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ANALYSIS OF FUNCTIONAL GENETIC POLYMORPHISMS IN PROSTATE CANCER AND TYPE 2 DIABETES: GROWTH HORMONE RECEPTOR AND MUCIN 1

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ABSTRACT

Prostate cancer and type 2 diabetes are complex diseases, the genetic and environmental basis of which are not well established. Epidimiology suggests a link between the two diseases, with type 2 diabetes redusing the risk of prostate cancer, and faily history of prostate cancer reducing the risk of type 2 diabetes. Common genetic variations are believed to influence risk of both diseases, with very little overlap between the genes implicated.

Metabolism may be a link between the two diseases: diabetes is characterised by abberant utilization and storage of dietary energy, where as prostate cancer has a high demand for energy input. It is plausible that genes and thier variants which alter metabolic homeostasis influence risk of both diseases in the opposite directions.

In order to investigate whether this hypothesis is correct, we investigated common genetic variation of two genes, *GHR* and *MUC1*, in prostate cancer and type 2 diabetes. Bothe genes have previously been implicated in prostate cancer, with some evidence suggesting a role for MUC1 as a biomarker for prostate cancer. The GH-IGF-I-Insulin axis is key to metabolism, thus is likely ot be important in diabetes. The suggestion of a role for MUC1 in type 2 diabetes is a novel.

A number of MUC1 isoforms, some of which have been implicated in various cancers, are determined by a SNP in exon 2. Functional differences between the variants are as yet unknown. A polymorphism in the GHR where by exon 3 is excluded is believed to have increased bioactivity compared to the full length form, although this is much debated.

In this thesis we demonstrated that the GHR exon 3 polyorphism reduces risk of type 2 diabetes, and is associated with increased BMI, CRP and IGF-I levels in diabetic subjects.

The variant allele of MUC1 was associated with an increased risk of type 2 diabetes and lower IGF-I levels. Subjects homozygous for the variant allele had increased LDL and CRP levels

In conclusion, these genetic variations of GHR and MUC1 have potential as biomarkers for type 2 diabetes, and its complications. Genetic variation of MUC1 in blood DNA samples does not influence prostate cancer risk or survival, however tumour-specific genetic alterations may be important. Sequence analysis indicates that MUC1 isoforms may have distinct differences.

LIST OF PUBLICATIONS

I. **Rona J Strawbridge**[#], Lars Kärvestedt, Chunde Li, Saud Efendic, Claes Göran Östenson, Harvest F Gu and Kerstin Brismar. GHR exon 3 polymorphism: Association with type 2 diabetes mellitus and metabolic disorder. Growth Hormone and IGF Research (2007) 17:392-8

II. **Rona J Strawbridge**[#], Monica Nistér, Kerstin Brismar, Henrik Grönberg and Chunde Li. MUC1 as a putative prognostic marker for prostate cancer. Biomarker Insights (2008) 3:303-315

III. **Rona J Strawbridge**[#], Monica Nistér, Kerstin Brismar, Chunde Li, Sara Lindström. Influence of *MUC1* genetic variation on prostate cancer risk and survival. European Journal of Human Genetics (2008)

IV. **Rona J Strawbridge**[#], Lars Kärvestedt, Harvest F Gu, Monica Nistér, Chunde Li and Kerstin Brismar. A *MUC1* SNP (rs4072037) influences risk and metabolic parameters of type 2 diabetes. *Submitted*

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OTHER PROJECTS

I. **Rona J Strawbridge**[#], Clive Osmond, Eero Kajantie, Riitta Koistinen, Monica Nistér, Chunde Li, Markku Seppälä, Johan G Eriksson and Kerstin Brismar. Influence of GHR exon 3 polymorphism on metabolic traits and childhood growth. *Submitted*

II. Christina Hägglöf[*], **Rona J Strawbridge**[*], Chunde Li, Monica Nistér and Arne Östman. Gene expression profiles of prostate tumour epithelium and stroma. *Equal contribution. *Manuscript*

III. **Rona J Strawbridge**[#], Monica Nister, Chunde Li, Kerstin Brismar. Effect of GHR stimulation and blockade on prostate cancer cell line proliferation. *Manuscript*

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CONTENTS

LIST OF ABBREVIATIONS

Aa	Amino acid
AD	androgen dependent
ADT	androgen depreivation therapy
AI	androgen independent
BMI	body mass index
BPH	benign prostatic hyperplasia
CAF	cancer associated fibroblast
CAPS	CAncer of the Prostate, Sweden
CHD	coranary heart disease
CRP	C reactive peptide
CVD	cardiovascular disease
DC	dendritic cell
DN	diabetic nephropathy
DR	diabetic retinopathy
DRE	digital rectal examination
ECM	extra cellular matrix
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FA	fatty acid
FAS	fatty acid synthase
FDR	first degree relative
FFA	free fatty acids
FPC	familial prostate cancer
GH	growth hormone
GHBP	GH binding protein
GHR	growth hormone receptor
GLP	glycagen peptide 1
H&E	hematoxylin and eosin
HDL	high density lipoproteins
HPC	hereditary prostate cancer
HT	hypertension
IGFBP	IGF binding protein
IGF-I	insulin-like growth factor I
IGT	impaired glucose tolerance
IMGU	insulin mediated glucose uptake
LADA	late autoimmune diabetes of the adult
LBM	lean body mass
LDL	low density lipoproteins
MA-GHBP	membrane associated GHBP
MI	mycoardial infarction
MMP	matrix metalloprotease
MODY	maturity onset diabetes of the young
MUC1	mucin 1
NGT	normal glucose tolerance
NIMGU	non insulin mediated glucose uptake

NK	natural killer cell
nPSA	nicked PSA
PC	prostate cancer
PIA	prostatic inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PRL	prolactin
PSA	prostate specific antigen
PUFA	poly unsaturated fatty acids
QoL	quality of life
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
SPC	sporadic prostate cancer
T1D	Type 1 diabetes
T2D	Type 2 diabetes mellitus
TACE	TNF alpha converting enzyme
TG	triglycerides
TGFβ	Transforming growth factor beta
tPSA	total PSA
UTR	untranslated region
VDR	Vitamin D receptor
VEGF	vascular endothelial growth factor
VNTR	variable number tandem repeat
WHO	World Health Organisation

NB gene names in accordance with NCBI GENE database.

1 AIMS OF THE THESIS

The interactions between prostate cancer and diabetes are unclear, but may be due to alterations in metabolism, whereby metabolic conditions which predispose to T2D protect from T2D, and vice versa.

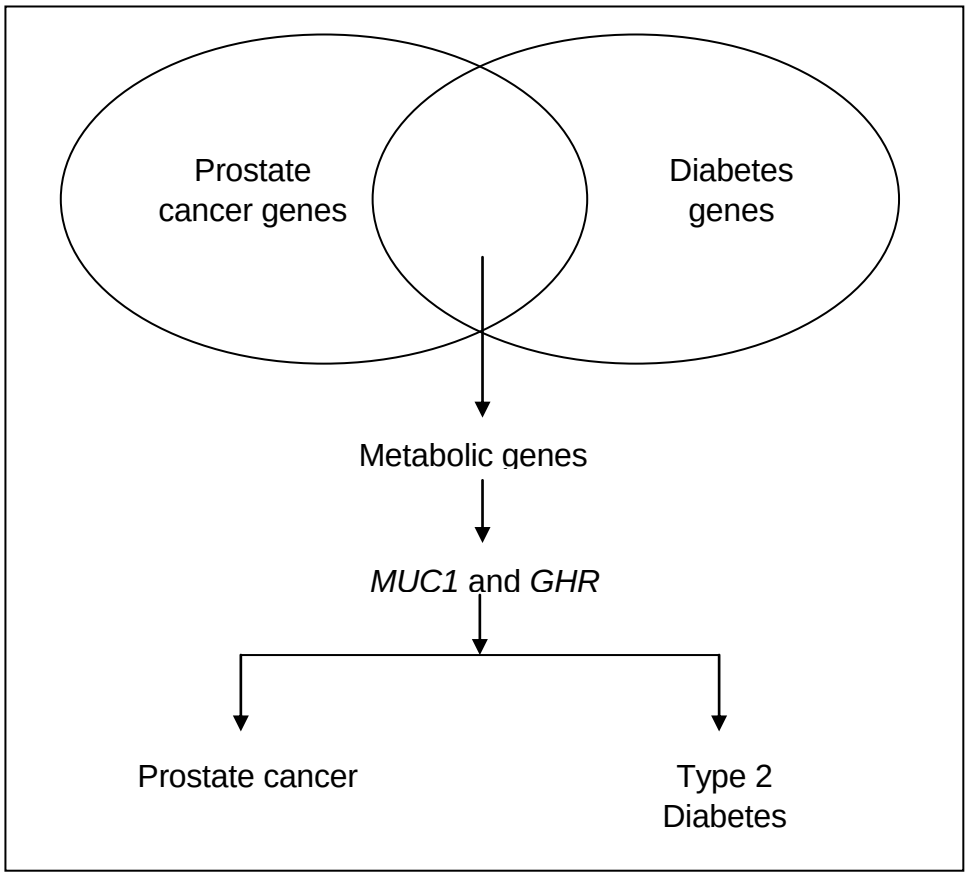

Figure 1: Proposed interaction between risk genes for T2D and PC, where genes involved in metabolism, for example MUC1 and GHR, influence disease, potentially via their effects on levels of IGF-I.

Common genetic variations are thought to be crucial in determining risk of common diseases such as prostate cancer or type 2 diabetes. This thesis aims to investigate whether genetic polymorphisms of *MUC1* and *GHR* influence prostate cancer and type 2 diabetes and if so, whether the opposite effect is seen in prostate cancer compared to type 2 diabetes.

Specific aims:

- To determine whether *GHR* exon 3 genotype influences risk or clinical parameters of type 2 diabetes
- To determine whether rs4072037 genotype of *MUC1* influences prostate cancer risk, whether there is any difference between blood and tumour DNA
- To investigate whether there is any potential for functional differences between the known isoforms of MUC1
- To determine whether genetic variation of *MUC1* and the surrounding region (in particular rs4072037) influences PC risk or survival
- To determine whether the SNP rs4072037 of *MUC1* influences risk or clinical parameters of type 2 diabetes

2 INTRODUCTION

2.1 TYPE 2 DIABETES MELLITUS

Diabetes mellitus is a very common metabolic disorder, with 3.2 million deaths every year attributable to diabetes or its complications and up to 15% of annual healthcare costs used in its treatment (WHO). If lifestyle and dietary habits do not change in the near future, the WHO predicts that health services will be crippled by diabetes and its complications. It has been accepted for several decades that diabetes mellitus can be divided into two diseases, Type 1 (insulin-dependant, T1D) and Type 2 (non insulin-dependant, T2D). The picture may be less clear, with intermediate phenotypes, such as those seen in Late Onset Auto-immune Diabetes of the Adult (LADA), and Maturity Onset Diabetes of the Young (MODY). In addition, the diagnosis of T2D in children is increasing, suggesting that diabetes mellitus might be one disease with a broad spectrum, where classical Types 1 and 2 diabetes are at the extremities (Figure 2S). From this point forward emphasis is placed on the classical Type 2, non insulin-dependant, Diabetes Mellitus (T2D).

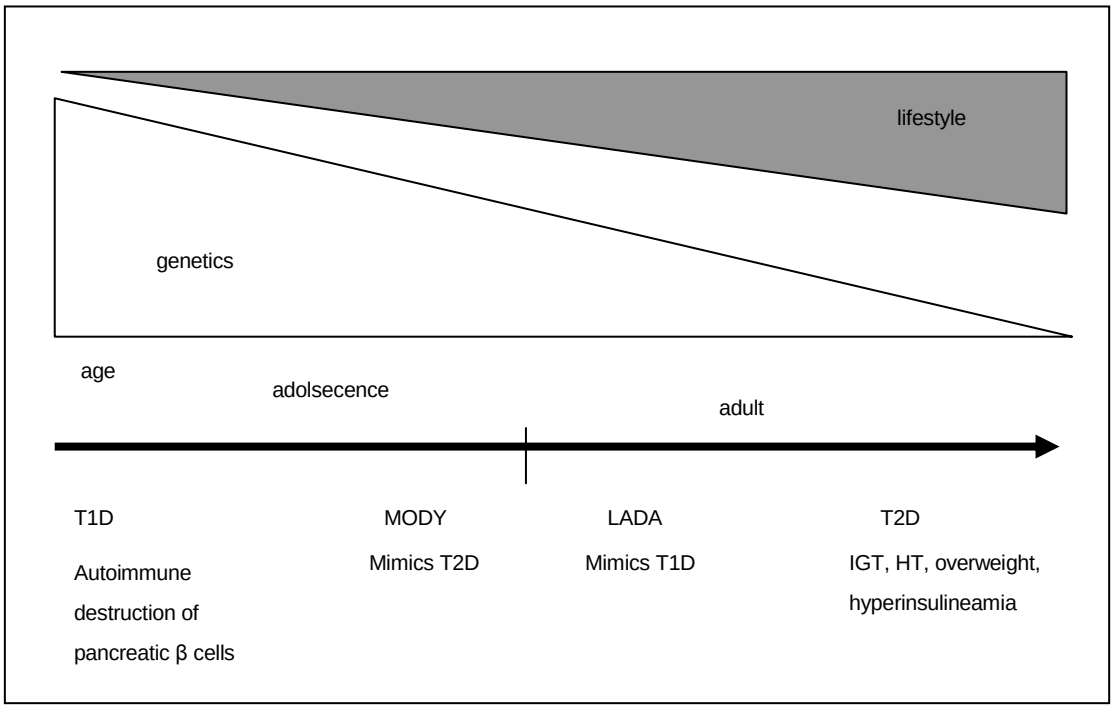

Figure 2: Schematic diagram demonstrating the relative influence of genetic and environmental components in risk of diabetes and the sliding scale of diabetes compared to the traditional types 1 and 2, where IGT impaired glucose tolerance, HT hypertension.

2.1.1 Glucose Homeostasis

At the most simplified level, diabetes is the inability to efficiently use dietary energy. More specifically, it is a decrease in insulin-mediated glucose disposal [1]. Normal metabolic control relies on dietary intake being broken down into glucose, transported to tissues in the blood and removed from circulation by cells. Glucose can be taken up by cells in two ways: Insulin-mediated glucose uptake (IMGU) and non insulin-mediated glucose uptake (NIMGU). Tissues differ in their ability to remove glucose from the blood, depending upon their demand for fuel for energy-dependant processes and ability to store glucose as glycogen. Skeletal muscle is the primary tissue for glucose uptake and storage [1].

2.1.1.1 Insulin-mediated glucose uptake (IMGU)

Insulin is crucial for the maintenance of glucose homeostasis and lipid profile [2]. Insulin levels are determined by factors such as energy balance, genetics and diet [3]. Insulin sensitivity is the degree to which certain cells respond to insulin [4] and is tissue specific.

Insulin is produced by pancreatic β cells and excreted in response to food intake. At target tissues, insulin binding to the tetrameric insulin receptor (IR) results in autophosporylation and signal transduction via mediators such as IRS1 and PI3K. the result of such signaling is tissue specific. The liver, skeletal muscle and adipocytes are the main targets of insulin signaling. In the liver, insulin inhibits the production of glucose (gluconeogenesis). In skeletal muscle, insulin stimulates uptake of glucose from the blood, for storage as glycogen. This is achieved by GLUT4 translocation to the cell surface. At the cell surface, GLUT4 is one of a number of glucose transporters facilitating glucose movement across the plasma membrane, which is the rate limiting step of glucose metabolism [1]. Insulin sensitivity is in part determined by the balance between the PI3K subunits P85 and P110, which influences the translocation of GLUT4. In adipocytes, insulin inhibits lipolysis and the subsequent release of free fatty acids.

Insulin release occurs in two phases. Phase I insulin release peaks rapidly (within minutes), where as Phase II release is of longer duration at a lower level. Phase I insulin is thought to prime target organs for phase II release. In subjects that have suboptimal phase I insulin release, the phase II insulin is less effective, suggesting a threshold level which must be achieved by phase I release.

2.1.1.2 Non insulin-mediated glucose uptake (NIMGU)

In addition to IMGU, cells have an endogenous ability to take up some glucose in a process termed non insulin-mediated glucose uptake. The degree to which cells respond high blood glucose levels is termed glucose sensitivity [4]. NIMGU is partly a response to high blood glucose levels, but can be stimulated by physical exercise.

2.1.1.3 Type 2 Diabetes

The characteristically high fasting blood glucose levels observed in diabetic subjects, indicate aberrations in glucose uptake, primarily IMGU. T2D has a large hereditary component which is influenced by environment.

Prolonged high glucose levels indicate insulin resistance (cells not responding correctly to insulin), which causes a compensatory increase in β cell secretion of insulin (hyperinsulineamia). Long term hyperglyceamia requires the β cell cells of the pancreas to be able to maintain production of raised insulin levels. Thus insulin resistance only leads to T2D when a high level of insulin production can not be maintained. β cell failure is progressive, and usually proceeds diabetes by about 8 years [5].

Insulin resistance is associated with redistribution of stored energy. Redistribution of lipids propagates a downwards spiral of reducing control over metabolism. Inability to correctly regulate fuel metabolism has knock-on effect on other systems, such as hormonal homeostasis.

Traditionally high glucose levels have been the focus of diabetes research, however more recently it has been recognized that lipid profile *per se* may be crucial to long term health, although some complications are still accepted as being glucose-related.

2.1.2 Definitions + Diagnosis

The World Health Organization set criteria for the diagnosis of diabetes (WHO 1985). Fasting blood glucose and stimulated blood glucose levels are measured to assess basal glucose levels and the ability to respond to high glucose levels. Subjects with normal glucose metabolism are defined as having normal glucose tolerance (NGT), those subjects who are not diabetic but demonstrate aberrations are termed impaired glucose tolerance (IGT). NGT subjects demonstrate low basal blood glucose levels which increase after ingesting food, but are reduced to basal levels within 2hrs of eating.

NGT subjects have fasting glucose levels of <7.0mmol/L and plasma glucose levels <7.8 mmol/L 2 hrs after an oral glucose tolerance test (OGTT). IGT is defined as fasting plasma glucose <7.8 mmol/L and 2 hr plasma glucose levels 7.8-11.0 mmol/L and T2D was defined as fasting plasma glucose >7.8 mmol/L and/or 2 hr plasma glucose >11.0 mmol/L.

Body mass index (BMI, kg/m^2) is used to estimate body fat in comparison to height. Appropriate or normal BMI ranges between 20-25, with a BMI of 25-30 being defined as overweight and a BMI of >30 is defined as obese. Whilst not used when diagnosing T2D, BMI is considered when determining therapeutics and prognosis.

The symptoms of diabetes become less acute with increasing age, thus reported incidence in the elderly most likely underestimates prevalence. Diagnosis of T2D is frequently secondary to a concurrent medical condition or diabetic complication.

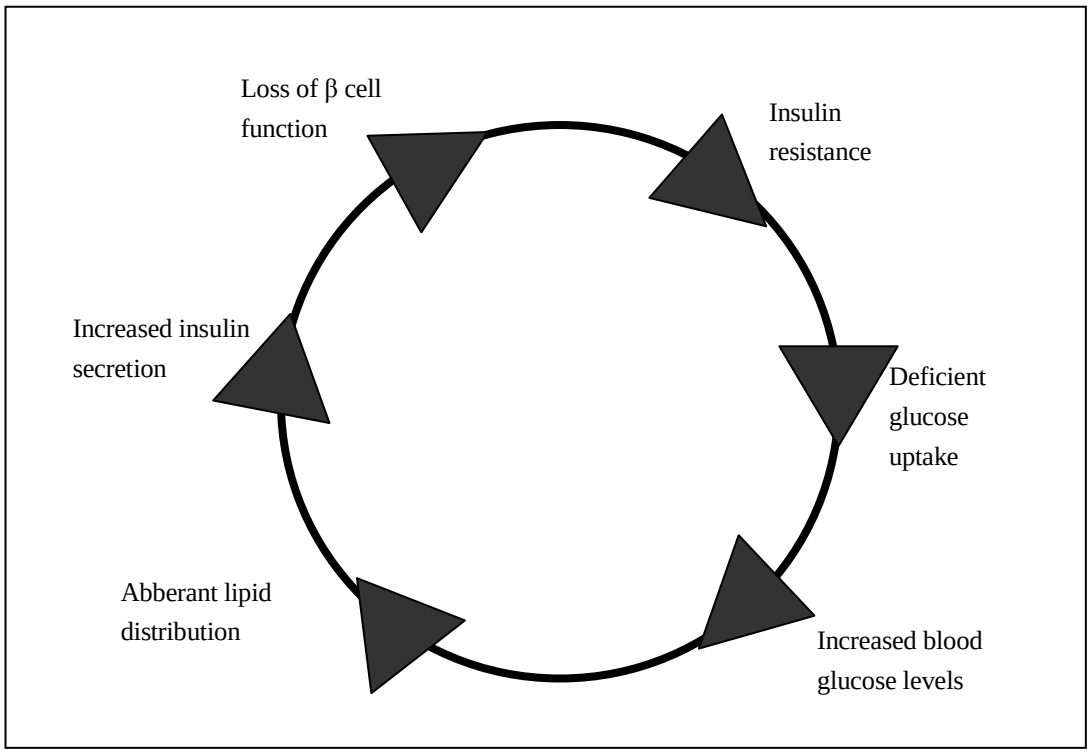

Figure 3: Hallmarks of type 2 diabetes

2.1.3 Therapy

The aim for T2D treatments is normalization of blood glucose levels as uncontrolled diabetes leads to complications that can drastically reduce quality of life and are a huge financial burden on healthcare systems. This can be addressed by lifestyle modifications of pharmaceutical agents.

2.1.3.1 Lifestyle alterations

Alterations in lifestyle allow approximately 20% of subjects to control their diabetes. Increased physical activity and reduced caloric intake typically results in a decrease in weight. Even without loss of weight, the modified hormonal status gives a much improved metabolic status.

Traditionally diabetes subjects were advised to reduce sugar intake, thus preventing high peaks in blood glucose levels, which require insulin. This is still advised, however it is becoming more evident (mainly from animal studies) that caloric intake *per se* is not as important as the source of calories. For example, the role of lipids in the maintenance of metabolic stability has been underappreciated. This may explain the increased insulin sensitivity observed with weight loss.

Diabetes subjects have suboptimal insulin responses, thus limiting IMGU. Physical activity increases NIMGU, thus in part compensating for the reduced IMGU function.

2.1.3.2 Pharmaceutical agents

Essentially the aim of pharmaceuticals is to supplement endogenous insulin levels.

Traditionally this has been with the use of insulin administered by subcutaneous injection. Insulin levels rise and fall under the influence of stimuli such as blood glucose levels and various hormones. Injected insulin is unable to achieve a physiological profile. The short duration of effect requires daily dosing, which can cause problems with compliance, particularly in the elderly.

Enhancing secretion of insulin by the pancreatic β cells is achieved by drugs such as sulfonureas. These drugs are rapidly effective, with little or no lag time [6]. Some level of β cell function is required, thus they are beneficial in early diabetes, however the stress of long-term increased insulin production reduces the lifespan of the β cells [6]. Exogenous insulin-like growth factor I (IGF-I) is administered to increase insulin secretion, peripheral glucose uptake, reduce hepatic glucose production and improve the lipid profile. Adverse side effects of IGF-I therapy include increased risk of some cancer.

Metaformin and similar drugs target the production of glucose. These drugs inhibit hepatic glucose production and are very effective, at least initially [6]. Other benefits such as reduced risk of cardiovascular disease (CVD) are observed, possibly due in part to this class of drugs ability to inhibit weight gain [6].

Other methods for normalizing glucose levels include the following:
Thiazolidiones act via PPARγ thus preserving β cell function. It is also protective of the vasculature [6]. Weight gain is common, thus this treatment is not suitable for obese patients [6].

Glucagen peptide 1 (GLP1) is a novel drug which specifically inhibits the absorption of dietary fats in the gastrointestinal tract. Currently in clinical trials, GLP1 shows promising results. Theoretically this should be suitable for obese as well as lean subjects.

2.1.4 Complications

Long term deregulation of metabolism leads to a number of complications, and it is these (rather than the elevated glucose levels) that are life threatening, thus attaining good metabolic control is crucial for long term health of diabetic subjects. The genes predisposing to complications are likely to differ from those of DM susceptibility [7], which in part explains the heterogeneity of progression. The following complications demonstrate the reduction in quality of life caused by long term glucose aberrations.

