

**IN VITRO MODULATION OF ANDROGEN AND ESTROGEN
RECEPTORS IN HUMAN PROSTATE CELLS BY
ESSENTIAL FATTY ACIDS**

BY

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SUBMITTED TO THE FACULTY OF HEALTH SCIENCES

SCHOOL OF MEDICINE

DEPARTMENT OF UROLOGY

UNIVERSITY OF PRETORIA

In partial fulfilment of the requirements for the degree ***Magister Scientiae with specialization in Reproductive Biology: Andrology***, Pretoria, South Africa, 2001



“Clearly there is need for a stronger evidence base, and more creative thinking on the part of the health-care profession, to help engage men of all ages in caring for their own health. Most importantly, it is essential that men themselves take an active part in the process of researching and developing their own care. The accomplishments of the women's health movements in recent years should provide ample stimulus and inspiration.”

The Lancet • June 9, 2001 • Vol 357 p. 1813 (editorial)



Hiermee erken ek met dank die finansiële hulp (beurs) wat ek van die "Struwig-Germeshuysen Kankemavorsingstrust" ontvang het vir die voltooiing van my studies. Menings wat in die publikasie uitgespreek word of gevolgtrekkings waartoe geraak is, is dié van die navorser alleen en strook nie noodwendig met dié van die "SGK-Trust" nie.



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ACKNOWLEDGEMENTS

On a personal note, I would like to extend my sincere gratitude to the following persons and organizations for the contributions they made in the fulfilment of this degree:

First of all, I would like to thank Dr. Casper van Aswegen for his patience in sharing his knowledge, insight and experience with me. I have learned that: "Any enterprise is built by wise planning, becomes strong through common sense, and profits wonderfully by keeping abreast of the facts." (Proverbs 24: 3,4 Living Bible). Thank you for your friendship and may you prosper in your future enterprises.

Dr. Marlene Fourie for her valued advice with my protocol and thesis and for many e-mails that made my day.

Prof. Riana Bornman for allowing me to study in her department.

Prof. Simon Reif, head of the Department of Urology for his support.

Prof. Dion du Plessis for his interest, encouragement and giving perspective and meaning to my project.

Miss Martie van der Walt and the staff from the Pre Medical Library for their friendliness and always excelling in obtaining the literature I needed. Smullerkie says thanks, NAMkie.

The Struwig-Germishuysen Trust for supporting me with a bursary for two years.

The Wolmarans Fund, Efamed SA, NAVKOM, CANSA, THRIP and the University of Pretoria for funding.

All my colleagues in the Department of Urology, including the secretaries, for their friendliness and Dr. Caren Hadders for helping me with my studies.

All my colleagues in the Department of Physiology, especially Dr. Petrus du Toit for teaching me cell culture work, Dr. Annie Joubert for her discussions and Prof. van Papendorf for the use of their facilities.

Mrs. Elize Gema and Prof. Leonora Dreyer from the Department of Anatomical Pathology who was so kind to assist me with immunocytochemistry.

Donovan Burger from Separations Scientific for helping me with valuable advice in the lab, Marinda van Kleef and Jeanni Fehrsen from Onderstepoort Research Institute for their interest and advice.

My parents, Piet and Elna Muller, my sister Marietjie and two brothers, Frikkie and Samuel, for always believing in me, for their support and for their love. Thanking you and loving you too!

My dear husband, Boet, for teaching this "slimjan" things about life that I shall carry with me always. Thanks Boet, I love you!

May you all be richly blessed with everlasting peace and joy!

To my Creator, Saviour and Encourager, I acknowledge Thee, I thank Thee and I praise Thee!

ABBREVIATIONS

A ₂₆₀	-	Absorbance at 260 nm
A ₂₈₀	-	Absorbance at 280 nm
ALA	-	α-Linolenic acid
AR	-	Androgen receptor
ARE	-	Androgen response element
bp	-	base pair
BP	-	Binding protein
BPH	-	Benign prostatic hyperplasia
°C	-	degrees Celsius
Ca ²⁺	-	Calcium catione
CAG	-	Cytosine Adenine Guanine
cDNA	-	complementary deoxyribonucleic acid
Ci	-	Curie
COX	-	Cyclooxygenase
CSPD	-	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5' chloro)-tricyclo[3.3.1.1 ^{3,7}]decan}-4-yl) phenyl phosphate
CsTFA	-	Cesium trifluoroacetate
DAB	-	3,3'-diaminobenzidine tetrahydrochloride
DHA	-	Docosahexaenoic acid
DTT	-	Dithiotreitol
ddATP	-	Dideoxyadenine triphosphate
ddNTP	-	Dideoxynucleotide triphosphate
ddUTP	-	Dideoxyuridine triphosphate
DEPC	-	Diethyl pyrocarbonate
DES	-	Diethylstilbestrone
DESdP	-	Diethylstilbestrone diphosphate
DHT	-	Dihydrotestosterone



DIG	-	Digoxigenin
DNA	-	Deoxyribonucleic acid
dpm	-	disintegrations per minute
E	-	Estrogen
E ₂	-	Estradiol
EMEM	-	Eagle's minimum essential medium
EDTA	-	Ethylenediamine tetraacetic acid disodium salt
EFA	-	Essential fatty acid
EFAs	-	Essential fatty acids
EGF	-	Epidermal growth factor-urogastrone
EPA	-	Eicosapentaenoic acid
ER	-	Estrogen receptor
ER α	-	Estrogen receptor alpha
ER β	-	Estrogen receptor beta
EpRE	-	electrophile/antioxidant response element
<i>et al.</i>	-	and others
EVS	-	Essensiële vetsure
FAs	-	Fatty acids
FAS	-	Fatty acid synthase
FCS	-	Fetal calf serum
FGF-2	-	Fibroblast growth factor 2
FSH	-	Follicle-stimulating hormone
G3PDH	-	Glyceraldehyde-3-phosphodehydrogenase
GAPDH	-	Glyceraldehyde-3-phosphodehydrogenase
GH	-	Growth hormone
GLA	-	γ -Linolenic acid
[³ H]-E ₂	-	Radiolabelled estradiol
[³ H]-T	-	Radiolabelled testosterone
HRE	-	Hormone response element
HRP	-	Horse radish peroxidase
hsp	-	heat shock protein



IGEPAL	-	(Octylphenoxy)polyethoxyethanol
IGF-I	-	Insulin-like growth factor I
IgG	-	Immunoglobulin Gamma
kDa	-	kilo Dalton
KGF	-	Keratinocyte growth factor
LH	-	Luteinizing hormone
LH-RH	-	Luteinizing hormone-releasing hormone
LA	-	Linoleic acid
M	-	Molarity
MDA	-	Malondialdehyde
ml	-	millilitre
mmol	-	millimoles
MOPS	-	3-[N-Morpholino]propanesulphonic acid
mRNA	-	Messenger ribonucleic acid
MUFAs	-	Monounsaturated fatty acids
NLS	-	Nuclear localization signal
nM	-	nano Molar
NO	-	Nitric oxide
NGS	-	Normal goat serum diluted 1 in 5 with TBS
NSAID	-	Nonsteroidal anti-inflammatory drugs
OA	-	Oleic acid
PAGE	-	Polyacrylamide gel electrophoresis
par	-	pararagraph
PBS	-	Phosphate buffered saline
PEP	-	Phosphoenolpyrovate
PIN	-	Prostatic intraepithelial neoplasia
PKC	-	Protein kinase C
pmol	-	picomoles
PMS	-	Premenstrual syndrome
PMSF	-	Phenyl-methyl-sulphonyl fluoride
PR	-	Phenol red



PSA	-	Prostate specific antigen
PUFAs	-	Poly unsaturated fatty acids
RIPA	-	Radioimmuno precipitation assay
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
RSHBG	-	SHBG receptor
SA	-	South Africa
SDS	-	Sodium dodecyl sulphate
SFAs	-	Saturated fatty acids
SHBG	-	Steroid hormone binding globulin
SH	-	Steroid hormone
SHR	-	Steroid hormone receptor
SH-SHR	-	Steroid hormone – steroid hormone receptor complex
SS	-	Saline solution
SSC	-	Saline sodium citrate
T	-	Testosterone
TAF	-	Transcription activation factor
TBS	-	Tris buffered saline
TBS-T	-	Tris buffered saline Tween 20
TE	-	Tris-EDTA
TEBG	-	Testosterone estradiol binding globulin
TeBG	-	Testosterone binding globulin
TGF- α	-	Transforming growth factor alpha
Tris	-	Tris(hydroxymethyl)-amino-methane
Tween 20	-	Polyoxyethylenesorbitan
UK	-	United Kingdom
uPA	-	Urokinase type plasminogen activator
USA	-	United States of America
μ l	-	microlitre
VEGF	-	Vascular endothelial growth factor

REAGENTS AND CHEMICALS

All Reagents were of analytical grade and were supplied by the following companies:

Amersham Pharmacia Biotech (Buckinghamshire, UK)

The ExcelGel 7.5 and 12.5 homogenous PAGE gels
Rainbow™ coloured protein molecular weight markers
Kodak Scientific imaging film, X-OMAT™AR AEC-Amersham (Kelvin, SA).

