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GENISTEIN

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**A CHEMOPREVENTIVE FACTOR IN
PROSTATE CANCER**

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CLINIC OF CANCER, SURGERY AND TRANSPLANTATION

DEPARTMENT OF UROLOGY

OSLO UNIVERSITY HOSPITAL

© **Bato Lazarevic, 2012**

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TO CLARA, OLIVER & REBECCA

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TABLE OF CONTENTS

LIST OF PAPERS	1
ABBREVIATIONS	2
1.0 INTRODUCTION	5
1.1 Epidemiology	5
1.1.1 Prostate cancer and risk factors	5
1.1.2 Prostate cancer epidemiology	5
1.1.3 Soy consumption and prostate cancer	6
1.1.4 Urgency to prevent prostate cancer	7
1.2 Nutrition and Cancer	8
1.2.1 Chemoprevention	8
1.2.2 Nutrition and prevalence of cancer	8
1.2.3 Nutrition and prostate cancer	9
1.3 Cancer	10
1.3.1 Background	10
1.3.2 Prostate cancer and androgens	11
1.3.3 Morphological changes in the progression of CaP	12
Gleason score	14
TNM-staging	14
1.3.4 Gene expression changes in the progression of CaP	14
Androgen related genes	15
Cell cycle related genes	16
Proliferative and apoptotic related genes	17
Neuroendocrine related genes	17
1.4. Genistein	18
1.4.1 History of the soy bean and its use	18
1.4.2 Background	18
1.4.3 Cellular and molecular effects	21
Hormonal effects	22
Estrogen	22
Androgen	22
Insulin	22
Thyroid	23
Anti-oxidant	23
Tyrosine kinases	23
DNA modulation	24
DNA topoisomerase II	24
Telomerases	24
Epigenetics	24
Peroxisome proliferator-activated receptors (PPARs)	25
Integrins, matrix metalloproteinases (MMPs) and angiogenesis	25
Major intracellular pathways	26
Akt/Protein Kinase B (PKB)	26
Mitogen activated protein kinases (MAPKs)	26
Cell viability	27
Cell cycle	27
Proliferation	27
Apoptosis	27
1.4.4 Clinical studies	28
1.4.5 Toxicity	28
Hormonal effects on men and female reproductive systems	29
Breast cancer	29
Prostate cancer	29
Infant leukemia	30
Thyroid disease	31
2.0 BACKGROUND AND AIMS OF THE STUDY	33

3.0 SUMMARY OF PAPERS	35
3.1 Paper I	35
3.2 Paper II	36
3.3 Paper III	37
3.4 Paper IV	38
4.0 METHODS	39
4.1 Pre-Clinical Study	39
4.1.1 Cells and treatments	39
4.1.2 Methodological aspects	39
4.2 Clinical randomized Trial	40
4.2.1 Accrual	40
4.2.2 Methodological aspects	40
4.2.3 Statistics	41
5.0 DISCUSSION	43
5.1 Genistein modulation of AP-1 may regulate ARG expression	43
5.2 Genistein may be a chemopreventive agent in early CaP	44
5.3 Genistein has no effects on sex hormones in circulation	45
5.4 Genistein has no effects on thyroid hormones in circulation	46
5.5 Genistein lowers serum cholesterol	46
5.6 Genistein phase II metabolism differs in blood and prostate tissue	46
5.7 Genistein is safe to use in CaP patients	47
6.0 CONCLUSIONS	49
7.0 FUTURE PERSPECTIVES	51
8.0 REFERENCES	53
9.0 APPENDIX PAPERS I – IV	65

LIST OF PAPERS

- I. **Lazarevic B**, Karlsen S.J, Saatcioglu F. Genistein differentially modulates androgen-responsive gene expression and activates JNK in LNCaP cells. *Oncol Rep.* 2008 May;19(5):1231-5.
- II. **Lazarevic B**, Boezelijm G, Diep L.M, Kvernrod K, Ogren O, Ramberg H, Moen A, Wessel N, Berg R.E, Egge-Jacobsen W, Hammarstrom C, Svindland A, Kucuk O, Saatcioglu F, Austlid Taskèn K, Karlsen S.J. Efficacy and safety of short-term genistein intervention in patients with localized prostate cancer prior to radical prostatectomy: a randomized, placebo-controlled, double-blind Phase 2 clinical trial. *Nutr Cancer.* 2011;63(6):889-98
- III. **Lazarevic B**, Hammarstrom C, Yang J, Ramberg H, Diep L.M, Karlsen S.J, Kucuk O, Saatcioglu F, Austlid Taskèn K and Svindland A. The effects of short term genistein intervention on prostate biomarker expression in patients with localized prostate cancer prior to radical prostatectomy. *Br J Nutr.* 2012 Mar 8:1-10
- IV. **Lazarevic B**, Moen A, Karlsen S.J, Egge-Jacobsen W. Disposition of synthetic genistein in humans induces genistein to genistein-phase II metabolite ratio differences in plasma and prostate tissue. Submitted.

ABBREVIATIONS

AE	adverse event
AR	androgen receptor
ARG	androgen regulated gene
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
CaP	prostate cancer
CgA	chromogranin A
ChT	charcoal-treated
CT	cycle threshold
DNA	deoxyribonucleic acid
EGFR	epidermal growth factor receptor
ECM	extracellular matrix
ER α	estrogen Receptor α
ER β	estrogen Receptor β
FCS	fetal calf serum
GM	genetically modified
GST	glutathione S transferase
IARC	International agency for research on cancer
JNK	c-Jun amino (N)-terminal kinase
KLK4	kallikrein 4
LH	luteinizing hormone
LNCaP	prostate carcinoma cell line
LRP	laparoscopic radical prostatectomy
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells

NKX3.1	NK3 homeobox 1
NSE	neuron specific enolase
NURSA	Nuclear receptor signaling atlas
PARP	poly (ADP-ribose) polymerase
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PIN	prostatic intraepithelial neoplasia
PKB	Akt/protein kinase B
PPAR	peroxisome proliferator-activated receptor
PSA	prostate specific antigen
PTEN	phosphatase and tensin homolog
RAR β	retinoic acid receptor β
RCT	randomized clinical controlled trial
ROS	reactive oxygen species
RR	relative risk
SELECT	Selenium and vitamin E cancer prevention trial
SHBG	sex hormone-binding globulin
q-PCR	quantitative polymerase chain reaction
STAMP2	six transmembrane protein of prostate 2
T3	triiodothyronine
T4	thyroxine
TPO	thyroid peroxidase
TRAMP	transgenic adenocarcinoma of the mouse prostate
TSH	thyroid stimulating hormone
U.S.	United States of America

1.0 INTRODUCTION

1.1 EPIDEMIOLOGY

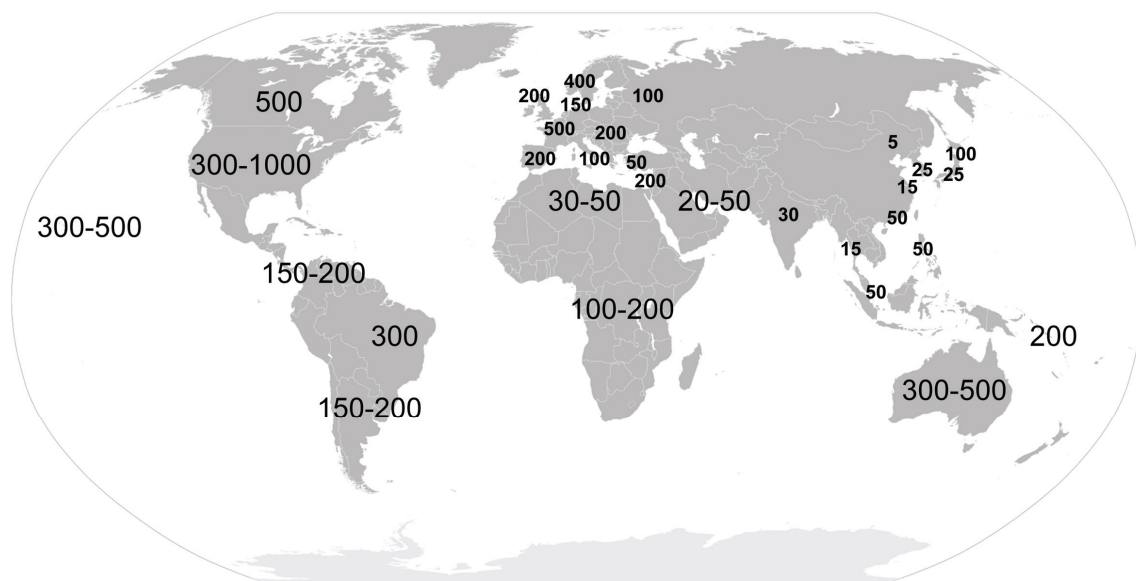
1.1.1 PROSTATE CANCER AND RISK FACTORS

Prostate cancer (CaP) is the most common non-skin cancer and the second most common cause of cancer death in Norwegian men (1). In 2009, 4299 new cases of CaP were recorded and 1048 died of CaP in Norway. The established risk factors of CaP are age, ethnic origin, heredity and geographic localization (2). There might be a weak association between obesity and CaP (3). Socioeconomic status has been a suggested risk factor, although unequal general health care among groups of residents may explain the difference (4). Occupation does not seem to be a risk factor in CaP (5).

1.1.2 PROSTATE CANCER EPIDEMIOLOGY

The earliest reliable cancer registries and epidemiological studies 40 years ago showed a distinct 40-fold difference in the incidence of CaP between U.S. African and native Japanese men (6). Latest registry data during 1998 to 2002 from the International Agency for Research on Cancer (IARC) show even higher differences (7). Figure 1 shows the CaP incidence per 100.000 men at 60 years of age in various countries around the globe. At 60 years of age, 1 % of U.S. black men will be diagnosed with CaP, while 0.5 % of Western European men, 0.025 to 0.1 % of Japanese men and 0.005 % of North-East Chinese men are diagnosed having the disease. The maximal difference is therefore 200-fold. There are also ethnic differences within the same region, like the higher incidence at 0.2 % for Israeli Jews as opposed to surrounding ethnicities which have incidence rates 4 to 10-fold lower. There are differences between countries having the same ethnic population, like a 2-3 fold lower incidence in Denmark compared to the rest of the Scandinavian countries, although the mortality rates are the same (8). When Chinese and Japanese men migrate to U.S., the incidence of CaP will increase to about half of that of the Caucasian population (7). The lifetime risk of a newborn today to be

diagnosed with CaP is for an U.S. Black 19.10 %, an U.S. White 16.48 %, an U.S. Asian 11.46 % and a Norwegian 12.7 % (1, 9).



Data source: The International Agency for Research on Cancer (IARC)/WHO. Cancer Incidence in Five Continents, Vol. IX (1998-2002)

Figure 1. Incidence of prostate cancer per 100 000 at 60 years of age.

1.1.3 SOY CONSUMPTION AND PROSTATE CANCER

High plasma levels of genistein have been found in men living in areas with decreased risk for CaP, whereas low levels are found in areas with increased risk. The difference in genistein levels may be more than 100-fold (10-12). Several studies have established an association between decreased CaP risk and high soy consumption or genistein plasma levels. In a cohort of 7999 men of Japanese ancestry in Hawaii observed from 1965 through 1986, increased tofu intake was associated with decreased risk of CaP (13). A later study on 5826 of these men concluded that the relative risk (RR) was 0.8 comparing high and low consumers of tofu, but it was not statistically significant (14). The Adventist Health Study in the U.S. (12,395 men) showed that once a day consumers of soy milk had a significant 40% reduced incidence of CaP while those who had several servings of soy milk daily had a 70% reduction, although it was not statistical significant (15). In the

Japanese Life Span Study cohort in Hiroshima and Nagasaki on 18115 men, total soy consumption was associated with a 20 % non-significant decrease of CaP (16). In the Japan Public Health Center–Based Prospective Study on 43509 men, a significant decrease of CaP risk (RR=0.52) in the highest compared to lowest consumers of soy food was reported (17). In a prospective nested case control study on 950 cases and 1042 controls participating in the European Prospective Investigation into Cancer and Nutrition (EPIC), a significant decreased risk (RR=0.74) of CaP was found in men with the highest circulating levels of genistein compared to the lowest (18).

1.1.4 URGENCY TO PREVENT CAP

The economic burden of CaP diagnosis and treatment, by drugs, radiation or surgery, is high in Western high endemic countries. Over- and under treatments of CaP are concerning issues (19). Although CaP occurs mainly in old age, most men receiving the diagnosis are still productive and the psychological effects may be devastating in respect to social life, family and work. The number of patients having CaP is expected to double or maybe even triple within the next decade in Western countries (20). It is therefore urgent not only to improve diagnosis and treatment of the disease, but also to improve and intensify preventive measures. There is strong epidemiological support for one or several environmental factors increasing or reducing the risk of CaP in a regional male population.

Currently, there is no highly specific CaP biomarker, which expression would be related to the aggressiveness of the disease. However, the level of prostate specific antigen (PSA) in blood gives a good indication of tumor load and progression when the disease has been diagnosed. PSA velocity has been significantly associated with outcome (21).

1.2 NUTRITION AND CANCER

1.2.1 CHEMOPREVENTION

Chemoprevention of cancer is the ability of certain molecules to inhibit (partially or totally) induction or progression of the disease. Ideally the molecule should be easily accessible, cheap, non-toxic and have a measurable clinical effect. Preferably it should be part of the normal nutrition in natural or enriched food. The hypothetical mechanisms of chemoprevention include that the agent; 1) acts as an anti-hormone and modulates hormonal receptors, e.g. tamoxifen in estrogen-dependent breast cancers, 2) acts as an anti-oxidant, thus reducing the damage on the genome by free radicals and relieving the cells own DNA repair machinery and 3) acts directly on intra-cellular signaling pathways, e.g. on important junctions for growth signaling like Akt/protein kinase B (PKB). A number of substances with all or some of these properties have been identified. During the last 30 years the basic and clinical research on the chemopreventive properties of these molecules has increased. Theoretically, CaP is a suitable target for chemoprevention due to the long latency of the disease and the usually slow progressive development into more aggressive stages.

1.2.2 NUTRITION AND PREVALENCE OF CANCER

Most people would recognize that there is a connection between what we eat and the prevalence of cancer, although we cannot estimate accurately the proportion of cancer attributed to nutrition factors. The reason for this is that we in most cases still don't know the causative agents. Even harder is the identification of protective agents. However, we know that acrylamide, a possible carcinogen, may be present in relative high concentrations in some prepared foods like French fries, although the epidemiological evidence is lacking for most cancers, except for a small risk increase in kidney cancer (22, 23). Nitrite, which is used for preservation in foods, is associated with stomach and esophageal cancer (24). Aflatoxin is associated with hepatocellular cancer (25). Also, some more or less diffuse connections between nutrition and cancer are known, e.g. obesity, energy intake, red meat, hot food and

alcohol (26). In a review in 1981, Doll and Peto concluded that their best estimate was that 35 % of cancer deaths were attributed to diet factors and that the range of acceptable estimates was 10-70 % (6).