Diabetic retinopathy (DR) results from an increased vasodilation, increased blood flow and neovascularisation of the retina which are thought to be a response to local hypoxia [5]. It is proposed that hyperglyceamia causes an increased oxygen consumption leading to hypoxia [5]. Most diabetic subjects will develop background retinopathy, while fewer will progress to blindness [8], although there are geographical variations in prevalence of DR. In Pima Indians retinopathy is frequent and linked to regions of chromosomes 3 and 9 [8], whilst in India, cases of DR are low, despite high levels of T2D [9]. Activation of the pentose phosphate pathway prevents poylol formation and protects from experimental diabetic retinopathy [7]. Accumulation of polyols has been associated with increased basement membrane thickening, loss of pericytes and vessel leakage and DR. [7]

A key molecule for angiogenesis is vascular endothelial growth factor (VEGF), which is up regulated by hypoxia [7]. Increased levels of VEGF have been observed in the aquaeous and vitreous fluid of 33% of patients with proliferative DR [7]. A G34C polymorphism of the 5'UTR of *VEGF* is associated with DR and is more significant in proliferative retinopathy. The C allele confers higher levels of VEGF, but it is not yet known whether local or systemic VEGF is important [7]. High serum levels of growth hormone (GH) have been associated with eye complications [10]. Other factors which may be involved are PON1, TGFβ, EDN1, PPARγ, GLUT1, PAI-1, MTHFR, and integrins.

Diabetic nephropathy (DN) is due to an imbalance between production and removal of extra cellular matrix (ECM) [8]. Chromosomes associated with susceptibility include 3, 7, 9, 18 and 20. DN is a complex disease [8] with many genetic and environmental factors. Familial clustering is seen in many racial and ethnic groups [8]. It is estimated that 35% of European Americans are at risk [8], with increased incidence in African Americans, Hispanic Americans and native Americans. Candidate genes with proposed influence are *NHE1* (1p36.1-35), *TGFβ* (19q12-13.31), *GH1* (17q24.2), *IGF-I* (12q22-23), *VEGF* (6p12), *RAAS* (17q23). Linkage has also been identified in African Americans to chromosome 3 (30% of cases), chromosome 7 (40% of cases) and chromosome 18 (65% of cases), whilst in Pima Indians, linkage to regions of chromosomes 3, 7q, 9 and 20 has been observed [8].

Subjects with T2D have a 200% increased risk of cardiovascular disease (CVD) and increased mortality from CVD compared to the general population. The recent decline in incidence of CVD in the general population is not observed in diabetic subjects. Age, duration of disease and degree of metabolic control are the major determinants of CVD risk in T2D subjects. Increased glucose levels, particularly for prolonged periods, increase CVD risk. In part this is due to hyperinsulineamia, which increase the risk of cardiovascular disease [11]. Both extremes of GH levels (deficiency or hyper secretion) have increased prevalence of CVD [12], indicating that to maintain cardiac health, GH signaling is tightly regulated. Down stream of GH, low serum IGF-I levels are associated with increased T2D risk [13]. IGF-I is also a mediator of atherosclerosis and

diabetic lesions [13]. Metabolic syndrome (one component of which is T2D) increases CVD due to visceral adiposity, insulin resistance and altered levels of atherosclerotic cytokines produced by adipocytes [6]. Aberrant processing of glycosylation metabolites results in the formation of advanced glycosylated end products (AGE). These are formed by non-enzymatic glycosylation of proteins and lipids [7]. Increased formation of AGE is associated with hyperglycemia and increased vascular cell permeability [7].

Hypertension (HT) has a definite hereditary component. IGF-I levels may explain some of the genetic component, as risk of HT is associated with adult height [14]. Diabetes subjects are also at increased risk of HT. Levels of some cytokines, in particular TNFα, are increased in subjects with HT [6].

Dyslipidaemia is the aberrant distribution of stored lipids as well as circulating lipids. IGF-I levels are inversely associated with triglycerides (TGs), Insulin and CRP levels. Dyslipidaemia is characterized by high TGs, low high density lipoproteins (HDLs) and small dense low density lipoproteins (LDLs) [6].

Impaired wound healing is a complication of diabetes which has possibly the highest impact on quality of life. Caused in part by aberrations in the coagulation system, small wounds do not heal and easily become infected, resulting in amputation. This is a frequent event in developing countries such as India, where a large proportion of the diabetic population live in rural areas with inadequate health facilities.

2.1.5 Genetics

Genetic variation is estimated to account for 50% of T2D risk. Loci associated with increased risk for T2D have been identified on chromosomes 1q21-24 [8, 15], 2q37 [8], 12q24 [8], 20 [8]. Mendelian forms of diabetes (such as MODY) have identified a number of genes implicated in regulation of β development and function [16]. The MODY genes are obvious candidates for T2D, however a recent study genetic variations of these demonstrated little or no association with T2D [16]. The genes implicated in T2D are listed in Table 2.

Risk of T2D increases with age, as do the number of mitochondrial mutations. Some evidence suggests maternal inheritance of T2D, supporting a role of mitochondrial DNA.

2.1.6 Inflammation

The Immune system is closely linked to the metabolic system [17]. American Indians have the highest T2D incidence in the world [17] which is widely thought to be due to differences in immune exposures and inflammatory responses compared to Europeans.

Chronic low level inflammation has been proposed as a component in T2D and may contribute to β cell destruction. CRP is a general inflammatory marker [12], which is inversely correlated with IGF-I levels [12].

2.1.7 Environmental Influences

50% of factors controlling insulin resistance are believed to be environmental. To date lifestyle (diet and physical activity) are the only known environmental/non-genetic factors implicated in T2D.

Recently, Exposure to sunshine and thus vitamin D levels have been implicated in metabolic disorder [18], in populations known to have high rates of T2D [9].

Table 1: Genes implicated in T2D

Locus	Gene	Function	Ref
1p13-p11	*ADAM30*	Weakly associated with T2D.	[19]
1p13-p11	*NOTCH2*	1 intronic SNP, rs10923931	[19]
1p36.1-35	*NHE1*	Implicated in diabetic nephropathy.	[8]
1q21	*ARNT*	Required for expression of genes involved in β cell function. Involved in β cell dysfunction. 90% reduced expression in islets of diabetic subjects compared to controls. Loss of *ARNT* is associated with reduced glucose tolerance and impaired insulin secretion.	[20]
1q21	*CASQ1*	Involved in calcium metabolism. Variants modulate T2D susceptibility.	[15]
1q21	*PKLR*	Up regulated by glucose. Variants modulate T2D susceptibility.	[15]
1q21	*RORC*	Nuclear hormone involved in immune response.	[21]
1q21-q22	*DUSP12*	Regulates enzymes within the glycolytic pathway. Located in a T2D region. Variants modulate T2D susceptibility.	[15]
1q22-q23	*RXRG*	Nuclear receptor involved in glucose and lipid homeostasis. Variants modulate T2D susceptibility.	[15]
1q31-q32	*IL10*	Anti inflammatory cytokine. Associated with T2D risk.	[17]
1q42-43	*AGT*	Angiotensin. Variations associated with diabetic retinopathy. Effects may be population specific.	[7]
2p21	*THADA*	SNP rs7578597 associated with T2D.	[19]
2p22-21	*SOS1*	Guanine nucleotide exchange factor.	[21]
2p25	*ACP1*	Implicated in T1D.	[21, 22]
2q32	*NEUROD 1*	Developmental transcription factor involved in regulation of β cell development. Involved in MODY.	[16, 21]
2q33-34	*IGFBP2*	involved in regulation of bioactive IGF-I levels.	[23]
2q36	*IRS1*	Signal transducer. Important for insulin action	[21]
2q37.3	*CAPN10*	Involved in β cell dysfunction. Confirmed as having a role in T2DM. Predictive of T2DM.	[20, 21]
3p25	*PPARγ*	Regulator of lipid and glucose homeostasis. Implicated in adipocyte function. A promising susceptibility gene. Variants may be predictive of T2DM. 1 variant is associated with increased serum insulin, reduced T2D risk. Replicated in a GWA study	[19-21, 24]
3p25	*SYN2*	Closest gene to the signal at rs17036101, thus implicated in T2D.	[19]
3p26-25	*GHRL*	Hormone involved in feeding and energy homeostastis	[21]
3q21-25	*AGTR1*	An angiogenic component found in the retina. May promote or initiate VEGF neovascularization	[7]
3q24-25.1	*GYG1*	Enzyme involved in glycogen synthesis	[21]
3q26.1-26.3	*SLC2A2*	GLUT2 glucose transporter. Involved in β cell dysfunction.	[20, 21]
3q27	*ADIPOQ*	A cytokine produced by adipose tissue. Involved in adipocyte function. Levels inversely correlate with insulin sensitivity.	[17, 20, 21]
4p15.1	*PPARGC1 A*	*PGC1*. Transcriptional co-activator involved in energy homeostasis.	[21]
4q12-13	*GC*	Vitamin D binding protein. Vitamin D binding protein involved in regulating insulin levels.	[21]
4q28-q31	*FABP2*	Transporter protein for long chain fatty acids. Involved in regulation of liver function.	[21]
5p13.1-cent	*NNT*	Nuclear-encoded mitochondrial gene, critical for glucose- mediated closure of an ATP-dependant proton pump. Involved in β cell dysfunction.	[20]
5q13	*PIK3R1*	Important for insulin action and glucose clearance.	[21]
5q15-21	*PC1*	Inhibitor of insulin signaling	[21, 24]
5q32-34	*ADRB2*	Associated with obesity and diabetes.	[21]
5q34-35	*GFPT2*	Hexosamine biosynthesis pathway	[21]
6p12	*VEGF*	Implicated in diabetic nephropathy.	[8]
6p21.3	*TNFα*	Proinflammatory cytokine, TNFα alters insulin action in peripheral tissues. Increased levels cause a hyper-coagulatory state via PKC and thromboxin	[7, 21, 25]
6p21.3	*AGER*	Activation of this receptor causes release of cytokines which may enhance progression of complications. 1 polymorphism may alter ligand specificity.	[7]
6p22.3	*CDKAL1*	A confirmed T2D susceptibility gene.	[23]
6q12	*VEGF*	Critical for breakdown of the retinal-blood barrier and neovascularization.	[7]
6q22-23	*ENPP1*	Inhibits insulin signaling. Variations modulate obesity.	[20, 21]
6q25.3	*SOD2*	Genetic variations associated with increased diabetic nephropathy.	[26]
7p15.2-15.1	*JAZF1*	Strong GWA evidence for intronic SNP rs864745 being involved in T2D.	[19]
7p15.3-15.1	*GCK*	*MODY2*. First enzymatic step in glycolysis. Involved in β cell dysfunction.	[4, 16, 21]
7p21	*IL6*	Inflammatory cytokine. Variations associated with altered insulin sensitivity.	[17, 21]
7q21.2-22	*PAI1*	Rate limiting step in coagulation.	[21]
7q21.3	*PON2*	Involved in regulation of fasting glucose levels	[21]
7q31.1	*PPPR3A*	Involved in glycogen metabolism and insulin action.	[21]

7q31.3	*LEP*	Leptin. Secreted by adipocytes. Levels may be associated with T2D.	[27]
7q33	AKR1B10	First and rate limiting step of polyol pathway. Involved in hyperglyceamia.	[7]
8p12-11.2	*ADRB3*	Involved in adipocyte function.	[20, 21]
8p22	*LPL*	A lipoprotein lipase.	[21].
8q24.11	*SLC3OA8*	Variations are associated with β cell function and decreased insulin secretion.	[23, 28]
9p21	*CDKN2A/ CDKN2B*		[23]
9q13-21.1	*FXN*	*FRDA*. Ion metabolism in mitochondria.	[21]
9q34.11	*SLC27A4*	*FATP4*. Transporter protein for long chain fatty acids.	[21]
10q21.3	*SIRT1*	Involved in repression of glycolytic genes, thus modulates energy homeostasis. Over expression increased insulin secretion by β cells.	[20]
10q23-24	*RBP4*	Modulates glucose homeostasis. Reduction in levels improves insulin action. Involved in adipocyte function.	[20]
10q25.3	*TCF7L2*	Involved in regulating blood glucose homeostasis. A nuclear receptor for β catenin. A variant may be associated with younger age of onset. May act via GLP1.	[20, 21, 23]
11p12-p11.2	*MAPK8IP 1*	Signal transducer.	[21]
11p15.1	*ABCC8*	Modulates ATP-sensitive potassium channels. Involved in β cell dysfunction and insulin secretion. Receptor for Sulfonylurea	[20]
11p15.1	*KCNJ11*	Involved in β cell dysfunction. A promising susceptibility gene.	[20, 21]
11p15.5	*INS*	Insulin. Important for glucose regulation and β cell function. A tandem repeat in this gene is implicated in T2DM.	[21]
11q13	*UCP2*	Mitochondrial transporter. Involved in β cell dysfunction.	[20, 21]
12p12.3-12.1	*IAPP*	Hormone involved in pancreatic glucose uptake and in β cell dysfunction.	[20, 21]
12p13	*GNB3*	Signaling molecule involved in obesity	[21]
12q12.1	*PDX1*	*IPF1*, *MODY4*. Promoter binding factor.	[4, 21]
12q13.1	*DCD*	Closest gene to the association peak at rs1153188, thus implicated in T2D.	[19]
12q14.1-q21.1	*TSPAN8*	SNP in the promoter, rs7961581 associated with T2D.	[19]
12q22-24.1	*IGF-I*	A hormone involved in growth and liver function. Mediates atherosclerosis and diabetic lesions	[13, 20, 21]
12q24.2	*HNF1B*	*TCF1* or *MODY3*. A transcription factor involved in β cell dysfunction and regulation of cholesterol homeostasis. 1 SNP associated with T2D.	[19, 21]
13q14.1	*FOXO1*	Involved in β cell dysfunction. Regulates insulin secretion and action.	[20]
13q34	*IRS2*	Signal transducer. Important for insulin action	[21]
15q21-23	*LIPC*	Hepatic lipase. Involved in lipoprotein regulation.	[21]
16q12.2	*FTO*	Associated with T2DM and obesity.	[23]
16q22	*AGRP*	Agouti related protein homolog.	[21]
16q22	*RRAD*	Involved in insulin sensitivity.	[21]
16q24.3	*FOXC2*	Transcription factor involved in regulating adipocyte metabolism.	[21]
17cen-q21.3	*HNF1B*	*TCF2 or MODY5.* 1 SNP protective against T2D in European, African and Asian populations.	[4, 29]
17p13	*SLC2A4*	*GLUT4*. Glucose transporter protein.	[21]
17q11.2-12	*NOS2A*	1 variation has been associated with diabetes in Indians.	[7]
17q23	*RAAS*	Implicated in diabetic nephropathy.	[8]
17q24.2	*GH1*	Implicated in diabetic nephropathy.	[8]
17q25	*GCGR*	Glucagon receptor involved in glucose homeostasis and liver function.	[20, 21]
19p13.3-13.2	*INSR*	Insulin receptor. Crucial for glucose regulation.	[21]
19q12-13.31	*TGFβ*	Implicated in diabetic nephropathy.	[8]
19q13.1-13.2	*LIPE*	Hormone-sensitive lipase. Involved in mobilization of fatty acids	[21]
19q13.3	*GYS1*	Involved in liver function.	[20, 21]
20p11	*FOXA2*	Regulates insulin secretion and action .	[20]
20q12.31	*PCK1*	Regulator of gluconeogenesis.	[21]
20q12-13.1	*HNF4A*	*MODY1*. Transcription factor involved in β cell dysfunction and in regulating hepatic glycogen stores. Confirmed as a susceptibility gene.	[4, 16, 20, 21]
20q12-13.11	*ADA*	Enzyme involved in purine catabolism.	[21]
Xp22	*ACE*	A key component in the rennin-angiotensin system. Circulating levels are determined by an insertion/deletion polymorphism.	[7]
Where: T2D type 2 diabetes, SNP single nucleotide polymorphism, GWA genome-wide association, T1D type 1 diabetes, MODY maturity onset diabetes of the young.			

2.1.8 Obesity

Obesity is a major risk factor for T2D, and is therefore the main reason for the predicted diabetes epidemic. Obesity has increased over the last 20 years [30] and is typically a result of caloric excess [31]. In particular a high fat diet coupled with physical inactivity appears to be the most common cause of obesity, especially in westernized countries. A number of T2D-associated genetic variants, such as *FTO* [32], only influence diabetes via obesity. Thus reducing obesity rates is a public health priority for reducing T2D incidence.

Obesity often demonstrates hormonal alterations [31] with increased estrogen and reduced testosterone levels [33]. Abdominal or visceral obesity releases FFAs into circulation where they may be taken up by the liver or muscle and utilised instead of glucose [33].

Obesity is usually concurrent with abnormally high GH levels, but levels of IGF-I and IGFBPs are normal [34]. Obese subjects also demonstrate increased hepatic GH sensitivity [34].

2.2 PROSTATE CANCER

Studies into the role of metabolic disorders in prostate cancer (PC) has been restricted to obesity and Diabetes Mellitus (although it is not always stated whether it is type 1 or type 2). A number of studies have investigated the relationship between PC and T2D, with some confusion of results being attributable to differences in stage of prostate cancer, lack of specificity with regards to type of diabetes and variations in therapies for both diseases. In addition, the complex interplay between obesity and diabetes is likely to have confounded some, if not all, studies. Meta analysis of studies indicates that T2D reduces risk of prostate cancer [35, 36], but that having T2D increased the morbidity of PC [35]. As mentioned in pervious chapters, lifestyle modifications are, to a large degree, capable of modifying and controlling T2D and risk of T2D. Therefore if a causal relationship exists between obesity, T2D and PC, understanding the mechanisms are important for the purposes of enhanced intervention and therapy.

2.2.1 The Prostate

The prostate gland is relatively small during childhood, and begins to develop during puberty, under the stimulus of testosterone [37]. At the age of 20 years prostate size ceases growth and remains stable until about 50 years of age, after which, in concert with the decreased production of testosterone, the prostate may involute [37]. The prostate accounts for 0.1% of body weight and has only a limited functional time period. The prostate gland produces fluid containing inorganic ions and a number of enzymes. Release of this fluid is synchronized with that of semen, and acts to raise the pH of semen thus improving motility and fertility of the sperm [37]. Aside from this, the prostate appears to serve no function, yet PC accounts for as many as 30% of male cancers [38].

2.2.2 Prostate Tumours

The prostate is comprised of glands surrounded by stroma (see Figure 3), which functions as structural support for the tissue. The stroma contains smooth muscle cells and fibroblasts interspersed with immune cells, vasculature and extra cellular matrix. A single layer of epithelial cells, which form the lumen of the gland, are separated from the stroma by a layer of basal cells and the basement membrane.

Tumours typically develop from the epithelial cell layer. An excess of cells grow into the lumen of the gland, causing disrupted function. Tumours may grow to the extent that they disrupt the basement membrane and invade the surrounding stroma. Cancerous tumours have the potential to exceed normal organ boundaries and eventually form metastatic leisons.

Benign prostatic hyperplasia (BPH) is a condition that presents in a similar manner to PC. BPH is a non-malignant enlargement of the prostate, characterized by increased size and number of cells, including all cell types. BPH is widely believed to lack the ability to progress to cancer.

2.2.3 Disease Detection

Urinary obstruction is a primary symptom of prostate cancer, although may also be caused by other factors. Confirmation of a tumour occurs by means of a digital rectal examination (DRE), and biopsies from a number of areas of the prostate are taken to determine whether the tissue mass is benign or malignant. Prostate cancer is often symptomless.

2.2.4 Pathology

A pathologist's analysis of biopsies is important in determining whether the sample is PC or BPH. Histopathological analysis is carried out on biopsied prostate material. Staining biopsy sections with hematoxylin and eosin (H&E) labels the nuclei and cytoplasm respectively. This allows the pathologist to assess the structure and organization of the tissue. The relative normality of tissue organization is assigned a grade, according to the Gleason grading system (Figure 1) [39].

2.2.5 Prognosis

PC tumours are highly varied at presentation, and even those presenting in a similar manner can have very different clinical progressions [40]. Autopsy-based studies estimate that 75% of men >85 years have PC foci [41], however a number of these remain undiagnosed at the time of death. Why some cancers progress rapidly and whilst others remain sub-clinical is not clear. It is debated whether all PC has the potential to progress to lethal cancer or whether the aggression of each tumour is predetermined. Microarray analysis of a large number of tumours have elucidated gene transcription patterns that define subsets of PC which correlate with clinical progression [42]. Whether these patterns determine, or are determined by, aggression of disease is not known.

For estimating prognosis and thus best treatment strategies, clinicians use two measures of disease progression, Gleason grade and stage. These are combined with age, general fitness, co-morbidities and levels of prostate specific antigen (PSA, discussed in detail later) in blood samples are also taken into account

2.2.5.1 Gleason grading

The Gleason score is the sum of the two most common patterns within the tumour (Figure 2), where a low score is < 3+3, medium score, is 3+4 and >3+4 is a high score [39]. A high Gleason score (thus >7) being an indicator of poor prognosis.

Thus the Gleason score is a measure of the effect of the tumour on the prostate organ, for example whether the cells are normal size, the glandular structure is normal, there is increased vasculature or there is infiltration of immune cells.

2.2.5.2 Stage

Stage of cancer progression is divided into 3 components, Tumour, lymph Node metastasis and distant Metastasis (TNM). The T score reflects the proportion of the prostate that is cancerous. The lymphatic system is similar to the blood stream in that it is a route for removal of cellular debris and waste products. As such it is also a route for metastatic tumour cells to spread to the rest of the body. Thus the N score reflects whether there is evidence of tumour cells in the lymph nodes. M reflects whether there is metastasis distant from the tumour.

Thus the Stage is a measure of the effect of the tumour on the organism, i.e. the patient.

It is important to note that TLM stage and Gleason grade do not correlate and that any grade can occur within a specific stage.

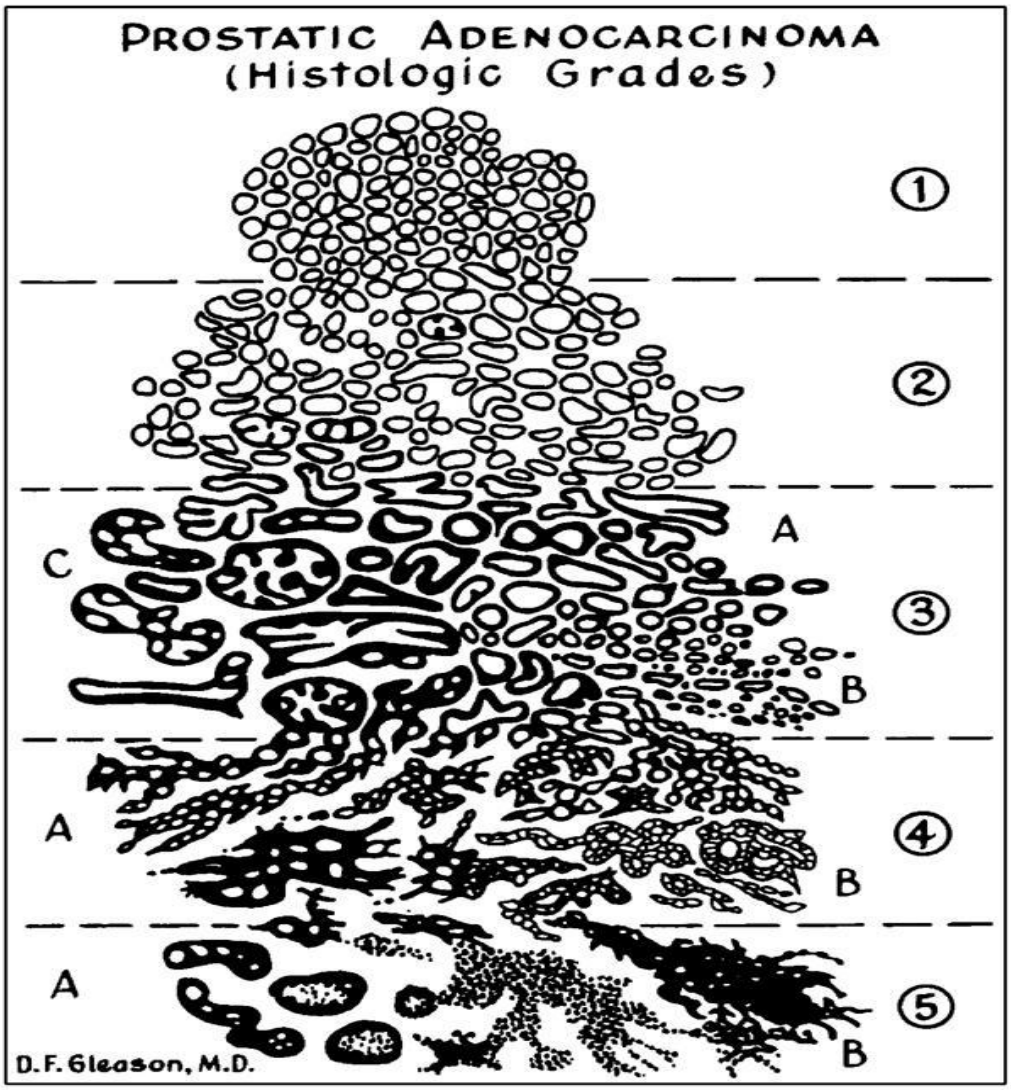

Figure 4: Gleason grading system [39].