Beckman (Irvine, USA)

Ready Protein⁺

BioWhitaker (Walkerville, MD, USA)

Trypsin

Bio-Rad Laboratories (Richmond, CA, USA)

Bio-Rad Protein Assay kit

Clontech Laboratories (Palo Alto, CA, USA)

G3PDH and β -actin probes

Corning Glass Works (New York, USA)

Corning culture flasks

Dako (Cambridgeshire, UK)

LSAB[®]2 (K0675)
DAB chromogen (K3466)

Delta Bioproducts (Kempton Park, SA)

Fetal calf serum

DuPont NEN (Boston, MA, USA)

[2,4,6,7-³H(N)]Estradiol-17 β (92 Ci/mmol)

[1,2,6,7-³H(N)]Testosterone (95 Ci/mmol)

Flow Laboratories (Irvine, UK)

Ham's nutrient F-10 mixture

Microsep (Bramley, SA)

Immobilon P membranes

Millipore Sterivex-GS filters

Millipore filters AP 15 and 20

NENTM Life Science Products (Boston, MA, USA)

3'-End Labelling Fluorescein kit with Antifluorescein-HRP

Oncogene Products (Cambridge, MA, USA)

ER and GAPDH probes

Novo Castra Laboratories (Newcastle upon Tyne, UK)

Mouse antihuman antibody for AR (NCL-AR-318)

Mouse antihuman antibody for ER α (NCL-ER-6F11/2)

Pierce (Rockford, Illinois, USA)

Super Signal West Picochemiluminescence Substrate

Roche Diagnostics (Boehringer Mannheim Roche Diagnostics SA)

TriPure isolation reagent, positively charged nylon membranes, DIG nucleic acid labelling and detection kits, washing, blocking, maleic acid and detection buffers.



Serotec (Oxford, UK)

Secondary antibody F(ab')₂ goat antimouse IgG, conjugated with Horseradish peroxidase

Sigma-Aldrich (Atlasville, SA).

Linoleic acid (LA), α -linolenic acid (ALA), γ -linolenic acid (GLA), arachidonic acid (AA), eicosopentaenoic acid (EPA) and oleic acid (OA), Testosterone and DES, PMSF, aprotinin, leupeptin, MOPS buffer, Tween 20. Primary rabbit antihuman antibodies for ER α and ER β , as well as the secondary monoclonal antirabbit immunoglobins, which were conjugated with Horseradish peroxidase, DEPC.

Sterilab (Johannesburg, SA)

DU-145 cell lines

Tec Med Imaging (Halfway House, SA).

Film developer and fixer solutions

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ABSTRACT

Cancer of the prostate is one of the leading causes of cancer related deaths in black and white men. An important role in the development of prostate cancer is played by androgens and androgen ablation is therefore currently used in cancer treatment. In the past, estrogens were widely used in treatment of prostate cancer, but there are indications that estrogens could also be involved in carcinogenesis. In contrast with saturated fats, some essential fatty acids (EFAs) are associated with a decrease in prostate cancer incidence. Lately, much research has been done on the modulation of the binding of steroid hormones to their receptors by essential fatty acids (EFAs), which could interfere with the steroid hormone's message. Therefore, the aim of this study was to determine in whole DU-145 human prostate cells the effect of EFAs and their metabolites on the binding and affinity of the estrogen receptor (ER) and androgen receptor (AR) to estradiol (E_2) and testosterone (T), respectively. Binding studies were carried out with radioactive T and E_2 with and without an excess of cold steroid. Data was analyzed according to the Scatchard method. Western and northern blotting were applied. The results showed that the EFAs under investigation inhibited the AR's capacity (B_{max}), in contrast to the ER's capacity, which was stimulated. However, the dissociation constants (K_d) of the AR and ER complexes in the presence of the fatty acids (FAs) were as follows. Except for eicosapentaenoic acid (EPA) which decreased the AR dissociation constant and EPA and alpha-linolenic acid (ALA) which increased the ER dissociation constant, the remaining FAs had no significant effect on the K_d values of both the AR and ER complexes. The decrease in the AR K_d and B_{max} values implied that EPA caused less T to bind to AR, but more tightly. In contrast, the increase in the ER B_{max} and K_d values implied that both EPA and ALA caused more E_2 to bind to ER, but more loosely. The overall inhibition of T binding to AR and stimulation of E_2 binding to ER by n-3 EFA may be beneficial during androgen withdrawal with or without estrogen therapy. Molecular studies showed that EFAs had no effect on the expression of the steroid receptors and further studies are necessary to



investigate the effect of EFAs on steroid receptor expression. According to these preliminary results it is concluded that men should benefit from a diet rich in certain essential polyunsaturated fatty acids although its function remains to be clarified.

KEY WORDS: Estrogen receptor α , Estrogen receptor β , Androgen receptors, Prostatic cancer, DU-145 cells, Essential fatty acids, Scatchard analysis, Northern blotting, Immunocytochemistry, Western blotting.

OPSOMMING

Prostaatkanker is een van die belangrikste oorsake van kankerverwante sterftes onder swart en wit mans. Androgene speel 'n belangrike rol in die ontwikkeling van prostaatkanker en derhalwe word androgeen onttrekking in die behandeling van kanker behandeling gebruik. In die verlede was estrogene algemeen gebruik in die behandeling van prostaatkanker, maar daar is aanduidings dat estrogene ook betrokke kan wees in karsinogenese. Onlangs is heelwat navorsing gedoen op die modulering van die binding van steroïed reseptore aan hulle reseptore deur essensiële vetsure (EVS), wat met die steroïed hormoon se boodskap kan inmeng. Daarom was die doel van hierdie studie om die effek van EVS en hulle metaboliëte op die bindingskapasiteit en die affiniteit van die estrogeen reseptor (ER) en die androgeen reseptor (AR) aan estradiol (E_2) en testosteroon (T) in heel DU-145 menslike prostaat selle onderskeidelik te bepaal. Scatchard binding studies, asook noordelike en westelike klad is uitgevoer. Die resultate het getoon dat die EVS wat ondersoek was die AR se kapasiteit (B_{max}) geïnhibeer het, in teenstelling met die ER se kapasiteit wat gestimuleer was. Die dissosiasie konstantes (K_d) van die AR en ER komplekse in die teenwoordigheid van EVS was as volg. Behalwe vir eicosapentaenoaat (EPA) wat die AR dissosiasie konstante verminder het en EPA en alpha-linolenaat (ALA) wat die ER dissosiasie konstante vermeerder het, het die oorblywende vetsure geen betekenisvolle effek op die K_d waardes van beide die AR en ER komplekse gehad nie. The afname in die AR K_d en B_{max} waardes beteken dat EPA veroorsaak het dat minder T aan die AR gebind het, maar stywer. In teenstelling daarmee, het EPA en ALA veroorsaak dat meer E_2 aan ER gebind het, maar lossier. Die algehele inhibering van T binding aan ER deur EPA en stimulering van E_2 binding aan ER mag voordelig wees tydens androgeen onttrekking met of sonder estrogeen behandeling. Hoewel geen effek op die uitdrukking van AR en ER verkry was nie, word verdere studies benodig om te bepaal of EVS die uitdrukking van steroïed reseptore sou affekteer. Volgens hierdie voorlopige resultate kan afgelei word dat mans bevoordeel sou



word deur 'n diëet ryk in sekere essensiële poli-onversadigde vetsure, hoewel die funksies nog opgeklaar moet word.