1.2.3 NUTRITION AND PROSTATE CANCER

As mentioned above, CaP is a good target for chemoprevention due to the long latency and slow progression of the disease, at least during the initial stages. The identification of protective agents is very difficult, due to the obvious small potency of agents that are supposed to fulfill the criteria of chemoprevention, foremost to be non-toxic during life-time use. Epidemiological studies are perhaps the strongest proof for the effects by these agents, although both molecular mechanisms and clinical trials are necessary to verify the evidence. It is not within the scope of this thesis to discuss all promising chemopreventive agents related to CaP. However, some should be mentioned. Resveratrol, a polyphenol, which is present in the skin of grapes has several similar molecular effects to genistein (27). It also has an epidemiological support by the lower incidence of CaP in the Mediterranean area compared to Northern Europe. Clinical studies are ongoing. The effects of Vitamin D are also supported by a South vs. North epidemiological difference, in addition to potent molecular effects (28). However, the toxicity in its present form makes it unsuitable as a chemopreventive agent. Lycopene, the red pigment in tomatoes, has a strong anti-oxidant activity and is also supported by North vs. South epidemiology (29). The effects of selenium, a mineral with a relatively high risk of toxicity, which is necessary for vital cellular functions, are supported by its anti-oxidant properties and by a phase II randomized clinical controlled trial (RCT) primarily investigating skin cancer, which showed a significantly less (RR 0.37) incidence of CaP (30). However, the phase III clinical trial, Selenium and Vitamin E Cancer Prevention Trial (SELECT) in the U.S. including more than 35.000 participants during 7 years of intervention with selenium, did not detect any significant benefit (31). Surprisingly, later analysis of its results shows that intervention with Vitamin E, a proposed anti-oxidant, induces a 17% statistically significant increase in CaP (32). In the Finnish Alpha-Tocopherol Beta-Carotene Cancer Prevention Study (ATBC), which primarily investigated the

chemoprevention of lung cancer in almost 30.000 male smokers, Vitamin E intervention resulted in 34 % lower incidence of CaP (33). These findings illustrate the difficulties in identifying the protective agents in cancer and the need for thorough epidemiological, molecular and smaller clinical intervention trials before venturing into large phase III/IV studies.

1.3 CANCER

1.3.1 BACKGROUND

Cancer is a common term for diseases caused by abnormal cells that grow without control and that are able to invade surrounding tissue. Figure 2 illustrates two ways of describing cancer, either by a chain of events or by biological capabilities. Cancer starts with subtle molecular changes before manifesting clinically, often spanning over many years. The following chain of events are suggested; 1) inflammation, which may create the prime setting for cancer, 2) premalignant cells, which with high probability will develop into malignant cells and 3) metastases, which represents the final and most advanced stage of cancer. These have also been the suggested targets of chemoprevention (34). Cancer may also be described as a collection of hallmarks of biological capabilities, which gives a more complex picture; 1) inducing angiogenesis, resulting in vascularization of the neoplastic cells. 2) evading growth or tumor suppressors, which may inhibit the cell cycle. 3) enabling replicative immortality by regeneration of DNA ends. 4) activating invasion and metastasis by a complex series of events, including cell to cell and cell to matrix interactions. 5) reprogramming of energy metabolism, shifting towards glycolysis producing lactate and becoming less sensitive towards hypoxic conditions. 6) sustaining proliferative signaling. 7) evading immune destruction and 8) resisting cell death (35). Increased mutability and inflammation may act as enabling characteristics in the progression of cancer.

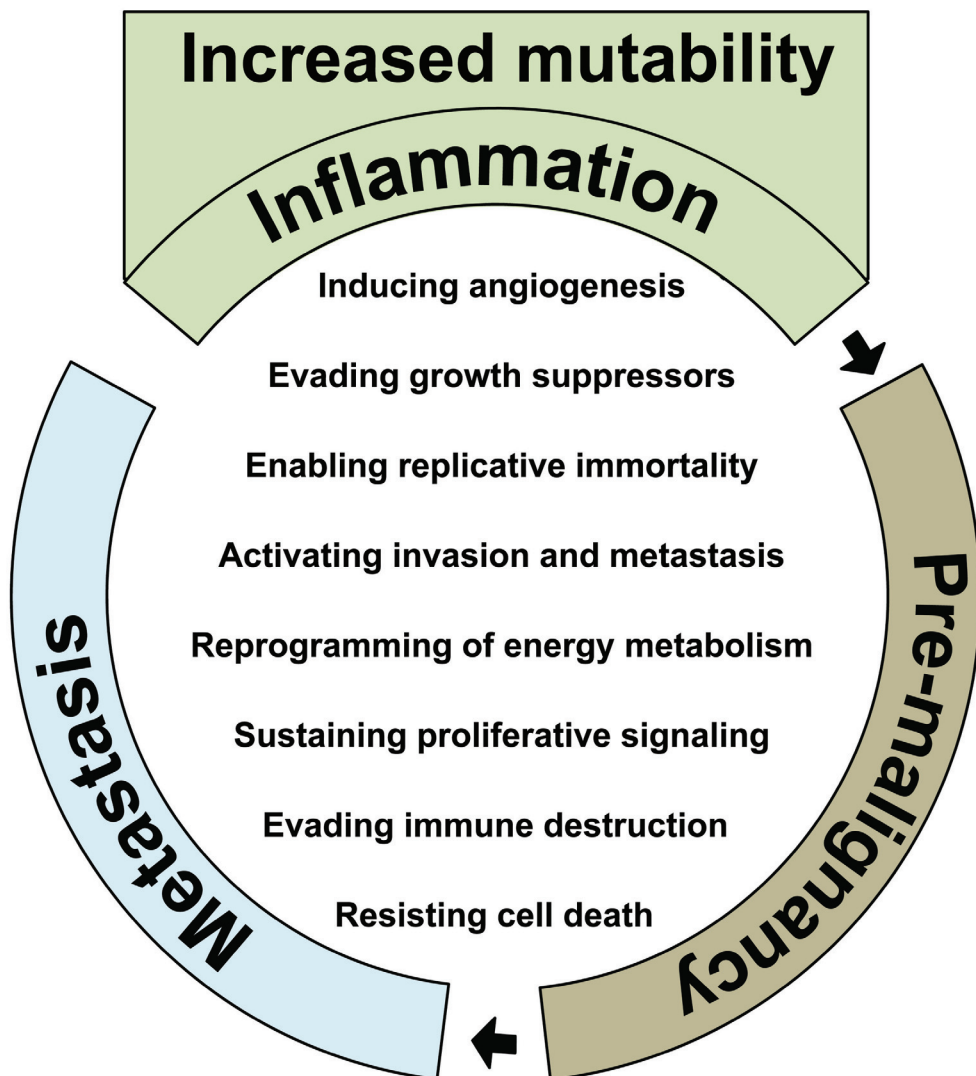


Figure 2. Cancer as a chain of events or biological hallmarks, including the enabling characteristics on top.

1.3.2 PROSTATE CANCER AND ANDROGENS

Since the 1940s pioneering and Nobel prize award winning work by Charles Huggins, androgens have been recognized as an important factor in CaP progression. Androgen depletion, either by orchiectomy or drugs, still stands as the

first line of treatment of advanced CaP (36). The androgens and the androgen receptor (AR) are important for prostate development and function, and AR positive cells are found both within the glands and the surrounding stroma. The AR gene is located on the X chromosome (37). It belongs to the nuclear receptor superfamily, which ligand-activated transcription factors are classified into three groups depending on the chemical property of their ligands, steroid, non-steroid or orphan (38). The AR is part of the steroid receptor subfamily and forms homodimers during activation. The structural composition of nuclear receptors contains an amino-terminal domain (NTD), a DNA-binding domain (DBD), a hinge region and a ligand binding domain (LBD). More than 100 cellular proteins, including co-activators and co-repressors that regulate AR function, have so far been identified (Nuclear Receptor Signaling Atlas (NURSA)). It was early recognized that not all advanced cases of CaP were sensitive to androgen depletion and that most CaP cells after a few years will become insensitive to androgen depletion treatment (development of castration resistant prostate cancer (CRPC)). It is still unknown how the CaP cells acquire this refractory trait, but the androgen signaling pathway seems to play an important role in most cases as mutations in the AR, changes in the activity of its cofactors, up-regulation of steroidogenesis and ligand independency are often observed (39).

1.3.3 MORPHOLOGICAL CHANGES IN THE PROGRESSION OF CAP

Several descriptive methods of the morphological changes in the progression of CaP have been developed and extended during the last decades. The top part of figure 3 illustrates 3 morphological methods of describing the progression of CaP, without any internal spatial specificity or temporal similarities between the methods. The simplest is to regard a transition from the normal epithelium to proliferative inflammatory atrophy (PIA), as a possible result of hormonal changes, physical trauma, viral, bacterial and/or oxidative damage (40). PIA has been observed in close relation to prostatic intraepithelial neoplasia (PIN), High grade PIN (HGPIN) and CaP. PIN is characterized by several cytological characteristics and HGPIN by further distinct characteristics, closely resembling those of CaP, like larger nuclei and increased chromatin content (41). Several molecular changes, like

chromosomal deletions, are also frequent both in HGPIN and CaP. It is somewhat controversial whether latent CaP is a special entity in the progression of CaP with not fully developed and senescent malignancy or if it simply is a developing CaP (42). Especially older autopsy materials revealed that many men in old age had prostate lesions resembling CaP, without being clinical apparent, indicating a prevalence of almost 40 % in 50 years old men (43). However, more recent studies have reported less than 1 % prevalence in 50 years old men (44). Symptomatic clinical CaP may present itself by lower urinary tract symptoms (LUTS), including urgency, obstruction, hematuria and infection. A tumor in the prostate may be palpable by digital rectal examination. The diagnosis is histologically verified by needle or resection biopsy. The final stage is metastases, nearly always first to regional lymph nodes and bone, although it may metastasize to other organs such as lung, liver, pleura, adrenal glands and brain (45, 46).

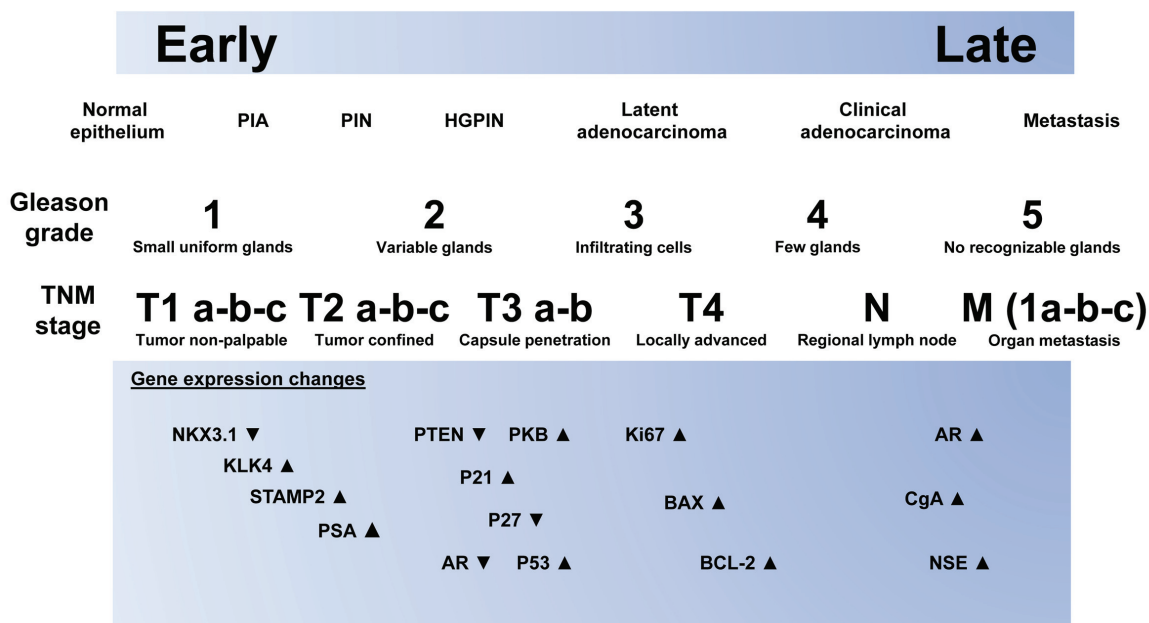


Figure 3. Morphologic and gene expression changes in the progression of CaP.

Gleason score

The Gleason grading and scoring system is based on morphologic glandular changes in the progression of CaP. The scoring is composed of a major primary grade and a secondary grade from 1 to 5. The grades 1 and 2 are rarely used as they can hardly be discriminated from normal glands (47). An additionally higher tertiary grade is sometimes used, although it is debated whether it is a factor of prognostic significance. Gleason grade 3 is the most common in CaP and is considered as well-differentiated. The glands may differ in size and some cells are invading the stroma, but each gland is surrounded by stroma. Gleason grade 4 is important as patients harboring this grade have a worse prognosis. The glands no longer form separate units with lumen and surrounding stroma. Gleason grading is widely used and is a strong prognostic parameter in CaP.

TNM-staging

The TNM-staging system of CaP represents the primary tumor load (T), presence of regional lymph node involvement (N) and distal organ metastasis (M) (48). At stage T1, the tumor is not clinically apparent and further divided into (a) and (b) for resected material and (c) for needle biopsy. At stage T2, the tumor is confined within the prostate and it is further divided into a, b and c depending on its involvement area of the prostate. At stage T3, the tumor extends through the prostate capsule and it is further divided into a and b depending on involvement of the seminal vesicles. At stage T4, the tumor is locally advanced and invades adjacent organ structures. The M stage (distal organ metastases) is divided into M0 (no distal metastases) and M1 (distal metastases), which is further divided into M1a (non-regional lymph nodes), M1b (bone) and M1c (other sites with or without bone). The TNM system is regularly revised and changes are likely to be proposed during coming years.

1.3.4 GENE EXPRESSION CHANGES IN THE PROGRESSION OF CAP

In addition to the close relation between CaP and androgens, the last decades of research have uncovered several gene expression changes. Some of these are appearing quite early in the progression of CaP, whereas others appear late.

However, the temporal relationship of the events and their significance regarding the development of CaP are mostly unknown (49). Those associated with our studies will be mentioned. The lower part of figure 3 illustrates the gene expression changes in relation to the progression of CaP, although the temporal and spatial differences in general still are uncertain. The changes will be detailed in the following chapters.

Androgen related genes

The androgen regulated genes (ARGs) represent a large group of genes connected to the AR by having an androgen response element (ARE), a conserved short DNA sequence matching the DNA binding site of the AR, in their promoters. Androgens regulate growth and differentiation of the human prostate. This is also mimicked at the gene level as AR-responsive genes linked to differentiation and cell cycle regulation are regulated as well as genes encoding enzymes of metabolic pathways. The expression of the AR itself is decreased with increasing Gleason grade (50). However, AR expression remains in most CaP and increases in CRPC compared to hormone sensitive CaP (51).

The androgen related gene expression changes appear mainly quite early in the progression of CaP. Among the best known ARGs are prostate specific antigen (PSA), also known as kallikrein 3, and NK3 homeobox 1 (NKX3.1). NKX3.1 is one of the earliest markers of prostatic luminal cells and is a suggested tumor suppressor in CaP (52, 53). Up to 60-80% NKX3.1 loss of heterozygosity has been reported in high grade CaP, although it also appears to a lesser degree in PIN (54, 55). However, although the nuclear protein expression of NKX3.1 is reduced in early CaP and continues to decrease with progressing CaP, it is not completely absent even in metastatic CaP (56). PSA is a widely used and the most important prognostic biomarker in CaP. Its main function is to liquefy the prostatic fluid by its serine protease activity and it is produced by differentiated luminal cells of the prostate. Although the serum level of PSA is increased during the progression of CaP, the intra-cellular expression of PSA is reduced with increasing Gleason grade (57). Kallikrein 4 (KLK4), encoding a serine protease neighboring KLK3 on chromosome 19, is also an AR target gene. In contrast to the genes mentioned above, KLK4 expression is increased in malignant cells and this may relate to is

putative role as a proliferative factor in CaP (58). The 6 trans-membrane protein of prostate 2 (STAMP2), also known as STEAP (six trans-membrane epithelial antigen of prostate) family member 4 (STEAP4)), is an androgen regulated metalloredutase that may also contribute to prostate cancer progression. Interestingly, STAMP2 has been implicated in adipocyte differentiation, inflammation and nutritional responses. The expression of STAMP2 is up-regulated early in many, but not all CaP tumors (59).