2.2.6 Treatment

When localized within the prostate, the primary aim of cancer therapy is removal of the tumour mass. Prostate cancer is invariably multi-focal, thus the entire prostate is removed surgically. Alternatively, radiation therapy aims to only kill the tumour cells, leaving the non-tumour mass intact. The size and extent of tumour cells is the main determinant of treatment modality. In addition, hormonal therapy (anti-androgens) is commonly used as an adjuvant to reduce tumour size before surgery and after surgery to minimize recurrence. Most tumours are initially responsive to hormonal therapy (androgen dependent, AD), with a 5 year survival rate of 60-100% (although this is geographically variable). The time taken for PC to become independent of androgen stimulation correlates positively with prognosis and inversely with aggression [43]. Tumours which exceed the normal boundaries of the prostate, which do not respond the hormonal therapy (androgen independent, AI) or have metastasized to distant sites are considered advanced. Advanced or metastatic cancers, which account for approximately one third of newly diagnosed PC [44] have a very poor prognosis. Current treatments are generally not effective (in terms of survival) against these tumours, thus the aim of therapy here is to minimize symptoms rather than curing the disease. The preferential site for distant metastasis is the bone [45]. This is invariably fatal, with less than 2 years survival from presentation [45].

2.2.7 Basic Cancer Biology

PC is the result of deregulated turnover of cells. Normal cells are prevented from inappropriate growth or dividing (proliferating) by contact with neighboring cells, lack of nutrients or growth factors and a toxic environment formed by metabolic waste products, as well as inherent regulatory mechanisms such as telomere shortening. When normal cells reach maturity they stop proliferating and only the processes needed to maintain cellular homeostasis (house-keeping processes) continue. Cancerous cells are characterized by their ability to override negative regulation, both internal and external. In addition, autocrine (within a cell) or paracrine (between neighboring cells) growth regulation allow continued proliferation in otherwise adverse conditions. These adaptations in essence mean the cells become immortal. Transformation of a normal cell to a tumourigenic cell is frequently accompanied by loss of polarity, thus the normal cellular architecture becomes disturbed by aberrant protein expression patterns.

Cancers are traditionally believed to develop from a single aberrant cell which displays growth and survival advantages over the surrounding cells. That PC develops as multiple foci argues against this model of clonal expansion. Another hypothesis involves stem cells [46], which become tumourigenic within or upon leaving the stem cell niche. It should be noted that these theories are not exclusive. Reports suggest genetic diversity between multiple tumour foci from a single prostate sample [47], which may indicate that the prostate is an inherently pro-tumourigenic environment.

2.2.7.1 Androgen Independence

Prostate cancer is initially dependant upon androgen stimulation (androgen dependant, AD) via the androgen receptor (AR) for growth and survival. Thus androgen depletion (by surgical or chemical castration with anti-androgens) initially prevents prostate growth and cause cells to undergo apoptosis, thus tumors regress or are halted for a time.

It has been observed that after androgen depletion the proliferation rate remains high yet there is a reduction in prostate tumour size. This is thought to be due to the death of 'bystander' cells, whilst a number of "true" cancer cells continue to proliferate at a high rate. It has been proposed that these "true" cancer cells are stem cells. Thus the survival time gained by androgen depletion is merely the lag time required for clonal expansion of cells with a mechanism for androgen independence (AI) to repopulate the 'tumour space'.

The mechanisms of AI are largely unknown. Adaptations to a low-androgen environment and establishment of mechanisms for bypassing the requirement of androgen are likely to be many and complex. It is plausible that some cancer cells have an innate ability to bypass androgen stimulation, whilst others need to acquire this advantage. Mutations of the AR which lead to a lower stimulation threshold, or alternative ligands (such as estrogen) and other steroid hormones) have been observed in the progression from AD to AI. It has been shown that in AI tumours, AR targets are still expressed, indicating that AR signaling has not been completely inhibited. This questions whether acquisition of somatic mutations is the only reason for accumulation of AR mutations, or whether anti-androgens target the AR specifically for mutations. Another, not exclusive, speculation is that androgen depletion allows for a mutator phenotype.

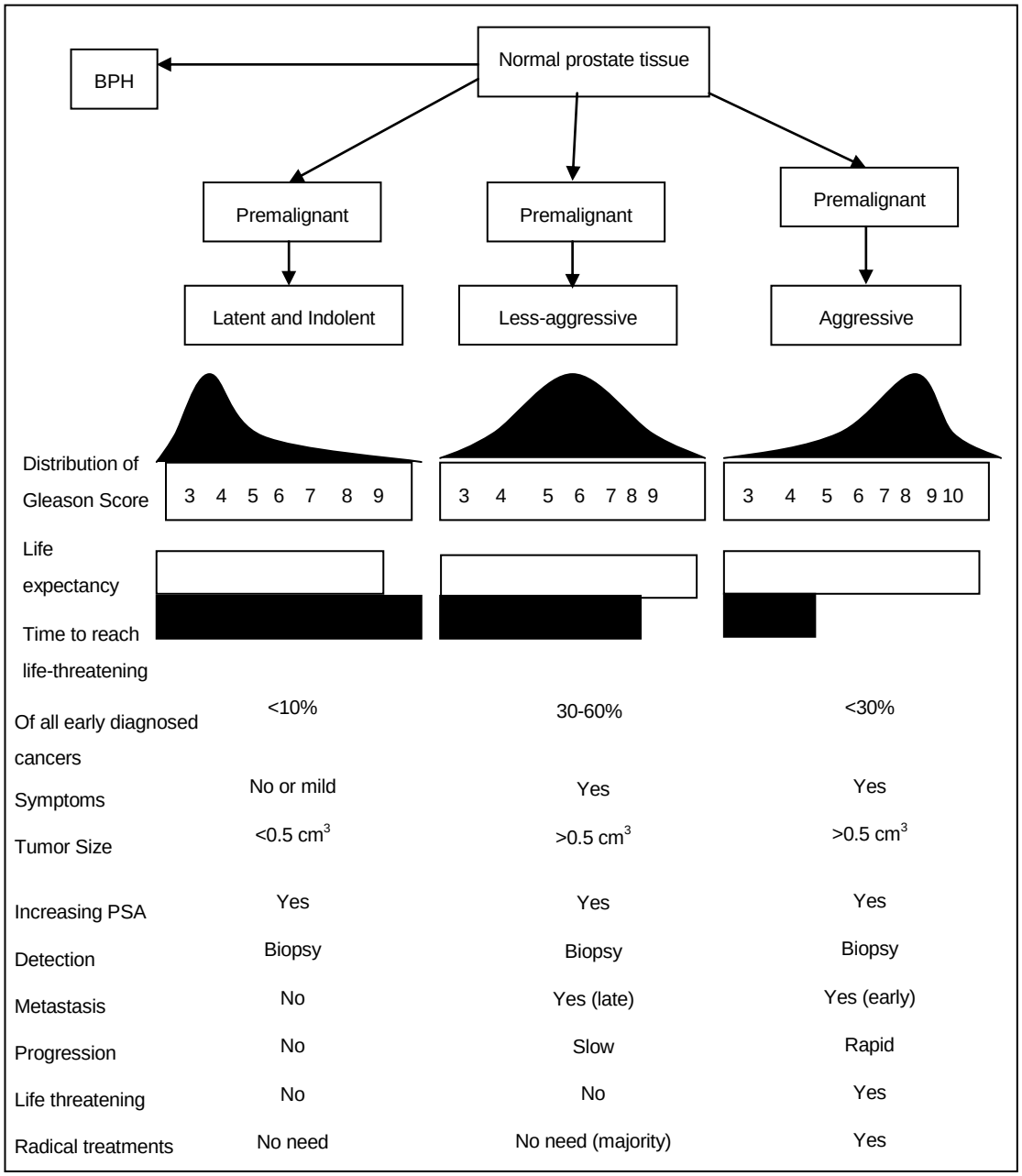

Figure 5: Proposed subtypes of PC by clinical features, courtesy of Dr Chunde Li.

2.2.7.2 Microenvironment

Classically it is believed that changes within the epithelium cause cancer. The resulting tumour then corrupts the normal organ architecture and alters the surrounding stroma [48]. This view is changing, with evidence accumulating to support the importance of stromal cells in promoting tumour growth [49]. It is known that diverse cell types need to interact for normal functions, therefore it is not surprising that the same interactions are required for cancer development [50].

Micro-dissection of the separate cellular compartments is a rapidly expanding field, which shows great promise. For example, whilst a number of papers have elucidated patterns of gene expression which change during progression [42, 51], these studies use total tumour RNA, rather than that of the separate cell types. Given the variable percentage of stromal tissue in samples, this approach is likely to reduce the ability to reproduce these results, and may mask important changes in the stroma. It has been observed that tumour epithelium demonstrates both down (76%) and up regulation of

transcription compared to normal epithelium [52]. In contrast, stroma genes in tumours are exclusively up regulated compared to normal stroma [52]. Perhaps surprisingly, the gene expression pattern from stroma was able to discriminate between tumour and non tumour samples [52].

2.2.7.3 Age-related Changes

Prostate cancer is multi-focal and heterogeneous [53]. That multiple tumours are detected within the prostate argues against the random occurrence of cancer-causing mutations within the epithelium as the main cause of these tumours. Rather, intrinsic as well as extrinsic changes of stroma are likely to influence tumourigenic potential [54]. Incidence of cancers, including that of the prostate, increases with age [54]. Age related changes occur not just in the epithelial compartment, but also in the stroma [55] and it may be that these changes in the microenvironment can promote or initiate cancer [54]. Whilst there are differences in potential, both senescent and cancer- associated fibroblasts are able to stimulate proliferation and invasion of initiated epithelium [54]. Accumulation of senescent cells is expected with age. However, whilst senescent fibroblasts are observed in BPH and prostate intraepithelial neoplasia (PIN), they are absent from invasive PC [55], implying that changes in stroma (specifically, cells re-entering the cell cycle) are an early event in prostate tumourigenesis.

The expression signature of senescent prostate fibroblasts includes proliferation and survival factors [54]. The influence of senescent stroma is thought to be largely via paracrine signaling [55]. For example, production of paracrine factors by fibroblasts are able to stimulate AR independent of androgens [54]. Thus increased mitogens from aged fibroblasts may compensate for age-related loss of androgens [54]. It has also been proposed that senescent cells may determine response to therapy [55].

2.2.7.4 Stromal Transformation

The ability of transformed epithelial cells to form tumours is inhibited (to some degree at least) when implanted in normal stroma. Modified or activated stroma is required for the full cancerous potential of the epithelium to be realized. This activated stroma may share characteristics of the normal stroma and of the transformed epithelium. Normal fibroblasts are distinctly anti-proliferative [49], with paracrine signals inhibiting characteristic traits of transformed cells [49]. Interactions between prostate epithelial and stromal cells are complex, with a temporal factor influencing the effects [56]. Stromal expression patterns are better than epithelial for discriminating between intra and extra- tumoural regions [52]. Initially tumour stroma inhibits PC growth but in later stages enhances cancer growth [56]. Thus it is likely that alterations of both the epithelial and fibroblast compartments are required for cancer development, and dormancy may be an effect of the balance between epithelial transformation and stromal inhibition [49]. For example, TGFβ-refractory fibroblasts result in PIN while TGFβ-responsive fibroblasts do not [46]. Normal fibroblasts overriding transformed cell signals may maintain tumours in a more benign state [49], and may be a reason for the low rate of cancerous tumours developing from those with transformation potential. In contrast, the activated stroma is believed to release paracrine stimulants thus contributing to tumour growth [49]. Cancer-associated fibroblasts (CAFs) demonstrate the same phenotypic changes as those observed during wound healing [49]. A part of normal wound healing involves recruitment of inflammatory cells, which inhibit apoptosis, stimulate proliferation by releasing of growth factors or cytokines, and morphogenesis [48]. This may in part explain the expression profiles of tumour subtype denoted Prostatic Inflammatory Atrophy (PIA) [42].

A limitation of cancer therapy is the ability for drugs to reach the tumour cells. Given the proximity of stromal cells to vasculature, they represent a better target than epithelial cells [52].

2.2.7.5 Basement Membrane

Polarity of epithelial cells, as well as proliferation and migration, is maintained by interactions with the extra cellular matrix (ECM) [48]. Alterations of the cell-cell and cell ECM interactions are observed with cancer progression [57]. In the prostate, the stroma is normally separated from the epithelium by the basement membrane [48]. Disruption of the basement membrane by tumour cells may be necessary not only for exceeding tissue boundaries, but also for releasing stimulatory growth factors normally associated with wound healing responses [49]. Some matrix metalloproteases (MMPs) increase cancer progression [58]. Constitutive activation of some MMPs in PC is thought to be due to a furin-like mechanism [58]. PACE4 processes proIGF2 to the active IGF2, which is able to stimulate proliferation [59]. PACE4 is associated with an increased invasive phenotype and MMP11 [59]. These interactions are vital in advanced cancer where a secondary site must be permissive of tumour growth for a metastatic cell to implant. It is interesting therefore that it has been shown that osteoblasts secrete cytokines (namely TGFβ) which cause cell migration and invasion [56]. Thus these cells appear to have been "primed" for supporting seed tumours, however this idea requires further investigation.

2.2.7.6 Practical Implications

Until recently most analysis, particularly of gene expression profiling, has focused on whole tumour material [40] which ignores the differences in cell types and functions. In addition, awareness of the inaccuracies resulting from varying degrees of contamination, as well as the power of analysing the compartments separately, is growing. Whole tissue approaches lack sensitivity to discrete/small changes [53]. The stromal compartment is reduced with increasing Gleason grade [53], thus changes in this compartment are likely to be lost, as the signal is drowned in the information available from the epithelial compartment.

2.2.8 Biomarkers

Therapies for PC have detrimental side effects, particularly where quality of life (QoL) is concerned, thus achieving the correct balance between early detection of aggressive disease *versus* over-diagnosis and treatment of latent PC is difficult. Biological markers are used to aid diagnosis, monitor treatment success or failure. Currently prostate specific antigen (PSA) is the only biomarker generally used, however restrictions in specificity and sensitivity of PSA testing demand a greater range of biomarkers.

2.2.8.1 PSA

PSA is a serine protease expressed almost exclusively by the prostate, under the regulation of androgens [60]. PSA is a component of seminal fluid, where is reaches concentrations of 0.3-3mg/ml [60]. PSA is not secreted into blood, rather the normal prostate architecture prevents all but a little leakage into the blood, where it can reach concentrations of 0.6ng/ml [60]. Aberrations of prostate structure cause increased leakage of PSA, thus increased levels (as much as 10-fold increases) observed in the blood of patients with PC [60]. Increased blood PSA levels are therefore a surrogate marker for abnormal growth, proliferation and structure of the prostate.

The PSA threshold for biopsy is currently debated, as no single cut-off achieves both high specificity and sensitivity [60]. Currently a cut-off limit of 4ng/ml is used. Levels

above this are considered to indicate risk [61] although this limit is not biologically founded [61]. Between 4-10ng/ml is a grey area, where only 25% of subjects have PC [61]. Age is the main factor in determining PSA levels, thus it has been proposed [60] that age-specific PSA ranges may improve diagnostic accuracy, however these have yet to be established [61].

PSA levels are reported to have predictive value in the long- as well as short-term [60]. PSA levels measured decades before diagnosis were able to predict advanced disease [60]. Furthermore, with BPH incidence increasing with age, the diagnostic ability of PSA levels declines with increasing age [60]. More PSA is released from PC than BPH tissue, thus PSA density (ratio of PSA concentration to prostatic volume) may improve differentiation between PC and BPH [60].

The dynamics, derivatives or complexes of PSA may give increased diagnostic information. PrePSA undergoes co-translational processing to proPSA, with a further removal of 7aa being needed to produce mature PSA [60]. Nicked PSA (nPSA) has an additional cleavage step (between lysines 145 and 146) [60]. When compared to total (tPSA), nPSA is better at discriminating BPH from PC [60]. Use of PrePSA, complexed or free PSA may enhance specificity over total PSA levels, however there is no consensus on this to date [61]. It has been suggested that undifferentiated PC may give normal PSA levels [62].

PSA velocity is strongly associated with PC diagnosis and recurrence [60] with velocities exceeding 0.75ng/ml/year being associated with increased PC risk [61]. High day to day variations make this measurement impractical [61] and it is unclear whether it adds to diagnostic or predictive accuracy [60].

It should be noted that in addition to malignancies and BPH, general stress and inflammation may also give increased PSA levels [61].

As yet, PSA screening is not widespread, although is fairly common in ‘at risk’ families (discussed further). As demonstrated by Figure 5, the number of cases potentially detected by PSA screening does not correlate with the number of life-threatening cases. Many regions are hesitant to introduce PSA screening in the general population. The risk of false positive diagnosis, resultant over treatment and reduction in quality of life, psychological impact of borderline diagnosis, workload and cost are believed to outweigh the minor gain in survival. A recent study indicates that, in the United Kingdom at least, predicted trends of prostate cancer have been exceeded since the introduction of even limited PSA screening [63]. Whilst the increase in detected cases between 1991 and 2000 is moderate (6%), it is significant [63].

2.2.8.2 Other Potential Biomarkers

A number of potential biomarkers are being investigated, to determine whether they outperform or compliment PSA levels with regards to diagnostic or prognostic information:

- HK2 is a serine protease with 80% homology to PSA [60, 61]. The promoter contains androgen-responsive elements [60] thus it is mainly expressed by the prostate [61]. HK2 levels are only 1% of PSA levels [61] and may be an independent marker for recurrence, particularly in those with PSA <10ng/ml [61]. Whilst this may be a good initial presentation marker, but is likely to be influenced by anti-androgen therapy [61].

- EPC is a nuclear matrix protein observed in association with PC [61]. PC biopsies more intensely stained than negative controls [61]. A majority of Japanese PC demonstrates EPC staining (94%), which may reflect early nuclear matrix alterations [61]. An assay of blood samples is both specific and sensitive [61].
- uPAR is part of the plasminogen activation cascade and thought to be mainly involved in ECM degradation during cancer progression. It's use for early PC detection is debated [61]. Increased predictive power is gained for assessment of uPAR fragments than total/intact uPAR, especially when combined with free PSA measurements [61].
- IL-6 levels are increased in PC and pretreatment levels are predictive of recurrence [61]. IL6 expression is inhibitory in AD tumours, but stimulatory in AI tumours [61]. This could suggest that its effects are secondary and dependant upon other cytokine/hormone signaling pathways.
- TGFβ expression has been associated with PC grade, stage and lymph node metastasis [61]. An association with extra-capsular extension and invasion is debated [61]. Pretreatment levels are predictive of recurrence [61].
- PSMA demonstrates highest expression in the prostate [61], but this molecule is not currently used as a marker.

2.2.9 Models of Prostate Cancer

Despite the research conducted into PC, there is as yet no general model for its development and progression [40]. Thus there is a large non-genetic or environmental component. The environmental component of PC risk includes diverse factors, such as exposure to sunlight or carcinogens and diet or physical activity.

The classical models of prostate cancer only take into account the genetic contribution: Hereditary prostate cancer (HPC) is defined as having either ≥3 relatives with PC in a nuclear family, PC in 3 successive generations (either maternal or paternal lineage) or 2 first degree relatives (FDR) diagnosed with PC ≤ 55 years of age [64]. Those patients with at least one FDR with PC but not fulfilling the above criteria are defined as having familial prostate cancer (FPC). Subjects which do not fit these criteria are classified as sporadic prostate cancer (SPC) [65].

A number of environmental effects have been studied in relation to PC risk, however the importance of lifestyle factors such as diet and physical activity have been underappreciated. With the current global focus on obesity and associated disorders, the interest in the role of metabolism is growing. In particular the possibility of modulating PC risk by lifestyle adjustments is of keen interest to healthcare providers worldwide. Using an additional classification of prostate cancers, i.e. those with concomitant metabolic disorders (here referred to as metabolic prostate cancer, MPC), may enrich for common factors (including genes) influencing disease occurrence and progression.

2.2.10 Family History

Clustering of prostate cancer within families suggests a strong hereditary component. Indeed it is believed that 40% of PC risk is inherited, whilst 60% is environmental [41]. Given that prostate cancer onset occurs after reproductive age, there is no obvious selection pressure against carriers of influential genes.

The etiology of PC is highly heterogeneous. At presentation it is not possible to differentiate between HPC and SPC, as clinical and diagnostic characteristics are very similar. The only exception is that younger age at onset may be an indicator of HPC. PC is a common disease, therefore most men diagnosed with prostate cancer will, by chance alone, have another member of the extended family with the disease. Thus it is difficult to determine whether each case of PC in a family is hereditary or sporadic. Enriching sample sets for HPC cases is particularly useful for genetic analyses.

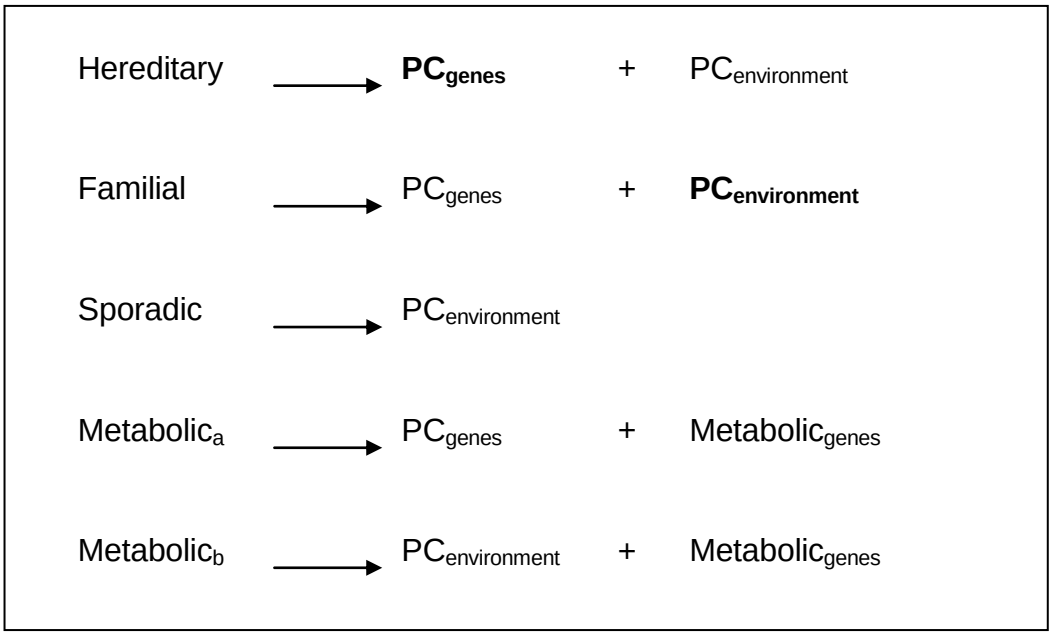

*Figure 6: Proposed classifications of PC and models of interactions between genes and environment, where **bold** type indicates the dominant effect.*

2.2.11 Genetics

Generally speaking, variations in genes may result in variable function or levels of the encoded proteins. Variations which exist in the general population at a frequency of >1% are defined as polymorphisms. Those at a reduced frequency are termed mutations. Commonly observed variations are amplifications, deletions and single nucleotide polymorphisms. It has been estimated that only 5-15% of cases are likely to be associated with highly penetrant genetic variations [66]. Rather, variations in each candidate gene have only a small effect, thus a combination of minor effects, acting together with the environment, are responsible for tumour development. This would explain the heterogeneity in clinical progression of PC.

The heritable fraction of PC risk appears to be constant among populations, however incidence and outcome differ [65]. Dominant, recessive and x-linked modes of inheritance have all been suggested. It has been proposed that genes predisposing to PC are distinct from those genes modulating aggression [65].

2.2.11.1 Germline Variations

These genetic variations are inherited and inherent in every cell of the body. Large chromosomal regions have been linked to PC risk, however the exact genes involved have yet to be identified or confirmed. Regions of chromosomes 1, 8, 17, 20 and X have been implicated in PC [41].

- Hereditary prostate cancer region (HPC1) is a region spanning chromosome 1q24-25 [67]. Familial association studies indicate male to male transmission of

risk variants which are associated with early age of diagnosis [67]. More important to those of African descent [68].

- HPC2 on chromosome 17p11 is implicated in HPC [65, 67].
- HPC20 is located at chromosome 20q13 [67]. The strongest association for this region is observed with fewer than 5 family members diagnosed after the age of 65yrs [65]. Male to male transmission is not observed [65].
- HPCX is chromosome Xq27-28 [67]. No male to male transmission observed [65]. No candidate genes have been identified.
- PCAP at Chr1q42.2-43 is associated with a younger age of diagnosis [65].

