A2	20	20	1.25 (0.61-2.56)	11	7	0.81 (0.27-2.37)	9	13	1.79 (0.67-4.78)
A2 + A1/A2	30	45	1.88 (1.05-3.37) *	17	24	1.79 (0.8-4.01)	13	21	2.01 (0.86-4.68)

A1-T nucleotide (homozygote), A2- C nucleotide (homozygote), A1/A2- T or C nucleotide (heterozygote). CI- Confidence interval, given in brackets for each allele or allele combination. "Reference" presents OR ratio for A1 allele. MC- Malaysian Chinese. OR- Odds ratio. *- significant.

This study has shown that there is significantly increased risk of PC in participants with A1/A2 allele compared to the A1 allele when data for men from both the ethnic groups were combined (OR = 3.14; 95% CI = 1.39-7.08). This significance was also present in the combination of A2 + A1/A2 alleles compared to the A1 allele (OR = 1.88; 95% CI = 1.05-3.37). Risk of PC was more prominent and statistically significant in the Caucasians (OR = 3.6; 95% CI = 1.24-10.41) than the MC (OR = 2.48; 95% CI = 0.68-9.07) with A1/A2 allele. Comparing the risk of PC in the presence of A2 allele to the baseline A1 allele, there is a non-significant increase in risk in the MC group (OR = 1.79; 95% CI = 0.67-4.78) compared to the Caucasians. A2 + A1/A2 allele showed a non-significant increase risk in both ethnic groups (Table 4.21).

In summary, this study showed that (i) A1/A2 or a combination of A2 + A1/A2 alleles of the SNP -34T→C in CYP17 gene increases the risk of PC in Caucasians and MC overall (ii) there is increasing trend in the risk of PC in MC in presence of A2 allele (iii) there is significant increase in PC risk in Caucasians in presence of A1/A2 allele. All the comparison was against the reference A1 allele.

4.3.3.2 Coincidental polymorphism at position 135C→T of CYP17 gene

Section 1.3.3 described amplification and sequencing of 500bp of CYP17 gene. Within this 500 bp, we were able to investigate not only ethnic-group specific occurrence and frequency of CYP17 SNP -34T→C, but also identify coincidental polymorphisms. This study identified a coincidental polymorphism at the position 135C→T consistently in gDNA. This polymorphism occurred in the CYP17 coding region but it did not result in an amino acid change and therefore was denoted as a 'silent' polymorphism. CYP17 DNA

sequence in our study contained either nucleotide C, nucleotide T or a dual peak indicating presence of both, C and T which is a wild-type heterogeneous polymorphism.

Difference in the frequency of the 3 variants in the Caucasian and the MC groups was not statistically significant when the data of both, cancer and control groups were combined. The MC group had a 2% higher prevalence of C and T allele whereas the Caucasians had about 4 % increased frequency of the C/T allele. The frequency profile was not significant statistically (Pearson Chi-Square and Fisher's exact tests of P = 0.768 and 0.875 respectively) (Table 4.22).

Table 4.22 Association of variant on ethnicity in the CYP17 gene at position 135C→T.

Ethnic Group	**N**	**Type of polymorphism (Allele)**			**P-value**
		C	**T**	**C/T**	
		n	n	n	
Caucasian	88	59 (67%)	18 (20.5%)	11 (12.5%)	0.768*
MC	97	67 (69.1%)	22 (22.7%)	8 (8.2%)	0.875**

C-C nucleotide (homozygote), T- T nucleotide (homozygote), C/T- C or T nucleotide (heterozygote). N- number of participant in the ethnic group. n- number of participant with polymorphism. Ethnic group- combination of cancer and control cases. *Pearson Chi-Square test **Fisher's Exact test.

This coincidental SNP in the CYP17 gene was present in cancer and control cases of both ethnic groups in this study. Frequency of polymorphism (C + C/T) was 33.7% (n=31) in the cancer group and 30.1% (n=28) in the control group when Data men from Caucasians and MC ethnic groups were analysed together. Comparison between the control and cancer cases overall showed that there was a non-significant increase (by 3.6%) in

frequency of the polymorphism in the cancer group compared to control group when Caucasian and MC participants were analysed as one dataset (P = 0.601 and 0.638 using Pearson Chi-Square test and Fisher's Exact test) (Table 4.23). Except for the frequency of the C/T allele which was almost double the frequency in cancer cases compared to controls, the other allele were not significantly different between the two groups (Table 4.23).

Table 4.23 Frequency of different alleles of the SNP in the CYP17 gene at position 135C→T in cancer and control groups.

Group	**N**	**Type of polymorphism (Allele)**				**P-value**
		C	**T**	**C/T**	**T + C/T**	
		n (%)	n (%)	n (%)	n (%)	
Control	93	65 (69.9)	21 (22.6)	7 (7.5)	28 (30.1)	0.601*
Cancer	92	61 (66.3)	18 (19.6)	13 (14.1)	31 (33.7)	0.638**

C-C nucleotide, T- T nucleotide, C/T- C or T nucleotide. N- number of participant in the ethnic group. n- number of participant with polymorphism. Group- All men combined (Caucasian + Malaysian Chinese). *Pearson Chi-Square test **Fisher's Exact test.

As in SNP -34T→C above, the OR and 95% CI was calculated to determine association between risk of PC and this polymorphism. It was noted that, there were no statistically significant risk associated with any of the allele at position 135C→T of the CYP17 gene (Table 4.24). It should be noted that the above data should be treated with caution due to a relatively small number of participants in this study.

Table 4.24 Association of SNP 135C→T in CYP17 gene with risk of prostate cancer.

Type of polymorphism	All ethnic groups			Caucasian			MC		
	Control	Cancer	OR (95% CI)	Control	Cancer	OR (95% CI)	Control	Cancer	OR (95% CI)
C	65	61	1.0 (reference)	29	30	1.0 (reference)	36	31	1.0 (reference)
T	21	18	0.91 (0.44-1.88)	11	7	0.62 (0.21-1.8)	10	12	1.39 (0.53-3.66)
C/T	7	13	1.98 (0.74-5.29)	4	7	1.69 (0.45-6.4)	3	5	1.94 (0.43-8.76)
T + C/T	28	31	1.18(0.64-2.19)	15	14	0.9 (0.37-2.2)	13	17	1.52 (0.64-3.61)

C- C nucleotide, T- T nucleotide, C/T- C or T nucleotide. CI- Confidence interval, given in brackets for each allele or allele combination. "Reference" presents OR ratio for A1 allele. MC- Malaysian Chinese. OR- Odds ratio.

To summarise, results presented in this chapter demonstrate that:

(i) A heterogeneous A1/A2 SNP in CYP17 gene at the position -34T→C was more frequent in Caucasians. There was increased risk of PC in case of A1/A2 and/or A2 + A1/A2 alleles when data for men from both ethnic groups were combined. Caucasians had a higher risk of PC in the presence of A1/A2 allele whilst MC ethnic group had higher risk of PC in the presence of A2 allele.

(ii) A new polymorphism was identified at the position 135C→T of CYP17 gene. Data of this study do not allow to conclude whether this polymorphism is associated with PC or whether it is ethnic-group specific due to a relatively low number of participants.

4.3.4 Identification of SRD5A2 polymorphism

SRD5A2 gene encodes the enzyme steroid 5α-reductase type 2 which mediates irreversible conversion of testosterone to a more metabolically active DHT (Hsing *et al,* 2001). This chapter focuses on determining whether the presence and frequency of SRD5A2 DNA polymorphisms reported in the literature are ethnic-group specific and whether frequency of these polymorphisms varies between control and cancer groups. A specific focus was on two polymorphisms as detailed below.

It is known that SRD5A2 DNA polymorphism at position 261C→G of the coding region results in amino acid substitution. Namely, Val is replaced for Leu at codon 89, and this has been defined as V89L variant. This amino acid change has been reported to reduce activity of SRD5A2 enzyme (Makridakis *et al,* 1997).

SRD5A2 DNA polymorphism at position 140G→A of the coding region induces another amino acid substitution. The A49T variant is produced where conversion of the amino acid Ala to Thr at codon 49 takes place due to the above 140G→A polymorphism. This amino acid change results in increased SRD5A2 enzymatic activity (Makridakis *et al,* 2000).

PCR reaction resulted in 500bp band which is consistent with the size of amplified SRD5A2 region. The amplification was performed on samples from the Caucasian cancer, MC cancer, Caucasian control and MC control participants. The PCR products from different groups were run on the same gel (Figure 4.16)

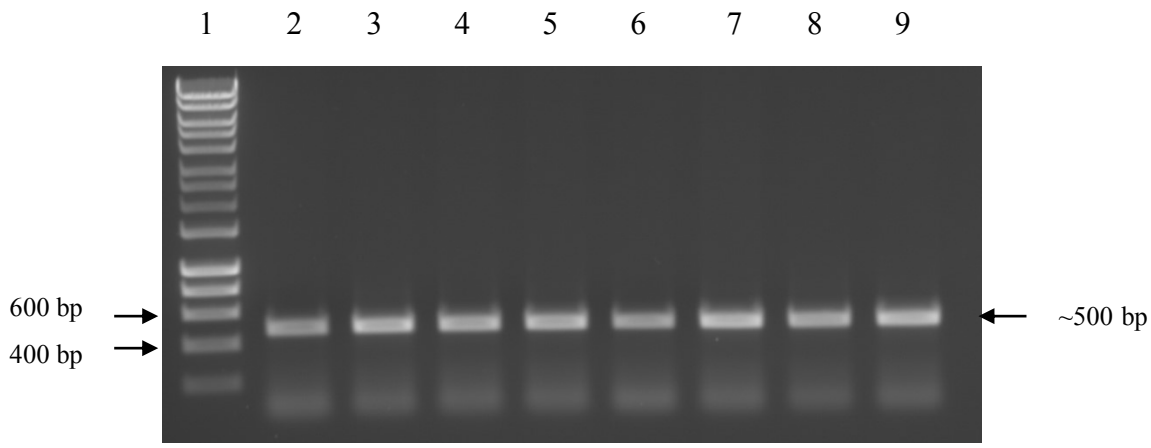

Figure 4.16 UV transilluminator image showing successful PCR amplification of a region of SRD5A2 gene.

Lane 1 DNA markers, Lane 2 & 6: Caucasian cancer, Lane 3 & 7: Malaysian Chinese cancer, Lane 4 & 8: Caucasian control, Lane 5 & 9: Malaysian Chinese control. bp- base pair.

The position of the polymorphism in the coding region in exon 1 of the SRD5A2 gene for the 140G→A (rs9282858) and 261C→G (rs523349) is shown in Figure 4.17 below.

Figure 4.17 Position of SNP 140G→A and 261C→G in SRD5A2 gene gDNA sequence (ref NG_008365.1).

SNPs 140G→A (rs9282858) and 261C→G (rs523349) is located 140 bases and 261 bases from the start codon in Exon 1. Numbering on the left indicates the reference point from origin of sequence of gDNA used for primer design. ATG indicates the position of the start codon.

4.3.4.1 Analysis of polymorphism 261C→G (rs523349) in SRD5A2 gene

The SRD5A2 SNP studied here was referenced to as rs523349, which is 261C→G substitution at the coding region in exon 1 resulting in amino acid Val to Leu substitution (Makridakis *et al,* 1997).

This study identified the presence of a baseline homozygote with nucleotide C (no polymorphism), a polymorphic homozygote with nucleotide G and heterozygote C or G nucleotide in the DNA sequence. Both, the G nucleotide and C or G nucleotides were denoted as polymorphic in this study which is in agreement with data of the literature (Reichardt *et al,* 1995, Makridakis *et al,* 1997). Frequency of polymorphisms at the position 261C→G (rs523349) of SRD5A2 gene in the Caucasians and the MC groups (combining cancer and control cases) is shown in Figure 4.18.

It was noted that frequency of G nucleotide was almost 33% higher in the Caucasian group compared to MC group. The study also showed that the MC ethnic group had 21.6% and 11.3% higher frequency of C nucleotide and C or G nucleotides respectively when compared to the Caucasians.

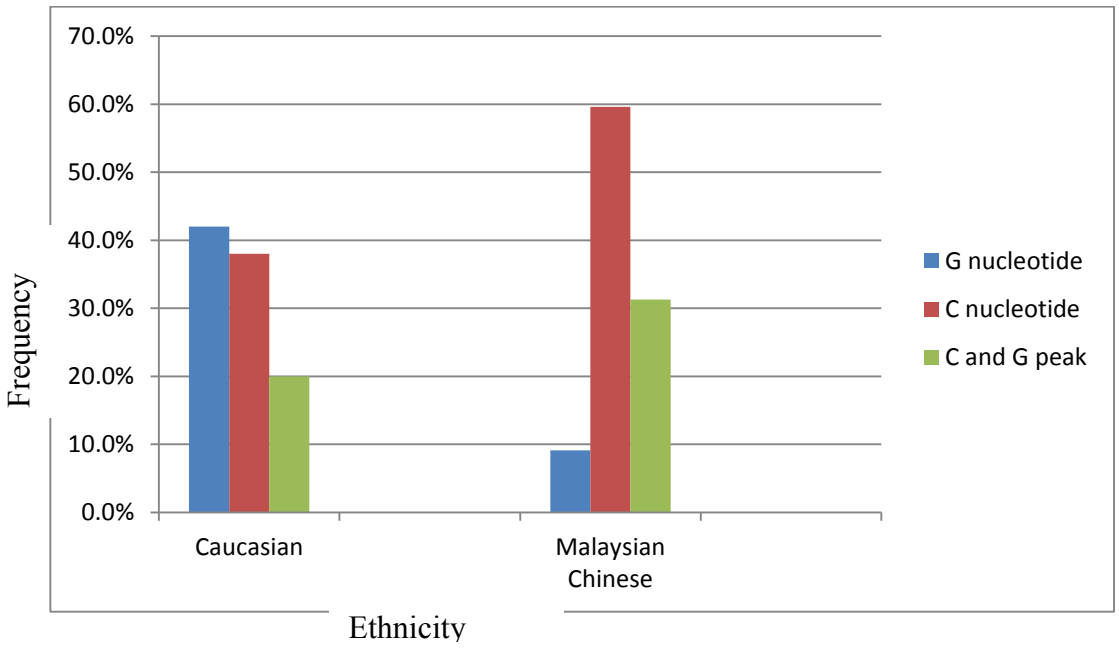

Figure 4.18 Frequency of polymorphism at position 261C→G of SRD5A2 gene in the Caucasian and Malaysian Chinese groups.