Cell cycle related genes

The cell cycle is a tightly regulated process leading to the duplication and division of a cell (60). Progression throughout the cycle is controlled by binding of specific cyclins to cyclin-dependent kinases (CDK). In the G₀-phase, which is a resting phase, progression is mainly regulated by the gatekeeper, the retinoblastoma tumor suppressor protein (RB), which assembles repressor complexes on promoters of genes needed for progressing. The next phase, G₁-phase, is when mitogenic stimuli have overcome RB repression and the cell size increases. Here different cyclin D's are produced, which activate CDK4 and CDK6, which in turn phosphorylate and initiate the inactivation of RB. Further phosphorylation and complete inactivation of RB is mediated by CDK2. The CDK inhibitor 1A (p21^{Waf1/Cip1}) is a negative regulator of the cell cycle and its expression is mostly related to increasing levels with progressing CaP (61, 62). p21^{Waf1/Cip1} is associated with the checkpoint in the G₁-phase, where it inhibits CDK2. Cyclin A and E are responsible for the activation of CDK2 and they will also initiate the S-phase, where the DNA is replicated. In the following phase, the G₂-phase, cell growth will continue and the progression is controlled by the levels of CDK1 (in humans named CDC2) with cyclin A and B complexes until the final phase, the M-phase (mitosis), where the cell divides. The cell cycle machinery controlled by cyclin-CDK complexes is further counterbalanced by mechanisms during cellular insults. The CDK inhibitor 1B (p27^{Kip1}) is associated with several cell cycle checkpoints, by acting both on CDK2 and CDK1 (63, 64). p27^{Kip1} acts as a tumor suppressor and its expression is decreased in CaP. The phosphatase and tensin homologue (PTEN) is not directly related to the cell cycle, but acts as a major suppressor of protein kinase B (PKB, also known as Akt) signaling, which is a major pathway of growth receptor signaling. PKB activation is mediated by phosphatidylinositol 3-kinases

(PI3K), whereas PKB inhibition is mediated by PTEN (65). Its gene expression is commonly absent or reduced in approximately 60-80% of CaP cases, resulting in a constitutive activation of PKB by the PI3K pathway (66, 67). PKB is connected to p27^{Kip1} through AFX-like Forkhead transcription factors and PKB activation will result in reduced p27^{Kip1} levels (68). Tumor protein 53 (p53), is a tumor suppressor protein, also known as the genome watchman. It is associated with genome damage and the checkpoint in G1-phase before DNA replication. Its expression is also associated to p21^{Waf1/Cip1} and p53 expression is increased in later stage CaP (61, 69).

Proliferative and apoptotic related genes

Cellular proliferation, pro- and anti-apoptotic mechanisms are common in all cancers, also in CaP. Several molecular changes have been reported. The nuclear antigen Ki67 is associated with proliferating cells and its expression is noticeably induced in a few percent of epithelial cells in early CaP and its expression increases with CaP progression (70). Both the anti-apoptotic B-cell CLL/lymphoma 2 (BCL-2) and the pro-apoptotic B-cell CLL/lymphoma 2-associated X protein (BAX) protein expression levels are reported to be increased in CaP (71). The BCL-2 subfamily has several important functions in the regulation of apoptosis, including controlling the release of cytochrome c from the mitochondria, which in turn will activate the caspases, which will carry out the proteolysis during the demolition part of apoptosis (72).

Neuroendocrine related genes

The human prostate is composed of a glandular compartment with luminal and basal epithelial and scattered occasional neuroendocrine (NE) cells. There is also a stromal compartment composed mainly of stromal cells and to a lesser degree of endothelial cells, lymphocytes, fibroblasts and smooth muscle cells. The role of NE cells is still unknown, but it is proposed that they may have a paracrine function, supporting the growth, secretion and differentiation of the glandular epithelial cells (73). NE cells may be identified by the expression of chromogranin A (CgA) and neuron-specific enolase (NSE). Increased numbers of NE cells is associated with late stage CaP, especially CRPC. Also, in rare cases CaP may first appear as an aggressive small cell carcinoma expressing NE antigens (74).

1.4 GENISTEIN

1.4.1 HISTORY OF THE SOY BEAN AND ITS USE

The soy bean plant is one of the oldest known crops to mankind and may have been domesticated already 9000 years ago (75). The exact origin of domestication is still unclear, although the archeological evidence indicates Northern China, Korea and Japan. These are also the areas in which the populations consume the highest amount of traditional soy bean products, such as soy sauce, tofu (soy cheese), miso (soy bean paste soup) and edamame (boiled young soy beans in their pods).

The word soy is derived from the Japanese word for soy sauce “shoyu” and it originated from the Dutch and Portuguese trade with Japan during the 16th century. The Europeans adopted the taste of soy sauce and it spread quickly across the continent with similar names; soy, soja, soia, soja or soya. At the end of the 18th and beginning of the 19th century scientists were interested in the presence of proteins, and the soy bean was identified to be rich in proteins. In fact, soy bean plants give the highest protein yield of all known cultivated crops per area.

The independence of nitrate fertilizers makes the soy plant suitable for growing in poor soil (76). Most of the soy bean production today is used for soy oil, which accounts for 50 % of all plant oils. A large proportion is also used in animal food. Nearly all U.S. and most of Brazils, which are the two biggest producers, soy plants have been genetically engineered to withstand pesticides. Therefore, soy has a rather bad reputation for ecological and environmental preservation.

1.4.2 BACKGROUND

Its name was derived when its glycoside form, genistin (5,7,4'-trihydroxyisoflavone-7-glucoside) (Fig. 4), was isolated from the plant *Genista tinctoria* (Dyer's Broom) in 1899 (77). The reason for its isolation was to examine its usefulness as a textile dyer. Genistein, which is the aglycone form of genistin, was first synthesized in 1928 (78). Genistein belongs to the flavonoids, which are polyphenol compounds in plants with a yellow color. It is further divided into the isoflavonoids and finally isoflavones, which in addition to genistein also consist of daidzein and glycitein.

Additionally, genistein may also be regarded as a phytoestrogen. The two principal classes of phytoestrogens are the isoflavonoids (including coumestans) and lignans (79).

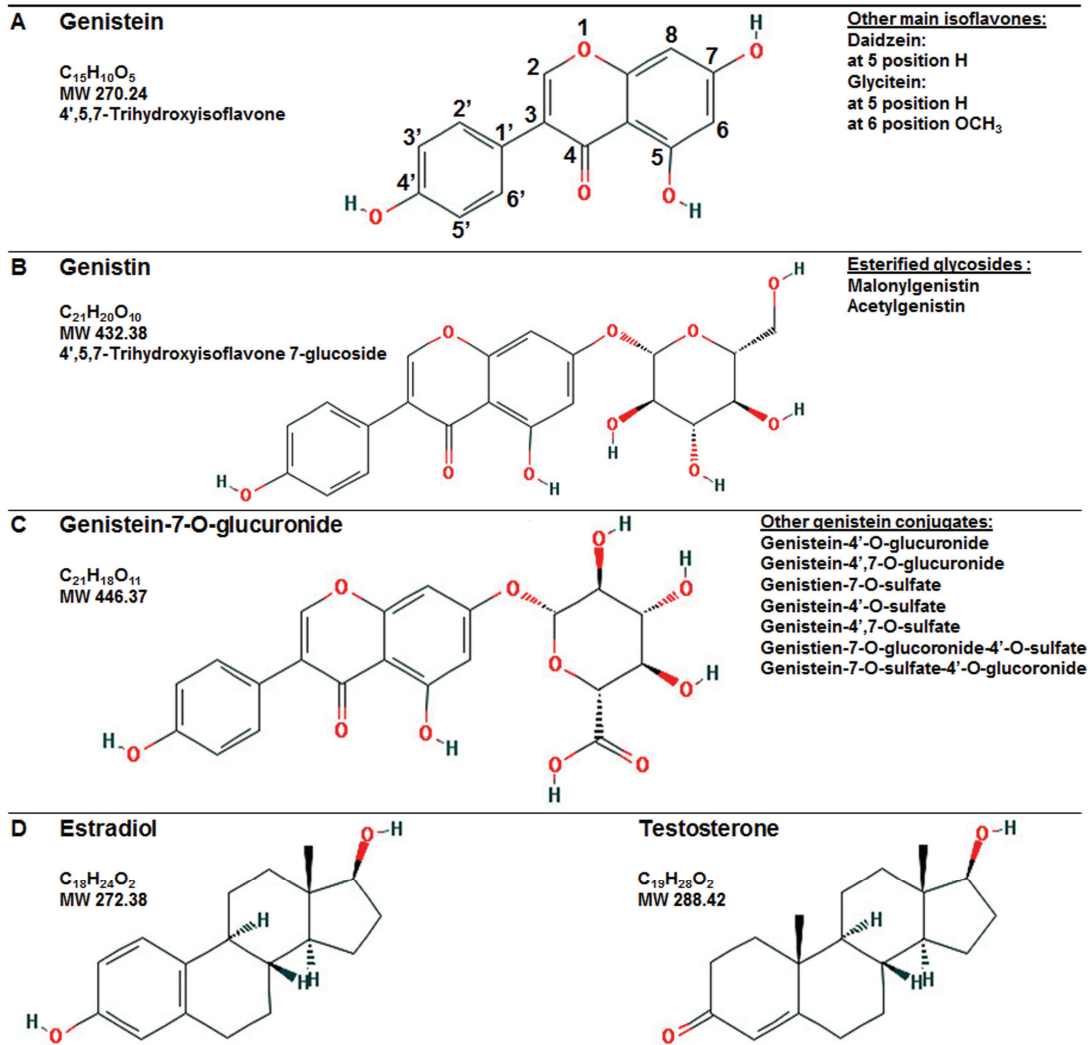


Figure 4. Molecule characteristics.

In 1931, genistin was shown to be present in soy beans (78). In fact, soy beans are the principal human source of genistin, the prevalent natural form, which requires the bacterial flora in the gut of the consumer or an external fermentation

process to metabolize it to genistein before absorption (80). In addition, later studies have shown the existence of two additional glycoside conjugates consisting of 6"-O-malonyl-b-glucoside and 6"-O-acetyl-b-glucoside, although their existence may depend upon food processing conditions (81). Small amounts of genistein may be found in other plants (82). Table 1 shows the content of genistein in different soy based products.

	mg/100 g
Soy oil	0
Soy sauce	1
Soy-based infant formula	2
Soy milk	6
Tofu	20
Miso soup	25
Roasted soy beans	65

Source: USDA-Iowa State University Database on the Isoflavone Content of Foods - 1999

Table 1. Content of genistein

The metabolic processes of absorbed genistein are elusive. One of the first findings was that genistein-glucuronide was detected when genistein was incubated with rabbit liver microsomal fractions and UDP-glucuronic acid (83). A more complex picture has later emerged. For the xenobiotic phase II metabolism, genistein is conjugated in the intestine and the liver during or after absorption by uridine 5'-diphospho (UDP)-glucuronosyltransferase and -sulfotransferases into glucuronides and sulfates (84). As shown in figure 4, two conjugating sites on the genistein molecule localized at positions 4' and 7 have been suggested and a total of nine different forms may exist after absorption (85, 86). They include the unconjugated genistein and the eight metabolites; 4' or 7 mono- and disulfates, and mono-, di- and sulfate-glucuronides. Most genistein is conjugated after absorption and unconjugated genistein represents only a few percent of total plasma genistein

(84). In addition, there is also a phase I metabolism of genistein. *In vitro* studies have revealed involvement of cytochrome P450 superfamily (CYP) enzymes CYP1A2 and 2D6 generating hydroxyl metabolites, including orobol (87). However, the extent of phase I metabolism of genistein has been indicated to be much less than the phase II metabolism (88).

Genistein and its metabolites are quite rapidly cleared from the body mainly by urine and partly by bile excretion (89, 90). The half-life of genistein in humans after oral administration is approximately 8 hours (91). Finally, there are major differences in genistein metabolism and conjugation both within and between species (92, 93). All these factors contribute to make exploring the metabolic processes of genistein difficult.

1.4.3 CELLULAR AND MOLECULAR EFFECTS

The cellular and molecular properties of genistein are several, diverse and not always coherent. Some of the properties are associated with high or very high concentrations of genistein ($> 10 \mu\text{M}$), whereas others can be seen at physiological relevant plasma levels in high consumers of soy products ($0.5 - 5 \mu\text{M}$). In addition, some of the effects have been reported as biphasic with stimulation at nanomolar and inhibition at micromolar concentrations. Several studies have used different mixes of soy extracts, containing variable amounts of genistein. The effects described in the present manuscript will focus on genistein alone. Undoubtedly, genistein is very pluripotent and the sheer scale of effects reported makes it impossible to discuss every finding. The division of effects made here does not represent any exclusivity and some of the effects may fit in several places. For example, genistein's inhibiting effects on proliferation can be described by hormonal effects of androgens, by DNA modulation of DNA Topoisomerase II, by modulating cell cycle regulation or apoptosis, by effects on intracellular signaling pathways or even effects by its tyrosine kinase inhibiting properties on growth receptors.

In plants, genistein is a chemical attractant for the nitrogen fixing bacteria of the *Bradyrhizobium* genus, activating so called nod genes needed for the symbiotic relation (94). It also acts as a selective antibiotic, inhibiting growth of

some bacteria such as *Staphylococcus aureus* and *Bacillus anthracis* (95, 96). In addition, its estrogenic properties may act as part of a fertility modulation defense against herbivores (97).

HORMONAL EFFECTS

Estrogen

Genistein is a phytoestrogen, meaning that it is an estrogenic compound found in plants. Its polyphenol structure has several characteristics similar to 17 β -estradiol (Fig 4). However, its estrogenic activity *in vitro* differs by having only 4 % of 17 β -estradiol binding affinity for Estrogen Receptor α (ER α) and 87 % for Estrogen Receptor β (ER β), while not having any marked antagonistic activity similar to tamoxifen (98). In CaP, ER β is associated with a protective role against abnormal proliferation of prostate epithelial cells by direct or indirect inhibition of ER α (99). The difference between receptor specificity is also suspected to mediate positive health effects such as increasing bone mineral density and in women reduction of menopause associated hot flushes, without negative effects such as affecting endometrial thickness (100-102).

Androgen

Several reports indicate that genistein modulates the activity of wild-type AR by transactivating it and inducing a biphasic effect wherein nanomolar concentrations stimulates and micromolar concentrations depress its activity (103, 104). However, a well-known AR point mutation (T877A), frequently present in CaP and present in the CaP cell line LNCaP, changes the receptor ligand binding affinity and transactivation activity of AR to estrogen in addition to androgen (103, 105). Additionally, genistein depresses steroid synthesis and metabolism by inhibiting 17 β -hydroxysteroid dehydrogenase and 5 α -reductase (106, 107).

Insulin

Genistein is able to enhance the insulin secretion in insulin-secreting cell lines at nanomolar concentrations, independent of ER and inhibition of tyrosine kinase, by increased cyclic adenosine monophosphate (cAMP) and activated protein kinase A (PKA) (108). Increased insulin- and insulin growth factor (IGF) levels have been

associated with CaP and will act through the insulin receptor or IGF receptor (IGFR) on cells by sending a tyrosine kinase dependent mitogenic signal through PI3K to PKB (109). However, late results from clinical studies have challenged the idea that IGFR inhibition reduces CaP (110, 111).