2.2.11.2 Somatic Variations

Other genetic variations are not inherited, rather they occur in discrete cellular populations during the subjects lifetime. Most frequent changes seen are 8p and 13q, with early gains of 8q and Xq implicated [66]. Chromosomes 4, 6 and 7 have been implicated but not yet replicated [65]. Amplification of 8q is associated with poor survival [69]. More recently, three independent regions at 8q24, one at 17q12 and one at 17q24.3, have been confirmed as being associated with PC risk [70]. Further more, when the genotypes of these regions are combined with family history, those subjects at increased risk (OR 9.46) can be identified [70]. Despite most of these genes being of unknown function/effect, this may be an effective clinical test. Specifically, rs443076 (17q12, risk genotype TT), rs1859962 (17q24.3, GG), rs1447295 (8q24 region 1, CA/AA), rs16901979 (8q243, region 2, AA/AC) and rs6983267 (8q24 region 3, GT/GG) when combined with family history give a population attributable risk of 46% [70].

Loss of DNA is 5-fold more common than gain [66] in primary tumours. Thus it is likely that loss of tumour suppressors are an early event in PC [66]. Activation of oncogenes are also implicated, but in later stages of PC [66]. Loss of heterozygocity of chromosome 5 is associated with PC stage [67].

Despite extensive studies into the regions and genes responsible for risk, few common variants have been confirmed as important. Several attempts at replicating association of genes with PC risk have failed to confirm previous findings. Reasons for this include the heterogeneity of PC cases included in the studies, the populations included in the studies and small numbers of cases and controls resulting in a lack of statistical power of detection. In addition, the diverse genetics of different populations cause differences in allele frequencies [71]. For this reason combining different genetic backgrounds may dilute the effect of any one population. For example, the Japanese population demonstrates a higher frequency of RAS mutations (25%) than Caucasians (almost never). In contrast, advanced PC in Caucasians often has P53 aberrations, whilst these mutations are very rare in the Japanese population. A study on a large and relatively homogeneous population was carried out recently, to systematically replicate many implicated genetic variations [72], providing important data on candidate genes.

Table 1: Genes implicated in prostate cancer

Locus	Gene	Function	Ref.
1p13	*HSD3B*	Part of the AR pathway .	[65]
1p13.3	*GSTM1*	Involved in metabolism. A homozygous deletion not associated with PC risk	[65, 72]
1p13.3	*GSTM3*	A deletion investigated, but not associated with PC risk	[72]
1q21-23	*CRP*	A general inflammatory marker. High levels associated with poor prognosis and seen in bone metastasis. Elevation in metastatic cancer attributed to nutritional decline. PSA associated with CRP levels.	[57, 73]
1q24	*MUC1*	Mucin 1 or KL-6. Cell surface glycoprotein. Aberrant expression levels and cellular localization in PC. Loss of heterozygocity with mutations in 71% of remaining alleles.	[66]
1q25	*RNASEL*	Located within HPC1. Antiviral and pro-apoptotic activity. Truncating mutations observed in PC.	[65, 66]
1q36	*EPHB2*	Ephrin receptor B2. Also known as prostate cancer-brain cancer associated protein. Predisposes to younger age for PC diagnosis in families strong for inheritance of these cancers. Replication has not been possible as there are only a few such families	[65, 67, 68]
1q42-43	*PCTA1*	Located within the PCAP region. Variants do not appear to be functional and no major role has been described. May be associated with younger age at diagnosis.	[65]
2p21	*CYP1B1*	5 SNPs investigated, none associated with PC risk.	[72]
2p23	*SRD5A2*	A component of the AR pathway. Influences levels of testosterone product which may be linked to PC risk. A missense variant is not associated with PC risk, but the variant allele of 2 SNPs (rs676033 and rs523349) are associated with PC risk.	[65, 71, 72]
2q37.1	*TRPM8*	Predicts PSA relapse.	[40]
3p26.2	*OGG1*	2 SNPs studied, neither was associated with PC risk.	[72]
4q13-21	*IL8*	Increased in AI and metastatic PC. 1 SNP investigated, not associated with PC risk.	[61, 72]
5p13	*AMACR*	Over expressed in PC compared t normal prostate. Auto antibodies to AMACR detected. 4 SNPs not associated.	[42, 61, 72]
5p13-12	*GHR*	Growth hormone receptor. Not expressed in adult normal prostate epithelium, observed in BPH and PC. Maps to a chromosomal region implicated in PC linkage studies. Variation not associated with PC risk.	[74]
6q25.1	*ESR1*	Estrogen receptor α. Adverse effects of estrogen via this receptor. Antagonists can reduce PIN and PC in mouse and human. 1 SNP not associated with PC risk.	[72, 75]
6q25-26	*EZR*	Ezrin. Mediates androgen-dependent invasion of PC.	[76]
7p13-12	*IGFBP1*	High levels are a risk factor for PC, especially with low IGFBP3 levels.	[77]
7p13-12	*IGFBP3*	Low levels are a risk factor for PC, especially with high levels of IGFBP1.	[77]
7p15.2-15.1	*JAZF1*	rs864745 implicated in PC risk	[19]
7p21	*IL6*	Inflammatory cytokine. May be used as a serum biomarker.	[61]
7q35-36	*EZH2*	Protein expression increased from benign to metastatic. When combined with normal markers, better predictions of recurrence.	[40]
7q36	*SHH*	Over expression increases PC proliferation. Use of an antagonist inhibits growth. Increased in PC compared to BPH.	[78]
8p22	*NAT2*	Involved in metabolism. 1 polymorphism not associated with PC.	[65, 72]
8p23.1-23.3	*NAT1*	Involved in metabolism. 1 SNP (rs15561) demonstrated border line association with PC.	[65, 72]
8q21	*NKX31*	Haplo-insufficiency may cause inactivation, thus implicated in PC.	[66]
8q21	*TCEB1*	Amplified in 23% AI tumours.	[66]
8q21.3	*CYP7B1*	Regulates estrogen biosynthesis and ERβ levels.	[75]
8q22-23	*MSR1*	Loss of heterozygocity is associated with PC. Of 6 SNPs investigated, only one (IVS5-59C>A) was associated with PC. Functional significance of SNP unknown.	[65, 72]
8q23	*MF3-p40*	Amplified and over expressed in PC cell lines and 30% of AI and 18% of primary tumours. Amplification associated with high Gleason grade	[66]
8q24.21	*comic*	Amplified in 70% AI tumours. mRNA levels not changed, thus posttranslational regulation. Needed for both AD and AI PC growth. Potential therapeutic target.	[79]
9q22	*HSD17B3*	1 SNP investigated but not associated with PC.	[72]
10q23.3	*PTEN*	A late stage deletion. Haplo-insufficiency could explain loss of function. Loss is associated with an increased stem cell-like population of PC cells.	[46, 66]
10q24	*PLAU*	Urokinase. Inhibition may be advantageous for tumour cells increased expression in PC	[66, 80]
10q24.3	*CYP17*	Involved in testosterone biosynthesis. Disputed that variations increase risk in European Americans but not those of African descent. A SNP (rs743572) associated with PC. A 5' UTR variant protective again PC.	[65, 72]
11p11.2	*FOLH1*	Prostate specific membrane antigen. Potentially a serum biomarker.	[61]
11q13	*CCND1*	1 SNP not associated with PC risk	[72]

11q13	*GSTP1*	Involved in metabolism. Inducible factor preventing oxidative damage and macromolecular damage. Carcinogens may be substrates for this enzyme. Loss of GSTP1 allows accumulation of alterations. High levels observed in PIA, but not normal prostate epithelium, PIN or PC. Loss of GSTP1 in a PC cell line results in increased DNA damage and reduced oxidative stress-induced cell death. 1 SNP not associated with PC.	[72, 81]
12q13.11	*VDR*	Vitamin D receptor. Involved in metabolism. Poly A>18 increases risk of PC. Inconsistent replication of SNP associations with PC risk.	[72, 82, 83]
12q22-23	*IGF-I*	Insulin-like growth factor 1. Mitogenic growth factor implicated in many cancers. 1 polymorphism investigated but not associated with PC. High normal levels give 2 fold increased risk.	[72, 84]
13q12.3	*BRCA2*	BReast Cancer Associated 2. Variations give increased risk of PC, especially at a young age	[65, 68, 85]
14q11-12	*CIDEB/ LTB4R2*	The variant allele of an SNP (rs2144493) in the 3' UTR of CIDEB and the promoter of LTB4R2 increases the risk of PC by 55%.	[86]
14q23.2	*ESR2*	Estrogen receptor β. Expression levels of ERβ correlate inversely with Gleason score, but may also be observed in metastatic lesions. Expression commonly lost in high grade PC due to promoter methylation. A promoter SNP (-13950 C/T) gives an increased risk of 35% for localized PC.	[75, 87]
15q21.1	*CYP19A1*	Responsive to inflammatory cytokines. 1 SNP not associated with PC risk. Additional promoters used in prostate cancer stroma compared to normal prostate stroma.	[72, 75]
15q21-24	*CYP1A1*	1 SNP not associated with PC risk.	[72]
16q22	*CDH1*	E cadherin. Required for ca^{2+} dependant cell adhesion, normal cell growth and development. 25% cases demonstrate reduced expression. Increased cleavage in neoplastic prostate tissue. Potential serum biomarker.	[66, 88]
17cen-q21.3	*HNF1B*	TCF2. 1 SNP associated with PC	[19, 29]
17p11.2	*ELAC2*	LOH seen in a limited number of HPC families, but little evidence for SPC. 2 SNPs not associated with PC risk.	[65, 66, 72]
17p13.1	*TP53*	Tumor protein p53. Few mutations seen in Caucasian primary tumours, increased frequency in later stage tumours. Dependant upon population, with increased occurrence in Asians. 1 SNP investigated was not associated with PC.	[66, 72]
17q21	*BRCA1*	Breast Cancer associated protein 1. A founder mutation with population frequency 2% doubles PC risk.	[65, 66]
17q21.1	*ERBB2*	Also denoted HER2/NEU. Contraversial role in prostate cancer. Over expression causes AI, and indicates synergism with AR in the context of low androgens	[66]
19p12	*CRLF1*	Homozygotes carrying the variant allele of an SNP (rs7250623) in the 3' UTR of CRLF1 increases the risk of PC by 37%.	[86]
19p13.2	*ICAM 1*	Maintains tissue architecture. Located within a PC-associated region. Variations associated with PC in African Americans. Not associated with PSA level, severity or age of onset.	[57]
19p13.2	*ICAM 4*	Maintains tissue architecture. Located within a PC associated region.	[57]
19p13.2	*ICAM 5*	Maintain tissue architecture. Located within a PC associated region. Mainly expressed in distinct areas of the brain. Rs1056538 and rs2228615 associated with PC predisposition.	[57]
19p133	*FCER2*	An SNP (rs753733) 5' of the FCER2 gene appears to protect against PC.	[86]
19q13	*PLAUR*	Urokinase receptor. Potential as a biomarker	[61]
19q13.1	*TGFB1*	Increased expression associated with grade, stage and LN metastasis. Has potential as a biomarker.	[61]
19q13.3-13.4	*IL11*	Increased in AI and metastatic cancer. Potential as a serum biomarker.	[61]
19q13.41	*KLK2*	Involved in metabolism. Similar to PSA. May be used as a biomarker	[61, 65]
19q13.41	*KLK3*	PSA. IGFBP3 protease. Currently the only clinically accepted biomarker for treatment response and recurrence.	[84]
20q12	*NCOA3*	Variations in repeat region not associated with PC risk.	[72]
20q13	*CSEIL*	Located within the HPC20 region. Over expressed in PC	[65]
20q13	*MYBL2*	Located within the HPC20 region. Over expressed in PC	[65]
20q13	*STK15*	Located within the HPC20 region. Over expressed in PC	[65]
20q13	*ZNF217*	Located within the HPC20 region. Over expressed in PC	[65]
21q22.3	*COL18A1*	1 SNP not associated with PC risk.	[72]
22q11.3	*GSTT1*	Involved in metabolism, genetic deletion associated with PC.	[65, 72]
22q13.1	*CYP2D6*	Involved in metabolism	[65]
Xq11-12	*AR*	Androgen receptor. Receptor for androgen signaling. . Amplified in 30% of AI tumours. CAG repeat length inversely correlates with risk, This has recently been confirmed, at least in the CAPS study	[66, 71, 72, 89]
Xq13	*PGK1*	Variations not associated with PC risk.	[72]
Where: AD androgen dependent, AI androgen independent, PC prostate cancer, SNP single nucleotide polymorphism, HPC hereditary prostate cancer, SPC sporadic prostate cancer.			

2.2.12 Epidemiology

Prostate cancer is primarily a disease of the elderly, with typical clinical prostate cancer occurring in the 6^{th} - 7^{th} decade. Clinical diagnosis before the age of 65 years is rare, thus these patients are considered young. Information as to family history is frequently lacking, due to the age of the patients. Conclusive information is missing because of less specific diagnosis in previous generations or because relatives are deceased.

Large sample sets are used to study genetic and environmental factors alike. Whilst minimizing false positive findings, important effects in a subset of the population may be missed. HPC subjects are normally separated from SPC subjects, although sporadic cases may occur in HPC families. The use of familial cancer grouping is not widespread, although used in some cases.

As with most cancers, increasing age is associated with increasing risk. As yet, the only other well established risk factors for PC are family history and race.

2.2.12.1 Socioeconomic Factors

Studies of socioeconomic factors have mainly been carried out in the USA, thus the structure of their healthcare system should be considered when interpreting the findings. These factors include education, access to healthcare and lifestyle. Complex interactions between factors hinder the efforts to elucidate causative factors.

Reduced literacy is associated with increased stage at presentation [90]. Less affluent areas are likely to have a poor diet of generally lower quality and lack the beneficial foods [91]. Chronic diseases, stress and untreated depression may influence hormone levels and ability to respond to infection [91]. Increased incidence of overweight is observed in affluent areas [91], and adipose tissue is the main store for environmental toxins [91].

2.2.12.2 Ethnicity

Variations in clinical practice are estimated to account for half of the differences observed between populations [81]. Much of the research into PC is carried out in the USA, where the healthcare system is biased towards the affluent. This does not necessarily give an accurate picture of PC incidence, treatment or treatment success.

Caucasian men have a 34% higher incidence of PC than Asian men, whilst incidence in men of African descent is 200% that of Caucasian men [71, 81]. The highest incidence is thought to be in sub-Saharan Africa [90], although the lack of registries hinder accurate assessment.

American blacks are less likely to have radical surgery and radiation therapy than whites [90] and black men present higher grade within the same stage [90]. However this does not explain the difference in mortality between black and white men, as similar treatment gives similar results [90].

Ethnic variations in AR polymorphic CAG repeat length may influence PC [71, 89]. African Americans have shorter repeats, which correspond to lower levels of androgen needed for AR stimulation.

2.2.12.3 Environment

The most obvious evidence to suggest that environmental effects have a role in determining prostate cancer risk is the racial and geographical distribution of this

disease. The impact of each environmental effect is hard to establish due to the complexity and variability of interactions as well as increased mobility of populations.

Migration studies have proved very useful in determining the effects of genetics versus environment on risk of PC. Cancer incidence in Japanese migrants to Hawaii increases to similar rates to those of natives [71], but childhood exposures appear to be important as the same level of cancer risk is not achieved until after the first generation [71].

The geographical distribution of PC risk indicates that a factor lacking in polar regions compared to equatorial climates may be influential in disease occurrence. Exposure to sunlight appears to be important. An inverse relationship is observed, where areas of the USA with more sunlight exposure have a reduced incidence of PC compared to areas with fewer hours of annual sunlight [83]. Vitamin D (D3) and its receptor (VDR) have been proposed as the modulator of this effect. It has been shown that 1,25(OH)2D3 levels vary with ethnicity, with white men having increased levels compared to their black counterparts [92], which may explain in part the increased risk of PC seen in black men compared to white men [92].

2.2.12.4 Lifestyle

Unlike many other cancers, lifestyle factors such as tobacco use, physical inactivity and alcohol do not seem to increase the risk of prostate cancer [65]. However some studies indicate that physical activity suppresses tumour growth [30].

Sexual activity has been more controversial, with some reports indicating that it has no effect [65], whilst others highlight a potential role for sexually transmitted diseases and consequent chronic inflammation in the development of tumours [81].

The "western" lifestyle has been associated with PC [41], however this comprises many factors including physical inactivity, poor diet and other components. The exact factors involved, and the mechanisms of effect, are not clear.

It is hard to analyse and replicate investigations into lifestyle, as these factors also cluster in families [65], making an effect difficult to distinguish from hereditary factors.

2.2.12.5 Occupational

Assessing occupationally increased risk has indicated that only farming increases an individual's risk of prostate cancer, and that this effect is very mild [65]. Whether this is due to an unknown exposure specific to farmers has not been clarified. The high level of physical exercise and relatively high exposure to sunlight could be expected to decrease risk. However, traditional diets rich in meat and general robust health of farmers may counter the positive lifestyle effects.

2.2.12.6 Infection and Inflammation

That chronic infection and inflammation can result in cancer has been shown for gastric cancer. Support is gathering for a similar mechanism in PC.

It has been suggested that sexually transmitted infections may increase prostate cancer risk [81], presumably through inflammation. An investigation into genetic variation of genes involved in inflammatory pathways has recently noted that variants in at least 3 genes alter the risk of prostate cancer [86]. Whilst the same study confirmed the initial findings in an independent sample set, the effect of these SNPs on the function of *CRLF1*, *FCER2* and *CIDEB/LTB4B2* protein products is as yet unknown [86].

Furthermore, a strain of fatless mice that demonstrate overt diabetes and inflammatory markers is prone to cancer, indicating that systemic inflammation and altered metabolism, rather than fat, is permissive of cancer [30]. PSA levels are increased by infection, and CRP is a general marker of systemic inflammation, so it is interesting that CRP and PSA levels are associated with metastatic PC or poor prognosis [73].

Retrospective studies suggest that anti-inflammatory steroids may have a role in preventing prostate cancer by inhibiting chronic inflammation. Targeting chronic inflammation may be a prevention strategy [73], however pprospective intervention studies have yet to report on any benefit.

With regards to metabolic alterations stemming from inflammation, aromatase expression, and thus increased local estrogen production, is stimulated by inflammation [75]. This is intriguing, as local estrogen increases would be expected to give reduced local testosterone levels, thus could help protect against prostatic growth. Further investigation into this is required.

Diet

A large part of the global variation in PC risk is thought to be attributable to diet [93]. Not only is metabolic imbalance (greater intake than expenditure) implicated in PC, but specific food groups appear to be important in determining risk. Metabolic homeostasis involves a complex network of signaling mechanisms, therefore the individual components are hard to pinpoint. A number of well designed studies have elucidated some components, however it remains extremely difficult to carry out accurate and reliable assessment of dietary patterns. It remains to be seen whether dietary alterations will reduce the risk of PC. However, it should be noted that PC has a long period of latency, so any beneficial effects will not be seen for some time.

The uptake of certain beneficial compounds, such as phytoestrogens, is dependant upon gut microflora, which ay be modified by dietary fat, antibiotics, alcohol [94] and other environmental exposures.

Caloric restriction has been shown to reduce cancer risk [95], although it is unclear whether this is due to food, or rather via indirect effects on body composition or hormones including testosterone, leptin or adiponectin [95].

1. **Fat**

   Dietary fat is associated with PC, independently of total energy intake or BMI [96]. Dietary fat may influence androgen and IGF-I levels [96]. Metabolites of fatty acid oxidation produce reactive oxygen species and pro-inflammatory factors [96]. Saturated animal fat consumption has been associated with PC, particularly when from red meat [65]. Other studies suggest that total fat intake is more influential that saturated fat *per se* [81].

2. **Meat**

   Red meat contains high levels of zinc, required for testosterone production [65], which correlate with an increased risk of PC [65]. Cooking meat at high temperatures causes formation of polyaromatic hydrocarbons [65], which have been implicated in a variety of cancers. A confounding effect may be the lack of protective vegetables in a diet high in red meat [65, 81].

3. **Fish**

Salmon is a major source of omega-6 and omega-3 poly unsaturated fatty acids (PUFAs), which promote and inhibit PC growth respectively [45]. Omega 6 PUFAs are associated with progression, increased proliferation and reduced apoptosis [45]. Intake of omega 3 PUFAs associated with decreased risk of metastatic PC [45], although it make be the ratio of omega 3: omega 6 PUFAs, rather than specific intake, which is crucial [45]. In light of bone being a preferential site for PC metastasis, it is of interest that bone marrow is rich in lipids, especially omega 6 PUFAs [45].

4. Vegetables

Vegetable consumption reduces PC risk [65, 81]. Not all studies have seen this association, therefore it has been proposed that certain vegetables, rather than total intake are important [93]. Which fruit, vegetables or vitamins are responsible for the protective effect is debated, although a role for vitamins A [83] has been proposed. A recent study found that PC risk demonstrated no association with vitamin A or β carotene, but a weak association with vitamin C vegetables and a non significant association with cooked tomatoes [93].

5. Dairy Products

High consumption of dairy products is also a factor of the western lifestyle and has been associated with an increased occurrence of prostate cancer [41]. Calcium from dairy products may increase PC risk [97] through modification of the Vitamin D homeostasis [65]. Milk consumption has been associated with increased risk of progressive disease [97].

6. Other Dietary Factors

Soy products and derivatives are a major source of phytoestrogens, which is believed to account for some of the difference in PC risk between Western and Asian populations [94].

A few studies have reported beneficial effects of mineral supplementations such as selenium [41, 97, 98], Vitamin E [97, 98] and α-tocopherol [41]. These micronutrients reduce PC occurrence by 66% and 40% respectively [41]. Lycopene also reduces PC risk [41], and believed to be the “active” component accounting for the protective effect of tomatoes [98]. The bioavailability of lycopene differs between raw tomatoes compared to cooked tomatoes [97].

2.3 INTERPLAY BETWEEN T2D AND PC

2.3.1 Cancer Metabolism

Alterations in metabolism have long been associated with cancer [96]. The increased cellular proliferation demands an increased supply of energy, normally with an increased rate of glycolysis being an indicator of malignancy. This is the basis of some imaging techniques, such as FDG-PET [96]. In the case of PC this particular technique has not proven successful, suggesting that glycolysis is not a major source of energy for malignant prostatic cells [96]. It has been proposed that fatty acid metabolism is the main energy source for PC [96]. Fatty acid synthase is required for fatty acid metabolism, in particular for biogenesis of cell membranes [96], and is over expressed in PC with highest levels being observed in AI bone metastasis [96]. Sterol coenzyme A desaturase is lost in PC compared to normal prostate epithelium [96]. This results in a reduction in TGs synthesis and storage and promotes fatty acid oxidation [96]. AMACR is an enzyme found in both the peroxisome and mitochondria [96]. The activity of AMACR is a prerequisite for β oxidation of fatty acids [96] and is consistently up regulated in PC [96]. There are a number of AMACR variants, some of which are associated with increased PC risk [96].

2.3.2 Obesity

It has been proposed that insulin resistance and the resulting hyperinsulinaemia precede obesity, diabetes and PC. Indeed, obesity may be considered a 'pre-diabetes' state. The influence of obesity on PC is controversial, with conflicting reports indicating both protective [99] and promoting effects [100], as well as a differential effect of obesity on low and high grade PC [35]. The finding that fatty acids rather than glucose are the primary metabolic substrate for prostate cancer [96] adds to the evidence in favor of a direct link between prostate cancer and obesity. Obesity has been shown to increase the risk of PC in older subjects [99], where as a protective effect is given by obesity in early adulthood [99].

Obese subjects typically demonstrate low testosterone [31] and high estrogen levels [100]. In obesity, the main precursor of androgen is converted to an estrogen precursor rather than androgen [99]. Thus with increased fat mass, global levels of testosterone may be reduced. As testosterone and androgen stimulate prostatic growth this reduction in testosterone would be expected to protect against PC. Furthermore, mutations of the AR allow for stimulation by ligands such as estrogen, providing a casual explanation for the differential effect of obesity on AI and AD PC [35].

Adipose tissue secretes cytokines such as adiponectin and leptin, which influence tumour growth [30]. Subjects with obesity demonstrate increased leptin, but reduced adiponectin levels [100]. It has been shown that leptin increases proliferation and reduces apoptosis of AI prostate cancer cell lines [101] and increases cell migration in a MAPK and PI3K dependant manner [101]. Furthermore, leptin stimulates increased expression of TGFβ1 and VEGF, and to a lesser degree, increased bFGF levels in a dose dependant manner [101]. Levels of IGF-I, a known mitogenic factor, are also increased in obese subjects [100].

2.3.3 Detection Bias

Obesity has been associated with worse prognosis after surgery and is associated with increased mortality of some cancers [30, 101] including PC [35]. It is estimated that obese patients are 34% more likely to die from PC [31] and up to 14% of all male cancer deaths are attributable to obesity [101].

Diagnosis and treatment of PC is biased against obese subjects. The prostate is enlarged by obesity which makes the DRE harder to perform. BMI is positively associated with an increased risk of progression after surgery [31] as increased fat mass leads to smaller surgical margins, which increase the likelihood of recurrence [31].