Data for Caucasians and Malaysian Chinese cancer and control groups pooled together. G and C nucleotide are homozygotes, and C and G peak is heterozygote.

When results for men from both ethnic groups were analysed together, it was found that frequency of C to G polymorphism in SRD5A2 in control group was only 1.3% higher than in cancer patients. The cancer group had a 12.6% higher frequency of C nucleotide compared to the control group .The frequency of C or G nucleotide was 11.3% higher in the control group when compared to cancer (Figure 4.19).

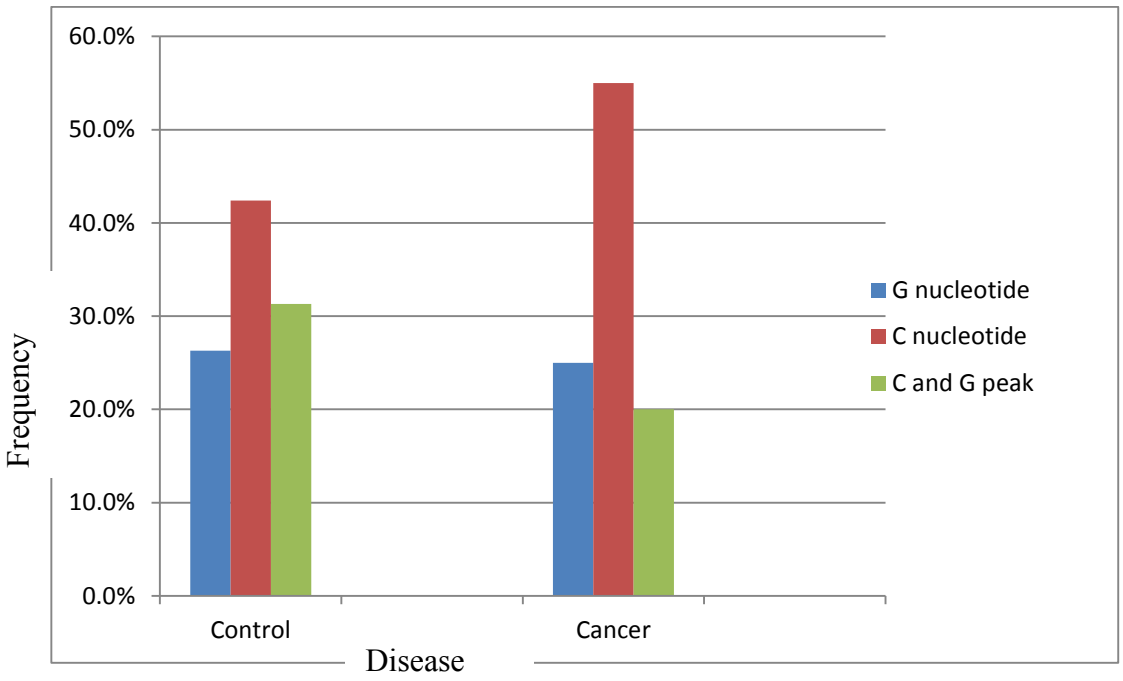

Figure 4.19 Frequency of polymorphism at position 261C→G of SRD5A2 gene in the cancer and control groups.

Data for both Caucasian and MC groups pooled together. G and C nucleotide are homozygotes, and C and G peak is heterozygote.

The SRD5A2 DNA sequence obtained in this study was translated into amino acid sequence using Geneious software as described in section 3.3.7. The amino acid sequence was then aligned with SRD5A2 protein sequence reported in the database. It was shown

that polymorphism at the position 261C→G results in amino acid substitution Val (V) to Leu (L) at the codon 89. Depending on the nature of changes in nucleotides in the position 261, three variants of amino acids were noted (i) Val (denoted as VV genotype), (ii) Leu (denoted as LL genotype) or (iii) a heterozygote L/V type of amino acid change for a case when both C and G nucleotide peak were observed.

Comparison between the 2 ethnic groups investigated revealed that Caucasians had a 33% higher frequency of VV genotype than the MC men. They also had a 20% and 10% lower frequency of the LL and the heterozygote L/V genotype than the MC group. This ethnic-groups difference in the frequency of DNA polymorphism resulting in amino acid substitution was found to be highly significant statistically ($P < 0.001$). However, the above polymorphism was not significant for cancer and control groups when ethnic groups were pooled together (Table 4.25).

Table 4.25 Frequency of genotypes due to functional 261C→G polymorphism of SRD5A2 in Caucasians, MC, control and prostate cancer groups.

Group	**VV**		**LL**		**L/V**		**P-value**
	N	%	N	%	N	%	
Caucasian‡	42	42.0	38	38.0	20	20.0	<0.001*
MC‡	9	9.1	59	59.6	31	31.3	
Cancer°	25	25.0	55	55.0	20	20.0	0.127*
Control°	26	26.3	42	42.4	31	31.3	

N- number of participants, MC-Malaysian Chinese, VV- Val change, LL-Leu change, L/V- Leu or Val. ‡ indicates data for both cancer and control cases. ° indicates data for men of both Caucasian and MC groups pooled together. Significant when P-value < 0.05. *Pearson Chi-Square test

It was also noted that the Caucasian cancer group had a significantly ($P < 0.001$) higher frequency of functional polymorphism resulting in VV genotype when compared to the MC cancer group. However, no significant difference was found between the Caucasian cancer and the Caucasian control group. The incidence of the VV genotype was also very low in the MC control group compared to the Caucasian controls and it was almost at the same frequency as for MC cancer patients. The LL genotype frequency did not differ significantly between the MC cancer and Caucasian cancer groups and the L/V genotype was higher in frequency in the MC cancer patients compared to the Caucasian cancer patients (Table 4.26).

Table 4.26 Association of V89L genotype with risk of prostate cancer.

Genotype	All ethnic groups			Caucasian			MC		
	Control	Cancer	OR (95% CI)	Control	Cancer	OR (95% CI)	Control	Cancer	OR (95% CI)
VV	26	25	1.0(reference)	22	20	1.0 (reference)	4	5	1.0 (reference)
L/V	31	20	0.67 (0.31-1.47)	17	3	0.19(0.05-0.76)	14	17	0.97 (0.22-4.32)
LL	42	55	1.36 (0.69-2.69)	11	27	2.7 (1.07-6.82)	31	28	0.72 (0.18-2.96)
L/V + LL	73	75	1.07 (0.57-2.02)	28	30	1.18 (0.53-2.61)	45	45	0.8(0.2-3.17)

VV- Valine, LL- Leucine, L/V- heterozygote with both Leu or Val. CI- Confidence interval, given in brackets for each allele or allele combination. "Reference" presents OR ratio for A1 allele. MC- Malaysian Chinese. OR- Odds ratio.

The OR and 95% CI was calculated to analyse impact of the polymorphism in SRD5A2 gene at the position 261C→G, that results in V89L genotype, on the risk of PC. The polymorphic genotypes were compared to baseline VV genotype which was used as a reference. It was noted that the L/V genotype was not associated significantly with the risk of PC when results for all the men in this study were considered together. A significant reduced risk of PC was observed in the Caucasian group with L/V genotype (OR = 0.19; 95% CI = 0.05-0.76) when compared to the MC group (OR = 0.97; 95% CI = 0.22-4.32). The LL genotype was noted to be associated with increased risk of PC in all the men but the results were not statistically significant (OR = 1.36; 95% CI = 0.69-2.69). However, there was a significant increase in risk of PC with in the Caucasians with LL genotype (OR = 2.7; 95% CI = 1.07-6.82) compared to the MC. In case of polymorphic genotypes (L/V or LL), it was noted that there was only small and non-significant increase in the risk of PC in the case when Caucasian and MC data were analysed together and in the Caucasians men when compared to the MC.

In summary, the results presented in this chapter demonstrated that:

(i) LL and L/V genotypes are more common in the MC compared to the Caucasians when the data for all the participants were pooled together.

(ii) There were no differences in the frequency of any of the genotypes between cancer and control participants when data for both ethnic groups were analysed as one dataset.

(iii) LL genotype and L/V genotype were associated with higher and low risks of PC respectively in Caucasians but not in MC.

4.3.4.2 Analysis of polymorphism 140G→A (rs9282858) in the SRD5A2 gene

The second SNP in the SRD5A2 gene that has been extensively studied in relation to PC, is A49T variant which resulted in Ala to Thr substitution at codon 49 due to the SNP at position 140 from nucleotide G to A. Presence of this polymorphism is supposed to increase the rate of conversion of testosterone to DHT due to increased activity of 5-α reductase type 2 (Makridakis *et al,* 1997).

In this study, the mentioned above polymorphism was not detected neither in control, cancer, Caucasian nor MC groups.

4.3.5 Assessment of number of CAG repeats in androgen receptor gene

The number of CAG repeats in Exon 1 of AR gene is known to be inversely related to the risk of PC development (Giovannucci *et al,* 1997). Prostatic cell division and possibility of carcinogenesis are controlled by interaction between testosterone and DHT with the androgen receptor which, in turn, affects function of AR gene (Chamberlain *et al,* 1994). This chapter presents results of study on a number of polymorphic CAG repeats in Exon 1 and the relationship between the number of CAG repeats, ethnicity and PC incidence.

Table 4.27 Association between mean number of CAG repeats in AR gene with ethnicity, disease status and interaction between these parameters.

	Group	N	Mean	SD	P-value
Ethnicity	**Caucasian**	100	21.80	3.13	0.212
	MC	100	22.37	3.37	
Disease status	**Cancer**	100	22.36	3.15	0.236
	Control	99	21.81	3.35	
Interaction	**Ethnicity vs. Cancer &Control**	199	85.21	45.68	0.111

*** Significant at P-value <0.05. N-number, SD- standard deviation, ANOVA- analysis of variance, MC-Malaysian Chinese, AR- Androgen Receptor.**

This study showed that a mean number of CAG repeats was not significantly (P = 0.212) different between the Caucasian and MC groups when control and cancer data were considered as one dataset. Comparison between the cancer group and control groups (when Caucasians and MC were considered as one data set) did not reveal significant differences in mean numbers of CAG repeats either (P = 0.236). The number of CAG repeats in each ethnic group was noted to be in the range of 18 to 29 in Caucasian cancer, 8 to 27 in Caucasian control, 12 to 31 in MC cancer and 15 to 30 in MC controls. There was no significant interaction between ethnicity and the disease status (Table 4.27).

4.4 Relationship between hormone concentration and DNA polymorphisms.

This chapter provides analysis of relationship between (i) polymorphisms in the CYP17 (-34T→C and 135C→T) and SRD5A2 at position 261C→G (V89L variant) genes and concentration of sex hormones. The A49T variant genotype was not analysed as it was not present in any of the participants in this study. (ii) number of CAG repeats in the AR gene and the concentration of sex hormones.

4.4.1 Relationship between testosterone concentration with genetic polymorphism.

The mean concentration of testosterone in the circulation in presence or absence of polymorphism in CYP17 and SRD5A2 genes are shown in Table 4.30. This study did not establish significant relationship between circulating testosterone concentration and presence or absence of polymorphism in both the positions of CYP17 gene (-34T→C and 135C→T) across all the groups investigated.

Presence of SRD5A2 (261C→G) SNP was only significantly related to the mean circulating testosterone concentration in Caucasian control group (P = 0.038). There was no significant relationship between this polymorphisms and testosterone concentration in Caucasian cancer, MC cancer and control groups (Table 4.28).

No significant relationship has been found between the number of CAG repeats and circulating testosterone concentration in all the sub-groups in this study.

Table 4.28 Relationship between polymorphisms and circulating testosterone concentration

Group	Presence of polymorphism	-34T→C (CYP17)			135C→T (CYP17)			261C→G (V89L variant)		
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
Caucasian Cancer	Yes	3.71	1.87	0.234	3.40	1.46	0.881	3.35	1.07	0.386
	No	3.22	0.88		3.48	1.53		3.59	1.75	
Caucasian Control	Yes	3.89	1.33	0.652	3.99	1.29	0.966	4.51	1.42	0.038*
	No	4.09	1.47		3.97	1.49		3.38	1.39	
MC Cancer	Yes	3.05	2.98	0.394	2.66	2.56	0.911	4.11	1.07	0.28
	No	2.44	2.04		2.75	2.50		2.78	2.64	
MC Control	Yes	4.51	1.39	0.746	4.38	1.46	0.551	3.16	1.21	0.086
	No	4.71	2.03		4.75	2.01		4.92	1.93	

Yes- one and/or two allele/genotype present, No- absence of polymorphism, SD- standard deviation, MC- Malaysian Chinese. * significant when < 0.05 (Independent t-test). Mean in ng/ml.

4.4.2 Relationship between dihydrotestosterone concentration with genetic polymorphism

This study did not find significant relationship between CYP17 (-34T→C) polymorphism and the concentration of circulating DHT in Caucasians cancer, Caucasian controls, MC cancer and MC control groups (Table 4.31). A significant relationship was noted in Caucasian controls in the 135C→T position of CYP17 gene but the significance of this finding needs further evaluation.

SRD5A2 polymorphism at the position 261C→G (V89L variant) was not positively related to DHT concentration in the groups studies (Caucasian cancer, Caucasian control, MC cancer and MC control) (Table 4.29).

There was no relationship between the number of CAG repeats and the hormones studied in the sub-groups in this study.

Table 4.29 Relationship between polymorphisms and circulating DHT concentration

Group	Presence of polymorphism	-34T→C (CYP17)			135C→T (CYP17)			261C→G (V89L variant)		
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
Caucasian Cancer	Yes	0.62	0.28	0.684	0.63	0.24	0.758	0.64	0.27	0.545
	No	0.59	0.28		0.60	0.32		0.59	0.30	
Caucasian Control	Yes	0.57	0.28	0.098	0.50	0.23	0.008*	0.67	0.25	0.903
	No	0.70	0.23		0.72	0.27		0.66	0.22	
MC Cancer	Yes	0.49	0.34	0.503	0.47	0.26	0.718	0.54	0.22	0.696
	No	0.44	0.27		0.44	0.37		0.48	0.33	
MC Control	Yes	0.74	0.19	0.076	0.69	0.20	0.357	0.60	0.27	0.820
	No	0.59	0.27		0.61	0.28		0.63	0.26	

Yes- one and/or two allele/genotype present, No- absence of polymorphism, SD- standard deviation, MC- Malaysian Chinese. * significant when < 0.05 (Independent t-test). Mean in ng/ml.