Thyroid

Starting at low micromolar concentrations, genistein inhibited thyroid peroxidase (TPO) activity in rats up to 80 %, without affecting serum levels of thyroid stimulating hormone (TSH), triiodothyronine (T3) or thyroxine (T4), suggesting that the remaining TPO activity is sufficient for retaining TSH production or a separate mechanism (112)

ANTI-OXIDANT

Reactive oxygen species (ROS) and oxidation of vital proteins and DNA are suspected to be important factors in carcinogenesis (113). Genistein has been shown to inhibit the formation of hydrogen peroxide (H₂O₂), 8-Oxo-2'-deoxyguanosine (8-oxo-dG) and malondialdehyde in skin of hairless mice exposed to ultraviolet B (UVB) radiation, either through indirect inhibition of neutrophil recruitment or direct quenching of ROS (114).

TYROSINE KINASES

Tyrosine kinases are involved in multiple cellular pathways and regulate the signaling activity of several cell growth receptors, such as epidermal growth factor receptor (EGFR), IGFR and fibroblast growth factor receptor (FGFR), which are of major interest in present-day cancer research and treatment (115). Genistein is a general inhibitor of tyrosine kinases and *in vitro* dose-dependently inhibits EGFR activity even at low micromolar concentrations, although complete inhibition required more than 100 μ M (116). This finding has been disputed and it is on the other hand proposed that it is the EGFR protein level that is reduced by genistein

(117). However, genistein inhibits the activity of multiple tyrosine kinase regulated receptors (118).

DNA MODULATION

DNA topoisomerase II

DNA topoisomerase II is an enzyme which unwinds and rewinds the DNA by breaking and re-ligating DNA during replication and transcription, so that the DNA becomes available for DNA polymerase (119). Topoisomerase II poisons are well established anti-cancer treatments, e.g. Doxorubicin. Genistein inhibits DNA topoisomerase II activity and induces single strand breaks at low micromolar concentrations and double strand breaks at high micromolar concentrations, possibly by competing for an Adenosine-5'-triphosphate (ATP) site (120). It has been suggested that the cytotoxic effects seen by genistein at very high micromolar concentrations are due to inhibition of DNA topoisomerase II activity (121).

Telomerases

The ends of the linear DNA in eukaryotic cells get shorter with each replication, resulting in genetic instability and senescence, which is prevented by telomerases adding a short repetitive DNA sequence (122). For immortality, cancer cells therefore have strong telomerase activity. Genistein at 30 μM inhibited telomerase activity in prostate cancer cell lines LNCaP and DU-145 (123). However, a more recent finding indicates that genistein induces a biphasic effect with increased telomerase activity in LNCaP, PC-3 and prostatic intraepithelial neoplasia (PIN) of the transgenic adenocarcinoma of the mouse prostate (TRAMP) at concentrations less than 1 μM , whereas a concentration at 50 μM depressed telomerase activity (124). The effect was coupled to the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3), mediating the effects of interleukin 6 (IL-6) and c-Src, a tyrosine kinase.

Epigenetics

The regulation of genes above the level of DNA sequence which is propagating, self-sustainable and gives a transcriptional effect is called epigenetics (125).

Examples are the different phenotypes arising from the fertilized egg or stem cells. Modulators of epigenetics include microRNA (miRNA), DNA methylation and histone modifications. Starting at low micromolar concentrations, genistein reversed hypermethylation and reactivated the mRNA expression of retinoic acid receptor β (RAR β), tumor suppressor B-cell translocation gene 3 (BTG3) and other silenced genes in human cancer cells by inhibiting DNA methyltransferase and modulating histone deacetylase activities (126, 127). High micromolar concentrations have also been shown to induce the tumor suppressors aplasia Ras homolog member 1 (ARH1) and phosphatase and tensin homolog (PTEN) in human CaP cells by epigenetic mechanisms (128, 129)

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORS (PPARs)

The PPARs major forms, PPAR α , PPAR β and PPAR γ , are a group of receptors within the nuclear receptor family of ligand-activated transcription factors, involved in inflammation, lipid and carbohydrate metabolism (130). Genistein has been shown to transactivate PPAR α at 10 μ M and PPAR γ at 5 μ M (131, 132). This may partly account for genistein's ability at physiological levels to inhibit lipogenesis and at high concentrations to enhance lipolysis in cell studies and lowering serum lipids in humans (133, 134).

INTEGRINS, MATRIX METALLOPROTEINASES (MMPs) AND ANGIOGENESIS

The integrins attach the cell to the extracellular matrix (ECM) or other cells and the MMPs is a family of proteins capable of degrading proteins in the ECM. Modulation of both families in a complex manner is necessary for cell adhesion, angiogenesis, tumor growth, invasion and metastasis (135, 136). Genistein at physiological levels has *in vivo* and *in vitro* been shown to increase cellular adhesion and dose-dependently inhibit the expression of MMP-2 starting at a dose as low as 0.1 μ M. This may account for reports that genistein inhibits the formation of metastasis in mice (137-139). Also, genistein inhibition of angiogenesis may be related to integrins and MMPs, although genistein also has a direct effect on endothelial cell

proliferation and expression of several other angiogenic factors at physiological levels (140, 141).

MAJOR INTRACELLULAR PATHWAYS

Akt/Protein Kinase B (PKB)

The PKB signaling pathway is important for cell growth, differentiation and apoptosis by interlinking cell surface receptors with cell cycle regulators, such as p27^{Kip1}, and transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (142). Genistein at 30 μ M inhibited the activation (phosphorylation) of PKB, the transactivation of NF- κ B and NF- κ B DNA binding activity in the prostate cancer cell line PC-3 (143). Additionally, genistein at 50 μ M induced the expression of PTEN, which expression was methylated and silenced in LNCaP and PC-3 cells (129).

Mitogen Activated Protein Kinases (MAPKs)

The MAPKs consist of a core of 3 sequential levels of serine/threonine-specific protein kinases where a MAPK3 activates a MAPK2 by phosphorylation, which in turn activates a MAPK, consisting of the 3 major conventional MAPKs; extracellular signal-regulated kinases (ERK) 1 and 2, c-Jun amino (N)-terminal kinases (JNK) 1-3 and p38 isoforms α - δ , in addition to several atypical MAPKs (144). The MAPK signaling cascades are activated in a complex manner by a wide variety of extracellular stimuli. Briefly, ERK has been implicated in growth responses, p38 in stress/inflammation and JNK in growth/apoptosis. Several reports show that genistein modulates all 3 major typical MAPKs at micromolar doses in human cancer cells, indicating that genistein have differential effects depending on cell line, although ERK and JNK are activated and p38 inhibited by genistein in CaP cells (137, 145, 146).

CELL VIABILITY

Cell cycle

Genistein has been shown to induce both G1 and G2/M cell cycle arrest, indicating that it has several effects on cyclin dependent kinases (CDKs) and/or cyclin dependent kinase inhibitors (CDKIs), which regulate the cell cycle (147, 148). Clearly, there are differences of reported induction on G1 or G2/M arrest not only between different cell lines, but also within the same cell line, e.g. LNCaP. The reason for this discrepancy is unclear, but technical differences in methodology or passage number of the cell lines may be suspected. Treatment of LNCaP cells with 20-40 μM genistein have been shown to up-regulate the expression of the CDKIs p21^{Waf1/Cip1} and p27^{Kip1} and the “genome watchman” p53 (148, 149).

Proliferation

Genistein, starting at upper physiological concentrations, has been shown *in vitro* to dose-dependently inhibit the proliferation of human prostate-, breast-, liver-, leukemia-, lymphoma-, myeloma-, thyroid-, oral-, ovarian-, pancreas-, lung-, renal-, and gastrointestinal cancer cells (118, 150-160)

Apoptosis

Programmed cell death, apoptosis, is induced in several cell lines by genistein at very high concentrations ($> 25 \mu\text{M}$). It has been detected by multiple assays such as microscopy of nuclear bodies, tunnel assays, flow cytometry, caspases and poly (ADP-ribose) polymerase (PARP) cleavage (153, 161, 162). The B-cell lymphoma 2 (Bcl-2) family consists of both pro-apoptotic proteins (Bax, Bad and Bak) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) and is considered to be the main regulators of apoptosis. The cell cycle associated genes p21^{Waf1/Cip1} and p53 may also be involved in the regulation of apoptosis (163). Genistein up-regulates Bax and down-regulates Bcl-2 in prostate and breast cancer cells (147, 162, 164).

1.4.4 CLINICAL STUDIES

The use of pure genistein in clinical studies has been rare until now, but is expected to increase with increasing availability. Pure genistein has been tested in phase II studies for menopausal symptoms and osteoporosis (100, 102). In CaP, there are no previous studies published which use pure genistein. However, there are several phase II studies using various mixes of soy based products and extracts. None of these were true RCT with placebo. All had prostate specific antigen (PSA) outcome as endpoint, although with various measurements. They may be sorted according to the stage of CaP. In early CaP, two studies with 76 and 29 subjects for 1 and 3 months of intervention showed a stabilization or significant reduction of PSA respectively (165, 166). In early to late CaP, three studies with 39, 52 and 20 subjects for 3 to 6 months of intervention showed reduction of PSA in early CaP and reduced increase of PSA in late CaP (167-169). In late CaP, one study with 20 subjects for 12 months of intervention showed less increase of PSA (170). Generally it seems that the reduction in PSA by soy administration is greater in early compared to late CaP.

1.4.5 TOXICITY

Negative effects of genistein and soy isoflavones on female and male reproductive systems, breast and prostate cancer, risk of infant leukemia and thyroid function have been suggested based on cell and animal studies. These will be detailed in the following paragraphs. Some of these adverse effects have been attributed to genistein's genotoxicity properties, which occur at high, non-physiological concentrations (171). Molecular suggested negative effects or toxicity by genistein have generally not been supported by clinical or epidemiological findings. No significant adverse effects were found in 248 Americans after 20-35 years, who were fed soy formula as infants compared to 563 who were fed cow-milk formula (172). However, a recent U.S. National Toxicology Program evaluation of genistein and soy isoflavones has concluded that "there is minimal concern for adverse effects on development in infants who consume soy infant formula", i.e. graded as 2 on a 5-level scale of concern (173).

Hormonal effects on men and female reproductive systems

In men, increased exposure to environmental estrogens, such as soy foods have been suggested to be related to reduction in sperm quality (174). Likewise, in premenopausal women, estrogenic effects in the form of elongation of the menstrual cycle, decreased serum progesterone, SHBG, FSH and LH have been suggested (175). Several soy and genistein intervention studies have not detected any statistically significant alternations of menstrual cycle length or endometrium (176, 177). Neither are there any significant effects on the male sex hormones by soy intake (178).

Breast cancer

The estrogenic properties of genistein have been suggested to promote the development or progression of breast cancer in mouse models (179). However, this is not supported by neither the molecular effects as genistein preferably attaches to ER β nor epidemiology as Asian high soy bean consuming countries have lower incidence of breast cancer (7). A recent phase II RCT with soy extracts on pre- and post-menopausal women in risk of breast cancer indicated a significantly higher proliferative index of breast gland epithelial cells in pre-menopausal, but not in postmenopausal women, compared with placebo (180). On the contrary, three recent large cohort studies have reported no adverse relation between breast cancer and soy intake (181-183). In fact, they suggest possible benefits for breast cancer survivors.

Prostate cancer

As opposed to the majority of chemopreventive reports of genistein treatment in CaP, there are mouse models showing increased metastasis caused by pure genistein. Genistein treatment in an orthotopic CaP model, in which the metastatic human CaP cell line PC-3 was implanted into the prostates of nude mice, resulted in a 2-fold increase in the size of para-aortic lymph nodes due to tumor infiltration (184). A similar experiment comparing pure genistein and a soy isoflavone mixture

composed of 43 % genistein, 21 % daidzein, 2 % glycitein and other components showed that the isoflavone mixture, as opposed to pure genistein, did not induce increased size of metastatic lymph nodes (185). The combination of daidzein and genistein has later been shown to have the same effect as the isoflavone mixture, indicating that daidzein is the mediator (186). Further, genistein treatment of a human metastatic CaP implanted in a Severe Combined Immunodeficiency (SCID) mouse model showed a highly significant increased frequency of metastases, proliferation and increased phosphorylation of EGFR (187). The implanted tumor had several passages with testosterone treatment in SCID mouse until it was highly metastatic. In another experiment in mice, genistein treatment of TRAMP with PIN induced a 70 % increase in lymph metastases (188). However, other mouse models indicate less tendency for metastases and the discrepancy of the results compared to those of other experiments may be related to different genetic profiles of mice, tumor or methodology (138, 139). Lakshman *et al* showed that genistein treatment of nude mice with orthotopic implanted PC3-M cells in prostate reduced lung metastases by 96% and that there were no difference in the weight of lymph node metastases (138). They also proposed that the anti-mobility effect by genistein might in fact increase attachment of orthotopic implanted CaP cells in nearby lymph nodes, whereas metastases are reduced at distal sites, i.e. a chemopreventive effect. In addition, Setchell *et al.* raised doubts about the use of rodent models for gaining insight into the effect of isoflavones in humans due to differences in metabolism of genistein (93).

Infant leukemia

Genotoxicity studies *in vivo* and *in vitro* have shown that genistein may increase breakage of genetic material, possibly by inhibiting DNA topoisomerase II (189). Children fed with soy based formula may reach the highest genistein concentrations, but may also be affected by maternal consumption during pregnancy. Genistein has been suggested to promote infant (less than 1 year) leukemia, a rare disease (190). One U.S. study using questionnaires linked maternal consumption of dietary DNA topoisomerase II inhibitors, including fruits, soy, green tea and coffee, to infant acute myelogenous leukemia (AML). However,

the author warned that the data were based on very few numbers (191). The rarity of the disease makes epidemiologic studies difficult. However, the incidence rate of leukemia in all age groups is less than half in Japan compared to U.S. (7).

Thyroid disease

Genistein dose dependently inhibits TPO in rats, and individuals with iodine or thyroid hormone deficiency may be more susceptible to the development of thyroid disease when including soy in their diet (112). However, no clinical adverse effects on thyroid function by genistein intervention have been found (192).

2.0 BACKGROUND AND AIMS OF THE STUDY

The AR plays an essential role in growth of most CaP cells. Genistein is a pluripotent molecule that may regulate AR activity through multiple mechanisms. In the late 1990s, it was shown that genistein down-regulates the expression of PSA, an androgen regulated gene (ARG), in the human prostate cell line LNCaP (193). Later it was shown that genistein down-regulates both AR mRNA and protein expression in addition to inhibiting both promoter activity and binding to androgen response elements (AREs) in LNCaP cells (104, 194, 195). However, other reports indicated that genistein acted as an agonist in CaP cells and induced conformational changes in AR, increased its nuclear localization, activated the mutant AR T877A receptor and enhanced promoter activity (103, 196). At least at micromolar concentrations, genistein has been regarded as a general inhibitor of ARG, although the extent of inhibition is unknown. The transcription factor AP-1, consisting of c-jun and c-fos, may also act as a co-factor to AR and modulate its transcription. This may be a possible mechanism for genistein inhibiting ARG expression (197).

Although genistein has shown a multitude of chemopreventive effects in cellular and molecular studies, no clinical studies on pure genistein alone in CaP has previously been performed. The reasons for this have been that pure genistein has not been easily commercially available in larger quantities and that several of the clinical studies which were performed on soy based products or isoflavones emphasized that the health benefits were from the isoflavones collectively, even though most cell- and molecular studies used genistein alone. Previous clinical studies, which all can be characterized as open-labeled or randomized phase II trials without placebo, indicate that soy isoflavones may lower the CaP surrogate end-point PSA in blood (164-170).