Other components of the metabolic syndrome, for example T2D, may mask symptoms of PC. Patients may mistake urinary obstruction for a diabetic complication such as nephropathy. Subsequently such cases may be diagnosed as cancer at a late stage, adding to the poor prognosis. The higher stage at presentation (compared to lean counterparts) does not explain all of the increased risk associated with obesity [31].

2.3.4 Epidemiology

The coincidence of high rates of PC and T2D in western countries indicates that environment and lifestyle are important. Countries which are becoming more "westernized" in terms of diet and lifestyle are starting to observe the same increase in T2D [102, 103].

2.3.5 Metabolic Syndrome

The clustering of a number of pathological conditions which makes up the metabolic syndrome may be a link between T2D and PC. Physiology of T2D promotes cancer, with increased GH, IGF-I and Insulin levels [30]. When considering only subjects with T2D, the consensus is a slightly lower risk of PC [36]. In all T2D patients this is a 17% reduction in risk, however if only those using insulin to control their condition are considered, the reduction is 51% [36], an association which is independent of PSA testing, age, stage and Gleason score [36]. Diabetic men are also less likely to have a family history of cancer than non diabetics [36]. Diabetic men normally have lower IGF-I and testosterone levels [36], both of which are stimulants for prostatic growth.

2.3.6 Androgen Deprivation Therapy

In addition to metabolic alterations influencing cancer, therapy for cancer can also have adverse effects on metabolism. The resulting hypogonadism in men treated with hormonal therapy can lead to several complications, some of which are thought to be secondary to the increased adiposity produced by low testosterone levels.

It has recently been reported that approximately half of men with prostate cancer die from non-cancer causes [104], indeed PC patients treated with androgen deprivation therapy (ADT) have a higher non-cancer mortality rate than the general population [104, 105]. It s reported that ADT is associated with increased incidence of T2D, coronary heart disease (CHD), myocardial infarction (MI) and sudden cardiac death [105]. Indeed, 27% of ADT-treated PC patients have fatal CVD, making it the most common non-cancer cause of death in this population [106]. The risk of the above diseases increases with short-term ADT and persists, but does not increase further, in long-term ADT [105]. In comparison, surgical castration increases the risk of T2D but not CHD, MI or sudden cardiac death [105]. In a recent study 51% of long-term ADT patients were diagnosed with metabolic syndrome compared with 20% of non-ADT treated PC or healthy controls [104].

Hypogonadism is associated with increased BMI and fat mass, reduced lean body mass (LBM) and increased risk of metabolic syndrome [104]. Thus it is quite plausible that ADT causes adverse metabolic changes leading to an increased risk of diseases such as (but not limited to) T2D and CVD. Testosterone has insulin sensitizing effects [62] and

levels are inversely correlated with fasting glucose, insulin and leptin levels [106] and these parameters are increased in ADT-treated patients [106]. Low testosterone levels are associated with hypertension and risk of T2D [104]. It has been shown that ADT causes increased fat mass [105], increased fasting insulin levels [107] with reduced insulin sensitivity [105, 107], and obesity is associated with increased PC mortality [35].

Testosterone is known to be immunosuppressive [108] and short term gradual repression is associated with increased levels of TNFα, IL6 and SIL6R [108]. AI cancers rarely demonstrate increased IL6, IL4 and IL10 [108]. Long term ADT did not demonstrate increased inflammatory markers compared to controls [108]. Testosterone replacement reduced inflammatory TNFα and IL1b as well as increasing anti-inflammatory IL10 [108]. The alterations in inflammatory status are interesting given its role in concurrent diseases such as T2D.

Low levels of sex hormones, such as those suddenly produced by ADT, are a risk factor for osteoporosis [109]. ADT also reduces bone mineral density thus increasing the risk of osteoporosis [109]. Whilst hip fractures in men are less frequent (in the general population) than in women, they are associated with higher morbidity in men [109]. Greater awareness of the effects of ADT on obesity and bone strength is required [109].

2.3.7 IGF-I

Insulin-like growth factor I (IGF-I) produced predominantly by the liver, which is a target for GH and sex steroid signaling [110]. IGF-I has important for maintenance of β cell mass and thus insulin production [111]. The most studied function of IGF-I is in promoting postnatal growth [112] and influencing proliferation, apoptosis, angiogenesis and metastasis [84]. Whilst insulin regulates short term metabolic changes, IGF-Is influence on metabolism is longer term but requires larger influences such as dietary restriction [112]. IGF-I bioactivity is regulated by the IGF binding proteins (IGFBPs) [112], with IGFBP3 being the predominant binding partner in blood, which sequesters IGF-I and prevents its action on cells [112]. IGF-I is believed to be locally produced by cancers including PC [112]. P53 binding to the IGFBP3 promoter represses transcription, thus IGF-I is more bioactive in P53 null cells [112]. PSA is a protease which targets IGFBP3, reducing the affinity of IGFBP3 for IGF-I allowing for enhanced IGF-I signaling [112].

IGF-I may be a link between prostate cancer and T2D. The effect of IGF-I is opposite in the two diseases, with high levels being a risk factor for prostate cancer [113], but protective of T2D [114].

2.4 GENES OF INTEREST

2.5 MUCIN 1

Mucin 1 (MUC1, also designated CD227, EMA, H23AG, MAM6, PEM, PEMT, PUM) is the best studied member of an extensive family of a large cell surface and secreted glycoproteins. It has been observed that MUC1 is genetically altered, over expressed or aberrantly modified in a large variety of cancers [115-118]. MUC1 has been investigated at length from many angles, however, due to a plethora of different cellular systems, reagents and methods utilized, reports are conflicting thus confusion remains.

2.5.1 *MUC1*

MUC1 is encoded by a single gene, *MUC1*, which has been mapped to 1q21. The human *MUC1* demonstrates homology with other mammals, namely 77% identity with bovines (*Bos Taurus*) and 76% with mice, rats and dogs (*Mus musculu, Rattus norvegicus and Canis familiaris respectively)* (Homologene, [119]). *MUC1* consists of 7 exons (Figure 7, B), all of which contain coding sequence.

2.5.2 Genetic variation

There are 66 SNPs within MUC1 (SNPper database, [120]), of which 26 are in downstream and 3'UTR sequences. 10 SNPs are within introns, 2 are on intron boundaries, 23 are in the promoter sequence and 5 are within coding exons. Of those within coding exons, 3 result in an amino acid substitution. Genetic alterations in non coding sequence may alter the binding of transcription factors, coactivators and corepressors, thus expression levels of mRNA may be influenced by DNA sequence variations. Variations in the coding sequence may alter the structure and therefore function of the protein.

The SNP which determines a number of isoforms, denoted rs4072037, is in exon 2 of MUC1. Whilst this polymorphism is synonymous (i.e. there is no amino acid change), it is in strong LD with another SNP, rs2066981. The common allele (A) of rs206698 is predicted to be preferentially bound by the transcription factor GATA1, compared to the variant allele (G) being preferentially bound by GATA2 (Transfac database), a factor associated with risk of cancer (GENE database [119]).

2.5.3 Protein Structure

Mucins are a large family of glycoproteins that characteristically consist of ≥ 50% o-linked glycosylation. The mucin family comprises 15 members which are either expressed at the cell membrane of glandular/secreatory epithelia, or are secreted into the lumen [144]. Secreted mucins are a major component of mucus.

MUC1 is a type 1 transmembrane glycoprotein, secreted by most glandular/secreatory epithelia. After processing the two fragments remain as a non-covalently associated hetero-dimerized bound at the cell membrane by the transmembrane fragment, until either endocytosis recycles the protein or cleavage releases MUC1n into the extra cellular space.

2.5.3.1 MUC1n terminal (ectodomain, α subunit)

The N terminal of the mature MUC1 contains a variable number tandem repeat region (VNTR), accounting for the majority of the extra cellular domain. A 20aa sequence is repeated in tandem between 30-125 times. Whilst this region is highly polymorphic, the number of repeats fall into two classes, consisting of either 45- 60 repeats (designated here as a short VNTR), or between 90-125 repeats (designated here as a long VNTR). Given that little recombination is observed between the two alleles, it is speculated that the 2 size categories may have resulted from a duplication event, with replication slippage explaining the variation noted [117]. The variable length and variety of possible modifications give heterogeneity to this fragment, with molecular weights in excess of 450KDa. The core peptide of MUC1 has elements which closely resemble Lewis epitopes, and are recognition sites for selectin and ICAM binding.

2.5.3.2 MUC1c terminal (transmembrane domain, β subunit)

The C terminal fragment of the mature MUC1 has a molecular weight of 25kDa [121] and consists of a 58aa extra cellular domain, a 21aa transmembrane domain and a 72aa [121] cytoplasmic tail. MUC1c contains serine-rich motif and phosphorylation motif. Indeed, MUC1 is a substrate for phosphorylation by several kinases, including Src, GSK3β and PKCδ. The cytoplasmic fragment of MUC1 includes 7 cystiene residues highly conserved with those of the cytokine receptor family, suggesting a signaling role for MUC1.

The role of the transmembrane domain of MUC1 is poorly described, however it is reputed to reduce levels of reactive oxygen species (ROS) within the cell, by an unknown mechanism.

2.5.4 Modifications

The importance of post translational modifications in regulation of protein function is now generally accepted and these changes seem to be as important as expression alterations in cellular transformation. Given that the cell is dynamic, with rapid turnover of cellular components, it is possible to speculate that there is no 'final' MUC1 product, rather it is continually being altered in response to cellular signals. With this in mind, when discussing the 'mature' protein this denotes any protein that has the minimal requirements for being expressed on the cell membrane.

The VNTR is rich in motifs for post translational modifications, with each repeat having 5 potential sites [118]. Modifications of glycosylation [122], sialylation [123, 124] and palmitylation [125] have been reported. The most studied modification to date is glycosylation. Generally, two adjacent proline or serine residues are required for o-linked glycosylation [126], whilst one report indicates that glycosyl residues are added to a NXS/T sequence [122]. A high prevalence of threonine/serine outside of the tandem repeat translates into another 5 potential glycosylation sites [124], between the tandem repeat and the C terminal [127]. Although 5 possible sites N- linked glycosylation sites have been reported [126], the most abundant modifications are O-linked [124].

Glycosylation patterns are determined by the repertoire of glycosyl transferases present in the cell [123]. In addition, most modifications take place in the gogli aided by chaperone proteins, which may influence the type and extent of glycosylation that occurs [123]. Furthermore, differential expression of transferases is observed between cellular compartments and cell types [123]. In breast tissue for example, 50% by weight of the MUC1 is composed of carbohydrates, in comparison to the pancreas, where

carbohydrates make up 80% of the weight of the protein [127], despite high similarity in the core proteins.

Palmitylation has been shown to be necessary for recycling, but not for endocytosis of MUC1 [125]. There is no consensus motif for this modification, but evidence suggests a stretch of 8aa between the TM domain and the c terminal[125].

Processing and interactions with the ECM or cells can be changed, depending on the modifications present. Modifications may mask binding sites or locally alter the conformation and properties of the protein. For example, O-linked glycosylation of the DTR motif within the VNTR causes a conformational change resulting in a more rigid protein [128]. The modifications are bulky and negatively charged, thus form a steric and electrochemical barrier around the core protein [124]. Glycosylation at adjacent sites has been demonstrated to be a prerequisite for the action of some enzymes [123]. That the intra cellular domain of MUC1 can be heavily glycosylated [129] implies that modifications may also alter the signaling function of MUC1. It is speculated whether palmitylation is responsible for modulating nuclear trafficking [125].

The patterns of MUC1 modifications and the structures of these modifications differ between normal and transformed cells [124]. Indeed, cancer associated modification patterns have been reported. For example, in prostate cancer high levels of sialylated MUC1 may be a poor prognostic marker [124].

2.5.5 Isoforms

According to current literature there are several isoforms of MUC1, however studies of expression patterns and functions of each isoform are somewhat limited. Further confusion arises from the lack of systematic nomenclature of the isoforms.

MUC1/REP The full length, membrane-tethered protein, consisting of a signal peptide, cytoplasmic, transmembrane and extra cellular domains, including an extensive VNTR. This is considered to be the "normal" protein.

MUC1/A,B,C,D These four isoforms are variants of MUC1/REP, in that they contain extra cellular, transmembrane and cytoplasmic domains as well as a VNTR, that are determined by a splicing event 5' of exon 2. MUC1/A and B are determined by an SNP (rs4072037) in the exon 2 splice acceptor site resulting in 9aa upstream being used for A compared to B [117]. Interestingly, the length of the VNTR appears to be in linkage disequilibrium with this SNP [117]. G at this position corresponds to a long VNTR, A to a short VNTR. Little is known about isoforms C and D, although differential expression has been observed between cancer and normal tissues [118].

MUC1/X,Y,Z These isoforms are determined by splice acceptor sites 3' of exon 2. They are transmembrane proteins, but lack the entire VNTR region, thus are not classical mucins. At least one report [130] suggests that isoforms X and Z are one and the same. Alignment of the protein sequences listed on the Ensembl website contradict this finding.

MUC1/S This isoform is highly homologous to MUC1/Z however lacks 95aa in the extra cellular and transmembrane domains. Little has been reported about this isoform.

MUC1/SEC This is a secreted form of the MUC1 protein, which lacks the transmembrane and cytoplasmic domains. This isoform is the major component of

mucus produced by the epithelium. Conflicting reports describe both shedding of MUC1/SEC from MUC1/REP and translation of MUC1/SEC alone, which is supported by further reports that MUC1/SEC possesses a unique C terminal section not present in either MUC1/REP fragments. As this isoform is not tethered to the cell membrane, thus is unlikely to undergo extensive cycling into the golgi, it is probable that it demonstrates a lower level of glycosylation compared to membrane bound isoforms.

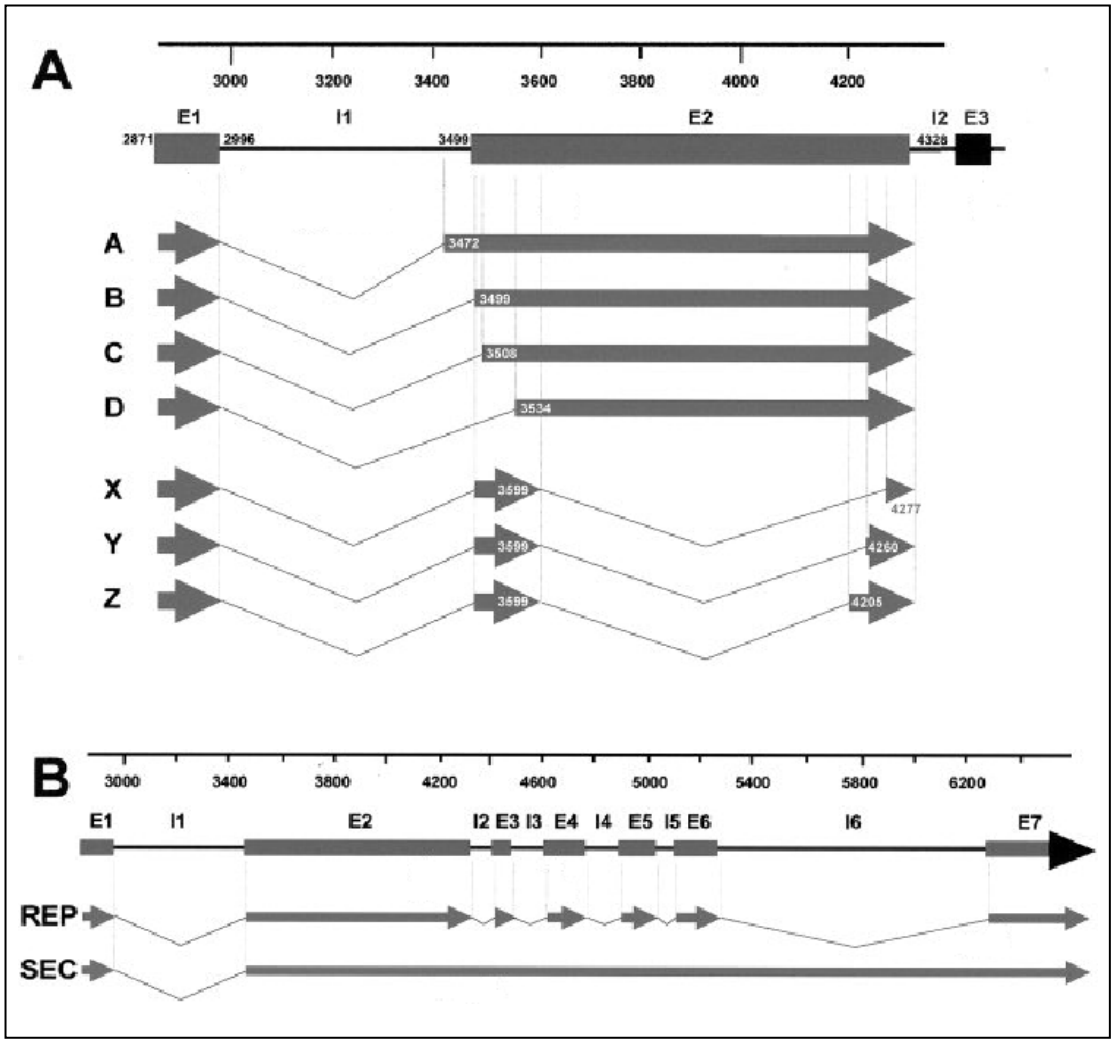

Figure 7: Splicing of MUC1 isoforms [118], where E denotes exon, I intron.

In an effort to avoid confusion, unless otherwise stated, by MUC1 I refer to MUC1/REP, the "normal" protein isoform. Given that splicing generates some of the variety of isoforms in each tissue, the abundance of splicing factors probably determines specific expression patterns, thus tissue, gender, developmental stage and individual expression patterns could be expected.

2.5.6 Expression

Typically expressed in glandular epithelium, MUC1 is also expressed in T cells, B cells and mononuclear bone marrow cells, although epithelium is the best studied. Expression is normally restricted the apical or luminal surfaces and cytoplasmic vacuoles of cells [124], although MUC1 has been demonstrated on activated T cells with expression levels highest on the leading edge of these cells [131]. Specific elements for transcription in T and B lymphocytes, hepatocytes, hemopoietic and muscle cells [130] are present in the MUC1 promoter, however as yet little is known

about its role in the immune system and nothing has been reported on MUC1 in muscle.

In transformed epithelium, MUC1 demonstrates global expression [116, 132] and is up regulated in approximately 70% of newly diagnosed cancers. Indeed MUC1 is a common tumour marker for breast cancer [133], with aberrant expression noted in >90% of patients [134]. Associations have been found between high MUC1 levels and low grade/poor prognosis in this disease [134]. It has been reported that individual cancers appear to have unique expression patterns [135].

A limited number of studies have assessed isoform expression patterns. In ovarian cancer, MUC1/Y and MUC1/SEC are co-expressed in benign tumours, with loss of MUC1/SEC observed in malignant tumours [118]. In fact, MUC1/Y has been shown to increase the tumourigenic potential of many epithelia, but not fibroblasts ([136, 137]. MUC1s/A,D,X,Y and Z have all been observed in malignant ovarian tumours. Up regulation of MUC1 appears to be a late stage in colorectal cancer, and predicts poor outcome [138] as well as correlating with lymph and liver metastasis [139]. Breast cancer cells selectively express MUC1/A and D, with an absence of MUC1/B and C correlating with invasion [140]. Interestingly, the co expression of MUC1s/A and D corresponds to a GG genotype (rs4072037). Epithelial tumours demonstrate MUC1/Y expression, however adjacent normal tissue is negative for this isoform. Pancreatic cancer studies have noted that loss of either the cytoplasmic tail or the VNTR correlate with increased invasiveness, whilst over expression of the full length MUC1/REP decreases invasiveness. It is tempting therefore to speculate that MUC1/REP functions as a tumour suppressor (therefore up regulation is frequently seen as it is an 'early' tumourigenic event), and only becomes an oncogene when the ectodomain is lost to the intracellular space. Taken together with the data from breast cancer, it seems that isoforms B and C correlate with decreased invasiveness, this suggests that MUC1s/Band C release the MUC1n less readily. Obviously this hypothesis and its implications for the other isoforms should be thoroughly tested.

2.5.7 Regulation

Not so much is known about regulation of MUC1 expression, and even less is known about regulation of specific isoforms. Transcription factor binding sites in the promoter of the gene can elucidate some mechanisms for transcriptional regulation and the tissues in which the gene could potentially be expressed.

MUC1 contains a glucocorticoid responsive element (GRE) and is up regulated by ligand activation of the androgen receptor (AR) [141], indicating that MUC1 could be involved in regulation of growth and/or development of the prostate.

EGR1 does not appear to directly regulate expression of MUC1, however signaling via this factor seems to amplify AR signaling, thus MUC1 is indirectly increased [141].

Carbonic anhydrase 9 (CA9) is up regulated by chronic hypoxia, with an associated increase in MUC1 and EGFR [142]. CA9 reduces CO2 levels by producing carbonic acid. Acidity of the ECM causes angiogenesis and is a marker for resistance to radio therapy and aggressive tumours [142].

2.5.8 Functions: protection

Glandular tissue is subject to a variety of stresses that threaten epithelial integrity, including shear and abrasive forces [115].

The soluble form of MUC1 is a major component of mucus, which is produced by cells to lubricate, protect and thus maintain the lumen. The soluble MUC1 is believed to inactivate foreign bodies and potentially immune cells.

The extensive length of membrane tethered MUC1s minimise exposure of the cell membrane to potentially harmful agents. The dense glycosylation of the extra cellular domains may act as a mesh in which to immobilize pathogens, with charge repulsion increasing efficiency. Cell surface MUC1 regulates blastocyst attachment in the uterus, demonstrating that it is not only prevents foreign body adhesion to the epithelium, but also 'inappropriate' contact between two host cells. When un-glycosylated the core MUC1 protein extra cellular domain demonstrates binding sites which are predicted to function as decoy binding sites for infectious agents [143].

Another potential function of the MUC1 protective barrier is to modify the cellular surroundings. It is speculated that the mesh and/or gel formed by both tethered and secreted mucins may act as a filtration unit or a storage unit for cytokines and other molecules [144]. Tumour cells could utilize this to enable maintenance of the optimal tumour microenvironment, particularly metastatic cells in the bloodstream.

2.5.9 Functions: Signaling

MUC1 is involved in protecting cells from chemical and genotoxic effects, indirectly by functioning as a signal transducer. In particular it would appear that following hypoxia or oxidative stress, up regulation of MUC1 contributes to shifting the cellular balance towards that of cell cycle arrest (P53 dependant) rather than apoptosis (P53 dependant and independent) following cellular stress [145].

It has been suggested that as with other cytokine receptors, the MUC1 gene encodes both the receptor (membrane tethered isoforms) and the associated ligand (soluble isoforms). Indeed, the cytoplasmic domain of MUC1 is highly homologous to that of the cytokine receptor super family. It has been observed that MUC1/SEC associates with and stimulates phosphorylation of the intracellular domain of MUC1/Y [136], however, whether this also occurs with the other isoforms is not yet known. Such interactions may form autocrine or paracrine signaling loops [144]. The extra-cellular status of MUC1 (cleavage *vs* no cleavage) seems to influence signaling by the cytoplasmic domain, thus is it plausible that MUC1 transduces information about the extra cellular environment to the interior. Furthermore, phosphorylation of the cytoplasmic tail coincides with altered MUC1 localisation, cell migration and adhesion.

2.5.9.1 Nuclear targeting

MUC1 is proven to form complexes with many different partners via the cytoplasmic tail. Whilst the functions are not clarified, it is likely that these interactions, particularly those observed in the nucleus, are functional.

2.5.9.1.1 FOXO3a

FOXO3a is indirectly activated by MUC1 [146]. Activated FOXO3a regulates transcription of a set of genes including catalase, SOD1 and SOD2, which reduce endogenous H_2O_2 levels and thus reduces oxidative stress in the cell [146].

2.5.9.1.2 P53

Phosphorylation of MUC1 allows direct interaction with P53 [145], a transcription factor that determines (albeit not alone) whether a cell with damaged DNA enters

growth arrest or apoptosis. The result of this interaction is P53 co-localization in the nucleus with MUC1. P53 regulates transcription of many cell cycle and apoptotic genes, in a differential manner that depends upon the co- activators or repressors available. MUC1, when bound to P53, is targeted to the nucleus by importin β [145], where it functions as a P53 modulator, up regulating cell cycle arrest factors, for example by recruiting coactivators to the P21 promoter (in a P53 responsive element-dependent manner), but repressing apoptotic factors for example by reducing binding of basal transcription cofactors to the Bax promoter [145].