SLEUTELWOORDE: Estrogeen reseptor α , Estrogeen reseptor β , Androgeen reseptore, Prostaat kanker, DU-145 selle, Essensiële vetsure, Scatchard analise, Noordelike klad, Immunositochemie, Westelike klad.

CHAPTER 1

1. INTRODUCTION

The living body is receptive to a wide variety of external disease-causing agents and also to the fallibility of its own composition, which increases with age. These factors are demonstrated in one of the most common diseases of modern society, cancer. Prostate cancer is the most commonly diagnosed male cancer and one of the leading causes of cancer-related deaths in men over 50 years. Each year in the United States, approximately 200 000 new cases of prostate cancer are diagnosed and approximately 40 000 men dies because of it¹. In South Africa prostate cancer is increasing in black men in the cities and this tendency might be due to the shift from a traditional to a Western diet and lifestyle². It is widely accepted that prostate cancer incidence is related to environmental factors such as lifestyle or diet, as well as to energy metabolism and balance, which is related to body height and fat stores. Furthermore, prostate cancer exhibit various genetic discrepancies, some of which can be linked to prostate cancer in the family history or to racial differences^{3,4}. Both genetic and environmental factors may alter hormone metabolism and in this way affect prostate cancer risk. The importance of hormones in the development and progression of prostate cancer has been realised since the 1940's when Charles Huggins and his colleagues laid the scientific basis for treatment of prostate cancer with androgen ablation therapy⁵. At onset, most human prostate cancers are androgen sensitive and respond well to hormonal therapy, while others present temporal remission, followed by relapse to a hormone refractory state.

1.1. ANDROGEN AND ESTROGEN RECEPTORS

In addition to the nervous system, hormones and hormone receptors are part of the communication network in the body. Communication plays an essential role in normal daily living, not only in the modern external environment, but also

in the human body itself, between different body parts, between neighbouring cells, as well as intracellularly. It revolves around sending, receiving and responding to different signals or messages. Ineffective or defective communication can create immense hazards in the outside modern world as well as inside the living body. In fact, cancer develops when the signalling control of a single cell becomes distorted, leading to uncontrollable proliferation of the modified or tumour cell⁶. The ability of specific target cells to receive and respond to a specific signal is dependent on the binding of a ligand or messenger to a receptor found in or on the target cell. Ligands are usually secreted in the cellular environment or in the circulating blood, and can travel over distances to reach local or distant receptors. On the other hand, receptors are only found in the cells that respond to specific signals.

1.1.1 Structure

Androgen and estrogen receptors are both classified as members of the class I nuclear receptor family since they all interact with heat shock proteins, they tend to form homodimers or heterodimers and bind DNA on specific sites⁷. All steroid hormone receptors in class I generally share a high degree of structural homology with each other and each receptor protein are divided into the transactivation, DNA binding, hinge and ligand binding domains according to the different functions of each domain (Fig. 1. 1)⁸.

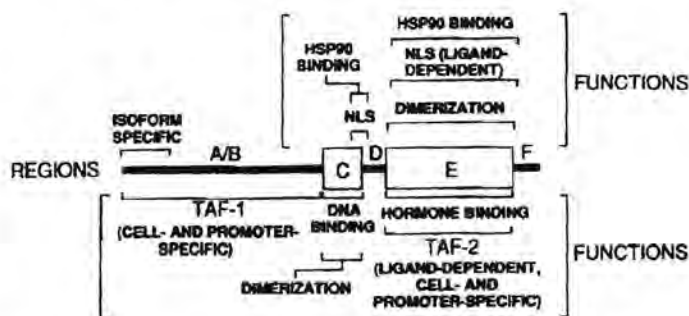


Fig. 1. 1. Schematic illustration of the structure/function of steroid hormone receptors. The conserved regions C and E are indicated as boxes, a black bar illustrates regions A/B, D and F. Domain functions are depicted above and below the scheme. NLS, nuclear localization signal; TAF, transcription activation function⁸.

The molecular weights of the AR, ER α and ER β proteins are 110 kDa, 67kDa and 65 kDa respectively. The genes encoding for the AR, ER α and ER β lie on chromosome Xq 11-12, 6q25.1 and 14q 22-24 respectively⁹. Each gene consists of 8 exons: exon 1 encodes for the N-terminal, exons 2 and 3 each encode for a zinc-finger, and exons 4-8 encode for the hormone binding domain (Fig. 1. 2). Structurally, the ER β amino acid sequence is highly homologous to the ER α amino acid sequence and like other steroid receptors, it possesses conserved functional domains, which are necessary for receptor function^{10,11}.

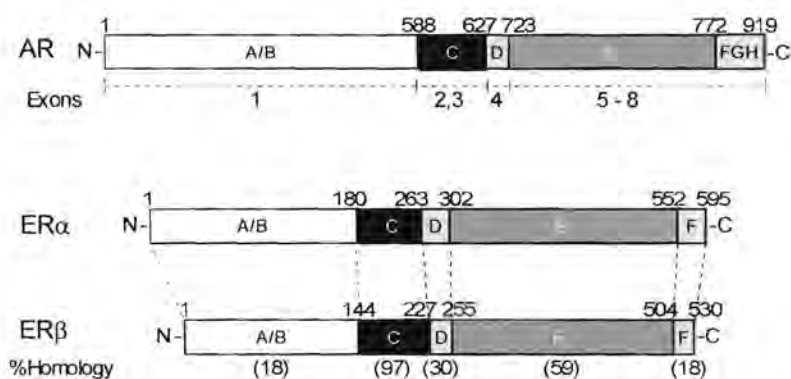


Fig. 1. 2 Diagram of the human androgen receptor (AR), estrogen receptor α (ER α) and estrogen receptor β (ER β) proteins. Exons are shown, but not the introns as well as the percentage of homology between ER α and ER β ^{12,13,14}.

Since the ER β is a novel ER, the difference between ER α and ER β is shortly reviewed. The high homology between ER α and ER β in the DNA binding domain (97%) suggests that these receptors interact with similar estrogen response elements on the DNA^{9,15}. However, ER α and ER β display different patterns of affinities for ERE, which can cause differential activation in the presence of estrogens^{15,16}. Important factors, which can determine the activation of particular estrogen responsive genes, are the nature of the ERE and the ratio of ER α and ER β subtypes in a particular cell or tissue¹⁶. Human and rat ER β can form homodimers or heterodimers with ER α ^{17,18}. Therefore, the cell can respond to estrogens in three different ways by forming three different types of dimers: ER α homodimers, ER β homodimers and ER α ER β heterodimers¹⁸. The presence of

homo- and heterodimers and their content in different cells, tissues and organs may explain the selectivity of estrogen action in different cells and tissues.

The relative high homology between ER α and ER β in the ligand binding domain (60%) imply that they have similar binding affinities for estrogen and estrogen related compounds. Binding affinities for estrogenic compounds such as estradiol, DES, estrone and 5 α -androstane-3 β ,17 β -diol, as well as for antiestrogens such as tamoxifen, 4-OH-tamoxifen and the synthetic antiestrogen ICI-164,384 are similar for ER α and ER β ¹⁹. However, phytoestrogens such as genistein, coumestrol and zearalenone have up to 10-fold higher affinities for ER β than for ER α as reported in cell transfection studies²⁰. Ligands that act as agonists on the one ER subtype and as antagonists on the other are being developed and may play a role in determining the different roles of ER α and ER β ²¹.

The low homology between ER α and ER β in the N-terminal A/B domain (18%) indicates different patterns of gene activation between these two receptors. Construction of ER β chimeras with the ER α A/B domain exhibit an improved transcriptional response to estrogens and antiestrogens and indicate that differences in the N-terminal end contribute to cell and promoter specific differences in transcriptional activity of ER α and ER β ²². Furthermore, ER β differs from ER α in the ligand activation profile at an AP1 element where 17 β -estradiol stimulates or inhibits transcription in the presence of ER α and ER β respectively²³.