Paper I

The aims of the pre-clinical study were in LNCaP cells to investigate whether genistein modulates:

- 1) the expression of well-known androgen regulated genes; PSA, AR, NKX3.1, KLK4 and STAMP2.
- 2) the MAPKs end targets JNK and c-jun.

Paper II and III

The aims of the RCT were to investigate in human patients having localized CaP:

- 3) if genistein modulates PSA in blood and prostate tissue.
- 4) if genistein modulates prostate pathology.
- 5) if genistein modulates biomarkers associated with development and progression of CaP, including androgen-, cell cycle-, proliferation-, apoptosis- and neuroendocrine related genes.
- 6) if genistein modulates blood lipids, sex- or thyroid hormones.
- 7) if total genistein in plasma change upon administration.
- 8) if the pure synthetic genistein intervention is safe in humans.

Paper IV

The aims of this study was to investigate the presence and compare the distribution of genistein aglycone and its main phase II conjugates in human plasma and prostate tissue compartments in patients with localized CaP.

3.0 SUMMARY OF PAPERS

3.1 PAPER I

Genistein differentially modulates androgen-responsive gene expression and activates JNK in LNCaP cells.

The extent of down-regulation in PSA and AR by genistein has varied between publications. The purpose of this study was to examine if genistein modulates gene expression in five distinct ARGs and activates JNK and c-Jun in LNCaP cells.

AR, PSA, KLK4, NKX3.1 and STAMP2 were examined at the messenger ribonucleic acid (mRNA) level with quantitative real-time polymerase chain reaction (qRT-PCR) and protein level with Western blotting in LNCaP cells. Total and phospho-JNK were examined with Western blotting whereas c-jun was examined with solid phase kinase assay with ³²P-ATP by using the fusion protein glutathione S transferase (GST) c-jun.

We showed that whereas there are inhibitory effects of genistein on the ARG protein accumulation, the effect on mRNA levels did not always coincide with this, suggesting that there are different mechanisms through which genistein affects the AR signaling pathway. Further, genistein directly activated JNK signaling transiently at 3 hours, which was evident both in the phospho-JNK and the solid phase assay.

Our conclusions were that genistein differentially modulates the ARG mRNA expression in LNCaP cells and inhibits ARG protein expression. Genistein activates the JNK pathway in LNCaP cells, which may in part explain the reduction of the ARG protein levels in response to genistein.

3.2 PAPER II

Efficacy and safety of short-term genistein intervention in patients with localized prostate cancer prior to radical prostatectomy: a randomized, placebo-controlled, double-blind phase 2 clinical trial.

There is no previous phase II RCT reported on genistein alone in CaP. The purpose of this study was to examine if genistein modulates the primary endpoints PSA, testosterone and biomarkers associated with development and progression of CaP. Secondary endpoints included safety, prostate pathology, genistein plasma concentration, blood lipids, sex- and thyroid hormones.

Forty-seven patients were randomized to daily intervention by 30 mg genistein or placebo for 3 to 6 weeks prior to prostatectomy. Seven patients were non-compliant to the study protocol.

Serum PSA decreased close to statistically significant level and the protein expression of PSA in prostate tissue was significantly higher in Gleason grade 4 tumor compared to normal tissue in the genistein arm. Total cholesterol was significantly lower in the genistein arm. There were no effects on thyroid or sex hormones. Adverse events were few and mild.

Our conclusions were that there may be a possible therapeutic effect by genistein in early CaP and that the anticancer effect by genistein suggested by our observations could explain the epidemiological data indicating a preventive effect of a diet rich in soy products. Synthetic genistein was safe to use.

3.3 PAPER III

The effects of short term genistein intervention on prostate biomarker expression in patients with localized prostate cancer prior to radical prostatectomy.

The purpose of this study was to examine if genistein modulates biomarkers associated with development and progression of CaP.

Thirty-nine prostates from patients randomized to daily intervention by 30 mg genistein or placebo for 3 to 6 weeks were examined. Glandular cells were isolated by laser capture micro dissection and the mRNA level of androgen related biomarkers (AR, NKX3.1 and KLK4) and cell cycle related genes (p21, p27, p53) were analyzed with qRT-PCR. Immunohistochemistry of androgen-, cell cycle-, proliferative- (Ki67), apoptotic- (BCL-2 and BAX) and neuroendocrine related biomarkers (NSE and CgA) was performed using tissue microarrays containing normal and Gleason grade 3 and 4 tissue.

We showed that genistein intervention significantly reduced the mRNA level of KLK4 in tumor cells and induced a non-significant reduction in androgen and cell cycle-related biomarkers, except for p27, whose expression in the nuclear compartment was increased.

Our conclusion was that genistein intervention at nutritionally relevant levels in patients with early CaP modulated several biomarkers which may be related to cancer prediction and progression.

3.4 PAPER IV

Disposition of synthetic genistein in humans induces genistein to genistein-phase II metabolite ratio differences in plasma and prostate tissue.

The purpose of this study was to investigate the presence and compare the distribution of genistein aglycone and its main phase II conjugates in human plasma and prostate tissue in patients with localized CaP.

Seventeen paired samples containing plasma and prostate tissue from patients treated daily by 30 mg synthetic genistein orally for 3 to 6 weeks (average 34 days) were analyzed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

We showed that the distribution of both genistein aglycone and its conjugates was significantly different between plasma and prostate tissue. Corroborating previous reports, there was very little unconjugated genistein in plasma. On the other hand, nearly half of the total content of genistein in prostate tissue was composed of the biological active aglycone. We also found a high content of genistein-sulfate in both compartments whereas genistein-glucuronide contents were low.

The results indicate that oral administration of genistein alone induces high ratios of the aglycone form in prostate tissue. The distribution may differ compared to a mix of soy isoflavones, leading to more genistein-sulfates and less genistein-glucuronides in both plasma and prostate tissue.

4.0 METHODS

Important aspects of the methods used in our studies are presented in this section. It also contains some information not added in the original publications. More detailed information on methods is given in the original publications.

4.1 PRE-CLINICAL STUDY

4.1.1 CELLS AND TREATMENTS

The androgen responsive human CaP lymph node metastatic cultured cell line LNCaP were used in all experiments and the passage numbers were between 10 and 20. The passage number for LNCaP is important as the cells develop AR independence in later passages (198). The cells were hormonally starved by using charcoal treated (ChT) bovine serum both before and during treatments to avoid any confounding effects (199). In order to achieve hormonal starvation before treatment, the cells were grown for 48 h in RPMI with 2% ChT fetal calf serum (FCS), followed by 24 h in RPMI medium with 0.5% ChT FCS. Consistently, 2 μ M, 10 μ M and 50 μ M genistein were used in all treatments.

4.1.2 METHODOLOGICAL ASPECTS

The solid phase kinase assay used GST fusion proteins coupled to glutathione agarose beads, which were incubated with treated whole cell extracts, resulting in a strong binding of JNK, if activated, to c-jun. After washing the beads extensively from excess proteins, addition of the radioactive isotope 32 P-ATP resulted in the phosphorylation of two serines on c-jun and the dissociation of JNK from the complex (200). The radioactive phosphorylated c-jun was thereafter separated on gel according to size and detected on radioactive sensitive film. The qRT-PCR cycle threshold (CT)-values were adjusted towards the CT-values of the reference gene ATP-6. The PCR efficiency correction model by Pfaffl was used for all calculations (201).

4.2 CLINICAL RANDOMIZED TRIAL

4.2.1 ACCRUAL

Table 2 shows the accrual data. Patient recruitment is depicted in figure 1 in paper II. During the study period 217 LRPs were performed. Nearly all study patients were recruited at the OUU outpatient clinic. A few patients missed at the outpatient clinic or diagnosed in a private clinic were recruited after a personal meeting with the principal investigator. About one quarter of all patients treated by LRP during the study period was recruited into the study. Our exclusion criteria were quite stringent. Patients with place of residence more than 150 km from Oslo were excluded to secure adequate follow-up in case of the occurrence of adverse events during the intervention period. The next common cause for exclusion was patients on anticoagulative treatment, due to uncertain interaction between genistein and the anticoagulants. About 1/3 of the LRP patients not included in the study were missed. The efficiency of the study (number of patients included/total number of eligible patients) was close to 50 %.

4.2.2 METHODOLOGICAL ASPECTS

We expected that most patients included in the study would be treated surgically within 3 to 4 weeks, which was the current waiting time for LRP at study startup. The average days of intervention in both arms turned out to be 33 days. There were 2 to 14 days (average 8 days) latency between recruiting and startup of the intervention, during which time base-line samples were analyzed, patients randomized and study drugs delivered. We expected that changes in the primary endpoints would be apparent within a few weeks of intervention and that further intervention for a few weeks was unlikely to have any major influence. The time of intervention was more likely to have an influence on the secondary endpoints of pathological analysis and the intervention time was stratified in respect to the respective endpoint. However, the numbers of patients were too low for statistical analysis.

Instead of ATP-6, the qRT-PCR cycle threshold (CT)-values were adjusted towards the CT-values of the reference gene delta-aminolevulinic acid synthase 1 ALAS1 in this study. Additionally, (TATA-binding protein) TBP and glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) were tested as housekeeping genes before deciding for ALAS1. The PCR efficiency correction model by Pfaffl was used for all calculations (201).

	Number	Percentage
Patients listed for LRP at OUU one week before surgery during 140507-040808.	217	100,00
Patients recruited to Genistein study	54	24.9
Exclusion criteria, reasons:		
Living in remote area	40	18.4
Anti coagulative treatment	33	15.2
Doctor/patient discretion	15	6.9
Surgery within 3 weeks	9	4.2
Hypothyreoid	4	1.8
Liver or pancreas disease	2	0.9
Intestinal disease	0	0
Extreme dietary habits	0	0
Hormonal therapy or chemotherapy	0	0
Hormone dependent malignancies	0	0
Allergy	0	0
Subtotal	103	47.5
Missed, reasons:		
Unknown	40	18.4
Suspicion of advanced cancer	12	5.5
Admitted through private clinic	8	3.7
Admitted before study start-up	1	0.5
Subtotal	61	28.1
Efficiency (Recruited/(Total-Exclusion))	54/114	47.4

Table 2. Accrual report

4.2.3 STATISTICS

In the power analysis (paper II), we assumed a monthly PSA rise by 15–30 % in pre-intervention arms with CaP and a mean PSA level by 10 µg/L, based on previous reports (167). However, the degree of monthly PSA rise (velocity) in localized CaP is disputed. Other authors have reported a yearly increase by 15 % in localized CaP and 30 % in advanced CaP (202). In our study the average PSA velocity in the placebo arm was 4.4 % over an average period of 33.9 days. We needed at least 17 patients in each arm to demonstrate a difference in PSA level by

2 µg/L at a significance level of 0.050 and a power of 0.80, assuming equal standard deviation of 2 µg/L in both arms. Having 23 patients in the genistein arm and 17 in the placebo arm this requirement was accomplished. In paper II, we tested differences between the two arms by 2 independent sample t-tests or Mann-Whitney test, whereas differences in distributions of categorical variables between the two arms were tested by Fisher's exact test. In paper III there were fewer samples due to losses during analysis and stratification. Therefore we tested differences between two related and independent samples by Fisher-Pitman permutation tests for paired and two-sample interval-scaled data. The p-values were based on 2000 simulations or permutations, which reduced the probability of false positive findings. The permutation tests do not require normality of data and are not dependent on the sample size. However, the study was limited by the small number of cases included and also by the relatively short time of intervention. Only 10 patients in the genistein arm and 12 in the placebo arm could be included for the mRNA measurements. Thus, the results are prone to statistical errors of type I and II in the investigation of multiple parameters.

5.0 DISCUSSION

5.1 GENISTEIN MODULATION OF AP-1 MAY REGULATE ARG EXPRESSION

Genistein is a pluripotent molecule with effects that may coincide with different pathways and in some respects also may be paradoxical, i.e. stimulative at nanomolar and inhibitory at micromolar concentrations. ARG expression is naturally regulated by or responsive to the AR at a significant degree, although it may also be regulated by other transcription factors. AP-1 is a transcription factor, composed of the MAPK end targets c-jun, activated by JNK, and c-fos, activated by ERK, that additionally may act as a co-repressor to AR by direct interaction between the DNA binding domains of AR and c-jun (197, 203, 204). Therefore, our finding in paper I that genistein transiently activates JNK in CaP cells is important in regard to AR function and ARG expression. Others have reported that among the MAPK end targets, JNK was not modulated by genistein, as opposed to p38 and ERK. Possibly this may explain the effects by genistein on growth and inflammation (137, 205). However, the reports are not unambiguous and there are several indications that JNK may be activated by genistein in some cell types (146, 206). It is therefore plausible to imagine a mechanistic signaling pathway for genistein. It may first affect tyrosine kinases to growth receptors, as a general tyrosine kinase inhibitor, above the MAPK chain. This in turn may modulate the complex signaling in the MAPK chain, resulting in a transient activation of JNK and c-jun because of genistein's rapid half-life and conversion to metabolites. Finally it may repress AR function and ARG expression through AP-1. Alternative hypotheses may be proposed. For example, LNCaP cells containing the T877A mutation in AR, which makes them sensitive to estrogens, may also make genistein an antagonistic ligand and induce a direct inhibitory effect on transactivation and transcription (207, 208). Additionally, the superfamily of nuclear receptors and other transcription factors in many cases share the same co-factors, which may result in more efficient recruitment of co-factors needed by AR during genistein stimulation (209, 210). Genistein may also modulate AR directly by any of the several hundred other co-factors (see NURSA at <http://www.nursa.org/>). In paper I we showed that genistein did not inhibit mRNA levels in all ARGs, which may indicate a non-AR dependent mechanism. The complexity of AR function and the pluripotency of genistein make it difficult to demonstrate a certain superior credibility of one hypothesis compared to another.

Most probably, genistein modulation of ARG expression depends on several effects.

5.2 GENISTEIN MAY BE A CHEMOPREVENTIVE AGENT IN EARLY CaP

In paper II we investigated the percentage change in serum PSA in patients with localized CaP. There was a close to statistically significant difference between the two study arms, with a reduction by 7.8 % in the genistein arm and an increase by 4.4 % in the placebo arm. In direct values, there was an 11.3 % decline in the genistein arm (1 µg/L / 8.9 µg/L) and a 1.2 % increase in the placebo arm (0.1 µg/L / 8.2 µg/L). Interestingly, this shows that the reduction in serum PSA levels reported by clinical studies using soy bean products can be caused by genistein alone (211). It also indicates, although does not prove, that genistein may have a measurable clinical effect as a chemopreventive agent.

We showed that there was a significant difference between the expression levels of PSA in prostate tissue of Gleason grade 4 in the two intervention arms. There was reduced expression in the placebo and normal expression in the genistein arm. However, the number of patients having Gleason grade 4 tumors was few, and the significance of this finding needs further research.

There was no association between the number of intervention days and the extent of decline in serum PSA in the genistein arm. This may be due to a low number of patients and the relatively short length of intervention time. Possibly an increase in these elements would have detected a more pronounced effect by Genistein on the level of PSA.