Table 4.30 Relationship between polymorphisms and circulating IGF-I concentration

Group	Presence of polymorphism	-34T→C (CYP17)			135C→T (CYP17)			261C→G (V89L variant)		
		Mean	SD	P-value	Mean	SD	P-value	Mean	SD	P-value
Caucasian Cancer	Yes	104.5	77.1	0.131	103.8	85.6	0.269	110.3	82.1	0.040*
	No	78.1	40.0		81.7	45.8		72.9	36.2	
Caucasian Control	Yes	112.8	53.4	0.043*	90.4	45.0	0.802	108.1	51.9	0.289
	No	88.3	30.3		93.6	30.0		89.8	28.5	
MC Cancer	Yes	55.7	25.6	0.152	61.9	25.6	0.697	67.1	53.4	0.586
	No	70.9	42.3		66.3	41.7		58.3	28.2	
MC Control	Yes	85.3	24.5	0.600	80.2	21.5	0.236	72.4	23.6	0.289
	No	90.9	35.1		92.7	35.3		87.9	27.4	

Yes- one and/or two allele/genotype present, No- absence of polymorphism, SD- standard deviation, MC- Malaysian Chinese. * significant when < 0.05 (Independent t-test). Mean in ng/ml.

4.4.3 Relationship between insulin-like growth factor-I with genetic polymorphism

No significant relationship was established between the mean circulating concentration of IGF-I in Caucasian cancer, Caucasian controls, MC cancer and MC control groups and CYP17 polymorphism at the position 135C→T (Table 4.30). Caucasian control was the only group that showed significant positive relationship between IGF-I concentration and CYP17 -34T→C polymorphism (P = 0.043).

Circulating IGF-I concentration was significantly elevated in the presence of SRD5A2 261C→G polymorphism in the Caucasian cancer group (P = 0.040) (Table 4.32). No relationship was found between the SRD5A2 polymorphism and IGF-I concentration in Caucasian control, MC cancer and MC control.

The mean number of CAG repeats was not significantly related to the concentration of IGF-I in any of the sub-groups in this study.

5 Discussion

This thesis explored ethnic group-specific differences in PC development with regards to the concentration of hormones and genetic polymorphisms that play active role in androgen metabolism. The hormones studied were testosterone, DHT and IGF-I in the circulation. The genes investigated were CYP17, SRD5A2 and AR. The ethnic groups investigated were Bristol Caucasians and Malaysian Chinese men.

The hypotheses tested in this project were: (i) circulating concentrations of androgens and IGF-I should be elevated in participants with PC compared to non-cancer participants within the same ethnic group; (ii) circulating concentration of androgens and IGF-I should be higher in a population with higher incidence of PC, such as Bristol Caucasians compared to the Malaysian Chinese; (iii) frequency of polymorphisms in CYP17, SRD5A2 and AR genes is associated with increased risk of PC. This study also included investigation of relationship between presence/frequency of DNA polymorphisms and the concentration of circulating hormones.

Our study was a case-control study with all the blood samples collected under similar condition for both ethnic groups. PC samples were obtained from patients who have been newly diagnosed with PC and the control group were selected from BPH patients with low risk of PC (see section 3.1.2) Blood storage conditions and experimental protocols were strictly uniform for all the samples. It should be highlighted that this is the first study which compared two completely different ethnic group tested under uniform conditions in contrast to most studies reported in the literature which are based on nested case-control studies for hormonal analysis (Dorgan *et al,* 1998, Sawada *et al,* 2010, Allen *et al,* 2007, Rowlands *et al,* 2012). Design of genetic part of our study was comparable to designs in majority of case-control studies (Karimpur-Zahmatkesh *et al,* 2013, Torkko *et al,* 2008,

Price *et al,* 2010) with a difference in blood storage time which was much longer in nested case-control studies, ranging from 8 to 20 years (Dorgan *et al,* 1998, Nomura *et al,* 1996).

This project provides important knowledge on androgens and growth hormone status of Malaysian Chinese group in comparison with the Caucasians which can form basis for a larger ethnic-group specific study. This study will be, for the first time launched in Malaysia in 2015/2016. The planned larger scale study will extend to participants from the other two major ethnic groups in Malaysia, namely Malays and Indians. Understanding ethnic-group specific mechanisms regulating PC is critical for effective PC diagnostics, management and avoiding unnecessary cost-consuming and stressful investigation and procedure for patients.

The discussion chapter consists of a number of sub-chapters which discuss different aspects of finding presented in this dissertation as follows:

i. **Participants' demography**: demography of participants from Caucasian and MC ethnic groups and ethnic group-related differences.
ii. **Effect of hormonal** concentration: discussion of results on hormonal status in different ethnic groups and in PC and control participants.
iii. **Effect of genetic polymorphisms**: this sub-chapter discusses results of identification of polymorphisms in CYP17, SRD5A2 and AR genes in MC and Caucasian cancer and control groups.
iv. **General discussion**: This sub-chapter critically discusses limitations and inferences which could influence results of this study.

5.1 Participants Demography

The participants in this study comprised of 100 Caucasians from Bristol and 100 individuals from the Malaysian Chinese ethnic group (50 individual in each group, with newly diagnosed PC and the other 50 were controls). The participants were all carefully selected based on strict inclusion and exclusion criteria discussed in section 3.1.2 of this thesis.

The Malaysian population is known to be heterogeneous and consisting of the three major ethnic groups, Malays, Chinese and Indian (Hirschman, 1987). For the purpose of this study, only Malaysian Chinese group was selected. The reason for this was the location of the University Malaya Medical Centre (a collaborator on this project) at the boundary of Petaling Jaya and Kuala Lumpur which is a catchment area for an urban group comprising more of the Chinese group. This ethnic group selection enabled us to conduct a quicker sample collection for this study.

In our study, it was noted that participants' age distribution significantly differed in some groups. The Caucasian participants were approximately of the same age with no significant difference between PC and the control groups. MC cancer group had higher mean age compared to the Caucasian cancer and MC control groups in our research. Overall, taking both ethnic groups into consideration, mean age of the PC patients was higher than the control group. The main reason for discrepancy in the age in different groups in our study is likely due to the effect of the age of MC cancer participants which was the highest. It should be noted that in the case of nested case-control studies reported in the literature, the age of participants was always well-matched between control and cancer groups (Chen *et al,* 2003, Gann *et al,* 1996). Margiotti *et al* (2000) grouped the PC and the recruited control participants into age ranges e.g. 51 to 60 years, 61 to 70 years, 71 to 80 years etc. However, a research paper by Giwercman *et al* (2005) on Swedish population, similarly to

our study, noted cancer patients group having much higher average age compared to control group. Therefore having age differences between participating groups is not an unusual situation.

In the present study, one reason for a higher average age of MC cancer group might be the fact that in Malaysia, an uptake of screening tests by the community is low due to various reasons including poor information received from doctors, language barrier with leaflets not written in languages of the minority ethnic groups, poor socio-economic status, fear of the unknown and also religious reasons (Farooqui *et al,* 2013). One of the studies conducted at Hospital Kuala Lumpur, Malaysia demonstrated that patients with PC usually seek consultation at later stage of the disease. Majority of the patients undergoing biopsy of the prostate in Malaysia are above 70 years of age (Selvalingam *et al,* 2007) which had made it very difficult to recruit participants of the same age across all the groups for the present study.

However, it is important to highlight that in our study, in case of age-difference between groups, adjustments for age were made during statistical analysis. These adjustments are important due to the fact that the concentration of sex steroids testosterone and DHT as well as IGF-I are known to decline with age (Gray *et al,* 1991) and these steroids are thought to be important in regulation of PC development

Our study also noted that there was difference in PSA level between the groups. The mean PSA level of our MC cancer group was very high compared to Caucasian cancer group (by 9.5 times). This could be due to the fact that MC cancer group included more patients with advanced PC compared to the Caucasian cancer patients. This indicates the early and localised stage of PC being frequently diagnosed in Caucasians in the UK, which is likely to be due to successful PSA screening programme and the high uptake of screening by Caucasians (Melia, 2005). Higher socio-economic status with excellent access to

healthcare can also contribute to the success of screening programmes in developed countries. This is in sharp contrast to Asian countries, where PC patients usually seen at hospital late and at more advanced stage of PC (Selvalingam *et al,* 2007). Higher grade (Gleason score) of PC in MC cancer patients can also be due to the same reason of late presentation of the cases in this ethnic group. PSA of MC control and Caucasian control groups were similar as they were deliberately matched as selection criteria (only those with PSA of less than 4 ng/ml were included in the study)

5.2 Effect of androgens and insulin-like growth hormone-I on prostate cancer

5.2.1 Effect of serum testosterone concentration

It is well known that androgens play an important role in regulation of normal proliferation and differentiation of prostate cells, as well as in aetiology of PC (Wu *et al,* 1995b, Buttyan *et al,* 2000, Takizawa *et al,* 2010). Wu *et al* (1995) demonstrated difference in concentration of androgens in different ethnic groups in their study in African-Americans, White-Americans and Asian-Americans. Buttyan *et al* (2000) and Takizawa *et al* (2010) showed that PC treatment outcome may be related to androgen status which can be manipulated by treatment with androgen deprivation therapy (ADT). Although there have been multiple studies on androgen concentration in different ethnic groups, including Americans, European Caucasians and Japanese, data of the literature on relationship between PC and androgen concentration are controversial. (Dorgan *et al,* 1998, Hsing and Comstock, 1993, Sawada *et al,* 2010). Gann *et al* (1996) found significant elevation in testosterone concentration and DHT/T in relation to PC as also Hsing and Comstock (1993).

In the study conducted in this thesis, the mean testosterone concentration of Caucasians and MC were not significantly different when both cancer and control groups were analysed together. Testosterone concentration between the control groups of both, Caucasian and MC, were also not significantly different. Therefore results of this study do not support the hypothesis that an ethnic group with a higher risk of PC has a higher testosterone concentration.

It is important to highlight that the 2 ethnic groups used in our study were from 2 countries (UK and Malaysia) which significantly differ in environmental condition, diet and lifestyle. We are aware of only one other study that had an experimental design similar to ours, namely comparing two ethnic groups (Dutch and Japanese) from different countries (de Jong *et al,* 1991). The majority of ethnic studies reported in the literature were performed on different ethnic groups within the same population which had the same environmental exposure. Among these studies, de Jong *et al* showed that there was no significant difference in testosterone concentration between control groups in the Dutch and Japanese populations. Litman *et al* (2006) found no ethnic difference in testosterone concentration between Black, Hispanic and White men in the USA. No difference in testosterone concentration was observed in the study by Kubricht III *et al* (1999) who analysed testosterone concentration in African-American and White-American patients undergoing PC assessment. Results of our study are in agreement with de Jong *et al* (1991), Litman *et al* (2006) and Kubricht III *et al* (1999). In contrast to our finding, Wu *et al* (1995) showed that African-Americans had the highest testosterone concentration compared to White-Americans, Japanese-Americans and Chinese-Americans. It is known that African-Americans have the highest risk of PC followed by the White-Americans and the lowest PC risk in Asian-Americans. It has also been reported that the Arabs and Chinese have significantly lower mean testosterone concentration compared to Black and

White Americans, Germans and Nigerians (Kehinde *et al,* 2006). One of the reasons for controversial data regarding ethnic group difference in testosterone concentration can be different age of participants which varied from 15 years old in the study by Kehinde *et al* (2006) to 60 or above in studies by Wu *et al* (1995) and Litman *et al* (2006). It must also be taken into consideration that the study by Kehinde *et al* (2006) on different ethnic groups were not performed in a single laboratory using the same laboratory techniques which might have affected outcomes of experiments. Therefore, the ethnic group differences in testosterone concentration observed by Kehinde *et al*. (2006) should be treated with caution.

It should be noted that our study was conducted on 100 participants per ethnic group. This number was adequate for statistical analysis but it was a relatively small number when compared to majority data of the literature based on data collected from up to 300 participants (Wu *et al,* 1995b, Kubricht III *et al,* 1999). Therefore we are not in a position to conclude whether ethnic-group differences in testosterone concentration would have been observed if the study was conducted on a larger number of samples.

It was noted in our study that testosterone concentration was able to differentiate PC from control cases when data for MC and Caucasian groups were pooled together, with cancer patients having a lower mean testosterone concentration compared to the control group. When our data were adjusted for age, the MC cancer patients still had a higher average testosterone concentration when compared to MC control but this was not the case for Caucasians where testosterone concentration was similar in cancer and control groups. Our finding on MC group is consistent with a report by Hoffman *et al* (2000) who analysed testosterone concentration in American patients undergoing PC treatment. Hoffman *et al* (2000) showed that low testosterone concentration was positively related to a higher Gleason score of 8 or above (i.e. positively related to a more advance stage of PC). As the

MC cancer group in our study had more patients with advanced stage of PC then Caucasians, this might be an explanation for testosterone concentration differences between control and PC in MC but not in Caucasians. Our results suggest that a low testosterone concentration might be an indicator of advanced PC.

In contrast to the mentioned above papers, a substantial number of publications did not observe significant variations in testosterone concentration between PC and non-cancer cases in Japanese-American man (Nomura *et al,* 1996), Finnish population (Dorgan *et al,* 1998), mixed-American population (Hsing and Comstock, 1993) and Japanese population (Sawada *et al,* 2010). There might be a number of reasons for controversial data of the literature regarding relationship between testosterone concentration and PC. These reasons could include (but not limited to) differences in the size of groups studies, the use of different laboratory techniques for testosterone analysis (radioimmunoassay (RIA) and electro chemiluminescence immune assay (ECLIA)). For example, Gann *et al* (1996) showed a significant positive relationship between PC risk and a higher circulating testosterone concentration in a nested case-control study in USA using the RIA laboratory technique. Chen *et al* (2003) showed that no significant testosterone association with PC in a study using the RIA technique. Severi *et al* (2006) showed no significant association of testosterone with PC in a study of more than 500 participants using the ECLIA technique in Australians.