1.1.2 Mechanism of action

Steroid hormones, which are the initial activators of ligand-activated nuclear transcription factors or steroid hormone receptors, are transported to the target tissues *via* the circulation¹². The average plasma testosterone concentration in the adult male is 11-33 nmol/l (mean, 22 nmol/l). Only 2% (0.5 nmol/l) exists in free form, while 98% is bound to plasma proteins²⁴. In the prostate testosterone is converted by the 5 α -reductase enzyme to DHT (Fig. 1. 3), which is the

principal androgenic hormone in the prostate. The formation of the AR-DHT complex regulates the activity of the androgen responsive genes that are responsible for prostate growth and function¹³. In contrast to testosterone, the daily production of estradiol in the male is in the range of 40-50 μg , of which only 5-10 μg is produced by the testes, the rest being formed in the prostate, adipose tissue, liver, brain and bone by aromatase enzymes (Fig. 1. 3)^{25,26}. Local production of estrogen in the male is of physiological significance throughout adult life, since it plays a role in spermatogenesis^{27,28}. Since estradiol levels do not decrease significantly in elderly men, it may play a protective role in preventing osteoporosis and Alzheimer's disease, which occur during estrogen deficiency²⁹.

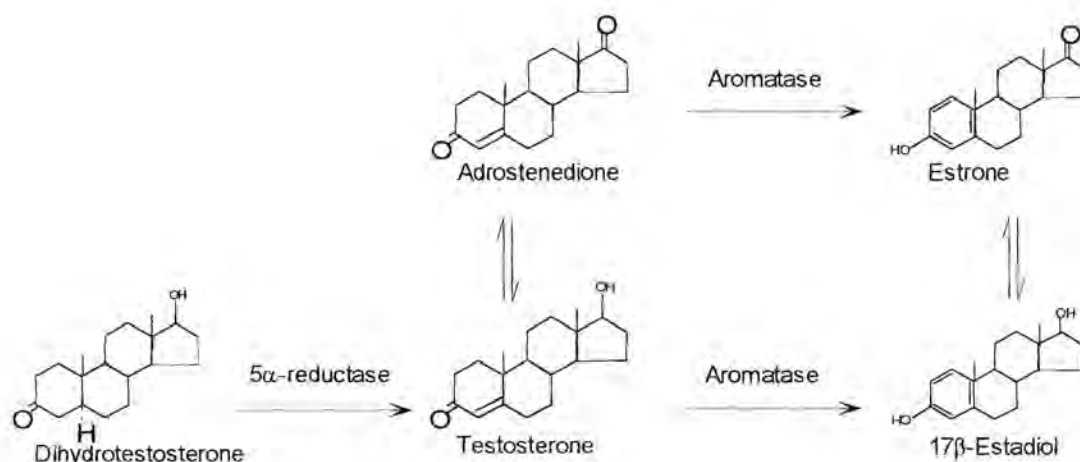


Fig. 1. 3 Synthesis of androgens and estrogens.

Current thought suggests that steroids be transported across the cell membrane at the target cell *via* passive diffusion. Previously it was thought that steroid hormones bind to steroid hormone receptors in the cytoplasm and then the SH-SHR complex is translocated across the nuclear membrane. However, cytoplasmic receptors may be an artefact of receptor assays and if this is true, ligands bind directly to nuclear receptors. The binding of the ligand to the SHR triggers a conformational change that unmarks the DNA-binding domain of the

receptor (Fig. 1. 4)^{30,31,32}. During the unmasking process, the heat shock protein 90 (hsp90) is released from the SHR and thereby reveals the two zinc fingers, which facilitate the high-affinity association of the complexes with the HRE. Another function of unmasking is to promote dimerization of the ligand receptor complexes, which provides stability to the process of gene transcription. Following phosphorylation of the SH-SHR, the complex binds to the HRE and the appropriate transcription factors associate with the complex on the DNA. Then the targeted gene is transcribed, the mRNA is processed *via* splicing and transported to the cytoplasm, where ribosomal complexes translate the message to produce the specific induced protein.

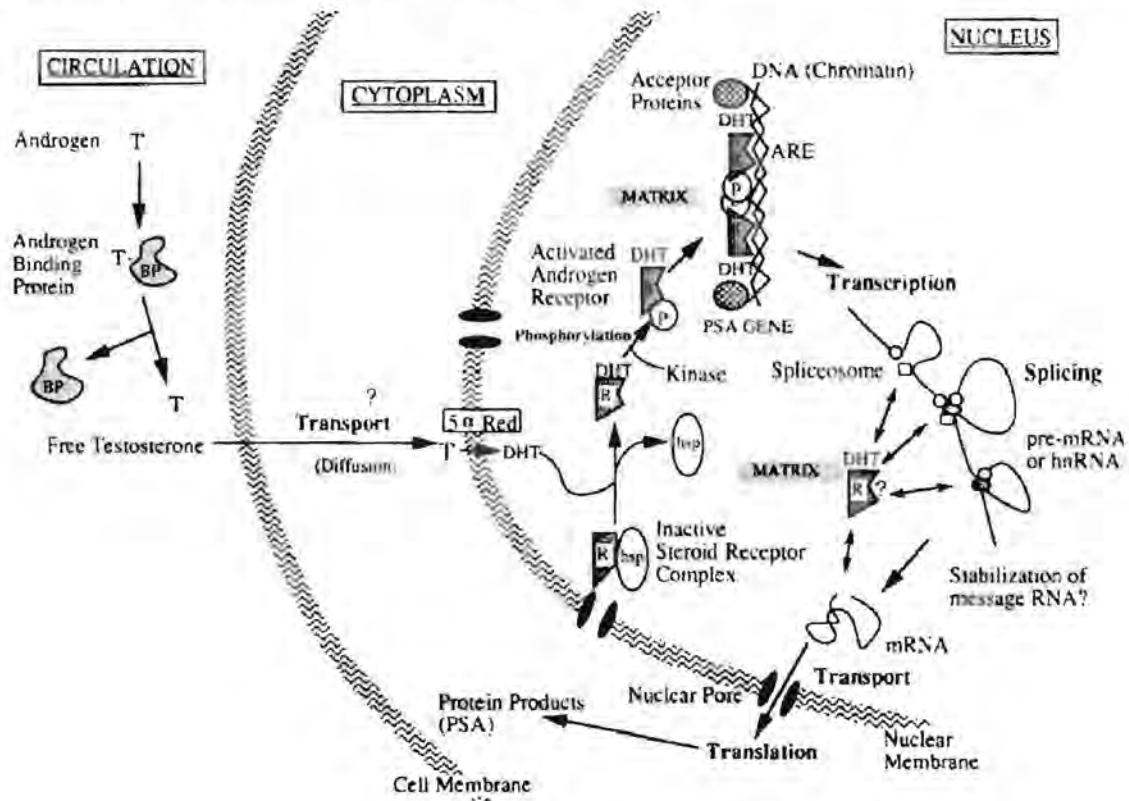


Fig. 1. 4. Mechanism of action of androgen in the prostate cell³³.

1.1.3 Prostate cancer

1.1.3.1 Androgens and estrogens

Androgens have been considered to play a crucial role in the development and progression of prostate cancer, since men castrated before puberty do not develop prostate cancer³⁴. Estrogens may too be culprits in carcinogeneity, since estrogen causes florid squamous cell metaplasia that can be offset by androgens^{34,35}. In the rat, chronic administration of estradiol in combination with low doses of testosterone caused low-grade carcinomas³⁶. Therefore, it seems that the combination of estrogens with androgens or the testosterone estradiol ratio is an important factor in prostate cancer ethiology.