In paper III we observed several interesting findings indicating possible chemopreventive properties of genistein. Firstly, there was a general reduction in the genistein arm of the androgen related biomarkers. This corroborates our *in vitro* findings in the LNCaP cell line (paper I). It also indicates that the administration of genistein in humans, at much lower concentrations than *in vitro* studies, but of longer duration, may have similar effects. Interestingly, in our LNCaP study (paper I) we showed that genistein did not reduce KLK4 mRNA, which on the contrary seemed to increase. In our *in vivo* studies however, (paper II), KLK4 mRNA was the only androgen related gene whose expression was significantly reduced. This may indicate cell specific effects by genistein, although the protein expression in both

studies was reduced. Secondly, the cell cycle related gene expression in general was reduced, except for p27^{Kip1}, whose protein expression in the nuclear compartment was increased compared to the placebo arm. We expected that genistein as a chemopreventive agent would increase the expression of the cell cycle related genes, which would corroborate previous *in vitro* based reports (148, 149). The increased expression of p27^{Kip1} in the nuclear compartment in the genistein arm and its reduction in the placebo arm may however support anticancer effects by genistein. Loss of PTEN function and/or constitutive activation of PKB by the PI3K pathway, commonly found in CaP, will reduce the expression of p27^{Kip1} (67, 68). Genistein inhibits PKB constitutive activation by reestablishing PTEN expression by its epigenetic modulation. Our result may therefore indicate the mechanism for its cycle arrest and proliferation inhibition of tumor cells (129, 143, 147). The longer duration of intervention in our clinical study compared to the short duration in cell studies, may also explain why the other cell cycle related markers were normalized.

To summarize, we showed that genistein intervention had measurable effects on biomarkers in both blood and prostate tissue in patients with localized CaP. As of today the most reliable biomarker in CaP is of course the surrogate end-point serum PSA. However, the other androgen related biomarkers may also be of importance as biomarkers of prediction and progression of CaP. We also showed that genistein *in vivo* may modulate cell cycle related biomarkers, explaining inhibitory effects of cell cycle and proliferation.

5.3 GENISTEIN HAS NO EFFECTS ON SEX HORMONES IN THE CIRCULATION

One could have suspected genistein to modulate PSA and androgen related genes by modulating testosterone levels. This is supported by cell- and molecular studies which have shown that genistein both inhibits 17 β -hydroxysteroid dehydrogenase and 5 α -reductase (106, 107). However, 17 β -hydroxysteroid dehydrogenase is simply one pathway for metabolizing androstenione to testosterone, whereas androstenediol may be metabolized to testosterone by 3-beta-hydroxysteroid dehydrogenase (3 β -HSD) (212). On the other hand, 5 α -reductase metabolizes testosterone to the much more potent dihydrotestosterone. We showed that genistein does not modulate testosterone levels in blood. We also investigated

luteinizing hormone (LH) and sex hormone-binding globulin (SHBG); the other sex hormones associated clinical biomarkers, without finding any significant effect by genistein intervention.

5.4 GENISTEIN HAS NO EFFECTS ON THYROID HORMONES IN THE CIRCULATION

Genistein has been reported to inhibit TPO in several species, including humans. In vivo effects by genistein on T3 and T4 in rats and cats have been reported. The reports however are not unambiguous (112, 213, 214). Several human studies with soy or genistein have reported no significant effects on thyroid function, corroborating our results (192, 215, 216). The discrepancies between species may be explained by different metabolic pathways and effects (93).

5.5 GENISTEIN LOWERS SERUM CHOLESTEROL

Soy has previously been shown to be strongly associated with lowering of serum cholesterol levels. It is not clear however whether this effect can be attributed to soy protein alone or isoflavones (134). Genistein may affect lipid metabolism through the PPAR or inhibition of the gene expression of hormone sensitive lipase or lipoprotein lipase (133, 217). No previous human clinical trials with genistein alone have reported a full lipid analysis, although HDL cholesterol was reported to be non-significantly increased in one study (218). In our study, serum total cholesterol was significantly lowered by genistein alone, although the average percentage reduction was only 4 %. The reduction in LDL- and HDL-cholesterol was non-significant. S-lipase and S-triglycerides were not affected.

5.6 GENISTEIN PHASE II METABOLISM DIFFERS IN BLOOD AND PROSTATE TISSUE

In paper IV we detected two interesting findings. Firstly, the ratio of genistein aglycone was significantly higher in prostate tissue compared to plasma. Secondly, genistein-sulfates were the dominating form in both compartments. The conjugated forms of genistein are not considered as bioactive as the aglycone form (219). Genistein-sulfates may be a resource for the aglycone form by the action of specific sulfatases, whereas the glucuronide form cannot be reversed (220). The expression of sulfatases in some cancer cells, including the CaP line LNCaP, may be increased

compared to normal cells (221). Our results differ from other studies which show the glucuronide form to be the dominating in plasma, tissue and urine (85, 86, 222). The reason for this discrepancy is unclear. However, there are no previous reports comparing the amount of genistein aglycone and all major conjugates in both blood and prostate tissue compartments. We are also the first to test genistein aglycone alone and not several isoflavones, which may have effects on conjugation and bioavailability by saturation of transferase enzymes. A recent small study on genistein and genistin supports our finding that sulfate conjugates are increased and glucuronide conjugates are decreased in plasma when consuming pure genistein (223).

5.7 GENISTEIN IS SAFE TO USE IN CAP PATIENTS

We defined an adverse event (AE) as any untoward medical occurrence in a patient or clinical investigation subject which was temporally related to protocol procedures, including the administration of a pharmaceutical product at any dose. It was not required for the occurrence to have a causal relationship to the treatment. The AEs were graded into a five-level scale including, mild (1), moderate (2), severe (3), life-threatening (4) and fatal (5). AEs grade 1-2 were considered as non-serious and AEs grades 3-5 as serious.

Only a few grade 1 AEs were reported, mostly of gastrointestinal origin. They were equally distributed in both intervention arms. In the genistein arm one patient experienced increased tiredness during the study period and one had heart palpitations. He had had this condition for several years and investigations did not reveal pathology. The intervention was completed as planned in both cases.

In the genistein arm we noted transient biochemical AEs in two patients. One patient had an increase in S-lipase, without other effects on blood lipids. Another patient had a slight transient increase in S-bilirubin without change in other liver parameters. The biochemistry were normalized in both patients. In summary, our results show that 30 mg genistein daily is well tolerated without yielding any marked AEs. This corroborates the results of previous studies on soy based products.

6.0 CONCLUSIONS

1. Genistein inhibits the protein expression of the following ARGs: PSA, KLK4, NKX3.1 and STAMP2 in LNCaP cells.
2. The MAPK end targets JNK and c-jun are phosphorylated by genistein, which may activate the AR cofactor AP-1 and inhibit AR in LNCaP cells.
3. Intervention with a daily dosage of genistein, easily acquired by food, for 3 to 6 weeks reduces serum PSA close to significant level in patients with localized CaP.
4. In patients with localized CaP, genistein intervention yields a PSA protein expression in Gleason grade 4 prostate glands at the same level as normal prostate tissue, whereas the PSA expression in non-treated tissue is weaker with increasing Gleason grade.
5. Androgen related gene expression of AR, KLK4 and NKX3.1 in prostate glands of patients with localized CaP is generally reduced by genistein intervention.
6. Cell cycle related gene expression of p21^{Waf1/Cip1}, p27^{Kip1} and p53 in prostate glands of patients with localized CaP is generally reduced by genistein intervention, whereas the protein level of nuclear p27^{Kip1} is increased.
7. Genistein does not modulate blood sex or thyroid hormones in patients with localized CaP.
8. Genistein significantly lowers blood total cholesterol in patients with localized CaP.
9. Genistein is differently metabolized in prostate tissue and plasma in patients with localized CaP.
10. Genistein is safe to use in patients with localized CaP.

7.0 FUTURE PERSPECTIVES

Genistein has been investigated as a chemopreventive agent for CaP during the last 20 years. However, commercially available pure genistein was not introduced until about 2005. It is still not marketed in European countries due to EU regulations labeling it as a novel substance. However, it is available in the U.S. Soy has been consumed for several thousands of years in some Asian countries, and it is clear that these countries have a considerable lower rate of both breast and prostate cancer. On the other hand, there are occasional warnings that genistein may have an opposite effect. Often these warnings are based on single research reports on cell or animal studies, performed under special circumstances with high non-physiological levels of genistein. In addition, there are differences in effects of genistein between species, which may be attributed to different metabolism, making animal studies unsuitable for deducing effects in humans.

The author believes, based on research reports declaring it safe and beneficial for health, that soy consumption and nutraceutical additives of genistein are likely to increase in Western countries in the future. The intake of pure genistein will probably reduce the risk of allergy compared to consumption of soy. Also the administration of pure genistein would be easier, considering the rapid clearance. Genistein and soy isoflavones already belong to the most investigated food derived molecules. Soy production has several positive environmental effects in general. Soy plants, cultivated in the appropriate way, are beneficial to the soil, rivers and lakes, reducing the use of artificial fertilizer of nitrates. Consumption of red meat is coupled to several cancers including CaP.

The final answer as to whether genistein is a chemopreventive agent in CaP cannot be resolved until a large phase III-IV RCT is performed over a long period of time. Unfortunately, this will probably not be realized during the next 5-10 years. Implementation of such a study will be very costly, and it will render necessary the participation by several countries. The disappointing results in the SELECT study may also be discouraging. However, genistein is one of the top candidates of natural chemopreventive agents in CaP. Its effects are clinically measurable in patients having localized CaP already after one month of administration. The effects

probably will be more pronounced after a longer treatment period and twice daily orally administration. Men having low grade CaP and treated by active surveillance will be an especially interesting group to investigate the possible efficacy of genistein. A phase II-III RCT at this stage of the disease may find that genistein reduces the progression of CaP and therefore the need for surgery or radiation. A major problem in such a study however will be patient compliance, due to the prognostic uncertainty felt by the individual patient. Another obstacle may be soy danger reports in the popular press or internet. The author regards it unlikely that genistein alone, at dosages that may be acquired by food, will have any effect on high grade CaP considered for surgery or radiation. Finally, further exploration on genistein effects in CaP, both *in vivo* and *in vitro*, may lead to novel understandings and treatment modalities of the disease.

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9.0 APPENDIX PAPERS I-IV

The effects of short-term genistein intervention on prostate biomarker expression in patients with localised prostate cancer before radical prostatectomy

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Abstract

Nutritionally relevant levels of genistein, the predominant isoflavone in soyabean associated with lower risk of prostate cancer (PCa), may modulate the expression of prostate tissue biomarkers associated with cancer prediction and progression. A phase 2 placebo-controlled, randomised, double-blind clinical trial was conducted in forty-seven Norwegian patients before prostatectomy. Intervention was 30 mg genistein or placebo capsules daily for 3–6 weeks. Luminal cells from malignant and benign glands were isolated with laser capture microdissection and the mRNA levels of androgen-related biomarkers (androgen receptor, NK3 homeobox 1, kallikrein-related peptide 4 (KLK4)) and cell cycle-related genes (*p21^{Waf1/Cip1}*, *p27^{Kip1}*, *p53*) were analysed with real-time semiquantitative PCR. Immunohistochemistry of androgen-, cell cycle-, proliferative- (Ki67 nuclear antigen), apoptotic- (B-cell CLL/lymphoma 2 (BCL-2) and BCL-2-associated X protein) and neuroendocrine differentiation-related biomarkers (neuron-specific enolase and cytoplasmic chromogranin A) was performed using tissue microarrays containing normal, Gleason grade 3 and grade 4 prostate tissues. There were no significant effects by genistein intervention on proliferation-, cell cycle-, apoptosis- or neuroendocrine biomarkers. Genistein intervention, however, significantly reduced the mRNA level of KLK4 in tumour cells ($P=0.033$) and there was a non-significant reduction in androgen and cell cycle-related biomarkers, except for *p27^{Kip1}*, whose expression in the nuclear compartment was increased. Genistein intervention modulated the expression of several biomarkers which may be related to PCa prediction and progression. The present study supports genistein as a chemopreventive agent in PCa. Further investigation is warranted in larger and longer-duration studies.

Key words: Biomarkers; Genistein; Immunohistochemistry; PCR; Prostate cancer

Although not an ideal biomarker, serum prostate specific antigen is the only predictive and prognostic prostate cancer (PCa) biomarker widely used in clinical practice. Several proteins have been proposed as candidate biomarkers exhibiting all or some of these predictive or prognostic properties^(1–5). Among these are androgen-, cell cycle-, proliferation-, apoptosis- and neuroendocrine differentiation-related biomarkers.

The soya isoflavone, genistein, is a promising chemopreventive agent in PCa based on molecular, epidemiological and clinical studies^(4,5). We have previously reported the clinical endpoints of a phase 2 clinical randomised trial with short-term genistein intervention in patients with localised PCa. Genistein reduced the level of serum prostate specific antigen without any effects on hormones⁽⁶⁾. The purpose of

Abbreviations: AR, androgen receptor; BAX, B-cell CLL/lymphoma 2-associated X protein; BCL-2, B-cell CLL/lymphoma 2; CgA, cytoplasmic chromogranin A; G3, Gleason grade 3; G4, Gleason grade 4; KLK4, kallikrein-related peptide 4; NKX3-1, NK3 homeobox 1; NSE, neuron-specific enolase; *p21^{Waf1/Cip1}*, cyclin-dependent kinase inhibitor 1A; *p27^{Kip1}*, cyclin-dependent kinase inhibitor 1B; *p53*, tumour protein p53; PCa, prostate cancer.

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Table 1. Primers for PCR

Gene	Forward primer	Reverse primer
AR	TGGAAGCTGCAAGGTCTTCT	AAGCCTCTCCTTCTCCTGT
KLK4	ATGGAAAAGCAATTGTTCTGCTC	CAGCACGGTAGGCATTCTCG
NKX3-1	GGCCTGGGAGTCTCTTGACTCCACTAC	ATGTGGAGCCCCAACACAGAAAAATG
p21	AGGGGACAGCAGAGGAAGA	CTTCTGTGGCGGATTAG
p27	TCAGAAGACGTCAAACGTAAACA	CCAACGCTTTTAGAGGCAGA
p53	CCCCAGCCAAGAAGAAAC	TTTTTATGGCGGAGGTAGA
ALAS1	CTGCAAGATCTGACCCTC	CCTCATCCACGAAGGTGATT

AR, androgen receptor; KLK4, kallikrein-related peptide 4; NKX3-1, NK3 homeobox 1; p21, cyclin-dependent kinase inhibitor 1A; p27, cyclin-dependent kinase inhibitor 1B; p53, tumour protein p53; ALAS1, aminolevulinate synthase gene.

the present study was to investigate the modulation of potential candidate PCa tissue biomarkers by genistein at a dose that can be obtained from a diet rich in soya-based food.

Experimental methods

Patients and study design

A total of forty-seven patients with localised PCa scheduled to be treated by radical prostatectomy were randomised during April 2007–August 2008. Next, a total of forty-one prostates were analysed and forty were considered as evaluable according to the study protocol. However, one specimen only contained enough tumour material for pathological analysis and could therefore not be analysed for biomarkers. The study was a single-centre, placebo-controlled, randomised and double-blind phase 2 clinical trial with two treatment arms⁽⁶⁾. One arm was given 30 mg synthetic genistein (geniVida^{®(7)}) daily and one arm received placebo. The intervention period was 3–6 weeks (average 33 d) before radical prostatectomy. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Norwegian Medicines Agency, the Regional Ethics Committee, the Privacy Ombudsman and the Prostate Biobank at the Oslo University Hospital, Aker. Written informed consent was obtained from all subjects/patients. The study has been registered in the ClinicalTrials.gov registry (study identifier: NCT00546039).

Tissue processing, laser capture microdissection and RNA isolation

After surgery, the prostates were immediately transferred to the pathology department and macro-dissected by a pathologist. Fresh tissue cubes, approximately 0.5 cm³, were cut from tumour and normal tissue and frozen at –80°C. Frozen 10 µm sections were stained with haematoxylin and eosin. At least 10 000 cells from histologically benign and cancer glands from each patient were selected and captured using laser capture microdissection (Leica LMD 6000; Leica Microsystems) and frozen at –80°C⁽⁸⁾.