2.5.9.1.3 ERα

MUC1 has been proven to interact with the pro-survival estrogen receptor alpha (ERα) [147]. In response to estrogen, these receptors dimerise and translocate to the nucleus where they bind to estrogen responsive elements (EREs), prompting transcription. MUC1 increases the stability of ERα therefore increasing the probability of ERα:estrogen complexes and therefore ERα's potential as a transcription factor [147]. Of note, the ERα-binding region of MUC1 is directly adjacent to that for β catenin, which has previously been observed to coactivate ERα [147]. This raises the question as to whether MUC1 is a coactivator of ERα or rather a bridging molecule that enhances β catenin recruitment [147]. Also of interest is that the widely used ERα-targeting drug, tamoxifen, has little effect on MUC1s interaction [147] suggesting that this pathway may be a bypass mechanism that allows for continued growth of breast cancer despite therapy.

2.5.9.1.4 EGFR

MUC1 demonstrates uniform distribution over the cell membrane, co-localized with another receptor, EGFR (at least in breast cancer cells), in what appears to be a constitutive association [148]. This apparent heterodimerisation indicates that MUC1 (lacking intrinsic tyrosine kinase activity) needs another receptor such as EGFR to initiate a signalling cascade [149]. A member of the HER family of receptors, EGFR is stimulated by factors such as EGF and TGFα [148] and can function as an oncogene via Src signalling [148]. EGFR activation causes phosphorylation of the MUC1 cytoplasmic tail, followed by Src and β catenin in a manner that reduces MUC1s affinity with GSK3β [148]. Whilst MUC1 modification is prompted by EGF stimulation, the association is ligand-independent [148]. Further studies are required to determine whether this interaction is restricted to breast or indeed transformed cells, or whether it occurs in other tissues under 'normal' conditions.

2.5.9.1.5 β Catenin

The WNT signaling pathway is accepted as being of importance for oncogenesis. The cellular pool of β catenin, a co-transcription factor for WNT signaling, is restricted by continuous turnover and ubiquitin-mediated degradation. Formation of this degradation complex depends upon GSK3β phosphorylation of several components, including β catenin [121]. MUC1 stabilizes β catenin by competing for binding to the same armadillo repeats as components of the degradation complex, APC and Axin [121]. As a complex, MUC1 and β catenin translocate to the nucleus, where they regulate transcription of WNT target genes, including down regulation of GSK3β. The affinity for β catenin is dependent upon the kinase by which (and presumably the residue on which) MUC1 is phosphorylated: for example, PKCδ phosphorylation increases the binding affinity, thus increasing complex formation, whereas GSK3β causes lowered affinity and dissociation. In this respect MUC1 is competing with β catenin as substrate for GSK3β, which combined with the reduced expression of GSK3β, results

in increased β catenin stability. This complex therefore has implications for cell adhesion, as is discussed at a later point. Taken together, these reports suggest that MUC1 integrates growth factor and WNT signaling cascades [121].

The β catenin repeats to which E Cadherin binds overlap with those of phosphorylated MUC1 [150], thus these two proteins compete for the same pool of β catenin [121].

2.5.9.2 Mitochondrial targeting

The MUC1c is necessary and sufficient to form a complex with HSP 70 and/or HSP 90, either at the cell membrane or in the cytosol [151]. Formation of a HSP90:MUC1 complex is Src dependant, whilst the HSP70:MUC1 complex is not [151]. Classically, HSP70 is involved in folding new proteins, and HSP90 targeting them to TOM70, where it is thought that MUC1 becomes embedded in the outer mitochondrial membrane (a TM domain-dependant function) and attenuates the stress-induced release of pro-apoptotic factors [151]. MUC1 also interacts with EGFR, which, when stimulated by heregulin, increases mitochondrial targeting of MUC1 by promoting complex formation with HSP90 [151]. Src phosphorylation of MUC1 promotes HSP90 complex formation at the same residue as that for β catenin, with lack of the latter in the mitochondrial membrane suggesting mutual exclusivity of these two binding partners [151]. It is speculated that transformation causes constitutive nuclear and mitochondrial localization of MUC1 [145].

2.5.10 Functions: Adhesion

Cells depend on constant stimulation from their environment, be it from neighboring cells, a distant tissue in the form of hormones, or the extra cellular matrix (ECM). Most cell surface molecules do not exceed 25nm in length, whilst MUC1 (even with only 3 repeats in the VNTR) measures 30nm. Given that it is typically greater than 125nm in length [132], it is the first cell surface molecule to come into contact with other cells, ECM, pathogens and immune or inflammatory cells. MUC1 is thought to influence both cell-cell and cell-ECM adhesion, primarily by virtue of its branched side chains which provide steric hindrance.

2.5.10.1 Cell-ECM

The core MUC1 protein encodes epitopes responsible for direct interactions with the extra cellular matrix 131]. Glycosylation of the core protein might mask these epitopes, therefore modification patterns of the protein have as much a part in this function of MUC1 as the primary sequence.

2.5.10.2 Cell-cell

The majority of adhesion between two cells is mediated by calcium-dependant E cadherin interactions, which constitute adherens junctions [152]. E cadherin is stabilized by the intracellular domain's association with α and β catenins [153]. In normal tissue, MUC1 is restricted to the cell's apical surface and E cadherin to the basal surface. Therefore the interactions between E cadherin and the limited cellular pool of β catenin are stable.

After loss of polarity, intracellular MUC1 resides in the same compartment as E cadherin. When phosphorylated, MUC1 demonstrates an increased affinity for β catenin [116] and competes for the same pool of β catenin as is bound by E cadherin. E cadherin and MUC1 interactions with β catenin are exclusive, as they bind to the same or overlapping armadillo repeats of β catenin [150]. Loss of β catenin results in

destabilized E cadherin and similarly destabilized cell-cell interactions [121]. Increased expression of MUC1 is associated with a significant decrease in cell-cell adhesion [141]. MUC1's ability to override E cadherin adhesion is dependant on the protein's length [132], presumably by increasing the distance between cells.

Cell adhesion also occurs via selectin and ICAM binding [141]. The core protein of MUC1 acts as a ligand for selectins [154] although these sites may be masked by post translational modifications such as glycosylation and sialylation. ICAM1 binding is of particular relevance to metastatic cells. Ligation of a cell to ICAM1 triggers partial retraction locally of the endothelium, allowing extravasation and trans-endothelial migration of the cell [131]. Importantly this interaction is strong enough to withstand the pressure of physiological flow rates [131]. Oscillations in calcium concentrations within the cell follow ICAM1 binding [131].

The different isoforms might participate to differing degrees in adhesion. For example those which lack the VNTR are not likely to influence cell adhesion extracellularly, but may well influence β catenin-E cadherin complexes.

MUC1 is also implicated in regulation of tight junctions [149], via interactions with Grb2 and SOS, which lead to Ras, Raf and eventually ERK1/2 signaling and decreased adhesion [149]. The complexity of signaling cascade means that MUC1s precise influence on tight junctions has yet to be elucidated.

2.5.11 Functions: Immunosupression

MUC1 is reported to disable the immune system through a number of mechanisms:

MUC1 is reported to have immuno- suppressive or regulatory action on T cells [141, 154, 155]. MUC1-coated cells are resistant to cytotoxic T cells [124], by reversibly blocking of T cell activation [135], at least in the case of MUC1/SEC. Whether other isoforms have the same effect is unknown.

Some chemokines rapidly induce MUC1 production and presentation on the leading edge of activated T cells [156] however, how and why this occurs is not known.

Tumour-derived unglycosylated VNTR fragments act as chemotactants for immature dendritic cells (DCs), in a similar manner to an inflammatory response [157], thus MUC1 positive tumours demonstrate locally increased levels of DCs. However it has been observed that MUC1 is able to inhibit monocyte maturation into immature DCs [157]. Thus the DCs present dysfunction and may not be able to prime the T cell population to attack the tumour. For example, incorrect processing of secreted MUC1 fragments by DCs prevents cleavage and consequently inhibits presentation [128], thus activation of T cells can not occur. Furthermore, semi-mature DCs which come into contact with T cells in the absence of co-stimulatory factors, may create tolerance towards the tumour. Conversely it has been reported that with short sialylated MUC1 fragments bind DCs, are rapidly internalised causing activation and maturation [157].

MUC1 is rapidly recycled and reprocessed, thus it is feasible that the modifications presented to the immune system for priming of cytotoxic T cells are different from those present on the tumour cell.

The cytotoxic capacity of natural killer (NK) cells in the immune system is believed to be activated by stress-induced ligands on target cells [157], although the signaling

pathways and specific ligands recognized are as yet unclear. Given that MUC1 is reported to reduce signaling in response to some types of stress, in part via P53, it is feasible that MUC1 may also inhibit or reduce expression of NK ligands on the tumour surface.

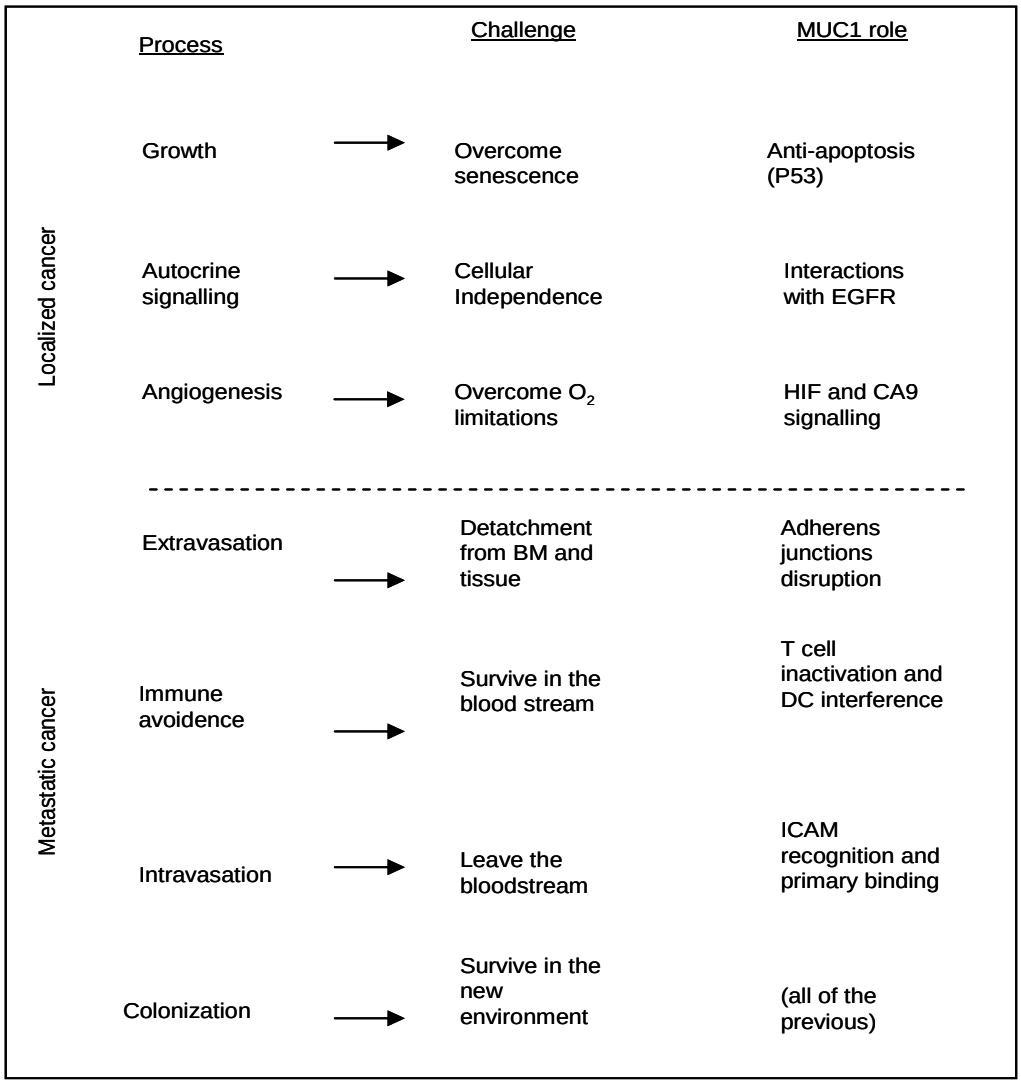

Figure 8: Potential roles of MUC1 in cancer

2.5.12 Diseases

2.5.12.1 Cancer

MUC1 has been suggested as a diagnostic or prognostic marker for various epithelial cancers, including prostate cancer [144], however it is not used in clinical use due to inconsistent results. Clinical trials are underway to assess the potential of MUC1 as a vaccine as well as anti-MUC1 antibodies for targeting tumour cells for immune recognition. The rate at which the MUC1 protein is recycled and modified as well as the extensive variety of possible side chains is likely to prove challenging and may limit the success of such therapies

MUC1 knockout mice demonstrate no developmental abnormalities or phenotype, however, when crossed against a tumour model tumour development is delayed [149], indicating a functional role in cancer development. Unfortunately, animal models that spontaneously develop prostate cancer do not exist, thus this type of experiment sheds no light on the role of MUC1 in prostate cancer.

Given its apparent role in protecting the cell from genotoxic and oxidative stress, assessment of MUC1 levels could prove useful. For example by combining MUC1 knockdown techniques with standard therapies may increase their efficacy.

2.5.12.2 Type 2 Diabetes

MUC1 is located within a region associated with T2D [15, 158], although to date this gene has not been investigated, and was first isolated from the pancreas. It is plausible that the aberrations of MUC1 expression observed in cancer also contribute to T2D: The progressive destruction of pancreatic β cells may be due to autoimmune attack or chronic inflammation. Chronic hypoxia leading to an environment rich in reactive oxygen species and thus cellular stress has also been suggested as causative in reducing β cell function. Therefore MUC1's roles in reducing inflammation, reactive oxygen species and infection by foreign agents could be vital to the integrity of the pancreas.

KL-6, has been reported to be increased in type 2 diabetes [159]. KL-6 is described as being a mucin-like protein [160], however the primary protein sequence demonstrates no significant similarity with that of MUC1 (sequences obtained from the NCBI protein database [119]).

2.6 GROWTH HORMONE RECEPTOR

Growth hormone (GH) is a major regulator of post natal growth and metabolism. GH exerts its endocrine actions by binding to its receptor (GHR) at the cell surface of target tissues. When bound by its ligand, the GHR undergoes a series of modifications resulting in signal transduction. GH effects can be generally classified into IGF-I-dependant and independent effects.

2.6.1 GH

GH is encoded by a single gene *GH1* on chromosome 17q22-24 and is expressed by the anterior pituitary [161]. The normal pituitary isoform (GH-N) has a molecular weight of 20KDa [161]. A second isoform, GH-V, is observed in other tissues such as the placenta [161].

Secretion of the mature GH protein into the blood stream leads to its endocrine action in regulating growth, carbohydrate, lipid and protein metabolism [10]. The main target tissue of GH is the liver. GH is has mitogenic effects [162], potentially in all tissues which express the GHR. In men, GH secretion occurs as sharp peaks at regular intervals, with a low baseline concentration. In women the pattern is slightly different, with more frequent, longer lasting peaks of lower intensity. GH has both insulin-like (acute) and anti-insulin (chronic) actions. GH indirectly signals via insulin-like growth factor (IGF-I) and is crucial for growth, metabolism and development of some tissues.

2.6.2 GHR

The GHR protein is encoded by a single gene [163] of 86Kbp [164], on chromosome 5p13-12 [163]. *GHR* consists of 10 exons [162], 9 of which are coding [163]. There are a number of non-coding exons 5' of exon 1 [165] providing the opportunity for alternative splice forms thus the primary transcript ranges from 4.5Kbp to 5Kbp [161].

Exon 1 and part of exon 2 contain the 5' untranslated region (UTR), the remainder of exon 2 contains a signal peptide whilst exons 3 to 7 encode the extra cellular domain [166]. The transmembrane domain is encoded by exon 8, with exons 9 and the majority of 10 encoding the cytoplasmic region [166]. Exon 3 is highly conserved in mammals, however it has an as yet unknown function [167].

2.6.3 Protein Structure

The GHR protein is conserved in mammals, with high amino acid homology when the human sequence is compared to *Pan troglodytes* (96.2%), *Canis familiaris* (82.3%), *Rattus norvegicus* (70.2%), *Mus musculus* (69.5%) (Homologene, [119]).

The GHR protein is a type 1 transmembrane cytokine receptor with the potential for heavy glycosylation. As illustrated in Figure 9, the protein is 638aa in length [168], consisting of a 246aa extra cellular domain, which includes a hormone binding interface and dimerisation domains, a single helical transmembrane domain [169] and a cytoplasmic domain of 350aa [163]. A signal peptide of 18aa ensures TM localisation. GHR demonstrates limited homology to other cytokine receptors, with the exception of the Prolactin receptor (PRLR) which is closely related to the GHR but lacks the equivalent of GHR exon 3.

2.6.4 Processing of GHR

The GHR is produced as an immature 110kDa protein which is modified in the ER to form a mature protein at 117kDa [162]. The increased molecular weight corresponds to

the addition of 2 glycosyl residues. Mature GHR receptors are thought to dimerise in the ER prior to transportation to the cell surface [170]. The receptor is not presented at the cell surface until it is fully mature [170]. Approximately equal quantities of the mature protein and the precursor proteins indicate a high rate of turnover of the GHR protein [170].

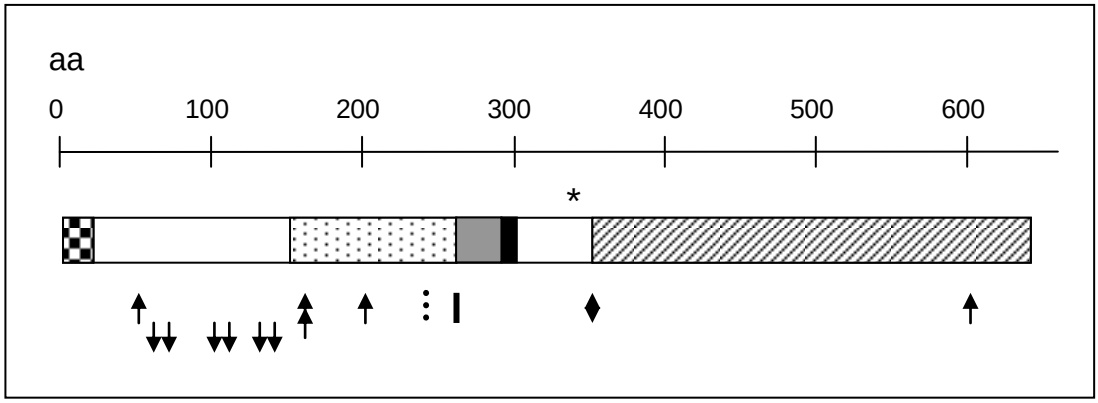

Figure 9: A schematic diagram of GHR protein architecture where: chequered, signal peptide; dotted, FNIII domain; grey, transmembrane domain; shaded, intracellular domain; black, box 1; arrows down, disulphide bonds; arrows up, sites for N-linked glycosylation; dotted line, ligand binding domain; solid line, cleavage site for TACE; diamond motif for endocytosis;, motif for ubiquitination. Adapted from [173].*

It is not yet clear whether JAK2 is merely a signalling molecule (signalling functions described later) which associates with GHR only when GHR is activated, or whether JAK2 is associated with the GHR receptor during/as part of receptor processing. It has been shown with other molecules that interaction with the FERM domain of JAK2 facilitates carbohydrate processing and efficient expression [171]. Other members of the JAK family regulate levels of their associated cytokine receptors; by acting as a chaperone through the secreatory pathway, though regulation of the rate of internalisation or by selectively stabilising the receptor at the cell surface [171]. Whilst JAK2 is not essential for the appearance of mature GHR, it does enhance the fraction of mature GHR at the cell surface [171]. GHR in JAK2 positive cells is more stable, possibly by inhibition of endocytosis [171]. GHR expression in JAK2 null cells may be stabilised by an alternative JAK molecule [172].

When present at the cell surface, GHR is available for active signalling. Tight regulation of GHR signalling is required and is achieved through regulation of mRNA transcription, reduction of protein translation, reduction of the number of receptors at the cell surface, inhibition of downstream signaling or removal of actively signaling receptors. The fates of GHR after presentation at he cell surface are presented in Figure 10.

2.6.4.1 Receptor Internalisation

The various methods for internalisation of the GHR may be differentially influenced by dimerisation, ligand activation and cleavage events of the receptor.

GHR activity is dependant in part upon the rates of internalisation and termination of signalling [173] as deactivation of signalling occurs intra-cellularly [174]. Activated GHR is internalised within 5 mins of ligand stimulation and not recycled [174].

The GH:GHR complex is internalised [167] with GH remaining bound until the complex enters the endosome. However internalised GHR could alternatively be destined for lyzosomes, the golgi apparatus, mitochondria or the nucleus [175]. Depletion of cellular K+ reduces internalisation by 50%, thus multiple pathways exist [175], as lack of K+ inhibits almost all clathrin-dependant internalisation [175]. Many cellular signalling molecules (including Src, cFyn, cCbl, Ras and STATS) are concentrated in calveolae [175]. It is possible that the 2 pathways determine different destinations, e.g. clathrin-coated pits lead to lyzosomal degradation and calveolae lead to an alternative compartment [175].

GHR is not recycled, rather it is degraded in lyzosomes. Dimers of GHR are internalised in a ubiquitination-dependent manner whilst single receptors are internalised independent of ubiquitin [176].

Prolonged GH treatment results in rapid deactivation of the GHR:JAK2 complex in a manner dependent upon ubiquitin and proteosome function [174]. This ligand-dependant internalisation is dependant on the ubiquitin and proteosome systems [173], whilst degradation targeting is independent of either system [174]. Ubiquitination of GHR occurs even in the absence of GH [173], possibly indicating that GHR is subject to continual turnover. Indeed, deactivation of an ubiquitin-activating enzyme causes accumulation of GHR at the plasma membrane [174]. CIS is also a requirement for GHR internalisation [173]. Dominant negative forms of CIS inhibit internalisation thus prolonging STAT5b signalling [174].CIS may recruit an E3 ligase to GHR, which may be sufficient for degradation targeting [174]. A CIS-independent mechanism is also observed [174]. There is evidence to suggest that GHR endocytosis may occur via calveolae or clathrin-coated pits [174], thus a CIS independent mechanism is not unexpected. Of note, calveolae are dependent upon membrane cholesterol levels [174].

GH, phorbol esters and glucocorticoids (GCC) decreases number of GHR at the plasma membrane but there is no alteration in total number [177], suggesting that GHR is redistributed [177]. Serine/threonine phosphorylation by PKC has been shown to regulate distribution of other receptors to the various cellular compartments [177] but is not thought to be involved in GH-induced internalisation [177]. Internalisation may not involve tyrosine phosphorylation of GHR. For example, PMA (a phorbol ester) internalisation of GHR does not appear to be due to increased general endocytosis, rather, it requires specific elements of the receptor [177]. JAK2 tyrosine phosphorylation and box 1 are essential for internalisation induced by GH but not DEX (a synthetic GCC) [177]. PMA internalisation appears to be distinct from either of these [177]. For maximal GCC effects, amino acids 455- 506 of GHR are required, along with tyrosines at positions 333 and 338 [177]. PMA internalisation requires PKC phosphorylation sites [177]. The regions necessary for DEX (a synthetic GCC) internalisation are not required for GH-induced internalisation [177]. Glucocorticoid (GCC) internalisation of GHR may be a stress response, inhibiting energy-demanding actions, such as growth and peripheral metabolism [177].

2.6.4.2 Receptor Cleavage

Cleavage of the GHR extra cellular domain (ECD) releases a soluble fragment, termed the GH binding protein (GHBP). The mechanisms regulating this cleavage are not fully elucidated.

The generation of GHBP occurs in concert with the disappearance of GHR from the cell surface. The fragment left after the cleavage of GHBP is termed the remnant. Depending upon the species (rodents have been the best studied), the remnant includes 8 or 9aa of the extra cellular domain, the transmembrane domain and the cytoplasmic domain [178]. The remnant is further processed by a γ-secreatase complex, containing cofactors APH1, PEN2 and Nicastrin, which is dependant upon the activity of the catalytic core, presenilin [178]. Whilst the remnant is predicted to be non-functional [179], the stub is thought to have signalling functions [178]. The stub is rapidly degraded in a proteosome dependent manner, although whether ubiquitination is required is unknown [178]. Cleavage by γ-secreatase may function to clear the remnant, as the stub is rapidly degraded [178]. The functions of the remnant and stub are of great interest, given that neither is formed by GH binding [178].