Another reason for a lower testosterone concentration in MC cancer group in our study when compared to MC control might be a higher rate of conversion of testosterone to DHT in the prostate gland in MC cancer patients and thus, reduction in circulating testosterone concentration. Further investigation is needed into this hypothesis. However, there are some data of the literature which suggest different rate of steroid metabolism in different ethnic groups as indicated by a research in a mixed USA population where the estradiol

concentration was found to be highest in non-Hispanic blacks followed by non-Hispanic whites compared to Mexican-Americans (Rohrmann *et al,* 2007). Another global ethnic study found that Black men (from USA and Tobago) had higher oestrogen levels than Caucasians and Asian men (Orwoll *et al,* 2010). Different pathological condition like breast cancer can influence sex steroids metabolism and the level of hormones such as estrogens and their derivatives (Hankinson *et al,* 1998). Hankinson *et al* (1998) described that there was strong positive relationship between post-menopausal oestrogens level and breast cancer. Increased risk of breast cancer was also noted with elevated concentrations of testosterone, androstenedione and dehydroepiandrosterone sulphate (Kaaks *et al,* 2005).

5.2.2 Effect of serum dihyrotestosterone and dihydrotestosterone to testosterone ratio

DHT is the other principal prostatic androgen which is produced from testosterone in a reaction catalysed by 5α-reductase (Feldman and Feldman, 2001). In this study, we analysed not only DHT concentration in control and PC groups of Caucasian and MC populations, but also the ratio of DHT to testosterone. This ratio indicates the amount of DHT which has been produced from available testosterone in the circulation. It can be used as an indicator of hormones interaction and as an indicator of 5α-reductase activity (Feldman and Feldman, 2001). Our research demonstrated that unadjusted (for age) mean DHT concentration was higher in the Caucasians than the MC when the cancer and control cases were analysed together. However, the mean DHT/T ratio in the MC ethnic group was higher than in Caucasians. We expected both, DHT concentration and DHT/T ratio to be elevated in the ethnic group with higher risk of PC, i.e. in Caucasians. Our result was in agreement with Litman *et al* (2006) who showed that DHT concentration was higher in black men when compared to Hispanics and white men in USA population which is

consistent with the higher incidence of PC in the black community in USA. This is not consistent with results of our study where DHT/T was lower in a population with higher incidence of PC (Caucasians compared to MC). Wu *et al* (1995) also reported that the DHT concentration was highest in African-Americans, followed by Japanese-Americans with the lowest values in white Americans and that these differences were statistically significant. On the other hand, de Jong *et al* (1991) group observed significant differences in DHT/T but not in DHT concentration in study on Dutch and Japanese man.

Our study also found that unadjusted (for age) DHT concentration was significantly higher in the controls than in the cancer group when results for men from both ethnic groups were pooled together. Similar to situation described above with testosterone, age factor played a very important role as confounding factor in the study on DHT. After adjusting for age, the noted above differences in DHT between control and PC and between the ethnic groups were not significant.

In our study, cancer patients had a higher DHT/T compared to controls when data of both ethnic groups pooled together. We also found that DHT/T was higher in the MC cancer patients compared to the Caucasian cancer group and the MC control group. This finding is not in agreement with some literature reports. Mohr *et al* (2001) did not find differences in DHT and DHT/T between cancer and control groups in a USA -based study using prediagnostic blood samples (i.e. samples collected during patients recruitment and analysed after establishing PC) from participants who were under observation for potential PC development for up for 8 years. A study by Dorgan *et al* (1998) in a Finnish population also did not show any difference in DHT and DHT/T between control and PC cases. Furthermore, Nomura *et al* (1996) did not find significant association between DHT and DHT/T with PC in a study on Japanese-Americans. Another study by Gann *et al* (1996) also showed no difference in DHT concentration among Caucasian-Americans. Our

study showed a significant increase in DHT/T in the MC cancer group but it remains unclear whether this is due to ethnic reasons. The presence of more advanced PC cases in the MC cancer group when compared to Caucasians (rather than ethnicity as such) could have influenced DHT/T ratio. This is an important finding in an ethnic group with low PC incidence like the MC. Future larger multi-ethnic studies may be needed to confirm effect of ethnicity and advanced PC on DHT and DHT/T and to evaluate suitability of DHT and DHT/T as potential markers for PC. However, there are also publications which did not find significant relationships between DHT concentration and PC but observed relationship between DHT/T and PC. For example, Hsing and Comstock (1993) demonstrated that there was no significant difference in the concentration of DHT but there was a non-significant increase in risk of PC with increasing DHT/T. DHT and testosterone are usually analysed together and the probable reasons for the inconsistency in the results by different authors are the same for both hormones as described in section 5.2.1 above. Some generic differences across all the studies above are also described in detail in section 5.5 below.

5.2.3 Effect of insulin-like growth factor-I

In addition to testosterone, IGF-I is viewed as an important factor which can be associated with PC. An association between circulating IGF-I concentration and PC was first documented in 1997 by (Mantzoros *et al,* 1997) followed by studies in larger population (Wolk *et al,* 1998, Harman *et al,* 2000). Results reported in this project has shown that there was an inverse relationship between IGF-I concentration and PC cases with lower IGF-I concentration in cancer cases when data for both the ethnic groups were pooled together. At the same time we observed a significantly higher mean IGF-I concentration in the Caucasian groups compared to the MC but this is likely to be an effect of age as the ethnic differences were not observed after adjusting for age. No significant differences

were observed between Caucasian controls and MC controls in our study. It is difficult to compare our findings with data of the literature as publications were scarce on direct comparison of non-cancer groups from population with different ethnic background. Most of the studies compared only cancer vs. controls by clumping the population together without ethnic breakdown. . The only study available on comparison of control groups is from Arab population which showed that the IGF-I concentration in control Arab group was lower when compared to control Caucasians (Kehinde *et al,* 2005). However, it should be taken into account that this study by Kehinde *et al* (2005) was not performed in a single laboratory and the comparison was based on information available in the literature. Caucasian cancer patients in our study had a statistically significant higher mean IGF-I concentration compared to the MC cancer. Although the mean IGF-I concentration was lower in PC patients of both the Caucasian and MC groups compared to their counterparts in the non-cancer groups, this IGF-I test was able to significantly differentiate the cancer cases in the two ethnic-groups from the controls in the same ethnic group. The results in this study did not concur with most of the studies published where there seem to be multiple evidences to show that increased circulating IGF-I is a good indicator of PC risk in the population studied (Stattin *et al,* 2004, Price *et al,* 2012, Platz *et al,* 2005, Chan *et al,* 2002, Chokkalingam *et al,* 2001). In particular, Chan *et al* (2002) showed in a mixed USA population that high IGF-I is a good predictor of advanced PC which is in contrast to our finding that showed lower IGF-I concentration in MC group which had the higher number of patients with advanced PC. Chokkalingam *et al* (2001) demonstrated positive association between PC risk and IGF-I in the same ethnic group (Chinese). A Swedish study by Stattin *et al* (2004) also reported similar strong positive association between PC and circulating concentration of IGF-I. Another USA study by Platz *et al* (2005) and a European study involving multiple countries concurred with the above literature reports.

Our study did not find significant difference in IGF-I concentration between PC and control in Caucasians. Furthermore, in the MC group we observed an inverse relationship between these two parameters. This is consistent with results of a number of publications that did not find positive association between IGF-I concentration and PC (Borugian *et al,* 2008, Chen *et al,* 2005, Allen *et al,* 2007). In particular, Chen *et al* (2005) and Borugian *et al* (2008) demonstrated that circulating IGF-I concentration was not associated with increased risk of PC in a mixed USA population. A multinational European study by Allen *et al* (2007) also did not find any relationship between increased IGF-I concentration and PC risk.

In our study, the statistical analysis were extended to investigate whether any of the hormonal assays (testosterone, DHT and IGF-I), either individually or in combination could be used to aid PSA as a screening tool and to establish ethnic-group specific screening test. Results of our study suggest that detection of testosterone in combination with IGF-I might potentially be useful for PC detection in Caucasians and MC groups. DHT/T also has a potential for being used as a PC screening test in MC ethnic group (but not in Caucasians). However, these results need to be treated with caution as they are true for a relatively small cohort used in the present study but need to be confirmed on a larger cohort. To the best of our knowledge, there are not data of the literature that analysed diagnostic capability of concentration of hormones either individually or in combination.

5.3 Effect of genetic polymorphisms on prostate cancer

Genes that are known to be polymorphic can be analysed to determine whether the presence or frequency of their variants are related to hormonal profiles (Wirén and Stattin, 2008). Experiments conducted in this thesis evaluated polymorphisms in the three genes

that encode for key enzymes controlling androgen metabolic pathway (Figure 2.12) as follows:

i. CYP17 gene that encodes for the enzyme cytochrome P450 17α-hydroxylase which is involved in the regulation of testosterone biosynthesis.
ii. SRD5A2 gene that encodes for the enzyme 5α-reductase which is important in the regulation of the conversion of testosterone to DHT.
iii. The number of CAG repeats in AR gene which affects activity of this gene.

The following part of the thesis will discuss the findings in our thesis and compare them to data of the literature and address the issues related to inconsistencies in the findings between studies.

5.3.1 Polymorphism in the CYP17 gene and its association with prostate cancer risk

In this experiment, the presence of genetic polymorphism in CYP17 gene at position -34T→C was examined in Caucasian and the MC groups. The presence of homozygous A1 allele and A2 allele or a heterozygous A1/A2 allele was determined. Our study demonstrated that there were no significant difference in the frequency of the above three alleles in the Caucasians and MC when the cancer and control cases were analysed together. The A1, A2 and A1/A2 alleles were seen in 59%, 18% and 23% of Caucasians compared to 65.7%, 22.2% and 12.1% of MC participants respectively when data for cancer and control participants were combined. These data suggest that -34T→C polymorphism in CYP17 gene cannot be considered as a good marker for ethnicity. Most of the studies reported in the literature investigated frequency of -34T→C polymorphism in CYP17 gene amongst PC and control within the same ethnic group. One of the studies on USA populations compared frequency of CYP17 polymorphism at position -34T→C between the American-Caucasians and American-Blacks (Lunn *et al,* 1999). Data of this

study were similar to our finding that there was no ethnic difference in the frequency of CYP17 polymorphism at position -34T→C.

A study in an Austrian population showed that A2 allele was more frequent in PC cases (23.8%) compared to the BPH control group (9.5%) (Gsur *et al,* 2000). Yamada *et al* (2001) also demonstrated in Japanese ethnic group that the A2 allele was more frequent in PC group (18.8%) than in control group (14.5%). Results of our study noted that MC PC patients had higher frequency of A2 allele (26%) compared to control participants (18.4%) in agreement with findings of Gsur *et al* (2000) and Yamada *et al* (2001). Our data are also in agreement with reports by Lunn *et al* (1999) who showed higher frequency of A2 allele in the Caucasian PC patients compared to the Caucasian control participants in USA, and with data of Karimpur-Zahmatkesh *et al* (2013) who reported higher frequency of A2 allele in the PC group compared to the control group in Iranian population. However, the Caucasians in our study exhibited higher frequency of the A2 allele in the control group (22%) compared to the PC group (14%). The frequency of A1/A2 allele in both the Caucasians and the MC PC groups in our study were higher compared to the control groups. These results was not in agreement with Lunn *et al* (1999), Gsur *et al* (2000) and Yamada *et al* (2001) who found no significant difference in the A1/A2 frequency between PC and control groups.

The present study established that presence of A1/A2 was associated with a significantly increased risk of PC development compared to the men with A1 allele only when data for Caucasian and MC groups were analysed together. Men with A2 allele, showed a non-significant increase risk of PC compared to those with A1 allele. When the data were analysed for each ethnic group investigated, it was found that association between A1/A2 allele and PC risk was significant in the Caucasians. We noted that the risk of PC in the MC group non-significantly increased in association with A1/A2 allele. In men with A2

allele, the risk of PC was non-significantly increased in the MC but not in the Caucasians. We also found that the combination of A2 or A1/A2 alleles increases the risk of PC in both Caucasians and MC but this association was not significant.

There are multiple epidemiological studies indicating that the polymorphism in the -34T→C position of CYP17 is associated with an increased risk of PC as detailed below. A study on Japanese population demonstrated that presence of A2 allele was positively associated with risk of PC development (Yamada *et al,* 2001). Association of A2 allele with increased risk of PC was also reported by Lunn *et al* (1999) in USA Caucasian population when compared to USA Black population. A significant association of A2 allele with PC was demonstrated by Karimpur-Zahmatkesh *et al* (2013) in a study on PC patients and healthy controls in Iran. Gsur *et al* (2000) reported a significant association between A2 allele and PC risk on Austrian population.

Our study did not find significant association between A2 and PC risk in MC and Caucasians. This does not agree with results reported by Sobti *et al* (2009) on positive association between A2 allele frequency and PC risk in a South Indian population. A study in a Tunisian population comparing PC patients and healthy controls showed that presence of A1/A2 or A2 allele increases the risk of PC by two-fold in this community (Souiden *et al,* 2011).

A study in a Chinese cohort (Madigan *et al,* 2003) did not find significant association between PC risk and any of the CYP17 genotypes but suggested an increasing trend in PC risk with increasing frequency of A1 and A1/A2 alleles. Our study partially agree with Madigan *et al* (2003) as we also found a significant association of A1/A2 allele with PC risk in the Caucasians and an increasing trend in MC group.

It should be noted that majority of the studies reported in the literature have been performed on control and cancer cases in a single population with an exception of Lunn *et al* (1999) who compared the American Caucasians and Black populations. This thesis is the first report when analysis of genetic polymorphisms was performed in two different communities from different living environment simultaneously. This might form a basis for further study on ethnic group-specific PC genetic markers.