Age is an important prostate cancer risk factor, which can partly be explained by the alteration in the ratio of androgen and estrogen levels (Fig. 1. 5)³⁷. In the case of testosterone, it is generally accepted that it is not so much the total serum testosterone levels that decrease with age, but rather the serum testosterone not bound to the sex hormone binding globulins, the bioavailable testosterone. The decrease in bioavailable testosterone has prompted the issue of androgen therapy in the elderly. In healthy men 55 and older, total serum testosterone may be lower in only 20% of men, while bioavailable testosterone may be decreased in as much as 50% of men³⁸. Total plasma estradiol levels in men older than 50 are increased. However, plasma levels of free estradiol stay the same, due to an age-related increase in the testosterone estradiol binding globulin (TEBG). This causes a 40% increase in the ratio of free estradiol/free testosterone, which could be important in development of BPH and prostate cancer^{39,40,41}. Altering hormone levels may not merely be increased or decreased, but irregular and asynchronous secretion of hormones such as LH and testosterone may play a major role⁴². An increase in the serum hormone binding proteins in the elderly reduces the bioavailability of androgens, estrogens and IGF-I⁴³. Although prostate cancer generally develops later in life, fetal exposure to estrogen and environmental factors may induce a greater propensity to develop cancer later in life³⁷.

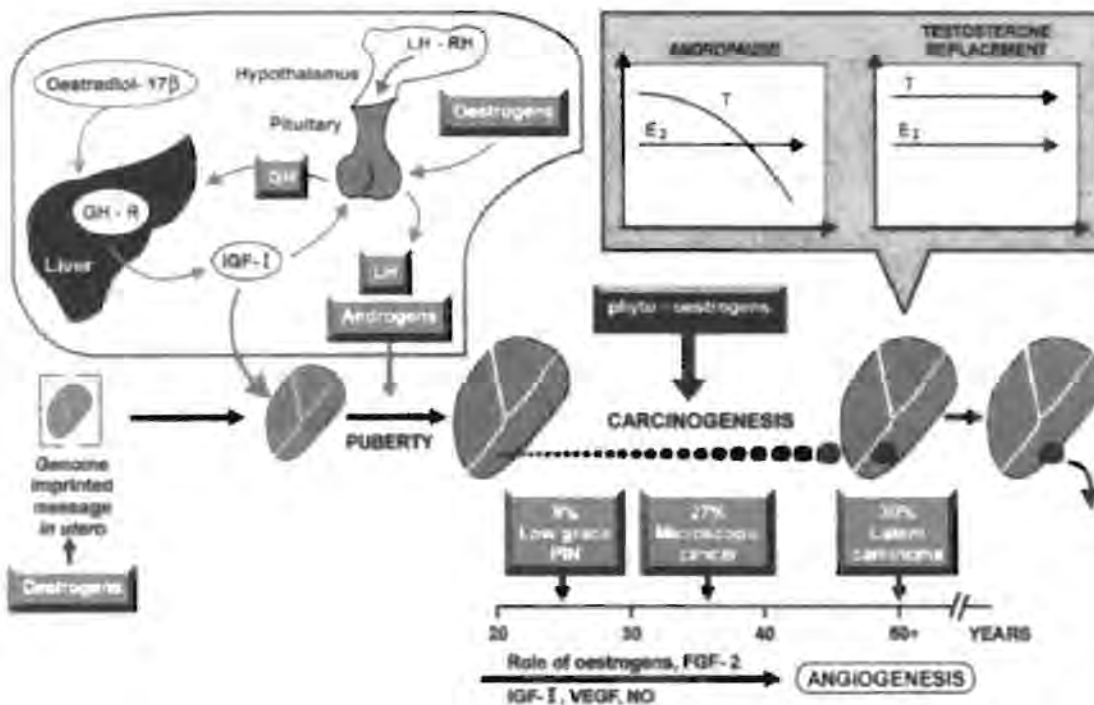


Fig. 1. 5 Estrogens in imprinting, puberty and early prostatic disease³⁷.

1.1.3.2 Androgen receptors

Since androgens and the AR play such an important role in development and progression of prostate cancer, the AR has been studied as a biomarker and prognostic factor in the progression of this disease. Prostate tumours can be either AR positive or AR negative. Most AR positive tumours respond to hormonal treatment, but some are resistant to therapy and progresses. Androgen receptor negative tumours usually relapse even though some of them may initially respond to endocrine therapy⁴⁴. In normal prostate epithelium, AR seems to be expressed by the secretory cells and the basal cells in culture⁴⁵. Since secretory cells degenerate during androgen ablation, it has been proposed that the presence of AR is associated with maintenance and survival of these cells¹³. The basal cells are the stem cells for the secretory cells and therefore the possibility exists that growth factors could stimulate basal cell differentiation and eventually AR expression to produce secretory cells³³.

Biochemical and immunohistochemical studies of AR content in relation to grade or stage of disease, as well as prediction of response to endocrine therapy

has been inconsistent. Despite the inconsistencies, nearly all primary prostate cancer specimens positively express AR protein, while some advanced stage prostate cancers, including metastases in bone, the epidural space periosteum and most of the metastases in pelvic lymph nodes also express AR protein^{46,47,48}. A fact that must be taken in consideration is that endocrine treatment may increase AR content in cancerous prostates, which may play a role in recurrence, progression or hormone resistance during therapy⁴⁹. In a study by de Vere White, the AR expression increased within higher Jewett stages and higher Gleason grades after combined androgen blockade⁴⁶.

Although some prostate tumours stain less intensely for AR than normal and BPH prostates, it was noticed that the tumours stain more heterogeneously for AR than normal and BPH prostates, which stain homogeneously⁵⁰. Since the variability of AR content per nuclear area increases with increasing grade and Gleason score, heterogeneity may act a diagnostic tool in staging the tumour⁵¹. In addition to diagnosis, variability of AR content can predict the outcome of endocrine therapy rather accurately and therefore AR heterogeneity may be important in choosing the best possible treatment⁴⁴. Prins *et al*⁴⁴ demonstrated that tumours, which consisted of highly variable or heterogeneous cell sub-populations, were associated with endocrine therapy failure, while more homogenous AR concentrations were associated with a favourable response to endocrine treatment.

Several AR aberrations in structure, function and content have been implicated during initiation and progression of prostate cancer (Fig. 1. 6). An AR aberration associated with initiation of prostate cancer include the (CAG)_n tandem repeat lengths in the N-terminal domain of the AR. Shorter repeat lengths are not only associated with increased transactivation, but with increased cancer risk, increased severity of the tumour and development at a younger age^{52,53,54,55}. Interestingly, black Americans, who are more prone to develop prostate cancer, also have shorter (CAG)_n tandem repeat lengths in the N-terminal domain of the AR than their white counterparts^{56,57}.

During androgen ablation therapy of advanced prostate cancer, tumour cells may adapt themselves to androgen deficient levels by utilizing the residual low levels of androgens more effectively in order for cell growth to be sustained in an androgen-independent manner⁵⁸. One mechanism is to increase AR gene amplification. In recurrent tumours, the average copy number of the AR gene varies substantially from 2.7 to 28 AR gene copies per cell, with up to 60 copies in some individual tumour cells⁵⁹. No mutations were reported in these cases. However, some hormone-refractory tumours, which exhibited AR gene amplification, correlated with an increase in p53 mutation^{60,61}. When p53 mutates, cancer risk increases, since normal p53 controls cell proliferation.

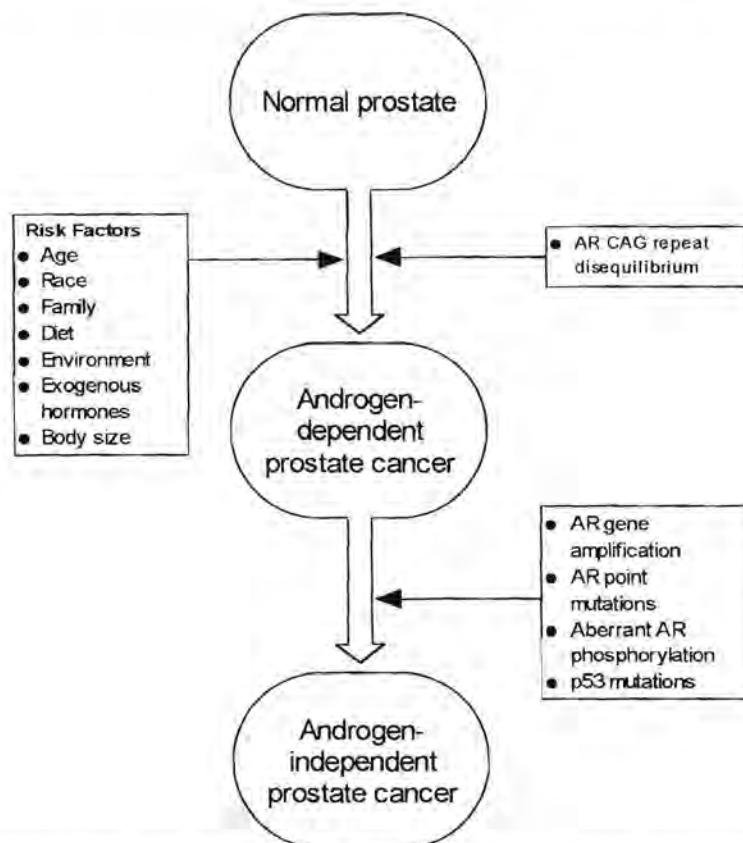


Fig. 1. 6. Androgen receptor aberrations in development and progression of prostate cancer⁶².