Total RNA was isolated using the Arcturus PicoPure RNA Isolation Kit (Molecular Devices) according to the manufacturer's protocol. The concentration of RNA was determined by measurements in the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Reverse transcription of

50 ng total RNA was performed with the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Although fresh prostate tissue was acquired from most patients, tumour was found in only ten study subjects from the genistein arm and twelve from the placebo arm.

Semi-quantitative real-time RT PCR

Primers were designed with Primer3 provided by the Whitehead Institute for Biomedical Research (http://fokker.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Sequences are listed in Table 1. Relative quantification of gene expression by semi-quantitative real-time RT-PCR was performed by analysing the expression of human housekeeping delta-aminolevulinate synthase gene. Real-time PCR was performed on the Bio-Rad Opticon DNA Engine. All amplifications were run as triplicates with standard curves. Here, 1 ng of template was used in each reaction following the manufacturer's protocol (Bio-Rad SYBR Green Master Mix). The same set-up was used for all primers (sixty-six cycles of 55°C annealing, 72°C extension and 90°C denaturing).

Tissue processing and immunohistochemical analysis

The prostates were fixed in 10% buffered formalin, macro-dissected and paraffin-embedded. Tissue microarrays were

Table 2. Antibodies for immunohistochemistry

Antigen	Clone	Supplier	Dilution	Treatment
AR	MS-443	Thermo Scientific	Prediluted	Benchmark XT
KLK4	Rabbit	F.S lab	3 µg/ml	Autoclave
NKX3-1	Rabbit	F.S lab	1 µg/ml	Autoclave
p21	EA-10	Calbiochem	0.25 µg/ml	PT-module*
p27	SC-528	Santa Cruz	1 µg/ml	PT-Module†
p53	Bp53-11	Roche	Prediluted	Benchmark XT
Ki67	30-9	Roche	Prediluted	Benchmark XT
BAX	2D2	Thermo Scientific	12.5 µg/ml	PT-module‡
BCL-2	124	Roche	Prediluted	Benchmark XT
NSE	E27	Roche	Prediluted	Benchmark XT
CgA	LK2H10	Roche	Prediluted	Benchmark XT

AR, androgen receptor; KLK4, kallikrein-related peptide 4; NKX3-1, NK3 homeobox 1; p21, cyclin-dependent kinase inhibitor 1A; p27, cyclin-dependent kinase inhibitor 1B; p53, tumour protein p53; BAX, B-cell CLL/lymphoma 2-associated X protein; BCL-2, B-cell CLL/lymphoma 2; NSE, neuron-specific enolase; CgA, cytoplasmic chromogranin A.

* Citrate buffer pH 6.

† Tris buffer pH 9.

‡ EDTA buffer pH 8.

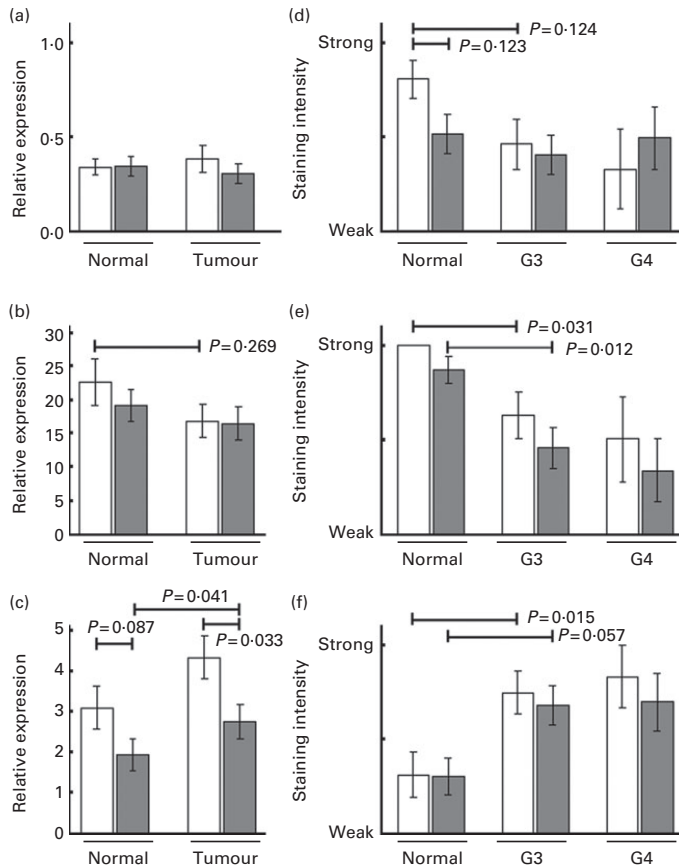


Fig. 1. Expression of androgen-related biomarkers. Real-time RT-PCR of the mRNA expression of (a) androgen receptor (AR), (b) NK3 homeobox 1 (NKX3-1) and (c) kallikrein-related peptide 4 (KLK4) in laser micro-dissected cells from normal and tumour areas of prostatectomy specimens from patients treated with either placebo (□) or genistein (■). The data were normalised to aminolevulinatase synthase gene and are shown as means, with their standard errors. The protein expression levels of (d) AR, (e) NKX3-1 and (f) KLK4 were determined by immunohistochemical staining of tissue microarrays containing normal and Gleason grade 3 (G3) and/or Gleason grade 4 (G4) spots of prostatectomy specimens. The figures show the mean staining intensity, with their standard errors.

assembled using the TMABooster (Alphelys) sampler. Normal, Gleason grade 3 (G3) and Gleason grade 4 (G4), if available, prostate tumours were marked on haematoxylin/eosin-stained sections. From each patient, three 0.6 mm biopsy cores were taken from normal prostatic tissue and each available Gleason grade tumour. The sampling size of three cores has previously been shown to be representative of PCa⁽⁹⁾. Next, 10 µm tissue microarray sections on glass slides (SuperFrost[®]) were treated according to the procedures listed in Table 2. 'Benchmark XT' used automated immunohistochemical staining (Benchmark XT; Ventana Medical Systems). The slides were treated in a dry cabinet for 60 min at 60°C, cooled and washed with a detergent before conditioning with cell conditioning 1 (CC1) buffer (Ventana Medical Systems). Detection of the bound primary antibody was visualised with the iView™ DAB Detection kit

(Ventana Medical Systems). The cells were counterstained with haematoxylin and Bluing reagent (Ventana Medical Systems) for 4 min. 'Autoclave' used citrate buffer pH 6.4 at 120°C for 40 min for antigen retrieval. Immunohistochemistry was performed according to the manufacturer's protocol (BioGenex Detection kit). The slides were visualised by diaminobenzidine staining followed by haematoxylin staining. 'PT-module' used different buffers according to Table 2. The slides were treated in 98°C for 25 min in the PT-Module (Lab Vision Corporation) for antigen retrieval. Immunohistochemistry and visualisation were performed according to the manufacturer's protocol (UltraVision ONE Detection System; Thermo Fisher Scientific). The cells were counterstained with haematoxylin. The stained tissues were then independently analysed by two consultant pathologists (A. S. and C. H.), who later made a consensus evaluation

on each sample. Samples not containing the designated Gleason grade were not analysed and treated as missing.

The staining intensities of nuclear androgen receptor (AR), cytoplasmic and nuclear NK3 homeobox 1 (NKX3-1), cytoplasmic kallikrein-related peptide 4 (KLK4), cytoplasmic and nuclear cyclin-dependent kinase inhibitor 1B (p27^{Kip1}) and cytoplasmic B-cell CLL/lymphoma 2-associated X protein (BAX) were scored as either weak/moderate (0) or strong (1), whereas the staining intensities of cytoplasmic B-cell CLL/lymphoma 2 (BCL-2), cytoplasmic neuron-specific enolase (NSE; also called ENO2) and cytoplasmic chromogranin A (CgA) were scored as negative (0) or positive cytoplasmic staining (1).

Sample sizes for the placebo arm were: sixteen containing normal glandular tissue, sixteen G3 and six G4 tumours; and for the genistein arm: twenty-three normal glandular tissue, twenty-two G3 and ten G4. In the placebo arm, one G3 disappeared due to technical reasons for AR and p27^{Kip1}. In the genistein arm, one G4 disappeared for NKX3-1 and cyclin-dependent kinase inhibitor 1A (p21^{Waf1/Cip1}).

Statistical analysis

Values are expressed as means with their standard errors for continuous and ordinal data. Differences between two related and independent samples were tested by Fisher–Pitman permutation tests for paired and two-sample interval-scaled data. The *P*-values were based on 2000 simulations and considered significant at *P*<0.050. The analyses were carried out in STATA/IC 11.1 for Windows (32-bit) and Sigmaplot 11.0 for Windows was used to create the figures.

Results

Androgen-related biomarkers

Genistein intervention significantly reduced KLK4 mRNA expression in tumour cells (*P*=0.033). The down-regulation of AR protein expression and KLK4 mRNA level in normal cells were not statistically significant (*P*=0.123 and *P*=0.087; Fig. 1(c) and (d)). There was a general non-significant tendency by genistein intervention to reduce the expression of androgen-related biomarkers (Fig. 1).

The AR and NKX3-1 nuclear protein expression in both study arms were reduced in higher Gleason grades, whereas both the mRNA and protein expression were increased for KLK4. AR mRNA expression was unchanged. Fig. 2(a) depicts a patient treated with placebo showing strong nuclear AR staining intensity in normal cells and weak intensity in the case of tumour. The cytoplasmic expression of NKX3-1 was equivalently increased (data not shown). Fig. 2(b) shows a patient from the genistein group with strong nuclear/weak cytoplasmic NKX3-1 staining intensity in normal cells and weak nuclear/strong cytoplasmic staining intensity in tumour. Fig. 2(c) depicts a patient treated with genistein showing weak cytoplasmic KLK4 staining intensity in normal cells and strong intensity in tumour.

Cell cycle-related biomarkers

Genistein intervention had no significant effects on p21^{Waf1/Cip1}, p27^{Kip1} or tumour protein p53 (p53) mRNA and protein expression (Fig. 3). There was a non-significant reduction in

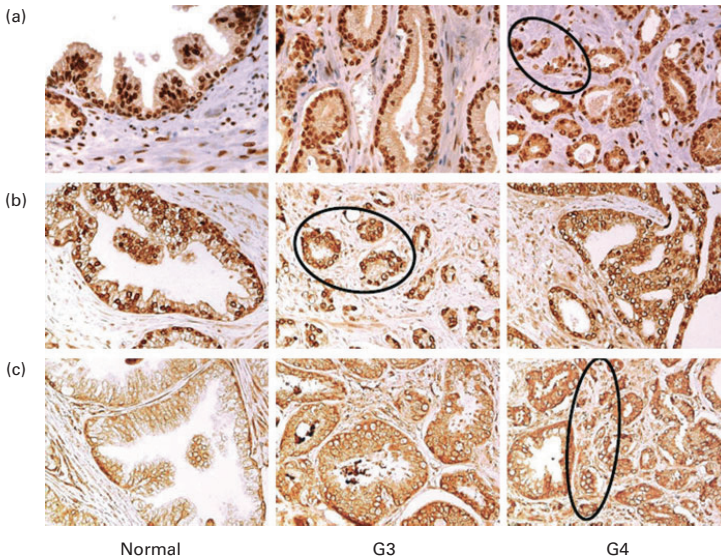


Fig. 2. Immunohistochemical staining of androgen-related biomarkers. Each row shows tissue microarray spots from a single patient containing normal, Gleason grade 3 (G3) and Gleason grade 4 (G4) prostate tissues. Encircled areas contain designated Gleason grade. Magnification $\times 400$. (a) Androgen receptor, (b) NK3 homeobox 1 and (c) kallikrein-related peptide 4.

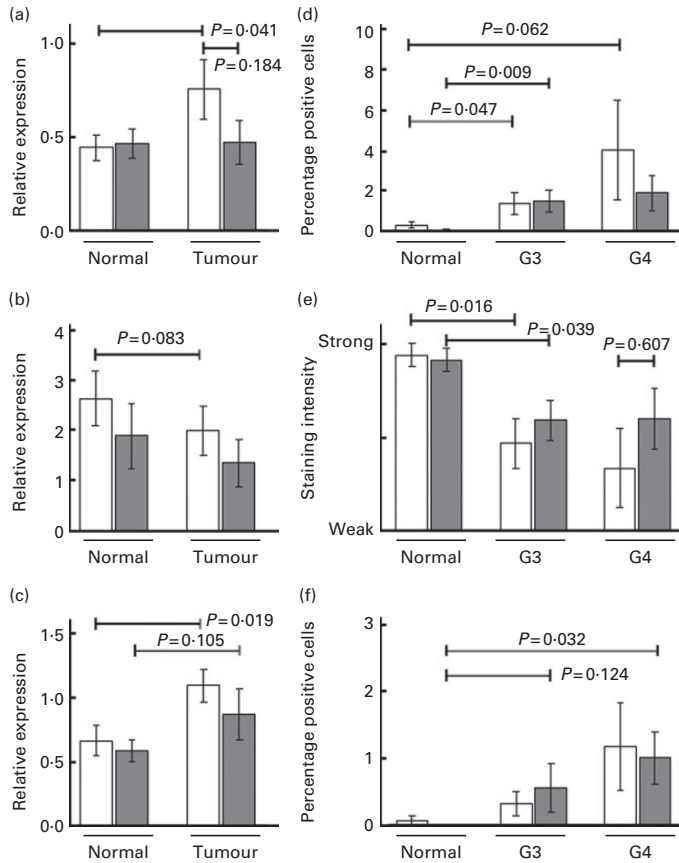


Fig. 3. Expression of cell cycle-related biomarkers. Real-time RT-PCR of the mRNA expression of (a) cyclin-dependent kinase inhibitor 1A (p21^{Waf1/Cip1}), (b) cyclin-dependent kinase inhibitor 1B (p27^{Kip1}) and (c) tumour protein p53 (p53) in laser micro-dissected cells from normal and tumour areas of prostatectomy specimens from patients treated with either placebo (□) or genistein (■). The data were normalised to aminolevulinatase synthase gene and are shown as means, with their standard errors. The protein expression level of (d) p21^{Waf1/Cip1}, (e) p27^{Kip1} and (f) p53 was determined by immunohistochemical staining of tissue microarrays containing normal and Gleason grade 3 (G3) and/or Gleason grade 4 (G4) spots of prostatectomy specimens. The figures show the mean staining intensity or percentage positive cells, with their standard errors.

the expression of p21^{Waf1/Cip1} mRNA expression in tumour ($P=0.184$) and a slight reduction in p27^{Kip1} and p53 mRNA expression, whereas p27^{Kip1} protein nuclear expression was slightly increased in G3 and G4 in the genistein arm compared with placebo.

The mRNA and protein expression results for the cell cycle-related biomarkers were coherent in both study arms, showing increased levels of p21^{Waf1/Cip1} and p53 and reduced levels of p27^{Kip1} in tumour tissue compared to normal tissue. The percentage p21^{Waf1/Cip1} positive cells was less than 1% in normal prostate tissue and increased with increasing Gleason grade to about 2% in G3 cells and 4% in G4 cells. Fig. 4(a) depicts a patient from the genistein group showing an increasing number of p21^{Waf1/Cip1} positive nuclei from normal tissue to G4. The nuclear expression of p27^{Kip1} was significantly reduced in G3 ($P=0.016$) compared to normal, whereas the

mRNA level was non-significantly reduced in tumour compared to normal cells (Fig. 3(b) and (e)). Fig. 4(b) shows a patient from the placebo group with strong nuclear/weak cytoplasmic staining intensity in normal cells and weak nuclear/strong cytoplasmic p27^{Kip1} staining in G3. Fig. 4(c) shows a patient treated with placebo with no p53 positive cells in normal tissue and with single p53 positive cells in G3 and G4 tissues.