It is important to highlight that Severi *et al* (2008) in study on Australian Caucasian group, dos Santos *et al* (2002) in study on Brazilian population and Chang *et al* (2001) in study on a mixed American population did not find association between the mentioned above CYP17 polymorphisms and PC risk. Two large meta-analyses have been published on association of CYP17 gene at -34T→C with PC risk among studies conducted in Caucasians, Asians and of African descent. The first report by Ntais *et al* (2003) showed no significant risk of PC associated with any of the alleles in this gene as was also described by another larger meta-analysis (Wang *et al,* 2011)when data from all ethnic groups were pooled together. In contrast, we found significant risk of PC in the presence of A2 or A1/A2 alleles in combination in our study. When the results were analysed by ethnic groups, Ntais *et al* (2003) and Wang *et al* (2011) concluded that A2 allele was associated with significant increase of PC risk in population of African descent and not the Caucasians and Asians. A1/A2 allele was reported to have no association with PC risk in any of the ethnic groups in the above two reports. Our study agrees that A2 allele was not associated with PC risk in Caucasians and MC but and increasing trend suggested in the MC needs to be looked at in future study to make a definite conclusion. Our report also contradicts the finding of the two meta-analyses above in that the A1/A2 allele in our Caucasian cohort was significantly associated with PC risk,

In our research, a coincidental SNP in CYP17 coding region were indentified during amplification and sequencing of a 500bp segment of CYP17 gene. The coincidental polymorphism was identified at the position 135C→T. There was no significant increase in the frequency of the C, T or C/T allele in the Caucasians compared to the MC in our experiments. When data from both ethnic groups were pooled together, no difference in frequency of the polymorphism was found between PC and control groups. As far as PC risk was concerned, no association was found with C, T or C/T allele in Caucasians and MC in our study. To the best of our knowledge, this polymorphism at 135C→T of the CYP17 gene has not been reported in any study in relation to the incidence or risk of PC. Therefore, we were unable to discuss our finding with data of the literature.

CYP17 gene is a highly polymorphic gene and other SNPs that have been studied with regards to PC risk including that analysed by Beuten *et al* (2009) at 5'-untranslated region rs284851, rs619824, rs10883782, rs1004467, rs17115144, rs2486758). No association between these SNPs and with PC risk was found in the Hispanic Caucasians, non-Hispanic Caucasians and African-Americans. Another African-American study also showed no significant association between PC risk and SNPs rs10883783 at intron 7, rs6163 at codon 65 of exon 1 (Sarma *et al,* 2008). There has also been interest in studying polymorphism at the position -34T→C of CYP17 gene in relation to its association with other diseases such as breast cancer. The study by Feigelson *et al* (1997) reported positive association between advanced breast cancer and frequency of CYP 17 A1/A2 and A2 allele. Further evaluation of relationship between coincidental polymorphism identified in our study and PC risk is required on a larger number of samples.

5.3.2 Polymorphisms in the SRD5A2 gene and their association with prostate cancer

5.3.2.1 Polymorphism at position 261C→G

SRD5A2 gene has been considered as one of the most important genes in PC development. It encodes for a key enzyme in the prostate gland (5α-reductase) that catalyses the conversion of testosterone to DHT in androgen metabolism (Coffey, 1993). The substitution of amino acid Val to Leu at codon 89 due to the SNP C to G nucleotide at position 261 is supposed to give protection from PC development (Makridakis *et al,* 2000). This is due to the postulation that this polymorphism reduces activity of the enzyme that converts testosterone to DHT thus reducing the circulating DHT concentration (Makridakis *et al,* 2000). It is therefore expected that a population with a higher incidence of PC should have a higher prevalence of Val whereas, population with low incidence should have a higher frequency of Leu.

Our study demonstrated that in control groups the frequency of VV genotype was higher in the Caucasians than the MC group. LL and L/V genotype was more frequent in MC than Caucasians. This ethnic difference was statistically significant. Our data are in agreement with a report by Ziegler-Johnson *et al* (2002) which demonstrated that LL or L/V genotype is most likely in Asians and least likely to be carried by the Africans. Intermediate frequency of the genotype was noted in Caucasians and African-Americans. Frequency of LL and L/V genotype follows the same ethnic group pattern as PC incidence with the lowest frequency observed in Asia (Ferlay *et al,* 2010a). Based on the finding of lowest frequency of the LL or L/V genotype in the Africans, the incidence of PC should ideally be the highest in African population but it is not the case because of the possible large underestimation of the number of cases reported in Africa (Osegbe, 1997). Comparison of

LL and L/V genotypes frequency in Asians, Caucasian and Black-Americans showed that American (Caucasian and Black) ethnic group had the world highest incidence of PC this ethnic group. Results of our work is also consistent with data of Li *et al* (2010) who reported the frequency of the LL or L/V genotype to be higher in Asians than in Caucasian. The risk assessment for PC development in our study showed that LL or the LL + L/V genotype had a non-significantly higher PC risk compared to VV genotype when data for all the participants were pooled together. Salam *et al* (2005) demonstrated the risk of PC was significantly higher in Hispanics and non-Hispanic Americans with the presence of the LL or L/V genotypes. Our findings showed an increasing trend in the risk of PC but it may be inappropriate to compare our finding with Salam *et al* (2005) because of the use of different ethnic groups in these two studies. A study in French Caucasian population (Cussenot *et al,* 2007) also demonstrated increased PC risk in men with LL genotype. Results of our study are consistent with data of Giwercman *et al* (2005) who reported an increased frequency of LL genotype in PC patients in Swedish-Caucasians and with results of meta-analysis undertaken by Wang et al. (2010) who observed increased risk of PC in European population (with higher frequency of LL) compared to the Asians and Africans. In contrast to our data, Margiotti *et al* (2000) had shown a reduced PC risk in an Italian population with high frequency of LL genotype. One of the reasons for this disagreement can be the fact that Margiotti *et al* (2000) worked on a smaller number of participants and that the participants were older (70-110 years) than in our study (40-80 years).

As far as Asian population is concerned, it was shown in a Japanese cohort that frequency of LL genotype was higher in PC cases than in controls (Yamada *et al,* 2001). This is in contrast to our study where a trend toward reduction of PC risk in the case of LL or LV genotypes was observed in MC group. A trend of PC risk reduction for LL or LV genotype was also described by Hsing *et al* (2001). This might be an indication of a protective nature

of this genotype in Asian groups as suggested by Makridakis *et al* (2000), Ziegler-Johnson *et al* (2002) and Li *et al* (2010).

In addition to the data of literature which found either positive or negative association between PC and LL or LV genotype, several authors have shown no association at all between these genotypes and PC risk. These reports were by Onen *et al* (2007) in Turkish population, Hayes *et al* (2007) in Australian Caucasians and Rajender *et al* (2009) in South Indians. Except for Hayes *et al* (2007), the other two reports were from small cohorts in their respective populations which might be one of the reasons for lack of associations. The other reasons for discrepancy of data of the literature could be age-differences of recruited patients, including PC patients with different stages of the tumour development and inconsistent use of controls (either BPH or healthy controls). In summary, the 261C>G polymorphism in SRD5A2 gene that results in Val to Leu substitution (i.e. LL genotype) is associated with increased risk of PC in Caucasian group. To the best of our knowledge our study is the first report which provides data on the SRD5A2 polymorphism in South-East Asian population. These data can form the basis for designing a larger study on the role of this polymorphism in PC risk in different ethnic groups in Malaysia.

5.3.2.2 Polymorphism at position 140G→A

Another important SNP in SRD5A2 gene is rs9282858 which was found in the exon 1 at codon 49. This SNP is a nucleotide substitution at the position 140G>A resulting in amino acid substitution (Ala (A) to Thr (T)) (Makridakis *et al,* 2000). Functionally, this amino acid substitution can increase the activity of the 5α-reductase enzyme, thus increasing the conversion of testosterone to DHT which can actively cause the cancerous changes in the prostate (Makridakis *et al,* 1997, Zeigler-Johnson *et al,* 2002). Hypothetically, frequency

of this polymorphism should be higher in a population with high incidence of PC and in individual with PC when compared to non-cancer cases.

Our study did not establish presence of T genotype in either Caucasians or MC; or cancer and control groups. This is in agreement with other studies when T genotype was not present in cancer or control cases in Japanese (Yamada *et al,* 2001), Turkish (Onen *et al,* 2007), Chinese (Hsing *et al,* 2001), or Italian populations (Margiotti *et al,* 2000) and in South Indian population (Rajender *et al,* 2009).

In a study on mixed American population, where the T genotype was present, no association between frequency of this genotype and PC was established (Torkko *et al,* 2008, Salam *et al,* 2005). This is consistent with data of a meta-analysis conducted by Li *et al* (2010) who did not find evidence of association between increased risk of PC and frequency of T genotype compared to A genotype.

In contrast to the above, Makridakis *et al* (1999) demonstrated in a study on African-American and Hispanic USA cohorts that PC risk increases by 7.2-fold and 3.6-fold in both population respectively in the presence of T genotype compared to the A genotype. A contradicting report was presented by Hayes *et al* (2007) on Australian population where 60% increased PC risk was observed in the presence of the A genotype compared to the T genotype.

Similarly to 261C>G polymorphism reported in the previous chapter, there is very limited information on frequency of 140G>A polymorphism in the SRD5A2 gene and its association with PC risk and incidence in Asian population. Although our study did not find any polymorphism at position 140G>A in both Caucasians and MC groups or in PC or control groups, it will be interesting to investigate further whether this polymorphism is

present in other ethnic groups, such as Malays and Indians in the multi-ethnic Malaysian population.

5.3.3 Polymorphism in AR gene in relation to prostate cancer

The androgen receptor (AR) is activated by binding of testosterone and, to a greater extent, (Huang and Tindall, 2002). The AR gene is located on Xq11-12 (Kral *et al,* 2011, Brown *et al,* 1989). This study assessed the number of AR trinucleotide CAG-repeats in PC and control participants in Caucasians and MC ethnic groups. It has been reported in the literature (although not consistently) that an increased number of CAG-repeats is likely to decrease transactivation and binding activity of AR which can lead to reduction of PC risk (Chamberlain *et al,* 1994). In contrast, reduction in the number of CAG-repeats may increase the risk of PC (Ekman *et al,* 1999, Nicolaiew *et al,* 2009). It has been reported that African-Americans generally have shorter CAG-repeats and higher PC risk compared to the Whites and Asians (Kral *et al,* 2011, Coetzee and Ross, 1994).

Our study demonstrated that the mean number of CAG repeats was slightly increased in the MC group compared to the Caucasians (both cancer and control participants) but statistical significance was not established. Our study also demonstrated a higher mean number of CAG-repeats in cancer patients compared to the control participants when data for all the men were pooled together. There was no interaction noted between the ethnicity and disease status of the participants which mean that there were no differences in the CAG repeat length between the 4 sub-groups (namely, Caucasian cancer, Caucasian control, MC cancer and MC control). Absence of ethnic differences in CAG repeat length in our study is consistent with data of the literature. Price *et al* (2010) did not find differences in CAG-repeat length between control and cancer groups in Hispanic White

population. In agreement with this study, Lange *et al* (2008) did not observe significant association between CAG repeat length less than 21 and PC incidence. Results of our study are also consistent with a report by Huang *et al* (2003) on Taiwanese population, and with a study by Edwards *et al* (1999) on British Caucasian populations who did not find differences in CAG repeat length between PC and control cases. Although all the studies above have shown no association between the CAG-repeat length and PC, there is significant number of studies that reported different results. One of the studies in Chinese population have shown that a smaller number of CAG-repeats (less than 22) (Zhai *et al,* 2014) increases the risk of PC, and another Chinese study (Hsing *et al,* 2000) showed increased PC risk in case of CAG repeats of less than 23. It should be noticed that there is inconsistency regarding the number of CAG-repeats associated with PC (a cut-off for CAG repeats). It is also not clear how the cut-off number was determined. A study on a mixed American population (Giovannucci *et al,* 1997) reported a cut-off at less than or equal to 18 CAG-repeats , and a study in non-Hispanic Whites (Ingles *et al,* 1997) reported a cut-off at less than 20 CAG repeats. Both studies reported significant association between PC at these cut-off points. An Iranian population study (Ashtiani *et al,* 2011) reported that the average number of CAG-repeat in PC patients was significantly smaller than control group. Krishnaswamy *et al* (2006) also agreed with the above author in a study on South Indian population where a number of CAG repeat was smaller in PC patients than in control. However, the average CAG repeat length was different in both Iranian and South Indian PC patients and was 19.9 and 17.0 respectively for PC compared to 22.8 and 20.7 respectively for controls.

A recently published meta-analysis demonstrated that AR with over 20 CAG repeats have a protective effect on PC risk and incidence (Gu *et al,* 2012). Taking into account controversial data of the literature on association between CAG repeats length and PC and

a relatively small number of participants in the present study, it will be interesting to evaluate further the above association on a larger group of patient across various ethnic groups using the same study protocol. This, in particular, refers to Malaysia, which is a multi-ethnic population.

5.4 Relationship between genetic polymorphisms and steroid hormone concentration

Rational for analysis of relationship between genetic polymorphisms and concentration of circulating hormones in this study was the hypothesis that increasing androgens and IGF-I concentration can increase risk of PC. It has been reported in the literature that -34T→C SNP in CYP17 gene can increase CYP17 activity in Caucasians thus, potentially increasing biosynthesis of testosterone (Carey *et al,* 1994). It was demonstrated in experiments on mixed-American ethnic groups in Los Angeles and Hawaii that SRD5A2 gene polymorphism in the position 261C→G causes amino acid substitution from Val to Leu at codon 89, This amino acid substitution is associated with reduced 5α-reductase enzyme activity, which could, in turn, reduce the production of DHT (Makridakis *et al,* 1997). The other polymorphism in the SRD5A2 gene at position 140G→A has been reported to result in Ala to Thr amino acid substitution at codon 49. This substitution is associated with increase in 5α-reductase enzyme activity and thus elevated DHT concentration is expected (Jaffe *et al,* 2000). Increase in CAG-repeat length in AR gene has been suggested to reduce the risk of PC by reducing transactivation activity of the AR after binding of DHT to AR (Brown *et al,* 1989). Defective binding of DHT to AR due to the polymorphic CAG-repeat length might affect the signalling of androgen response elements like PSA and growth factors (Heinlein and Chang, 2004).