A second mechanism of sustaining cell growth during androgen deficient levels is aberrant phosphorylation of AR by a protein A signalling pathway, polypeptide growth factors and cellular regulators such as IGF-I, KGF and

EGF^{13,63,64,65,66}. Here, the AR is activated in the absence of a ligand. A third mechanism is the presence of mutations. Mutations have been found in primary untreated, local and metastatic hormone-refractory prostate cancer as well as in LNCaP cells. However, mutations seem to be more common in advanced and hormone-refractory prostate cancers^{67,68,69,70,71,72,73,74}. This point is still debated. Several reports indicate that point mutations in the hormone binding domain increase binding specificity of the AR for other ligands such as estradiol, progestagens, adrenal androgens and even antiandrogens^{68,69,75,76,77,78,79}. Therefore, the AR could even be activated or hyperactivated during androgen ablation therapy, where residual adrenal androgens, estrogens or antiandrogens could still activate AR and tumour growth. Antiandrogens, which are used to eliminate the effect of residual adrenal androgens in combined androgen ablation treatment, could themselves cause progression of tumour growth. This could be a reason why the antiandrogen withdrawal syndrome is observed⁷¹. During this syndrome, mutated cancer cells start to feed on antiandrogens rather than testosterone or DHT. At withdrawal of the antiandrogens, the mutated cells die. Recently, evidence of the possible role of mutations in antiandrogen withdrawal was obtained when a mutation in codon 887 of AR was associated with antiandrogen withdrawal⁷¹. Mutations in other domains of the AR include the DNA binding domain and a phosphorylation site, which caused a decrease in transactivation and dimerization, respectively^{80,81}. Lastly, coactivators of AR transactivation such as ARA₇₀, ARA₅₅ and retinoblastoma could stimulate transactivation in the absence of T and in the presence of E₂, of hydroxyflutamide, an antiandrogen and c-erbB2/HER2/Neu^{82,83,84,85}.

1.1.3.3 Estrogen receptors α and β

The mechanism of action of estrogens on prostate cells is still largely unclear and three proposals of estrogen binding have been investigated recently. These include binding to the prostate cellular AR in the presence of an AR agonist, ARA₇₀, or binding to a mutated AR that has increased affinity for estrogens and other ligands, as already discussed⁷². Secondly, E₂ may bind to a SHBG-RSHBG

complex on the cell membrane and activate the AR and PSA expression⁸⁶. The third possibility is the binding of estrogens to a genuine ER in prostate cells⁸⁷. Type I and type II binding sites of the ER are present in prostate tissues, as well as in human prostate cell lines, LNCaP, PC-3 and DU-145^{87,88,89,90,91,92,93}. However, results have mostly been conflicting since all prostate cancer patients are not ER positive^{88,94,95}.

In comparing normal, BPH and prostate tumour specimens, differences in results have been obtained. In some cases, ER in prostate tumours was either lower^{96,97} or higher than in BPH specimens^{14,98,99} or not significantly different¹⁰⁰. Reduced levels or no ER was present in metastases^{91,101}. Some ER positive prostate tumours are associated with a diploid DNA pattern, which may be useful in predicting prognosis¹⁰². If present, ER mostly occurs in the prostatic stroma, although basal and secretory epithelium cells may contain some ER as well^{89,103,104,105,106}. Androgen ablation, which includes treatment with LH-RH agonists and flutamide, as well as estrogens, upregulates ER expression in a large number of stromal cells. In contrast, ER expression in epithelium cells is only occasionally upregulated, but not in carcinoma cells¹⁰⁵. The morphological changes, such as regression in both stromal and epithelium cells, which are induced by estrogens can be explained by a possible paracrine interaction between stromal and epithelium cells or tumour cells. The upregulation of ER in stromal cells could increase the production of growth factors, which could affect the neighbouring epithelium cells¹³. Another example of ER upregulation is displayed in the Dunning (R3327H) rat model, which was originally developed from a spontaneously occurring prostatic adenocarcinoma in the male rat. Here, DES treatment caused an increase in nuclear ER, which consequently lead to the increase in the number of ER that could be used in transcription^{107,108}.

The role of the newly discovered ER isoform, ER β , needs to be investigated since ER β may play a role in defining epithelial heterogeneity in the prostatic duct system, which may be the key element in estrogen-mediated events in the prostate¹⁰⁹. The rat prostate was one of the first organs in which ER β was discovered¹⁰. The human prostate possesses much less ER β than the human

testis and a variant of ER β , ER β 1, is detectable in human PC-3, DU-145 and LNCaP cell lines^{11,110,111}. The function of ER β in the prostate is largely unclear, but it has been suggested that ER β could act as a marker of epithelial differentiation of the rat ventral prostate¹¹². This hypothesis is based on the fact that normal ER β expression is low at birth and increases as epithelial cells differentiate into luminal epithelial cells, and increase even more with functional differentiation¹¹². Expression of ER β may be related to androgen levels, since ER β mRNA levels decrease during castration and can be restored with subsequent testosterone replacement. Interesting to note is that estrogens do not regulate ER β expression^{112,113}. Although the impact of the discovery of ER β has yet to be established, it may partly explain the complexity of selective estrogen and antiestrogen action in various tissues¹⁴. Therefore, it may be beneficial to re-evaluate previous research on estrogenic compounds in the light of this novel ER β .

The ER β may play an important role in the antioxidant pathway¹¹⁴. The quinone reductase enzyme mediates reduction of toxic and mutagenic quinones. Expression of quinone reductase and other detoxification enzymes are regulated by the electrophile/antioxidant response element (EpRE), which can be activated by antiestrogen/ER complexes. Estrogen receptor β activates EpRE to a greater extent than ER α , which suggests that ER β may be important in activating chemoprotective detoxification enzymes¹¹⁴. Since ER β is present in the prostate, it is suggested that ER β may play a role in protecting the prostate from developing cancer¹¹⁵.

1.1.3.4 Treatment

Treatments of prostate cancer for the various stages include radiation or radical prostatectomy for stages A (local, microscopic) and B (local, detectable with digital rectal examination) and radiation with or without hormonal therapy for stage C (spread to nearby tissue and seminal vesicles) prostate cancer¹¹⁶. Through the years, the method of choice in treatment of advanced stage D

(distant metastases) prostate cancer has been hormonal treatment to diminish or deplete testicular and adrenal androgen concentrations in the body¹¹⁶. In this way, an attempt is made to prevent the activation of the AR. Circulating testicular androgens can be removed by bilateral orchiectomy or secretion of androgens can be prevented by the administration of the female hormone diethylstilbestrol (DES) or LH-RH agonists. These compounds cause the down-regulation of the production of testosterone by paradoxical suppression. However, long-term androgen ablation may increase AR expression causing progression of the disease¹¹⁷. The action of adrenal androgens in target tissues can be blocked by antiandrogens. Antiandrogens directly inhibit the activation of the AR by competing with and displacing androgens from AR occupancy, preventing the secretory epithelium cells from surviving¹¹⁸. A combination of surgical or medical castration with an antiandrogen has been proposed to increase the survival of patients with metastatic prostate cancer¹¹⁹. However, this may be a controversial issue since meta-analysis of available data by the USA Department of Health and Human Services suggests that there is no statistically significant difference in survival at 2 years between monotherapy and combined androgen blockade. Limited data suggested that there is a slight significant difference favouring combined therapy over monotherapy in the survival at 5 years, but more data are needed for a better representation of clinical significance¹²⁰.