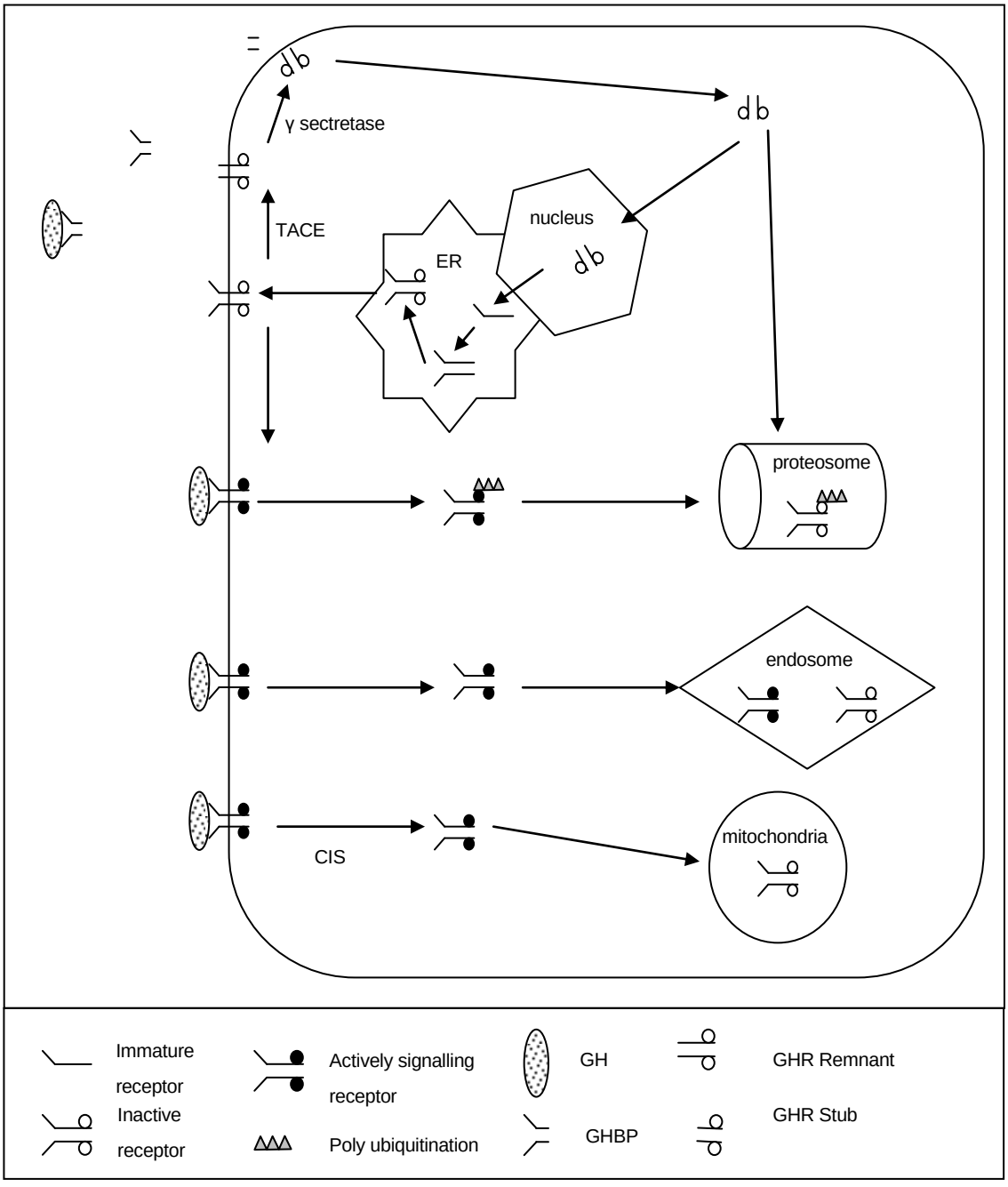

Figure 10: Schematic diagram of GHR processing and cellular fates.

Proteosome activity precedes endocytosis, thus it is feasible that cleavage of an inhibitor is needed before internalisation can occur [174].

It appears that constitutive (serum-stimulated) and induced cleavage utilise the same mechanism [180]. Inhibition of protein kinases reduced serum-stimulated cleavage by only 25-30%, thus there may be a component of serum that activates the GHBP sheddase [180]. Whilst cleavage of GHBP is resistant to many protease inhibitors [180], a metalloprotease has been implicated [180]. TNFα converting enzyme (TACE), a Zn2+-dependant metalloprotease [179] has been identified as the enzyme in question [181]. TACE binds GHR only when GH is bound to the receptor [170], however, when dimerised GHR is bound to GH it is resistant to TACE proteolysis [179].

Thus it appears that GH-inhibition of cleavage is not due to lack of TACE: GHR interaction [170]. The cleavage site for TACE would therefore appear to be distinct from the interaction site [170]. GH binding is believed to alter receptor conformation which may inhibit TACE enzymatic activity. It has also been suggested that shedding of GHBP is MAPK-dependant [180].

PKCa activation causes extra-cellular cleavage [180], with a resultant time- and dose-dependant increase in GHBP [180] and clearance of GHR from the cell surface [180]. PMA treatment causes PKCa activation [180]. PMA and PDGF treatment resulted in loss of GHR and the appearance of GHBP and a cytoplasmic remnant [178].

2.6.5 Function of GHBP

GHBP is a protein of 60kDa [179], which is observed at 80kDa when bound to GH [179]. While the affinity of GH for GHBP is lower than that of GHR [162], GHBP is thought to be a high affinity reservoir [179], with up to 50% of circulating GH being bound to GHBP. In addition, there is also a reported low affinity GHBP, which is bound by 5-20% of circulating GH [179], although little is known about this protein. The growth promoting effects of GH are increased by binding to GHBP, as this prolongs the half life of the hormone [181]. In addition to prolonging the half-life of GH, GHBP restricts bioavailability [173]. GHBP is primarily produced in the liver not the periphery [179], but may be retained in the vascular bed to inhibit GH binding in the periphery [179].

GHBP is evolutionarily conserved [169]. Some species translate the GHBP as a distinct protein from the GHR, whilst in humans it is believed that cleavage of the mature GHR gives rise to GHBP. One report in humans described a truncated mRNA that was predicted to represent the GHBP [182], but lack of confirmation has left scepticism. The redundancy of splicing and cleavage giving rise to this isoform further indicates that it is functionally very important in mammals.

A species of GHBP which remains cell-associated (membrane-attached or MA-GHBP) has been described in rodents [173]. MA-GHBP is formed by alternative splicing, where a hydrophobic tail is included instead of the transmembrane and cytoplasmic domains [173]. When located at the plasma membrane, MA-GHBP may act in a dominant negative fashion, by forming heterodimers with full length GHR, or may sequester GH [173]. Deficient GH levels in mice result in reduced GHR, GHBP and MA-GHBP, which can be corrected by administration of GH, where GHR, GHBP and MA-GHBP are produced at equivalent rates [173]. High levels (>500ng/ml) of GH

demonstrate greater increases in MA-GHBP production than GHR or GHBP [173], possibly as a mechanism for compensating for excess GH [173]. Two molecular weights of MA-GHBP have been observed, corresponding to different degrees of glycosylation [173]. Given that the smaller of these proteins is associated with intracellular membranes, particularly the endoplasmic reticulum, and the larger is observed at the cell surface [173], it is likely that the smaller protein is in an immature state. The rat MA-GHBP contains a "RGD" sequence, typically an integrin binding motif [173], thus it is likely to enable cell surface association [173]. This mechanism has not been noted in humans, perhaps due to technical difficulties.

2.6.6 Modulation of GHR Levels

GHR is ubiquitously expressed. With increasing age, GH levels decrease but as compensation, GHR levels in the liver increase. This is due to an increased number of binding sites, although impaired internalization.

Small nutritional changes alter GHR expression, but growth is not effected [165].

Chronic GH administration increases GHR levels while acute therapy has the opposite effect [165]. GH signalling is important for up regulation of GHR and membrane associated GHBP [183]. In view of the fact that GH binding to GHR is a prerequisite for cleavage, this is unsurprising.

Thyroid hormone increases GH and thus can potentially indirectly increase GHR levels [165], however it appears that Thyroid hormone and GHR levels are inversely correlated, with hypothyroidism demonstrating increased GHR levels [165].

Oestrogen increases GHR levels and it is predicted that testosterone reduces expression [165]. Continuous exposure to oestrogen in male rats alters the GH secretion pattern [183].

Trauma, surgery and spesis reduce expression of GHR and its downstream signalling [165], presumable as a method to divert energy into fighting infection and repairing damaged tissue rather than into longitudinal growth.

2.6.7 Functions: Signalling

Tight regulation of cytokine receptor activation and signalling is crucial for maintenance of normal cellular function [174]. The signalling potential of the GHR can be divided in to 4 categories: classical signalling pathways, autocrine signalling, paracrine signalling and direct transcriptional regulation.

GH signalling is crucial for growth in several tissues, including patterning and growth of the prostate. *In utero* GH signalling, and presumably GHR expression, is tissue specific [165].

GH signalling is a major determinant of postnatal growth, but this effect is not immediate. During the first couple of weeks after birth, GH is not the primary growth mediator, thus over expression of GH is not evident until after this period [165]. Speculation suggests that altered levels of Thyroid hormone may be the switch that induces GH signalling [165].

Animal experiments indicate that GH signalling via GHR is important for prepubertal growth and sexual maturation [184]. GHR disruption alters testicular endocrine function, reduces testes size and testosterone production [184] and results in delayed puberty and spermatogenesis [184]. The effects on initiation of male puberty are indirect and dependant upon IGF-I [184].

Acute (2hrs) stimulation of GHR signalling has an insulin-like effect, causing glucose transport, amino acid transport, lipogenesis, protein synthesis and increased AR, IGF-I and IGF-IR. Chronic or long term (more than 4hrs) effects of GH stimulation have anti insulin effects, promoting increased blood glucose levels, insulin resistance, lipolysis, inhibition of glucose transport and reduced AR and serine phosphorylation. GH and IGF-I promote LDL uptake [185].

2.6.7.1 Classical signalling

Classically, GH is produced by the pituitary gland and secreted into the blood, where it circulates to the relevant organs for its effects.

GH sequentially binds the first and then second GHR molecule to create a 1 GH: 2 GHR complex. The GHR binding sites of GH are on opposing sides of the molecule, but are offset rather than symmetrical. This causes a shift in the dimer so that the receptor tails to move closer [186]. When the GHR tails are brought closer by the dimer shift, associated JAK2 molecules are brought close enough to be able to trans-phosphorylate each other. Phosphorylation of JAK2 provides docking sites for signal transducers such as SH2B1 which binds phosphorylated tyrosine 813 to enhance signal transduction [186].

The proline-rich box1 of GHR binds one JAK2 molecule in a 1:1 ratio [186]. The FERM domain of JAK2 is necessary and sufficient for interaction with the GHR [172], although this appears to be by structural stabilisation of JAK2 rather then by direct GHR interaction [172]. The kinase domain of JAK2 is required for activation of, but not interaction with GHR [172]. The complex of JAK2:GHR does not depend on GH, and appears to be independent of tyrosine phosphorylation of either component [172].

STATs 5a and 5b are two molecules that rapidly associate with phosphorylated JAK2 and transduce signals to the nucleus. Activation of STAT5 requires JAK2 alone [186]. STAT5b is rapidly activated by pulses of GH, but down regulated by continuous stimulation [174].

GHR is also bound by other signal transducers, although less is known about the importance of these.

SRC can directly bind GHR and phosphorylate and activate the tyrosine residues to which STAT5 binds, independent of JAK2 [186, 187]. Activation of SRC is needed for RAL A/B and RAP1/2 signalling pathway activation [186]. Regulation of Ca2+ levels may be via SRC rather the JAK2 [186] and regulation of ERK1/2 is thought to be SRC-dependent and JAK2-independent [186].
JAK2 activation is required for activation of RALs A&B and RAPs 1&2 [186], the balance of which may determine the ratio of ERK1/2 to JNK signalling [186].

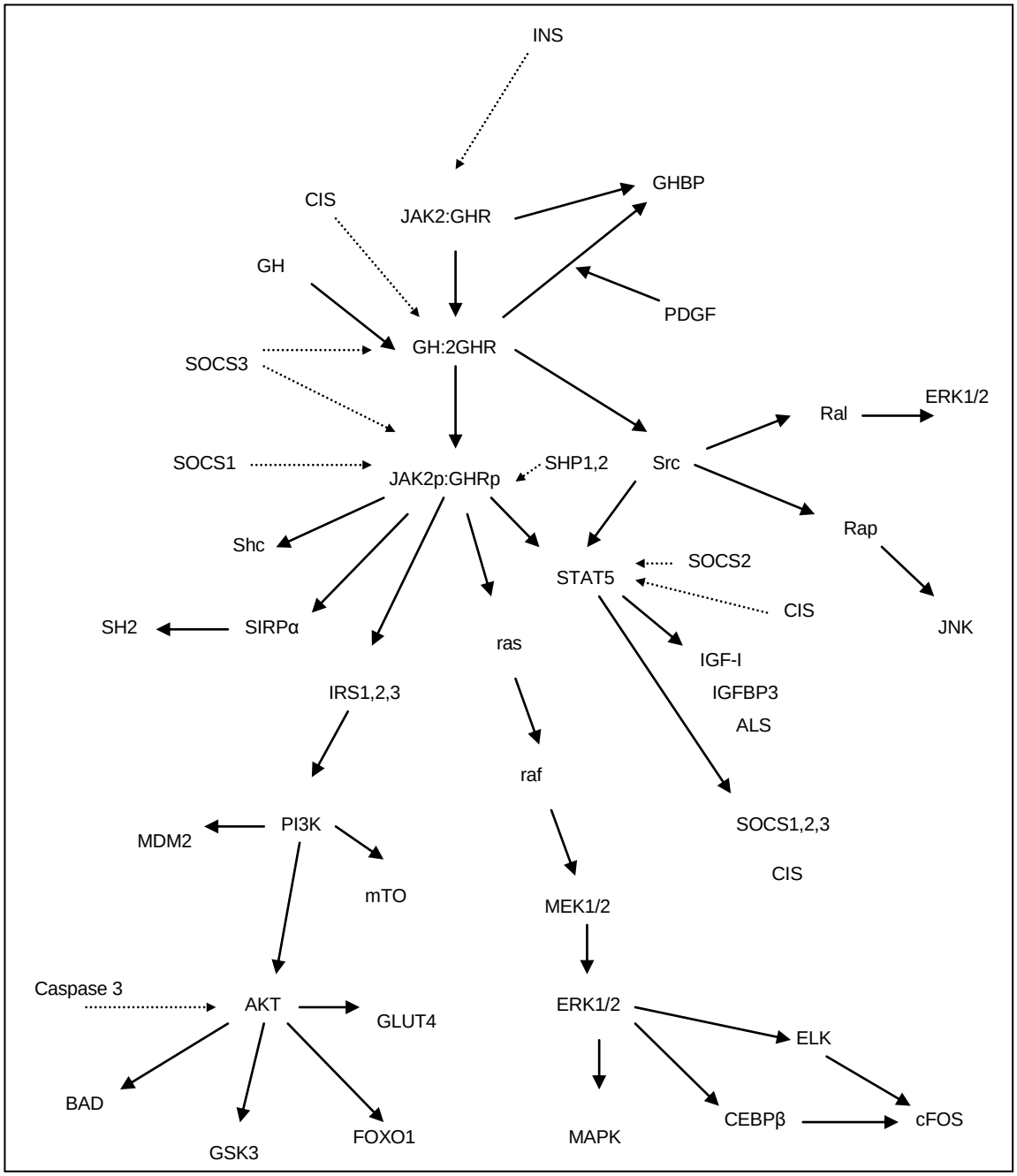

Figure 10: Main signalling pathways associated with GHR, adapted from [2, 186], where solid lines indicate promotion, dotted lines indicate inhibition.

The common γ chain in a complex with JAK3 has been shown to regulate the translocation of STAT5 [188]. GH fails to induce STAT5 phosphorylation in cells lacking γc, however wildtype γc replacement rescues this defect [188].

Cbl, the human homologue of the leukaemia virus, is involved in regulation of the actin cytoskeleton and cellular morphology in a PI3K-dependant manner [189]. Cbl is a negative regulator of several receptor tyrosine kinase signalling pathways [189]. Whilst its activity or DNA binding is not influenced by Cbl, STAT5's degradation is regulated by this protein [189]. In particular, over expression of Cbl reduces GH-stimulated STAT5 activity [189], although it is not known whether this occurs via direct interactions or adapter molecules.

In the kidney, GH causes the conversion of vitamin D 25(OH)D3 to 1,25 (OH)2D3 by 1α- hydroxylase [92]. These effects may be direct, or via IGF-I, which has been shown to directly stimulate 1,25 (OH)2D3 [92]. The rise in IGFBP3 as a result of 1,25(OH)2D3 stimulation may form a classical negative feed back mechanism for IGF-I levels, reducing the bioavailability and thus proliferative effects of IGF-I [92].

GH signalling via the GHR may influence AR signalling by crosstalk with intermediates or by transactivation of the AR at androgen responsive elements (AREs) [162]. GH increases transcription of prostate-specific C3 and probasin [161] in a similar manner to androgen. Furthermore, GH signalling increases mRNA levels of AR, IGF-I and IGFR, thus it is proposed that GH may regulate prostate function [162].

Supressors of cytokine signalling (SOCS) molecules are rapidly upregulated by GH signalling. SOCS and CIS (cytokine inducible SH2-containing protein) form the basis of the GH negative feedback loop. SOCS3 may inhibit JAK2 via the kinase domain [190]. SOCS1 may interact with cytokine receptors as a pseudo substrate, or may target JAK for ubiquitination and proteosomal degradation [190]. SOCS 1 and 3 bind phosphorylated tyrosine 1007 of JAK2 [186]. CIS inhibits STAT5b by competing for GHR/JAK2 binding sites and by another mechanism involving the proteosome [174]. CIS levels are themselves are regulated by STATs, thus forms part of a negative feedback mechanism [174]. CIS up regulation by prolonged GH treatment is observed at the level of mRNA within 2hrs of stimulation and remain at a high level for at least 24 hours [174].

IGF-I is a major downstream effector of GHR signalling. GH, via GHR regulates IGF-I expression, which is part of the negative feedback signal to inhibit GH secretion. IGF-I is mitogenic in several tissues including prostate epithelium. IGF-I is involved in several processes such as regulation of insulin sensitivity and growth.

2.6.7.2 Paracrine signalling

Paracrine signalling describes where a stimulus is produced by a cell physically close to the responding cell. Local production of GH is likely to have paracrine effects on the surrounding tissue. As a result it is feasible that production of GH may act to modulate the metabolism and proliferation of adjacent cells. With this in mind, it is of note that PC cells express levels of GH which correlate with aggression.

2.6.7.3 Autocrine signalling

Autocrine signalling is where the cell producing a stimulus is the same cell as responds to that stimulus. Most cell types express GHR, fewer express GH. If cells have the ability to express both proteins, the potential for autocrine signalling exists [191]. It has been proposed that the signalling pathways may differ between autocrine and paracrine signalling [191], although the details are unknown. It has been reported that co-expression of GH and GHR results in enhanced maturation of the GHR through the ER [191] (in terms of quality rather than rate of production), suggesting that GH is a ligand chaperone [191]. The complex of GH and GHR (whether in monomeric or dimeric form was unclear) may be actively signalling upon exiting the ER [191]. Upon arrival at the plasma membrane, further stimulation is not possible and endocytosis is likely to be rapid.

2.6.7.4 Direct effects

Recently the presence of GHR in the nucleus has been reported [192]. Whether the GHR directly binds DNA to influence transcription of target genes, or whether its

effects are indirect (via recruitment of cofactors) has yet to be elucidated. Injured tissues demonstrate levels of nuclear GHR which correlate well with proliferation rate. It is not yet known whether cellular GH response is dependent upon the origin of GH, that is, whether hormonal, paracrine or autocrine signalling have the same results.

2.6.8 Genetic Variation

2.6.8.1 5'UTR exons

GHR has 8 exons in the 5' UTR thus has great potential for splicing to form different mRNAs [165]. Little is known about the role of these exons as the GHR protein produced would appear not to differ [165]. The transcriptional regulation and functional differences of the alterative splice forms are as yet unknown. It is possible that the 5'UTR is used to recruit co-factors that alter transcriptional machinery binding, thus the probability of each mRNA species being transcribed may be dependant upon the cofactors available. This would suggest that transcription from *GHR* is regulated very specifically in a complex manner. The difficulty in distinguishing mRNA transcripts has hindered understanding of *GHR* regulation. Recently, some transcripts have been identified as being specifically up regulated in response to estrogen, at least in the liver. Further investigations are required to determine how 5' exon usage is controlled and what effect it has on protein function.

2.6.8.2 Single Nucleotide Polymorphisms

There are 807 SNPs noted for GHR (SNPper database [120]). Of these, 694 are located in introns, 41 SNPs are within the promoter, 45 are located in the 3' UTR or downstream sequence. The significance of these are unknown, however, as transcription factors, coactivators and corepressors of transcription bind to these sequences, expression levels of mRNA may be influenced by non-coding DNA sequence variations. 10 SNPs are on intron boundaries, thus may influence splicing of the mRNA transcript. 16 SNPs are located within coding exons, 4 of which are synonymous (no change in amino acid). The 12 SNPs which result in amino acid changes have the potential to change the proteins secondary structure (and thus the structure and function of the folded protein) due to the altered size and charge of the replacement amino acid.

2.6.8.3 Deletions

Truncations of the cytoplasmic domain have been observed (GHR_{tr}). $GHR_{(1-279)}$ is an isoform lacking 26bp of exon 9, so only 2.5% of the intra cellular domain is present [179]. Loss of the entire exon 9 is also seen, $GHR_{(1-277)}$ [179] and other cases where 75% of the cytoplasmic domain is lost have been observed [162]. It is speculated that GHR_{tr} may have a dominant negative effect [162], although so far this is merely speculation.

Generation of GHBP is regulated (at least in part) by splicing of an isoform with a truncated cytoplasmic region, GHR_{tr} [180]. GHR_{tr} is an inactive dominant negative receptor which is not internalised [180]. GHR_{tr} may be a mechanism for increased remnant and stub formation, thus greater nuclear localisation. GHR_{tr} released an equal if not greater quantity of GHBP than full length GHR, thus presence of the cytoplasmic domain is not required for PKCa-dependant cleavage [180].

Differential regulation of GHR isoforms may be a mechanism for regulating GHBP levels. GHR_{tr} is associated with increased GHBP levels [180, 162]. As cleavage required prior binding of GH, those isoforms with the most rapid GHBP formation

have the highest affinity for GH. It would follow therefore, that these GHBPs in the serum may sequester GH away from the cell.

2.6.8.4 Exon 3 Isoforms of GHR

Two common isoforms of GHR have been observed, which differ in the exclusion or retention of exon 3 [163]. The frequency of GHR exon 3genotypes in control/normal populations to date (September 2008) are shown in Table 3. Homologous recombination has been identified as the mechanism behind the origin of a genetic deletion of exon 3 of *GHR* [163]. The isoforms are believed to occur as a genetic deletion [163] as well as at the level of splicing [182]. It has been possible to amplify the exon 3 deleted (GHR_{d3}) GHR mRNA from full length GHR (GHR_{fl}) individuals, but no exon 3 fragment has been amplified from GHR_{d3} individuals, supporting both splicing and genomic deletion [181]. Another report demonstrates that both isoforms are equally likely to be expressed, with expression patterns mirroring genotype [193].

Table 3: GHR exon 3 genotype and allele frequencies in control samples .

			genotypes (%)			alleles (%)		
Author	Population	n	fl/fl	fl/d3	d3/d3	fl	d3	Ref
Pantel	French	150	58	33	9	75	26	[163]
Jorge	Brazilian	68	46	34	20	63	38	[194]
Audi	Spanish	289	27	58	15	56	44	[195]
Wagner	Polish	526	52	42	6	73	27	[196]
Tauber	Caucasian	193	51	41	8	72	28	[197]
McKay*	Swedish	1704	84	59	10	74	26	[198]
Mercado	Mexican	175	53	30	17	68	32	[199]
HapMap	CEU	60	53	42	5	74	26	[200]
Where; * rs6886047 used as a surrogate marker, CEU north Americans of European descent.								

The function of exon 3, which encodes a region close to the GH binding domain, is unknown [201]. Loss of exon 3 does not alter disulphide bridge formation, thus folding is not altered [201], however 1 glycosylation site is lost, and the exon 3 deleted (GHR_{d3}) protein has altered charge, size and hydrophobicity compared to the full length isoform [201]. Whilst there is no reported difference between the isoforms in terms of GH binding or internalisation, the absence of exon 3 is thought to confer an increased growth response to exogenous GH [201]. Indeed it has been suggested that GHR_{d3} has 130% of the signalling capabilities of GHR_{fl} [202]. There have been conflicting reports in this respect, which may be accounted for by differences in populations and treatment type and duration studied. How signalling differs between the 2 isoforms has not been studied but it is plausible that there may be a differential effect on some pathways. A conflicting study reports a dominant negative effect of GHR_{d3} on GHR_{fl} [203].