Our study did not find significant relationship between testosterone or DHT concentrations and CYP17 polymorphism at the position -34T→C in Caucasian and MC cancer and control groups. At the same time, the present study has noted a significant positive relationship between the above CYP17 polymorphisms and circulating IGF-I concentration in the Caucasian control group but not in the Caucasian cancer, MC control or MC cancer groups. Our results are in agreement with other study on a UK population which showed that serum testosterone concentration was not affected by CYP17 -34T→C polymorphism in men (Allen *et al,* 2001). This is consistent with report by Severi *et al* (2007) who found that serum testosterone concentration was not related to the CYP17 polymorphism at -34T→C in Australian cancer and control cohorts.

Similarly, a report from USA on a predominantly White population showed that the testosterone and DHT concentrations in participants with CYP17 A2 and/or A1/A2 alleles did not differ significantly between PC and control participants (Haiman *et al,* 2001). The research undertaken in this thesis demonstrated that there was no significant relationship between SRD5A2 gene polymorphism at the position 261C→G, circulating testosterone and DHT concentrations in Caucasian cancer, MC control and MC cancer groups. However, in case of Caucasian control group, we observed increased serum testosterone concentration in individuals with the mentioned above SRD5A2 polymorphism. This finding is consistent with a report by Hsing *et al* (2001) who also showed a higher testosterone concentration in the presence of the SRD5A2 polymorphism in control participants. This may be due to a lower rate of conversion of testosterone to DHT in the prostate gland. Testosterone that had not undergone the conversion in the prostate gland may leak back into circulation and increase serum testosterone concentration.

Our results are not in agreement with study by Allen *et al* (2001) who did not observe significant relationship between SRD5A2 polymorphism at 261C→G and serum

testosterone concentration in UK control population. Hsing *et al* (2001) and Hayer *et al* (2006) did not find significant association between the SRD5A2 polymorphisms and DHT or testosterone concentrations either. Our study established that IGF-I concentration in the Caucasian cancer group were significantly elevated in the presence of SRD5A2 polymorphism at the position 261C→G. We are unable to discuss this finding as, to the best of our knowledge, relationship between the above polymorphisms and IGF-I concentration has not been reported before. One of the reason for lack of information on this topic might be a low interest in looking at the relationship between IGF-I and the gene which is not directly involved in IGF-I biosynthesis.

Our study, did not establish relationship between the mean number of CAG-repeats length and serum testosterone, DHT or IGF-I concentration. These results are consistent with the study by Platz *et al* (2005) who reported no association between testosterone or DHT and the length of CAG-repeats in USA healthy men. However, an earlier study in the USA by Krithivas *et al* (1999) showed that testosterone concentration declined in individuals with a shorter CAG-repeat length but the report did not elaborate on the repeat length. At the same time, the study by Krithivas *et al.* (1999) did not find any association between the number of CAG repeats and DHT concentration (Krithivas *et al,* 1999). It should be noted that there is very limited literature on association of CAG repeats with concentration of sex steroids and therefore it is not possible to have a more extensive discussion of this topic.

5.5 Inferences and limitations of the study.

Research undertaken in this thesis was performed on Bristol Caucasians and MC men, and it is the first study which analysed a Malaysian ethnic group for all the parameters such as testosterone, DHT, IGF-I as well as genetic polymorphism in CYP17, SRD5A2 and AR in

comparison to a Caucasian group. This study was a prospective study designed carefully to include only cases selected using identical criteria in both populations. This study was also conducted using uniform blood storage and laboratory techniques to minimise potential effect of selection bias and heterogeneity of the study group.

Although, overall participant number of 200 is quite high and statistically had sufficient power to generate valid data, it should be noted that the subgroups (i.e. various ethnicity and disease status) comprised of smaller numbers. Therefore interpretation of relationships between hormonal status, genetic polymorphisms and PC with ethnic groups must be treated with caution. Nevertheless, the data generated in this study provide important baseline data for designing future studies on other or larger ethnic groups.

One of the challenges in analysing results of this study in the light of data of the literature is the fact that majority of literature reports, especially studies on hormonal assessment, are nested case-control studies which analysed samples stored for a long period of time (Sawada *et al,* 2010, Price *et al,* 2012) whilst our study analysed samples that were obtained after the diagnosis of PC. The PC and control samples in our study were not stored for more than 6 months before analysis. Furthermore, the samples used in the literature reports were pre-diagnostic (Chen *et al,* 2003, Rowlands *et al,* 2012) in contrast to our study where the samples were obtained post-diagnosis. Research on stored samples has established that stability of bio-molecules will not be affected if the storage condition is ideal. The stability of inflammatory factors like interleukins and coagulation factors was not affected when stored at -70^{o}C for a period of 6 years (Lewis *et al,* 2001). Samples that was stored at -20^{o}C and -40^{o}C showed degradation in some parameters like cholesterol, fibrinogen and creatinine compared to those stored at -80^{o}C or -180^{o}C (Elliott *et al,* 2008). Sex steroid hormones have been reported to be more stable than other proteins stored at -70^{o}C for up to 15 years (Barrett-Connor *et al,* 1990). Data of the literature with regards to

genetic studies were mainly case-control studies with blood sample collected from newly diagnosed PC cases similarly to the blood collection procedure in our study (Torkko *et al,* 2008, Krishnaswamy *et al,* 2006). The genomic DNA in our study was isolated within 2 weeks of blood collection which was similar to other studies and the genomic DNA stored at -20^{o}C till ready for analysis. Genomic DNA is known to be stable in blood stored at -70^{o}C giving good yield for up to 14 days (Polakova *et al,* 1989). Isolated DNA integrity has been reported to be stable for 7 years in solution form at -20^{o}C (Madisen *et al,* 1987). As far as hormonal study is concerned, we are of the opinion that a pre-diagnostic serum sample may not reflect the changes due to the influence of environment and lifestyle that preceded the diagnosis of PC, therefore a prospective sample collected may be a better way to obtain reliable results.

Another important aspect of our study is that during the participants' selection stage, we aimed to achieve the best possible age-match between the cancer and control groups and between Caucasian and MC groups. However, in spite of all the effort, the MC cancer group had older participants when compared to Caucasians and MC control. This was due to cultural differences between Caucasians and MC with MC normally seeing the doctors at later stages of PC and at older age (Selvalingam *et al,* 2007). In our study we attempted to take into account potential effect of older age of MC cancer group by including age as confounding factor in statistical analyses. Nested case-control studies are usually well-matched for age because the selection of controls is based on the average age of PC cases (Gann *et al,* 1996). When there is a discrepancy in the mean age between PC and control group, a statistical adjustment is made as reported by Giwercman *et al* (2005).

Another important feature of our study is that our control groups comprised of Benign Prostatic Hyperplasia (BPH) cases in which the possibility of having PC is generally low. This low risk of PC is based on analysis of PSA level of < 4 ng/ml, outcomes of normal

digital rectal examination and absence of family history of PC (Heidenreich *et al,* 2011). This, however, does not discount a possibility of presence of occult cancer because a low PC risk is still possible even in cases with the mentioned above criteria (Schroder *et al,* 1998, Schröder *et al,* 2000). Composition of our control group is quite different and, we believe, is more accurate and appropriate, when compared to control groups described in the literature. Some of the reported studies on PC use diverse control groups which include healthy individuals and some individuals with either symptomatic or asymptomatic BPH (Kehinde *et al,* 2005, Platz *et al,* 2005, Das *et al,* 2008). It is important to note that studies using healthy controls could be misleading, because the age of these participants should be over 45 years. It is known that prostate gland starts naturally enlarging from the age of 45 onwards and enlargement of the prostate gland itself does not mean predisposition to development of PC (Schatzl *et al,* 2002). It is very difficult to rule out BPH in these healthy individuals who are age-matched with the PC group, because prostatic enlargement invariably exists even when PC symptoms are absent as described by Lilja *et al* (2008). It is known that BPH is definitely not a risk factor for PC development (Lilja *et al,* 2008). Therefore, selection of BPH patient who have a low risk of occult PC is justified for their inclusion in control group in our study.

Data of the literature contain controversial results on hormonal changes in PC patients as described in section 5.2.1 and 5.2.2 above. One of the reasons for this controversy might be the use of different analytical methods and approaches. Some of the studies used radioimmunoassay (RIA) method (Wu *et al,* 1995b, Gapstur *et al,* 2002, Nomura *et al,* 1996) whilst others analysed hormonal status by ELISA (Kehinde *et al,* 2006, Borugian *et al,* 2008). ECLIA and IRMA methods were used by Sawada *et al* (2010) and Stattin *et al* (2004) respectively. This difference in the techniques used introduces a further variation factor and makes it difficult to conduct comparison of results obtained on different ethnic

groups. Our study ensured that the technique used for analysis of hormonal status in both ethnic groups was the same (ELISA).

It should be mentioned that, in our study we were not able to obtain samples of prostate tissue and therefore we were not able to compare the serum hormone concentration with the concentration of the same hormones in the prostatic tissue. It has been reported that the tissue concentration of testosterone, DHT and even IGF-I may be a more sensitive indicator of the concentration within the glands compared to the serum concentration (Cardillo *et al,* 2003, Titus *et al,* 2005). Collecting sample tissues must be explored in the future in experiments on a larger number of participants and in a wider range of ethnic groups.

The genetic studies conducted in this thesis were based on a relatively small number of polymorphisms that was identified based on their impact on PC in previous reports. Future studies should be directed to explore potential role of other genetic polymorphisms in various ethnic groups in relation to PC development. In particular, this refers to a multi-ethnic population with low PC incidence in Malaysia. The genes which might be interesting in relation to PC are Vitamin D receptor (VDR). It has been reported that sun exposure and polymorphism at 3' untranslated region in exon 2 of VDR gene may reduce the risk of PC in USA Hispanic and non-Hispanic (Caucasian and African descend) populations (John *et al,* 2005). VDR gene study in Malaysian population may give important information on PC risk and incidence due to the tropical nature of the climate. A Japanese population study (Habuchi *et al,* 2000) also confirmed the protective nature of the VDR gene polymorphism against PC. . Further research on relationship between VDR polymorphism, differences in lifestyle and dietary habits is required. Identification of variations in the concentration of androgens, and growth factors as well as identification of SNPs in genes controlling androgen metabolism might be an important tool for detection

of men with predisposition to PC, and for advocating any preventive medication or treatment strategy. It is rather surprising that studies in this field continue to produce conflicting data because there is very clear clinical evidence that patients with advance PC respond favourably to androgen deprivation therapy using either medical or surgical castration (Singer *et al,* 2008).

Our research has found ethnic differences in testosterone, DHT/T as well polymorphisms in CYP17 and SRD5A2 in a study conducted under identical conditions for different ethnic groups. Therefore, we feel there is immense potential in studying further the androgen and growth factor related pathway and their relationship with genetic mutations in PC as potential ethnic group-specific biomarkers for PC.

6 Conclusions

The main aim of the thesis was to investigate ethnic group-specific relationship between PC, androgens concentration and frequency of genetic polymorphism that play active role in androgen metabolism. The investigation focussed on the circulating concentration of testosterone and DHT as well as a growth hormone IGF-I. The genes studied were CYP17 and SRD5A2 which encode for enzymes that play crucial role in androgen metabolism. This study also investigated polymorphisms in AR gene that determines functionality of AR. **The key findings of this thesis are:**

1. The mean testosterone concentration did not differ significantly between Bristol Caucasian and MC ethnic groups (when PC and control data pooled together). To the best of our knowledge, this is the first communication based on direct comparison on the 2 ethnic groups in the same experiment.
2. The mean testosterone concentration in the MC cancer group was significantly lower than in Caucasian cancer and MC control group. Taking into account that MC group consisted of participants with a more advance stage of PC, the data suggest that an advanced cancer stage may result in reduction of testosterone concentration.
3. The mean DHT concentration did not differ significantly between Bristol Caucasians and MC ethnic groups (when PC and control data pooled together). This suggests that ethnicity has no impact on circulating DHT concentration.
4. Cancer group had a significantly lower DHT concentration than the control group (pooled data from Caucasian and MC group). However, this difference was not observed after taking into account the age factor.
5. Data adjusted for age, showed that DHT/T was increased in MC group compared to Caucasians (when PC and control data pooled together). There was no difference in

DHT/T between MC control and Caucasian control groups. Ethnic group difference in DHT/T should be further investigated on a larger cohort to confirm impact of ethnicity on DHT/T.

6. PC group had higher DHT/T when compared to control (when data from Caucasians and MC were pooled together) _. Sub-group analysis showed that MC cancer group had significantly higher mean DHT/T than Caucasian cancer and MC control groups. A larger number of cases with advanced PC stage in MC cohort might have contributed to these differences. These results suggest that PC grade may have an impact on the DHT/T ratio and therefore might be considered as a potential PC marker.
7. IGF-I concentration (unadjusted for age) was significantly higher in Caucasians than MC (when cancer and control were pooled together) but this difference was not observed for MC controls and Caucasian controls. This suggests IGF-I as a potential marker for PC. Caucasian cancer patients had higher IGF-I concentration compared to MC cancer group. MC cancer group had a significantly lower concentration of IGF-I than the MC control group.
8. Frequency of A1, A2 and A1/A2 alleles of CYP17 gene did not vary between Caucasian and MC group and therefore it is unlikely to be linked to ethnicity. A1/A2 allele frequency was positively associated with PC risk in Caucasians but not in MC. This is an important finding which suggests that this polymorphism can be used an ethnic group-specific marker in PC risk assessment. A coincidental polymorphism at the position 135C→T of CYP17 gene was established. There was no significant association between frequency of this polymorphisms and PC risk. Further assessment of this SNP on a larger cohort is required.