Two kinds of antiandrogens, steroidal and nonsteroidal antiandrogens have been used in treatment of various illnesses caused by AR dysfunction¹²¹. Nonsteroidal antiandrogens like bicalutamide, hydroxyflutamide and nilutamide directly inhibit androgen receptor action. Unfortunately the cell registers this as an androgen shortage, which causes an increase in LH-RH, LH and testosterone production. This increase in testosterone could cause a displacement of the antiandrogen from the AR and is therefore called a selective antiandrogen. In contrast, steroidal antiandrogens like cyproterone acetate, megestrol acetate, medroxyprogesterone acetate and chlormadinone acetate, inhibit AR action both directly and indirectly. Progestational activities of these steroidal antiandrogens explain the indirect action by means of sequential downregulation of LH-RH, LH

and eventually androgens. These compounds are called non-selective antiandrogens. Despite the benefits of antiandrogen treatment, differences in the severity of side effects must also be considered in the choice of treatment.

In contrast to the tumour promoting effects of estrogens, they are also being used in treatment. Estrogens such as diethylstilbestrol (DES) and diethylstilbestrone diphosphate (DESdP) are being used in endocrine therapy in advanced or aggressive prostate cancer to induce regression of tumours^{118,122,123,124,125}. The mechanism of action of DES was believed to be ER independent and *via* a negative feedback inhibition on the pituitary-hypothalamus axis, which leads to lower LH and testosterone levels. Recently there has been evidence that DES could have a direct effect on prostate and prostate tumour cells *via* inhibition of cell proliferation, cell cycle arrest and apoptosis¹²⁵. Epithelium, as well as stromal cell growth, may be inhibited by administration of estradiol, even in combination with testosterone¹²⁶. Recently phytoestrogens, which are biologically active plant compounds with estrogenic and antiestrogenic properties, have been found to have antitumour effects *in vitro* as well as *in vivo*^{127,128,129}. At concentrations between 10^{-7} M and 10^{-5} M, the phytoestrogen genistein acts as an antiandrogen by blocking PSA production in human prostate cancer cells^{129,130}. Since natural compounds may exert antiestrogenic and antiandrogenic action, new research in this field is essential. This brings us to the importance of essential fatty acids and its role in cancer.

1.2. Essential Fatty Acids

1.2.1. Dietary sources

Dietary sources containing essential fatty acids (EFAs) include plants, which possess the appropriate enzymes ($\Delta 12$ -, and $\Delta 15$ -desaturases) capable of inserting double bonds at the n-6 ($\omega 6$) and n-3 ($\omega 3$) positions of fatty acids (See par. 1.2.3). Animals may also act as source when they have ingested EFAs themselves previously. Linoleic acid (LA) is found in sunflower, mealies and

cottonseed, γ -linolenic acid (GLA) in oats, barley, evening primrose oil and in some meats, while fish, seals and whales are rich in eicosapentaenoic acid (EPA) and α -linolenic acid (ALA)^{131,132}. Arachidonic acid (AA) is found in egg yolks, meats, fish oils and seafood. Oleic acid (OA), which is not essential, is present in olive oil.

From the parent EFAs, LA (n-6) and ALA (n-3), all the other derived EFAs can be synthesised in the human body by desaturation and elongation enzymes (Fig. 1. 7). Desaturation steps of EFAs are usually slow and rate limiting, while elongation steps are usually quick¹³³. This means that the formation of GLA and EPA are slow, while they are rapidly metabolised. The n-6 and n-3 EFAs are competitive inhibitors of each other's metabolism at the desaturation steps. The n-3 EFAs are more effective at inhibiting desaturation of n-6 EFAs than *vice versa*¹³⁴. The non-essential polyunsaturated fatty acid, OA (18:1n-9), also compete with the n-3 and n-6 EFAs desaturation, but to a lesser extent.

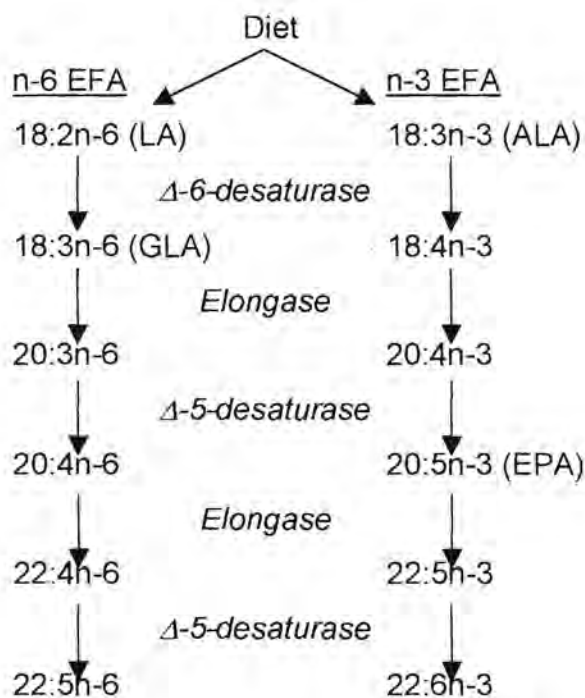


Fig. 1. 7 Metabolic pathways of the n-3 and n-6 essential fatty acids¹³³.

1.2.2 Definition

Currently the term EFA applies non-specifically to at least twenty-three interconvertible fatty acids that belong to the two related families of long-chain fatty acids, the n-6 and the n-3 poly-unsaturated fatty acids (PUFAs)¹³⁵. Strictly speaking, not all of these PUFAs are essential since humans can synthesize them from the two parent EFAs, as mentioned previously. Since the term EFA is broad and simple, it was proposed to reclassify the long-chain fatty acids according to more useful terms, which include dispensable, conditionally dispensable and conditionally indispensable FAs (Table 1. 1). The use of these terms is necessary because various conditions including age influence the capacity of the body to synthesize and conserve PUFAs as well as the rate of utilization or tissue incorporation of PUFAs. Since the conditional dispensability and indispensability of PUFAs in human prostate cancer are not clear, it was decided to use the term EFAs to describe the PUFAs used in this study.

Table 1. 1. Reclassification of some essential polyunsaturated fatty acids (PUFAs) as conditionally dispensable and conditionally indispensable at various stages during the life span*¹³⁵

Infancy, childhood, pregnancy and lactation		Adulthood (>20 years)	
Conditionally dispensable	Conditionally indispensable	Conditionally dispensable	Conditionally indispensable
Eicosapentaenoate [†]	Linoleate	Linoleate	α -Linolenate [‡]
	α -Linolenate	Arachidonate	
	Arachidonate	Eicosapentaenoate [†]	
	Docosahexanoate	Docosahexanoate	

* Since there is much information on the lack of dietary need for PUFAs during early development, a distinction is shown between infants and adults. Adolescence is omitted due to lack of sufficient data. Further research is needed to more fully identify the conditions (nutrient deficiencies such as Zn) and diseases (cystic fibroses, Zellweger syndrome) in which conditionally dispensable fatty acids become indispensable in the diet.

[†] Included with eicosapentaenoate are other intermediate PUFAs, i.e. γ -linolenate, dihomo- γ -linolenate, n-6 docosapentaenoate n-3 docosapentaenoate, and also C<18 and C>22 PUFAs.

[‡] α -Linolenate is listed as conditionally indispensable in adults due to its role in mitigating risk of chronic killer diseases, even though the specific mechanism is unknown.

1.2.3. Chemistry

Chemically an EFA consists of a terminal carboxyl group on a long hydrocarbon chain with more than one double bond. Nomenclature of all fatty acids can be derived from the number of carbons in the chain, the number of double bonds and the position of the double bond closest to the methyl terminus. For example, LA is designated as 18:2 n -6 (18:2 ω 6) meaning that there is 18 carbon atoms in the hydrocarbon chain, with two double bonds from which the double bond closest to the methyl terminal is 6 carbon atoms away (Fig. 1. 8). All unsaturated fatty acids synthesised by living organisms contain the *cis* stereoisomer of the double bond. *Trans* isomers are synthetically made and are widely used in the food industry since it is much more stable than the *cis* isomer. However, research has indicated that the *trans* isomers may be harmful to the consumer's health.