Analysis of predicted domains of the GHR_{fl} and GHR_{d3} isoforms indicate that loss of exon 3 is either associated with a loss of a type 3 fibronectin domain and regions of intrinsic disorder (SMART, [204]) or does not alter conserved domains (CDART). Type 3 fibronectin domains are present in 2% of all proteins and are thought to be involved in binding b chains of globular fibronectin (NCBI, [119]). Analysis of the amino acid sequence (ELM, [205]) indicates that GHR has a fork-head associated ligand binding domain, which is commonly seen in many proteins involved in signal transduction, cell cycle control and DNA repair. There are also 2 putative phosphorylation sites for casein kinase 1 and one for polo-like kinase. When exon 3 is

lost, a putative phosphorylation site for GSK3 is created. Loss of exon 3 does not alter disulphide bond formation.

It is debated whether there are tissue specific expression patterns of exon 3 isoforms [179, 181], or whether they are specific to each individual [182, 206]. The GHR_{d3} isoform has been mainly observed in the placenta [163], whilst the liver has been reported to express the full length protein [207] or both isoforms [203]. Some cell lines are reported to express the GHR_{d3} [203]. There is little evidence to suggest that the isoforms are developmentally regulated [179], except for the finding that the GHR_{d3} is expressed by the placenta [163], which is intriguing.

Why the placenta expresses GHR_{d3} is not yet known, although it may be due to the isoforms increased growth response to GH. It would be of interest to determine whether oestrogen increases expression of both GHR isoforms or whether it is a differential effect. Logically, either isoform may be preferentially increased by oestrogen: increasing GHR_{d3} in the placenta may cause a locally increased response to a globally stable GH signal during fertile periods, where as increasing expression of GHR_{fl} may provide a net increase in GH signalling that rivals basal GHR_{d3} signalling. How heterozygotes would be affected is thus likely to be complex.

2.6.8.5 Exon 3 isoforms of GHBP

GHBP isoforms derived from GHR_{fl} and GHR_{d3} are reported to differentially regulated [208], with $GHBP_{fl}$ being more abundant than $GHBP_{d3}$. Serum isoforms correlate with genotype [181]. Despite this, GHR_{d3} appears to be more readily cleaved, suggesting that the missing fragment alters conformation in such a way that it is more permissive of cleavage [181]. The full length form, when bound by GH, does not permit cleavage possibly by altered conformation moving the cleavage site away from the enzyme. Loss of exon 3 from the flexible region may reduce flexibility [181] thus it is feasible that with exon 3 missing the cleavage site is not moved away from the cleavage enzymes active site. In this way cleavage of GHR_{d3} is possibly by default rather than an active process. If it is indeed the case that GHR_{d3} produces a higher rate of GHBP than the GHR_{fl}, it is interesting to speculate as to whether this is due to increased GHBP production *per se*, or whether it is merely a mechanism for producing the signalling stub. Other possible mechanisms for the increased cleavage of GHR_{d3} compare to GHR_{fl} include loss of exon 3 allowing movement more easily and action than the full length isoform, or exon 3 being the binding site for factors that inhibit cleavage. Another possibility is that the change in hydrophobicity due to loss of exon 3 may alter the position of the receptor in the membrane therefore changing its movement through the membrane. The rate of presentation at the cell surface of the isoforms has not been fully investigated, nor have the differences in downstream signalling, thus further studies are required.

Expression of GHR but not GHR_{tr} differs between PC and BPH [162]. This isoform has been reported in normal tissue and the PC3 prostate cancer cell line but not other cell lines [161, 162]. At the protein level it is reported that expression of this isoform is too low for expression to be detected [162] although it may be that it is too rapidly recycled.

From an evolutionary perspective, it is curious that the PRLR is so similar to the GHR_{d3} isoform, and both are bound by GH. This may only function as a back-up system to enable GH signalling when there are defects in the GHR. Alternatively it may be that

the GHR_{d3} and PRLR are able to heterodimerise. How and why this may occur, as well as the effect of this interaction, would be interesting to investigate.

2.6.9 GHR_{d3} in Disease

2.6.9.1 Short stature and GH therapy

Most reports on the GHR exon 3 polymorphism have studied whether or not GHR_{d3} genotype influences response to GH therapy. The variety of conditions which result in short stature (thus the need for GH therapy), the therapeutic dosage, the genetic backgrounds of the subjects mean that the discrepancies observed between studies are inevitable.

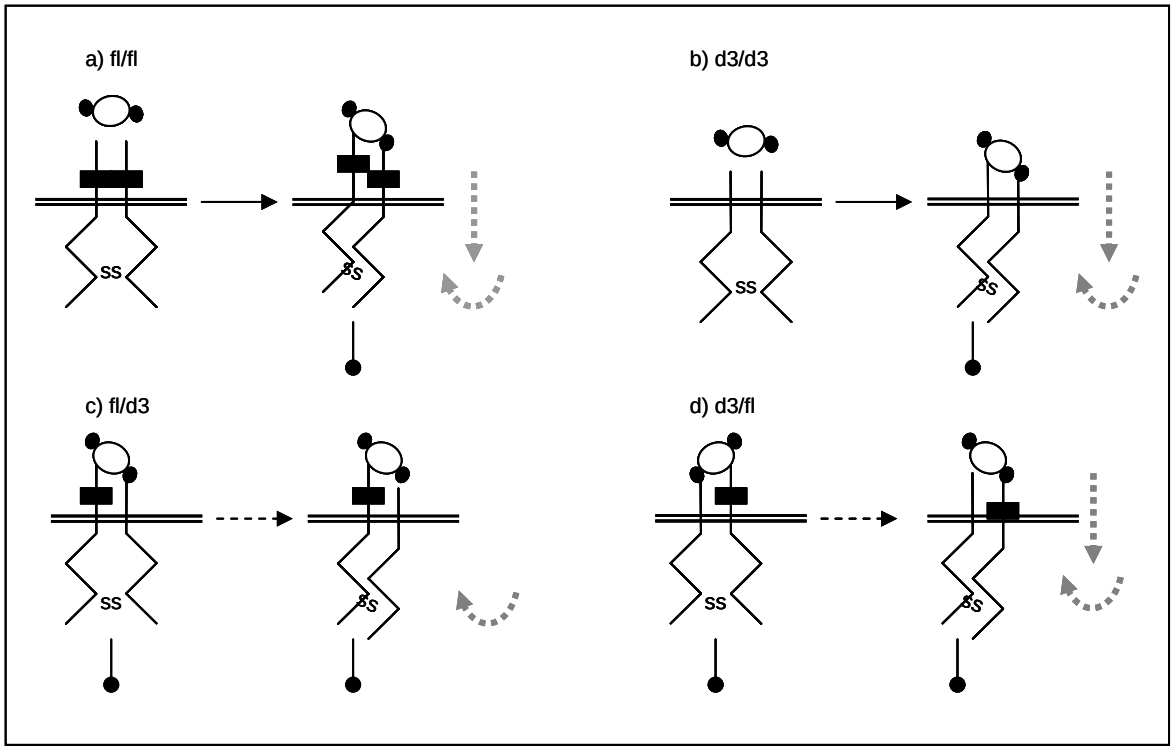

Figure 12: Schematic diagram possible mechanisms for activation of GHR dimers, where exon 3 is depicted as a black rectangle, growth hormone as white oval with black binding sites, and the plasma membrane as a double black line. A) the second GHR binding to the GH is offset, forcing one polypeptide to sink [209]. Additional rotation brings the receptors into close enough contact for trans-phosphorylation (depicted as a black ball/stick). B) where both polypeptides lacking exon 3, the same mechanism as with GHRfl/fl is likely to occur. With heterodimeric receptors it is more complicated and may depend on which isoform binds GH first: C) no movement, or only the rotation, may be sufficient for transphosphorylation or D) it may be that no movement is possible, thus receptor activation may not occur.

2.6.9.2 Diabetes and complications

Variations in GH and GHR have been implicated in diabetic complications such as hypertension (HT) and cerebral-vascular lesions (CVL) [14].

GH has a role in nephropathy independent of IGF-I with GHR and GHBP knockouts being protected from diabetic nephropathy [210] and GH over expression being associated with increased CV morbidity and mortality [185] as well as kidney complications [10].

Early changes in the kidney are increased size, volume and function [211] followed by increased proliferation, accumulation of ECM components and urinary albumin

excretion [211]. Increased size may be preceded by increased IGF-I, IGFBPs and IGF2R levels [211] although the role of GH in nephropathy seems to be independent of IGF-I [211]. It appears that it is GHBP which is crucial [211]. Reducing GH signaling (using GH antagonists) may be protective without altering metabolism (in animal models at least) [211].

Taken together, these suggest that if GHR exon 3 genotypes do demonstrate differential response to endogenous GH (rather than the exongenous GH used for treatment of short stature), this polymorphism may modulate risk of diabetic complications.

2.6.9.3 Obesity

Obesity increases serum GHBP [193], with triglycerides and BMI independently predicting $GHBP_{fl}$ levels [193]. It appears that adipose correlates with $GHBP_{fl}$, but not $GHBP_{d3}$ [193]. Furthermore, this association that may be modified by sex hormones levels, as a difference is observed between men and women [193].

Obesity is known to increase estrogen levels, thus increased GH in response to food intake combined with high estrogen levels could further disrupt aberrant metabolism [212].

Current knowledge implicates obesity in metabolic aberrations. That there is a difference in effect of obesity on the GHR_{fl} compared to the GHR_{d3} implies that certain genotypes may be more susceptible to the detrimental effects of obesity than others.

2.6.9.4 Prostate cancer

Both GH and IGF-I are mitogens for prostate epithelium [196], thus the level of GH signaling and the resultant increase in IGF-I could be important determinants of tumourigenesis.

GHR is located within a region implicated in hereditary PC [213]. Despite it's importance for prostate development, GHR is not expressed in the normal adult prostate [214], although it is expressed in BPH [161] and in PC [161, 162, 214]. Whether levels are comparable or higher in PC than BPH is still debated. GH and GHR are expressed by the PC cell lines PC3, DU145 and LNCaP [161]. It is interesting that the different cell lines are reported to express different isoforms: PC3 ($GHR_{fl/d3/tr}$), DU145 ($GHR_{fl/d3}$), LNCaP (GHR_{fl}) [161], However genetic variation of *GHR*, including the exon 3 deletion polymorphism, did not influence PC risk [74]. Truncations of exon 9 of GHR have been observed in the prostate, although no difference in levels was noted between PC and BPH [162]. The levels of GHBP secreted into media by PC cells is negligible, thus this is not a likely mechanism for local regulation of GH activity [161].

An isoform of GH expressed primarily by the placenta (GH-V) is also observed in LNCaP [161]. Whether stimulation by GH-V differs from that of GH-N (the more widely expressed isoform of GH) is not known. Nor is it known whether the affinity of GHR_{d3} and GHR_{fl} for GH-N and GH-V differs. However, it is unlikely to be coincidental that GHR_{d3} and GH-V are both expressed by the placenta. How this influences prostate cancer is unknown, but may be of importance.

GH releasing hormone (GHRH) and its receptor (GHRHR) are also expressed in PC [161]. Inhibition of GHRHR prevents PC growth [161], possibly by reduction of GH signalling.

In light of Gabreilsson *et al*'s [212] findings, increased estrogen in combination with GH may stimulate growth as well as aberrant metabolism. Androgen therapy results in increased estrogen levels and increased adipose tissue deposits, thus synergy between GH and estrogen may act as an androgen bypass mechanism for growth stimuli. This may help to explain why prostate tumours in obese patients are more likely to progress to aggressive cancers than those in lean counterparts.

3 DISCUSSION

Despite the success so far with elucidating genetic variations in PC, approximately 50% of population attributable risk is linked to unknown/unconfirmed variants. The heterogeneity of PC means that large numbers of cases are required to detect any effects of a particular polymorphism or exposure, thus only the most important effects are observed. Effects which are equally crucial, but only to a subset of cancers, are likely to be diluted and thus not detected. Separation of subsets such as hereditary from sporadic cancers has proven to be useful, despite the potential confusion caused by sporadic cases occurring within cancer-prone families. In a similar manner, it may be worthwhile dividing cases of PC with concurrent metabolic disorder from those who present as metabolically normal. This approach would enhance the probability of elucidating important mechanisms by enriching for similar genetic and or environmental backgrounds.

Genes known to be involved in monogenic forms of diabetes have been investigated for influence in T2D, with disappointing results. Any influence appears to be small, with little gain when a number of genetic variations are combined. Thus it appears that T1D and T2D are genetically less similar than previously believed. A further complexity is that the genetics involved in complications may be distinct again from those influencing risk. The effect of poor diet and physical inactivity may differentially influence certain genotypes, making it difficult to confirm T2D-associated genes, particularly in different populations.

The similarities between PC and T2D, even though they may be restricted to specific organs, are striking. The potential of chronic inflammation, altered glucose and lipid metabolism, vitamin D and growth factors such as insulin, IGF-I and GH to influence both diseases is well documented.

It appears that MUC1 is involved in many complicated networks, both within and between cells. MUC1 isoforms and functions are regulated in each cell by a) availability of splicing factors in the nucleus b) translation c) glycosyl transferase profile d) dimer stability/ectodomain cleavage potential. Given that loss of either the cytoplasmic tail or of the VNTR of MUC1 is reported to increase invasiveness of cancer cells, it seems that either fragment is capable of acting as an oncogene. MUC1s putative interactions with other receptors could further complicate MUC1s influence on cellular dynamics. The recent findings of a signaling role for MUC1 suggest that MUC1's roles may be more diverse than previously realized, adding further complexity to the functional differences between isoforms. It is clear that the importance of MUC1 isoforms has been underappreciated, and further investigation into MUC1 proteins could elucidate valuable information of clinical relevance.

Genetic variations in MUC1 that result in specific isoform patterns could be useful predictors of MUC1 functions and as such may be useful biomarkers of disease. Disease associations of such isoform patterns could elucidate functional differences between isoforms. Due to the number of parameters involved in isoform transcription, translation and modification, it is likely that cellular manipulation of isoform expression may not give a realistic view of isoform functions until more is known about regulation of these processes.

The influence of *GHR* exon 3 deletion on the protein function has not been well studied. That there is no apparent difference in GH binding affinity or internalization kinetics in surprising, particularly given that the deleted region is close to the GH-binding domain. Further studies are required to determine whether the deletion alters interactions with other ligands, receptors or interacting proteins. Differences in endogenous and exogenous (that is therapeutic) GH responses may be seen between the receptors. Whether the deletion has any influence on transphosphorylation of the GHR-associated JAK2 molecules or the intracellular binding partners of GHR has yet to be determined. As the signaling pathways initiated by GH depend on the signal transducers activated, this could be important to determining the functional significance of the deletion.

The majority of the studies into phenotypic differences associated with the GHR_{d3} polymorphism have been carried out on populations with growth deficiencies. The inconsistent results are unsurprising, as the reasons for growth deficiency and the corrective therapies differ between studies. There is limited knowledge as to the mechanisms of reduced growth in most of these disorders thus it is likely that there are multiple aberrations of metabolic and longitudinal growth pathways involved. That there is no reported effect on adult height of normal subjects suggests that the normal negative feedback mechanisms compensate for any increased signaling resulting from the loss of exon 3. This also suggests that in subjects with deficient growth there are aberrations of the negative feedback mechanism.

It is somewhat surprising that GH signaling is involved in many processes but studies have mainly been limited to longitudinal growth and a few on various cancers. The role of GHR_{d3} in metabolic homeostasis and the consequences of aberrant control appears to be an obvious area for investigation, however this has yet to be undertaken.

4 RESULTS

Paper 1
Deletion of *GHR* exon 3 has been associated with increased receptor bioactivity and thus IGF-I levels. To determine whether the GHR_{d3} genotype influences glucose tolerance, genomic DNA from subjects with NGT, IGT and T2D was analysed.

The frequency and genotype distribution of the GHR_{d3} allele in the Swedish population is similar to that in the other populations studied. T2D subjects demonstrated a significantly reduced frequency of GHR_{d3} homozygotes compared to NGT or IGT subjects. Adult height and BMI demonstrated differences between GHR_{d3} carriers and GHR_{fl} homozygotes. In T2D subjects, BMI and CRP levels were significantly higher in GHR_{d3} carriers. Non significant trends in HDLs, TGs and age-standardised IGF-I levels were also observed between genotypes. The seemingly protective role of the GHR_{d3} isoform against diabetes and its association with clinical parameters such as BMI are suggestive of functional differences between the two isoforms.

GHR exon 3 genotyping may be clinically useful as a biomarker for indicating those at increased risk of T2D as well as highlighting those T2D subjects most likely to develop severe complications. As 42% of Swedish T2D subjects carry the GHR_{d3} allele, aggressive treatment to prevent diabetic complications in these subjects would translate to significantly reduced health care costs and increased quality of life.

Paper II
A number of MUC1 isoforms are determined by a SNP (rs4072037). To determine whether the genotype of a SNP (rs4072037) in MUC1 influences risk of PC, subjects with sporadic or hereditary PC, BPH and control subjects were genotyped. The G allele was underrepresented in HPC compared to the other sample groups. Furthermore, to investigate whether differences between blood DNA and prostate tumour DNA, A fragment of DNA surrounding the SNP was sequenced in matched tumour and non-tumour samples (i.e. from the same patient). Loss of heterozygocity was observed in a number of cases, with the G allele being consistently lost in tumour DNA compared to blood DNA.

This variant is believed to determine a number of MUC1 isoforms, functional differences between which are not well known. The protein sequences of those isoforms available through the NCBI protein database and the Human Protein Atlas were subjected to *in silico* sequence analysis to investigate potential differences between the isoforms. Bioinformatics sequence analysis indicates that there are potentially important functional differences in phosphorylation and modification motifs. These are likely to influence the signaling capacity and determine the possible protein- protein interactions of the peptides.

Functional differences between MUC1 isoforms has so far been largely ignored in prostate cancer, where it is proposed as a biomarker for progression. Current techniques used for protein expression analysis of the MUC1 protein do not have the ability to differentiate between isoforms. Genetic differences in *MUC1* between DNA from tumour and non-tumour material suggests the importance of certain isoforms in tumourigenesis. Further investigation is warranted, given the small number of samples studied here.

Paper III

Paper II supports a role for genetic variation of *MUC1* in prostate cancer. The aim of this study was to determine whether rs4072037 and other common variants (i.e. with frequency >5% of the population) in and around *MUC1* are associated with increased PC risk.

In order to minimize the number of polymorphisms analysed, data from the HapMap project (CEU samples) was used to identify tagging SNPs. Thus 5 SNPs cover the common genetic variation in a 40Kb region surrounding *MUC1*. SNP and haplotypes frequencies were determined in control and PC subjects from the CAncer of the Prostate, Sweden study (CAPS). The assay for rs4072037 was not successful, however rs2066981 (which is in strong LD with rs4072037) was used as a surrogate marker.

No differences in genotype or haplotypes frequencies were observed between control and PC samples. Thus inherited variation in a 40kb region surrounding the *MUC1* gene does not influence risk of PC (sporadic or hereditary) or PC-specific survival. That these results did not concur with those in Paper II may be due to the sampling of a smaller and more homogeneous population of HPC families, compared to Paper III. Genetic variation which may be important in a limited number of families may be masked, or the effect diluted, when a larger and more heterogeneous population is studied. It should be noted that genetic changes within prostate tumours can not be assessed in studies such as this one.

Paper IV

MUC1 is localized within a T2D-susceptibility region which has been replicated in several populations. The aim of this study was to determine whether a SNP (rs4072037) of this gene, which determines a number of MUC1 isoforms, influences T2D.

Genotypes frequencies of T2D subjects did not fit the Hardy Wienberg Equilibrium, suggesting either importance for T2D susceptibility or a technical error. A number of samples were sequenced to confirm genotyping. All genotypes were confirmed. Genotype frequencies from T2D subjects were compared with those from the HapMap database (CEU samples) and the CAPS study.

The genotype and allele frequencies of T2D subjects were significantly different fro either the HapMap or CAPS subjects, with the variant allele (A) being overrepresented in T2D subjects. The A allele was associated with higher LDL and CRP levels, but lower IGF-I levels. In conclusion, this novel SNP has potential as a marker for those at increased risk of T2D.

5 CONCLUSIONS

This thesis supports our hypothesis that genetic variations in factors which influence metabolism have opposing effects on T2D and PC. That GHR is involved in metabolic homeostasis is well known, however the influence of the exon 3 deletion in processes other than longitudinal growth is a new field. The findings in T2D also shed light on the oncogenic role of GHR. Although MUC1 has been implicated in a variety of cancers, including prostate cancer, systematic assessment of genetic variation in tumour samples has previously not been performed. This thesis leads the field in addressing tumour specific genetic alterations of this gene and the potential for functional differences between MUC1 isoforms, as well as reporting a role of MUC1 in metabolic control.

Our hypothesis of opposite effects of genetic variants on PC and T2D would suggest that GHR_{d3} is a risk allele for PC. Given that ADT leads to an insulin-resistant state, carriers of GHR_{d3} may be at increased risk of T2D as a complication of treatment. Epidemiological studies support a role for this allele in increasing PC risk, via increased CRP and IGF-I levels. However, McKay *et al* found no such association [74], suggesting that if such an effect exists, it is at the transcriptional or post-transcriptional level, which is an area still to be addressed.

That the risk allele for HPC in our study is that same allele as that which reduces risk of T2D agrees with the hypothesis that genetic variants have opposing effects on T2D and PC. Given that glucose is not believed to be the main source for prostate tumours, the finding in Paper IV that the same allele of rs4072037 is associated with increased LDL levels is intriguing. If LDLs are indeed a substrate for PC, it would provide not only novel therapies for treating tumours, but also potential strategies for non-invasive diagnosis and monitoring.

The common feature of these two polymorphisms is that the variant allele increases levels of IGF-I, high levels of which are known to reduce T2D risk whilst increasing that of PC. The influence of these variants on clinical parameters of T2D may be via their influence on IGF-I or via functional differences in the encoded proteins; it is plausible, for example, that differences in mucus function of MUC1 isoforms determine uptake of LDLs or that IGF-I-independent effects of GH are able to influence BMI.

These results suggest that variants may influence both risk and the ability to control diabetes (thus influencing risk of complications), however the direction of the associations differ. This is in contrast to the suggestion that risk alleles are distinct from complication variants, but is plausible. For example, high IGF-I levels may protect form diabetes, but in the event of vascular lesions, cause inappropriate proliferation.

Further studies are required to confirm these findings and determine the prognostic value of these *GHR* and *MUC1* polymorphism as biomarkers. Mapping of the mechanisms by which the encoded isoforms differ will potentially allow the design of therapeutic manipulation of the isoforms.

6 POPULAR SCIENCE

Epidemiology indicates that there are common factors in prostate cancer (PC) and type 2 diabetes (T2D). Environmental effects, such as diet and lifestyle, appear to have the same effect on both diseases. However genetic risk factors seem to have opposing effects on the two diseases, with those that increase risk of PC decrease the risk of T2D, and vice versa. Variations in genes cause differences in the encoded protein's function, by altering the levels of the protein produced or changing the structure and thus function of the protein.

The aim of this thesis was to investigate the influence of common genetic variation of two novel genes, *MUC1* and *GHR*, on PC and T2D. GH signaling via the GH receptor (GHR) is involved in regulation of many processes including linear growth, glucose and lipid metabolism and tumourigenesis. MUC1 is involved in protecting the integrity of glandular structures and functions from mechanical, chemical and bacterial stress.

Comparison of DNA samples from blood of subjects with and without PC we have demonstrated that *MUC1* variations do not influence risk or aggression of PC. Nor is PC-specific survival influenced by common variation of *MUC1*. Analysis of a specific variant in PC tumours and blood from the same patient indicated loss of the variant allele, suggesting that changes within the PC tumour are of importance for tumour formation. The reduced prevalence of the variant allele in hereditary PC compared to sporadic PC or cancer-free subjects may indicate a protective effect, albeit in a small number of PC families. Predictions of the functional differences in the proteins resulting from the genetic variations indicate that the protective functions of MUC1 are changed, thus influencing disease risk.

The variant allele of this same genetic variation in *MUC1* is more frequent in subjects with T2D than control subjects, suggesting a functional role. Furthermore, carriers of the variant was associated with better lipid and inflammatory profile that non-carriers, indicating a reduced risk of diabetic complications.

Comparing subjects with normal glucose tolerance to those with impaired glucose tolerance or T2D demonstrated that a partial deletion of *GHR* appears to protect against T2D. Diabetic subjects with this variant demonstrated worse metabolic control compared to non-carriers, thus are at increased risk of diabetic complications.

Identification of those subjects at highest risk of diabetes or its complications would enable more efficient monitoring of vulnerable populations and the appropriate application of more aggressive prevention strategies. In conclusion, common variants in *MUC1* and *GHR* demonstrate potential as biomarkers for risk of T2D and its complications. It would appear that *MUC1* variations do not indicate PC risk status for the majority of subjects, however local genetic alterations within the tumour may be relevant to tumour biology.

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