Proliferation- and apoptosis-related biomarkers

Genistein intervention had no significant effects on the protein expression of Ki67, BAX or BCL-2 (Fig. 5).

In both study arms, Ki67 and BAX expression increased with increasing Gleason grade. Ki67 was expressed by 1% of normal epithelial cells and it increased significantly to 3%

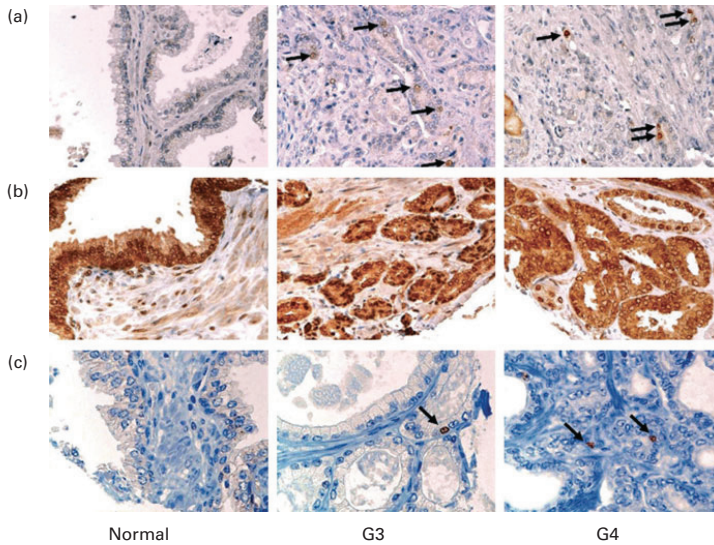


Fig. 4. Immunohistochemical staining of cell cycle-related biomarkers. Each row shows tissue microarray spots from a single patient containing normal, Gleason grade 3 (G3) and/or Gleason grade 4 (G4) prostate tissues. Magnification $\times 400$ for (a) cyclin-dependent kinase inhibitor 1A and (b) cyclin-dependent kinase inhibitor 1B. Magnification $\times 600$ for (c) tumour protein p53.

in G3 cells ($P < 0.001$) and further to approximately 5% in G4 cells (Fig. 5(a)). Fig. 6(a) depicts a patient treated with genistein showing increasing number of Ki67 positive cells from normal tissue to G4. BAX protein expression increased significantly ($P = 0.011$) in G3 compared to normal cells

(Fig. 5(b)). Fig. 6(b) shows a patient treated with genistein with weak cytoplasmic BAX staining in normal tissue and increasing intensity in tumour tissue. BCL-2 was in general not expressed in normal epithelial cytoplasm (Fig. 5(c)). The increased expression in malignant tissue was not statistically

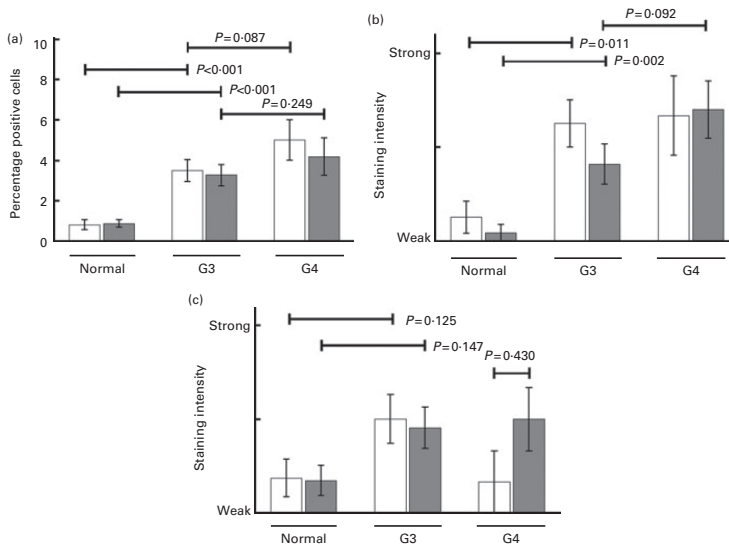


Fig. 5. Expression of proliferation- and apoptosis-related biomarkers. The protein expression of (a) Ki67, (b) B-cell CLL/lymphoma 2-associated X protein and (c) B-cell CLL/lymphoma 2 was determined by immunohistochemical staining of tissue microarrays containing normal and Gleason grade 3 (G3) and/or Gleason grade 4 (G4) spots of prostatectomy specimens. The figures show the mean staining intensity or percentage positive cells, with their standard errors. □, Placebo; ■, genistein.

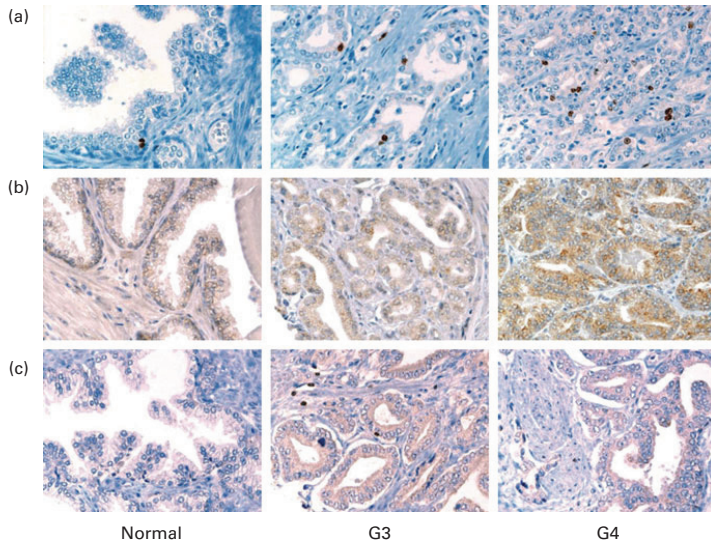


Fig. 6. Immunohistochemical staining of proliferation- and apoptosis-related biomarkers. Each row shows tissue microarray spots from a single patient containing normal, Gleason grade 3 (G3) and Gleason grade 4 (G4) prostate tissues. Magnification $\times 400$. (a) Ki67, (b) B-cell CLL/lymphoma 2-associated X protein and (c) B-cell CLL/lymphoma.

significant ($P=0.125$). Fig. 6(b) shows a patient treated with genistein with weak BCL-2 staining in normal tissue and stronger staining in tumour tissue.

Neuroendocrine differentiation-related biomarkers

Genistein intervention had no significant effect on NSE or CgA (Fig. 7).

In both study arms, the expression of the neuroendocrine differentiation-related biomarkers indicated reduced levels with increasing Gleason grade. Fig. 8(a) shows a patient treated with placebo with decreasing number of NSE-positive cells in G3 tumour. There was a clear presence of CgA-positive cells in normal tissue, which was completely abolished in G4 tissue in both treatment arms ($P<0.001$; Fig. 7(b)). Fig. 8(b) shows a patient treated with genistein with decreasing number of CgA-positive cells with higher Gleason grade.

Discussion

To our knowledge, this is the first study which exclusively investigates the effects by genistein alone in men with PCA. We showed that genistein intervention significantly down-regulated the mRNA expression levels of KLK4 in cancer cells in men with localised PCA. Genistein has previously been shown to decrease AR nuclear binding to the transcriptional binding site for AR⁽¹⁰⁾. We also report a decrease by genistein on most of the selected androgen-related biomarkers, corroborating the results of our *in vitro* studies⁽¹¹⁾. However, the average plasma level in the present study was at least 25-fold lower than the concentrations used in cell culture studies, indicating that *in vivo* nutritional relevant levels

of genistein may have comparable effects to high-dose cell studies. This may be related to longer *in vivo* exposure. The androgen-related biomarkers used in this study have been attributed roles in the development or progression of PCA. NKX3-1 is an androgen-regulated homeobox gene located

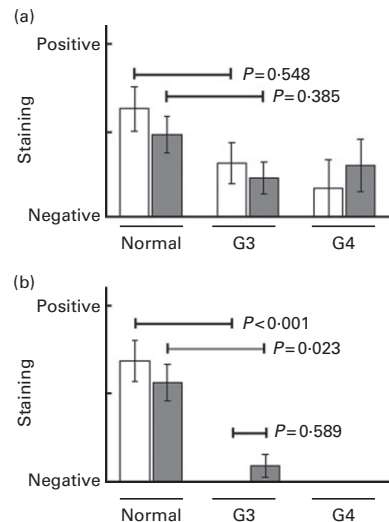


Fig. 7. Expression of neuroendocrine differentiation-related biomarkers. The protein expression of (a) neuron-specific enolase and (b) cytoplasmic chromogranin A was determined by immunohistochemical staining of tissue microarrays containing normal and Gleason grade 3 (G3) and/or Gleason grade 4 (G4) spots of prostatectomy specimens. The figures show the mean positive/negative stain, with their standard errors. □, Placebo; ■, genistein.

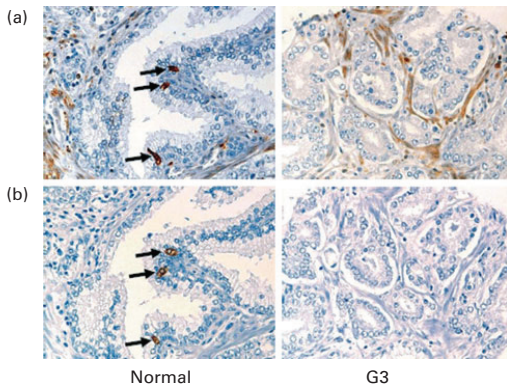


Fig. 8. Immunohistochemical staining of neuroendocrine differentiation-related biomarkers. Both rows show tissue microarray spots from the same patient containing normal and Gleason grade 3 (G3) prostate tissues. Magnification $\times 400$. (a) neuron-specific enolase and (b) cytoplasmic chromogranin A.

on chromosome 8p21.2, a region that shows a high degree of loss of heterozygosity in PCa^(12,13). Reports of its function and expression in PCa have been conflicting. However, most reports indicate that NKX3-1 acts as a non-classical tumour suppressor and that its expression is reduced in primary PCa and further reduced in metastatic PCa^(14,15). However, our immunohistochemistry results indicated a translocation of NKX3-1 from the nucleus to the cytoplasm and not a total reduction with increasing Gleason grade. Interestingly, increased cytoplasmic stain intensity in primary PCa was briefly mentioned by Gurel *et al.*⁽¹⁵⁾, although it was not taken into account during scoring. Different splicing variants of NKX3-1 and antibodies have previously been attributed for the conflicting results of NKX3-1 expression. Human kallikrein 4 (KLK4), a serine protease belonging to the prostate specific antigen-related kallikrein family, is over-expressed in PCa and might be a proliferative factor acting directly or indirectly on cell cycle regulators⁽¹⁶⁾. Increased KLK4 levels in PC-3 cells have also been associated with a transcriptional repression of E-cadherin and increased Matrigel motility⁽¹⁷⁾. Our results were consistent with previous reports showing an overexpression of KLK4 mRNA and protein in PCa.

There are no previous reports on *in vivo* studies on the effects of genistein on the cell cycle-related genes $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p53$. However, *in vitro* studies on prostate, breast and lung cancer cells report that genistein up-regulates them, in line with its inhibitory effect on cell cycle progression^(18,19). We did not observe any significant effects by genistein, but the mRNA levels of $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p53$ were slightly down-regulated. The reason for this discrepancy is not clear. Interestingly, we showed an increased expression of $p21^{Waf1/Cip1}$ and $p53$ mRNA and protein expression in G3 and that the expression may further increase with increasing Gleason grade. Navone *et al.*⁽²⁰⁾ showed that $p53$ accumulation is associated with late-stage PCa. With a cutoff

value at 5% of $p53$ positive cells, they detected no accumulation in nineteen patients with Gleason score 5–7, whereas nineteen of forty-two patients with Gleason score 8–10 showed accumulation. $p21^{Waf1/Cip1}$ may act both as a cell cycle negative regulator and anti-apoptotic mediator and its protein expression has been shown to correlate strongly with $p53$ in PCa^(21,22). The reduction of $p27^{Kip1}$ mRNA and nuclear protein expression in both study arms and the small reversal of protein expression in G3 and G4 by genistein intervention may have an interesting implication for the cell cycle modulating properties of genistein. Loss of phosphatase and tensin homologue function and/or constitutive activation of Akt/protein kinase B by the phosphoinositide kinase-3 pathway, commonly found in PCa, will reduce the expression of $p27^{Kip1}$ ^(23,24). *In vitro* studies on genistein indicate that it inhibits protein kinase B constitutive activation by re-establishing phosphatase and tensin homologue expression^(25,26). Although the increase of $p27^{Kip1}$ in G3 and G4 by genistein was not significant, this result may indicate the mechanism for its G₁ cell cycle arrest and proliferation inhibition of tumour cells⁽²⁷⁾. Further research is needed in this connection.

The proliferation biomarker Ki67 was clearly up-regulated in tumour tissue, although it was not modulated by genistein. Our detection of 1% Ki67 positive luminal cells in normal and 4–6% in tumour tissue corroborates with previous publications⁽²⁸⁾. The increasing levels of Ki67, $p21^{Waf1/Cip1}$ and $p53$ may indicate that loss of $p53$ function already is present in some G3 cancers.

The apoptotic biomarkers BAX and BCL-2 were both significantly or near-significantly up-regulated in PCa, corroborating a previous report on Gleason score 5–10⁽²⁹⁾. We observed no significant regulation of BAX or BCL-2 by genistein intervention.

The role of neuroendocrine markers as prognostic factors is controversial, although the serum level of CgA and NSE seems to have a prognostic value in castration-resistant PCa^(30–32). Genistein treatment of LNCaP human prostatic adenocarcinoma cells has been shown to induce several positive biomarkers for neuroendocrine differentiation including CgA⁽¹⁹⁾. We detected very few CgA- and NSE-positive cells in tumour tissue. The reduction of CgA-positive cells in tumour tissue corroborates earlier results from serum, showing a reduction in localised PCa and an increase in castration-resistant PCa⁽³¹⁾.

The ability of genistein to modulate the progression of existing PCa is not clear. In the present study, we have investigated the effects of pure genistein. As opposed to the majority of chemopreventive reports of genistein treatment, some recent studies on mouse xenograft models indicate genistein to promote increased metastasis of PCa, whereas isoflavones containing genistein and daidzein do not^(33–37). Other studies show that dietary genistein inhibits metastases of human cancer, including PCa, in mice and that the discrepancy may be related to methodology^(38,39). In addition, Setchell *et al.*⁽⁴⁰⁾ raised doubts about the use of rodent models for gaining insight into the effect of isoflavones in humans due to differences in the metabolism of genistein. We did not detect any signs of cancer-promoting effects in our study with pure

genistein intervention in our human study subjects having early localised PCa.

Overall, genistein intervention at nutritionally relevant levels in patients with early PCa modulated several biomarkers which may be related to cancer prediction and progression. Genistein may have an inhibitory effect on androgen-related biomarkers. We also suggest a possible mechanism as to how genistein may induce cell cycle arrest and inhibition of proliferation in PCa. A limitation in our study is the small number of cases included and also the relative short time of intervention. Further studies on the effects of genistein in PCa are warranted, including clinical studies examining biomarkers and *in vitro* studies investigating its mechanisms of action.

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