9. The frequency of SRD5A2 LL and L/V genotypes were significantly higher in MC compared to Caucasians. The presence of these genotypes may explain the low incidence of PC in Asian community.
10. An increased risk of PC was observed in the case of LL or LL + L/V genotypes and this risk was higher in the Caucasians compared to MC.
11. In contrast to data of the literature, no polymorphisms were observed at the codon 49 of SRD5A2 gene in both ethnic groups investigated. It was concluded that this polymorphism is a poor ethnic and PC marker in Caucasian and MC groups.
12. No relationship was found between the mean numbers of CAG-repeats in AR gene and ethnicity or PC incidence.
13. No significant relationship was found between CYP17 polymorphism at the position -34T→C and the concentration of testosterone and DHT in Caucasian or MC ethnic groups. No association was found between the CYP17 polymorphism and PC either. IGF-I concentration was related to the above polymorphism in Caucasian control group. Further investigation is needed to assess relationship between the CYP17 polymorphism and PC.
14. SRD5A2 polymorphism at the codon 89 was not related to testosterone and DHT concentration with exception of the Caucasian control group that needs further assessment. There was positive association between the SRD5A2 polymorphisms and IGF-I concentration in Caucasian cancer group.
15. The number of CAG-repeats was not related to testosterone, DHT and IGF-I concentrations in both Caucasians and MC cancer and control groups.

In conclusion, research presented in this thesis demonstrated ethnic groups- differences in the concentration of androgens and growth factor and ethnic-group-specific associations between these parameters and PC. The study also identified polymorphisms in CYP17 and

SRD5A2 that may play an important role in ethnic group-specific PC development. This study did not find significant relationship between a number of CAG-repeats in AR gene and PC in Caucasian or MC ethnic groups. Further evaluation of the results of this study on a larger cohort and in wider range of ethnic groups is needed

It has been suggested that testosterone and DHT/T has good potential to be used as indicators of advanced PC. Polymorphisms in CYP17 (A2 and A1/A2) and the V89L variant of the SRD5A2 gene can be potentially used as an ethnic based markers for PC development, although further studies should be performed to compare larger multi-ethnic populations within the same environmental conditions.

7 Future work

Findings of this research project will form the basis for future large-scale ethnic group-specific studies on hormonal and genetic association with PC. This includes the study of circulating androgens, IGF-I concentrations and polymorphisms in CYP17, SRD5A2 and AR in larger study in Malaysia which would involve all three major ethnic groups (Malays, Chinese and Indians) to establish variations within the same environmental conditions. Other future directions from this project would include:

1. Analysis of androgens, IGF-I concentrations and expression of CYP17, SRD5A2 and AR genes in prostatic tissues obtained by biopsy or surgical specimen from different ethnic groups in Malaysia. This will enable us to assess the intra-prostatic changes in the hormonal and genetic expressions that take place during cancerous changes of prostate gland.
2. To conduct analyses of genetic polymorphisms in CYP17, SRD5A2 and AR in PC patients facing multiple treatment options. This will assist with choosing the most effective treatment decisions.

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Appendix A: Data for Caucasian prostate cancer patients.

Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
CP1	42.90	1.85	2.91	0.74	0.26	75.60	A1	VV	20
CP2	39.40	1.93	5.15	0.94	0.18	132.16	A1/A2	LL	25
CP3	38.30	1.91	4.25	0.83	0.20	67.24	A1	LL	26
CP4	41.90	1.95	1.36	0.27	0.20	44.45	A1/A2	VV	21
CP5	69.90	1.81	3.44	0.87	0.25	100.19	A2	LL	29
CP6	64.50	1.78	5.01	0.73	0.15	67.24	A1	LL	24
CP7	129.50	1.90	3.14	0.38	0.12	70.04	A2	VV	25
CP8	77.40	1.89	2.72	0.65	0.24	75.60	A1/A2	VV	22
CP9	89.10	1.95	2.68	0.51	0.19	116.28	A1/A2	VV	20
CP10	41.60	1.96	3.34	0.72	0.22	70.04	A1/A2	LL	21
CP11	57.20	2.02	3.30	0.84	0.25	44.45	A1	LL	22
CP12	89.60	1.81	3.68	0.46	0.13	44.45	A1/A2	VV	21
CP13	63.60	1.85	3.34	0.54	0.16	150.47	A1	L/V	20
CP14	81.40	1.86	5.54	0.64	0.12	231.93	A1/A2	LL	20
CP15	174.80	1.91	3.26	0.81	0.25	97.49	A1/A2	LL	25
CP16	59.40	1.89	2.95	0.17	0.06	41.53	A1	VV	20
CP17	81.50	1.91	4.72	0.43	0.09	29.65	A1	VV	23
CP18	44.40	1.99	2.65	0.54	0.20	94.78	A1	VV	26
CP19	92.50	1.86	4.42	0.70	0.16	155.66	A1	VV	20
CP20	45.60	1.86	3.12	0.39	0.12	209.33	A1/A2	LL	21
CP21	77.00	1.87	3.22	0.61	0.19	143.13	A2	L/V	20
CP22	69.30	1.90	2.07	0.29	0.14	43.00	A2	VV	22
CP23	68.50	1.96	1.80	0.13	0.07	66.40	A1/A2	LL	26
CP24	42.40	1.82	2.74	0.06	0.02	88.71	A1	VV	19
CP25	57.50	1.93	2.99	0.27	0.09	107.25	A1	LL	18
CP26	65.80	1.93	3.21	0.42	0.13	63.13	A1/A2	VV	25
CP27	63.10	1.89	3.15	0.62	0.20	146.06	A1	VV	24
CP28	64.80	1.94	1.89	0.62	0.33	119.36	A1/A2	VV	24
CP29	56.70	1.85	4.54	0.56	0.12	379.75	A2	LL	23
CP30	77.50	1.87	2.74	0.69	0.25	88.71	A1	VV	22
CP31	35.60	2.01	2.78	0.52	0.19	91.83	A1	LL	21

Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
CP32	40.30	1.89	3.03	0.52	0.17	104.19	A1	LL	23
CP33	54.00	1.81	2.26	0.63	0.28	69.64	A1	L/V	24
CP34	79.40	1.90	7.33	1.06	0.14	113.33	A2	VV	23
CP35	67.20	1.83	5.37	0.61	0.11	55.47	A2	VV	24
CP36	80.70	1.91	3.68	0.80	0.22	63.92	A1	VV	20
CP37	69.70	1.89	3.24	0.96	0.30	55.74	A1	LL	25
CP38	42.60	1.87	2.79	0.83	0.30	35.18	A1/A2	VV	19
CP39	68.50	1.88	2.07	0.60	0.29	158.62	A1	LL	18
CP40	56.50	1.86	2.64	0.47	0.18	47.81	A1	LL	23
CP41	48.60	1.73	2.47	0.35	0.14	81.70	A1	LL	24
CP42	50.70	1.82	3.09	0.51	0.17	39.52	A1/A2	VV	24
CP43	51.30	1.84	9.82	1.41	0.14	59.20	A1/A2	VV	27
CP44	42.80	1.66	2.62	0.78	0.30	97.88	A1/A2	VV	21
CP45	50.20	1.72	4.83	0.81	0.17	12.25	A1	VV	21
CP46	39.30	1.81	3.83	0.48	0.12	100.68	A1/A2	VV	25
CP47	63.40	1.82	4.28	0.54	0.12	58.82	A1	VV	23
CP48	72.10	1.84	3.23	1.38	0.43	56.08	A1	LL	20
CP49	71.00	1.90	1.72	0.20	0.11	37.90	A1	LL	24
CP50	43.90	1.80	2.21	0.42	0.19	35.38	A1	VV	19

Appendix B: Data for Caucasian control patients.

Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
CC1	84.80	1.77	3.45	0.70	0.20	140.03	A1/A2	VV	27
CC2	41.20	1.88	5.50	1.07	0.19	116.28	A1	VV	24
CC3	120.80	1.97	4.54	0.86	0.19	78.36	A1	LL	21
CC4	76.80	1.86	4.47	0.89	0.20	121.59	A1	LL	23
CC5	128.00	1.91	4.99	0.84	0.17	264.22	A1/A2	VV	20
CC6	97.00	1.77	4.62	0.64	0.14	64.44	A1	VV	20
CC7	107.50	1.85	3.14	0.42	0.13	97.49	A1/A2	LL	19
CC8	109.30	1.91	4.47	0.96	0.22	189.05	A1/A2	VV	19
CC9	107.80	1.94	2.97	0.70	0.24	102.89	A1/A2	VV	14
CC10	68.40	1.94	3.60	0.78	0.22	113.61	A1	LL	18
CC11	61.10	1.92	7.08	0.76	0.11	113.61	A1/A2	VV	23
CC12	48.00	1.84	4.97	0.75	0.15	145.26	A1	VV	8
CC13	55.00	1.85	2.76	0.54	0.19	47.00	A1	L/V	25
CC14	64.80	1.85	5.33	0.64	0.12	41.53	A1	LL	19
CC15	56.90	1.81	4.51	0.81	0.18	64.44	A1	VV	26
CC16	87.00	1.91	4.70	0.47	0.10	55.95	A2	VV	23
CC17	80.20	1.86	4.18	0.56	0.13	55.95	A1	VV	20
CC18	85.60	1.82	4.69	0.85	0.18	70.04	A1	LL	19
CC19	44.10	1.90	3.06	0.80	0.26	64.44	A1	LL	18
CC20	81.30	1.85	4.39	0.63	0.14	75.60	2	VV	21
CC21	49.70	1.79	2.27	0.85	0.38	49.83	A1	VV	21
CC22	60.70	1.90	5.20	0.85	0.16	101.12	A1	VV	24
CC23	61.10	1.85	3.11	0.21	0.07	69.64	A2	VV	18
CC24	38.80	1.77	4.03	0.07	0.02	110.29	A2	VV	21
CC25	72.60	1.89	6.05	0.39	0.06	110.29	A1	VV	23
CC26	57.60	1.74	2.06	0.04	0.02	63.13	A2	L/V	20
CC27	49.80	1.82	2.65	0.15	0.06	88.71	A1	L/V	25
CC28	76.80	1.81	2.55	0.37	0.15	172.11	A2	VV	22
CC29	63.20	1.76	4.38	0.56	0.13	94.94	A1	VV	26
CC30	49.20	1.77	5.69	0.60	0.11	98.04	A1	L/V	24
CC31	89.40	1.79	3.96	0.79	0.20	134.28	A1	VV	18
CC32	31.60	1.75	5.53	0.99	0.18	113.33	A2	L/V	26

Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
CC33	48.50	1.79	0.52	0.17	0.32	143.13	A1	LL	18
CC34	54.00	1.83	4.11	1.18	0.29	98.04	A1	L/V	24
CC35	59.50	1.74	2.77	0.63	0.23	94.94	A1	LL	17
CC36	65.00	1.82	4.60	0.81	0.18	72.86	A1	L/V	27
CC37	71.90	1.91	5.03	0.93	0.18	119.36	A1	L/V	22
CC38	79.80	1.86	8.44	1.03	0.12	91.83	A1	VV	26
CC39	44.60	1.89	2.76	0.67	0.24	91.83	A1	L/V	18
CC40	65.30	1.90	4.15	0.64	0.16	104.19	A2	L/V	24
CC41	30.50	1.97	3.40	0.67	0.20	55.11	A1	VV	20
CC42	54.90	1.90	4.60	0.78	0.17	66.31	A2	L/V	22
CC43	54.80	1.88	2.44	0.50	0.21	129.41	A1	L/V	21
CC44	52.00	1.90	3.36	0.40	0.12	63.14	A1	L/V	19
CC45	44.90	1.89	5.44	0.70	0.13	35.14	A1	L/V	20
CC46	31.00	1.88	4.45	0.67	0.15	115.58	A1	L/V	19
CC47	69.00	1.89	3.28	0.76	0.23	62.40	A1	L/V	25
CC48	57.30	1.86	3.18	0.66	0.21	80.58	A2	LL	19
CC49	44.60	1.82	1.85	0.52	0.28	82.48	A1	LL	24
CC50	68.20	1.82	1.80	0.51	0.28	100.73	A2	L/V	18

Appendix C: Data for Malaysian Chinese prostate cancer patients.

Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
MP1	20.20	1.72	0.28	0.47	1.69	46.43	A2	LL	22
MP2	21.60	1.82	0.42	0.78	1.84	98.04	A1	LL	24
MP3	24.60	1.68	3.68	0.78	0.21	151.90	A1	VV	26
MP4	17.60	2.04	4.45	0.78	0.18	104.19	A2	L/V	23
MP5	20.10	1.84	7.26	1.10	0.15	49.83	A2	LL	18
MP6	41.20	1.87	0.20	0.33	1.68	39.30	A1	LL	16
MP7	23.10	1.97	3.28	0.19	0.06	86.25	A1	VV	24
MP8	13.10	1.94	3.99	0.55	0.14	118.28	A2	LL	23
MP9	15.60	1.84	3.37	0.65	0.19	94.09	A1	LL	15
MP10	7.40	1.97	0.15	0.02	0.12	33.31	A1	LL	28
MP11	33.20	1.59	0.72	0.10	0.14	86.19	A2	L/V	31
MP12	19.80	1.91	0.09	0.17	1.82	50.13	A1	LL	22
MP13	54.00	1.86	0.16	0.08	0.49	58.97	A1	LL	24
MP14	43.40	1.88	3.35	0.21	0.06	71.26	A2	LL	20
MP15	13.70	1.90	3.91	0.52	0.13	36.61	A1/A2	VV	27
MP16	23.50	1.88	4.47	0.79	0.18	35.33	A1	L/V	25
MP17	19.70	1.91	0.08	0.02	0.22	70.98	A2	L/V	23
MP18	28.70	1.91	5.03	0.86	0.17	56.02	A1	LL	27
MP19	31.80	1.85	0.27	0.34	1.26	23.05	A1	LL	22
MP20	33.50	1.79	0.05	0.06	1.25	36.64	A2	LL	20
MP21	32.70	1.92	0.55	0.09	0.16	83.92	A1	L/V	23
MP22	17.70	1.90	0.06	0.20	3.59	27.99	A2	L/V	21
MP23	30.30	1.76	5.31	0.66	0.13	39.35	A1/A2	LL	27
MP24	19.40	1.61	0.27	0.18	0.66	22.91	A1	LL	17
MP25	26.80	1.64	0.17	0.14	0.83	20.66	A1	L/V	21
MP26	37.40	1.91	5.46	0.42	0.08	44.76	A2	L/V	24
MP27	41.30	1.84	4.22	0.74	0.18	57.20	A1	L/V	16
MP28	21.40	1.67	0.11	0.26	2.44	70.05	A1	L/V	22
MP29	31.20	1.75	3.70	0.58	0.16	19.87	A2	VV	22
MP30	33.50	1.76	4.74	0.75	0.16	39.47	A1	LL	19
MP31	26.90	1.87	5.50	1.29	0.23	43.42	A2	LL	19

Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
MP32	51.20	1.79	0.14	0.31	2.28	50.49	A2	LL	23
MP33	17.70	1.84	3.89	0.58	0.15	67.29	A1	LL	24
MP34	47.60	1.83	1.47	0.28	0.19	94.34	A1	LL	24
MP35	19.20	1.81	2.38	0.18	0.08	57.75	A1/A2	LL	24
MP36	12.70	1.84	10.64	0.98	0.09	26.85	A1/A2	LL	24
MP37	10.50	1.94	0.17	0.43	2.62	62.11	A1/A2	L/V	12
MP38	31.60	1.75	0.33	0.56	1.72	52.69	A1/A2	L/V	22
MP39	50.30	1.86	0.24	0.34	1.39	85.07	A1/A2	L/V	15
MP40	13.70	1.71	2.86	0.52	0.18	133.05	A1	L/V	22
MP41	19.60	1.57	1.73	0.30	0.18	134.29	A1	LL	22
MP42	20.60	1.88	1.79	0.32	0.18	45.75	A1	LL	27
MP43	19.50	1.78	4.14	0.28	0.07	36.66	A1	LL	22
MP44	22.30	1.90	5.26	0.39	0.07	38.10	A1	L/V	21
MP45	20.40	1.86	5.45	0.61	0.11	46.60	A1	LL	21
MP46	9.40	1.69	3.52	0.79	0.23	72.26	A1	LL	26
MP47	23.20	1.72	0.65	0.30	0.47	201.14	A1	L/V	25
MP48	16.40	1.84	5.97	0.63	0.11	40.83	A1/A2	V	23
MP49	25.40	1.84	2.18	0.29	0.13	81.85	A1	LL	27
MP50	17.50	1.90	6.56	0.86	0.13	85.29	A1	L/V	19

Appendix D: Data for Malaysian Chinese control patients

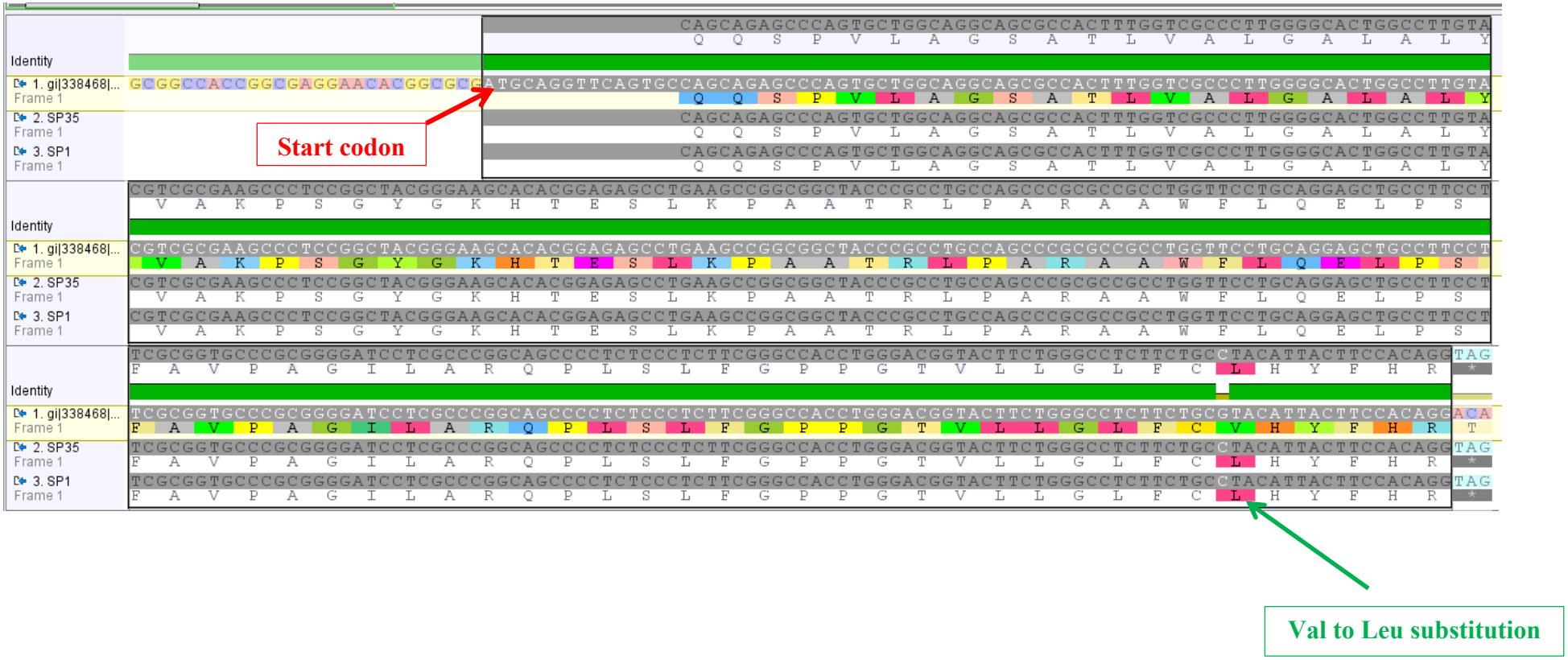
.Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
MP1	21.60	1.99	2.14	0.43	0.20	79.77	A1	LL	22
MP2	12.90	1.68	3.78	0.45	0.12	38.16	A1	L/V	24
MP3	17.00	1.90	3.30	0.25	0.07	82.31	A2	LL	20
MP4	23.30	1.84	3.70	0.03	0.01	82.83	A1	LL	26
MP5	21.30	1.93	3.14	0.44	0.14	104.13	A1	LL	23
MP6	22.50	1.94	4.84	0.62	0.13	139.06	A1	LL	26
MP7	14.90	1.95	2.88	0.07	0.03	133.06	A1	L/V	22
MP8	30.70	1.91	8.05	0.56	0.07	175.74	A1	LL	22
MP9	36.30	2.00	4.65	0.42	0.09	89.96	A1	LL	16
MP10	16.70	1.96	3.97	0.15	0.04	58.97	A1	LL	22
MP11	26.70	1.95	4.40	0.61	0.14	74.11	A2	L/V	24
MP12	15.50	1.82	4.36	0.72	0.16	63.64	A1	LL	21
MP13	30.60	2.03	6.60	0.87	0.13	75.21	A1	L/V	26
MP14	27.60	1.98	4.73	0.63	0.13	77.13	A1	LL	22
MP15	8.20	2.21	3.28	0.30	0.09	60.44	A1	L/V	24
MP16	16.80	2.03	2.89	0.51	0.18	62.79	A1	L/V	24
MP17	37.90	1.95	4.60	0.60	0.13	85.90	A1	L/V	22
MP18	58.10	0.80	4.75	0.47	0.10	56.38	No DNA		
MP19	40.10	1.99	7.77	0.89	0.12	53.06	A1	L/V	21
MP20	81.50	1.92	8.05	0.86	0.11	82.88	A1	LL	24
MP21	10.70	1.90	6.38	0.83	0.13	69.93	A2	LL	17
MP22	54.70	1.78	5.58	0.65	0.12	96.76	A2	L/V	20
MP23	68.40	1.90	3.11	0.81	0.26	111.30	A2	L/V	21
MP24	55.40	1.80	11.02	1.40	0.13	100.59	A1	LL	21
MP25	32.80	1.94	7.12	1.08	0.15	36.86	A2	LL	27
MP26	17.40	1.97	4.76	0.71	0.15	59.86	A2	VV	22
MP27	18.10	1.84	4.80	0.64	0.13	71.81	A1	LL	20
MP28	19.00	1.90	6.92	0.63	0.09	100.81	A1	LL	27
MP29	40.10	1.91	6.92	0.61	0.09	88.48	A1	LL	27
MP30	28.10	1.85	4.94	0.76	0.15	124.48	A1/A2	LL	22
MP31	41.80	1.88	3.81	0.93	0.24	62.00	A1/A2	LL	30
MP32	43.20	1.90	5.38	0.67	0.13	84.56	A1/A2	LL	26

Patient	DNA quality		Test (ng/ml)	DHT (ng/ml)	DHT/T	IGF-I (ng/ml)	-34T→C	V89L	CAG
	Conc. (ng/µl)	260/280							
MP33	48.20	1.95	4.33	0.69	0.16	73.00	A1	LL	17
MP34	64.10	1.88	3.82	0.95	0.25	119.68	A1	L/V	21
MP35	60.40	1.89	2.52	0.86	0.34	92.97	A1/A2	LL	25
MP36	44.40	1.97	4.43	0.50	0.11	77.31	A1	LL	19
MP37	26.20	1.99	4.44	0.75	0.17	106.29	A2	LL	24
MP38	63.00	1.94	1.69	0.27	0.16	109.81	A1	LL	23
MP39	57.30	1.83	5.46	0.67	0.12	64.37	A1	LL	15
MP40	19.50	2.02	4.88	0.72	0.15	58.31	A1	LL	24
MP41	24.10	2.06	4.62	0.64	0.14	105.80	A1	LL	22
MP42	29.10	1.99	7.00	1.00	0.14	142.69	A1	L/V	24
MP43	59.90	1.86	2.85	0.74	0.26	107.83	A2	VV	21
MP44	38.70	1.96	4.99	0.65	0.13	106.49	A1	LL	21
MP45	35.90	1.86	4.29	0.59	0.14	103.51	A1	LL	18
MP46	25.80	2.05	1.83	0.20	0.11	61.84	A1	VV	24
MP47	47.40	1.83	1.77	0.63	0.35	202.64	A1	L/V	26
MP48	25.50	1.62	3.20	0.76	0.24	60.15	A1	VV	23
MP49	30.70	1.87	2.77	0.68	0.25	52.63	A1	LL	21
MP50	70.40	1.86	5.24	0.62	0.12	111.25	A1	L/V	22

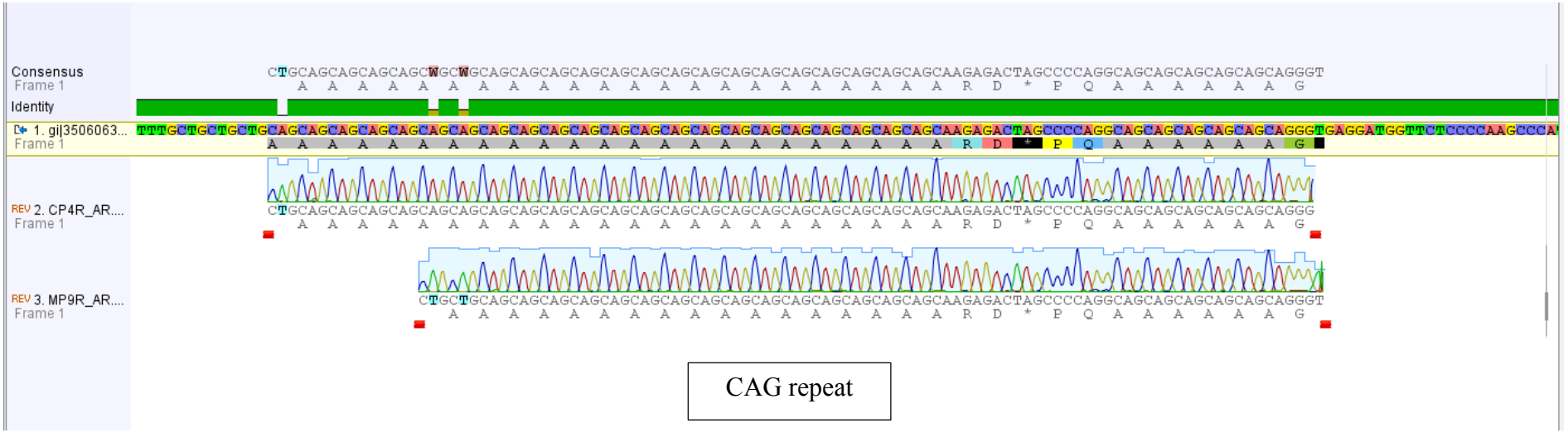
Appendix E: Sequence alignments

(i) Alignment of CYP17 gene for 3 patients indicating the start codon (ATG) (red).

(ii) Alignment of SRD5A2 gene for 2 patients indicating the start codon (ATG) (red) and Val to Leu substitution (green) at codon 89.



(iii) Alignment of AR gene showing the number of CAG repeat in 2 samples.



Appendix F: Abstract

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UP.034

Single Nucleotide Polymorphism (SNP) in CYP17A1 (rs743572) Gene as a Risk of Prostate Cancer Development – A Preliminary Report in Caucasian Group

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Introduction and Objectives: This study aims to sequence the established regions of CYP17A1 (rs743572) from Caucasians and to compare them with imported DNA from Malaysian Chinese group to investigate presence of ethnic-group specific single nucleotide polymorphism (SNP). This research will generate initial data for future clinical studies for development of biomarkers in ethnic-based prostate cancer screening.

Materials and Methods: Blood sample obtained from newly diagnosed and biopsy confirmed prostate cancer patients from Caucasian men. Controls are confirmed Benign Prostatic Hyperplasia patients with normal digital rectal examination, with a PSA of < 4.0ng/ml and with no family history of prostate cancer. Genetic polymorphisms were investigated by isolation of genomic DNA from whole blood and subsequent PCR amplification and DNA sequencing. Sequencing result aligned with reference gene using software (Geneious) to identify SNPs and anomalies in the translated protein.

Results: A preliminary result for 40 prostate cancer and 25 control patients among the Caucasians showed polymorphic T to C substitution in the 5'-untranslated region of the CYP17A1 (rs743572) gene in 50% (n=20) and 36% (n=9) respectively. The mean age of participants in both groups was 66 years. The median PSA was 6.7 and 2.1 ng/ml respectively. This polymorphism was seen mainly in cancer cases with Gleason grade ≥7 (n=15) (75%).

Conclusion: The rs743572 polymorphism appears to be more common among the cancer group, however, statistical significance will only be ascertained once all the Caucasian samples has been analysed. Imported Malaysian samples will also be tested using similar protocol in order to provide further evidence in testing ethnic variation of the above polymorphism.

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