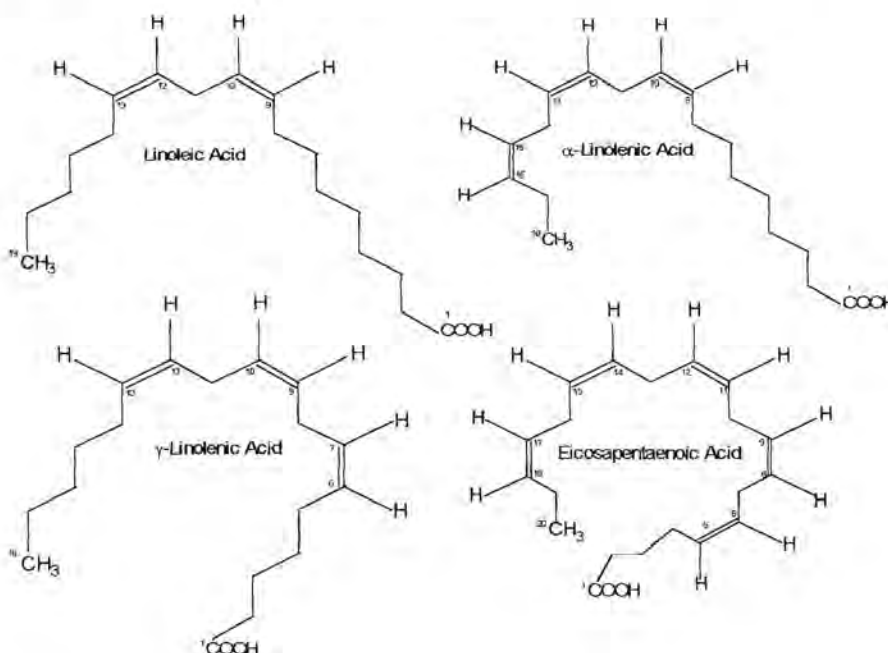


Fig. 1. 8 Molecular structures of linoleic acid, α -linolenic acid, γ -linolenic acid and eicosapentaenoic acid.

1.2.4. Function

The EFA is required for the structure of all membranes in the body (Fig. 1. 9)¹³⁶. Phospholipids, the basic structural component of biological membranes, usually contain EFAs on the second position and occasionally in both positions¹³⁷. Membranes consist of a phospholipids bilayer, which may contain EFAs either in one layer or in both layers¹³⁷. Physically the double bonds of EFAs confer a bend in the chain that increases fluidity compared to saturated fatty acids. This phenomenon is important in cellular membranes since it enables EFAs to influence membrane properties such as fluidity, flexibility and permeability. Other functions of EFA include the production of eicosanoids, prostaglandins, leukotrienes and thromboxanes, cholesterol transport, glucose and insulin responses, impermeability of the skin and normal brain function¹³⁴.

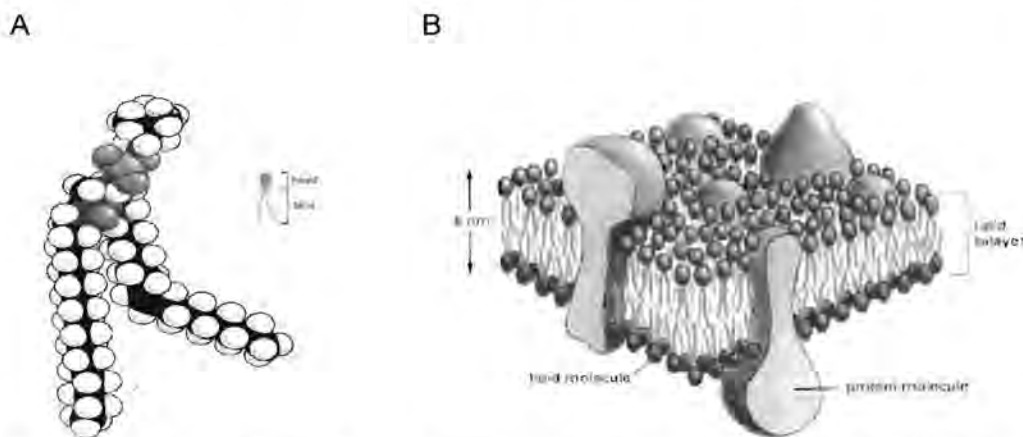


Fig. 1. 9. Fatty acids are part of membranes in the body: A: Phospholipid molecule have a hydrophobic tail, containing the fatty acids, and a hydrophilic head, which consist of phosphatidylcholine and glycerol. B: A cellular membrane consists of a phospholipid bilayer, which contains the membrane proteins¹³⁶.

1.2.5. Essential fatty acid effects on prostate cancer: human, animal and *in vitro*

1.2.5.1 Humans:

Over the years, several studies, some summarised in table 1.1 have been undertaken to investigate the correlation between dietary fats and prostate cancer. In humans, long-term intervention trials of the type used in experimental

animals are not practical. Therefore, other methods such as short-term trials or observational epidemiological research, which include ecological (correlation) studies, case-control (retrospective) studies and cohort (prospective) studies, are applied¹³⁸. Obviously, studies on relation between the intake of fatty acids are complex in design and in interpretation. Interviews and the completion of questionnaires for the assessment of dietary fat intake of people with and without prostate cancer rely heavily on reports of recalled diets in the preceding year or so before diagnosis. When prior knowledge of the hypothesis under investigation is unknown to the participants, this method is usually reliable and correlates with diet as measured by other techniques¹³⁹. These techniques include fatty acid measurement of plasma lipoproteins, cell membranes and adipose tissues that represent relative intake over short, medium and long-term periods respectively. Correlations between dietary intake of FAs and FA concentrations in both adipose tissue and plasma are greatest for PUFAs, intermediate for SFAs and lowest for MUFAs. The lower correlation of both SFAs and MUFAs with dietary FA intake can be explained by the fact that unlike PUFAs, these fatty acids are produced by the body itself¹³⁸.

According to epidemiological data, levels of *per capita* fat consumption correlate highly with prostate cancer incidence (Fig. 1. 10)¹⁴⁰. Epidemiological studies have shown that incidence and mortality rates of prostate cancer vary widely throughout the world and are strongly associated geographically with affluence and dietary factors associated with affluence¹⁴¹. For example, the United States has a 120-fold greater prostate cancer incidence than China¹⁴². Migrants from low-risk countries such as Japan and Poland to the USA have a large increase in prostate cancer risk, which increases even further over time^{143,144}. Therefore, it is clear that exogenous factors, which include diet, are important determinants of prostate cancer. However, a drawback of many early studies is the fact that fat intake was not separated into the different types of fats ingested.



Fig. 1. 10 Age adjusted death rate from prostate cancer compared to *per capita* dietary fat consumption in selected countries¹⁴⁰.

From Table 1. 2 it can be observed that both the total amount of fat and the type of fat ingested affect prostate cancer risk. Total, saturated and animal fat intake, generally increase prostate cancer risk, while PUFAs and the n-3 and n-6 EFA intake decrease or increase prostate cancer risk in different studies. It must be noted that most studies did not compare the combination and different ratios of n-3 and n-6 EFAs in the diet with prostate cancer risk. Combination of different ratio's of n-3 and n-6 EFAs is important in maintaining normal health and may play a role in prostate cancer^{145,146}.

Table 1. 2. Human studies on dietary fat intake and prostate cancer risk.

Type of fat, Study	Population	Results	
		Significance	Association
Total dietary intake			
Whittemore <i>et al</i> , 1995 ¹⁴⁷	1655 case-subjects (531 blacks, 515 whites, 2283 Chinese-Americans, 326 Japanese-Americans), 1645 age- and race-matched control subjects, USA and Canada	+	Total fat and advanced prostate cancer risk
Gann <i>et al</i> , 1994 ¹⁴⁸	51 529 US men, 300 new cases, 126 advanced cases out of 47 855 initial participants, age- and energy-adjusted	+	Advanced prostate cancer risk
Giovannucci <i>et al</i> , 1993 ¹⁴⁹	Health Professionals follow-up, 51 529 US men, 300 patients including 126 advanced cases	+	Advanced prostate cancer incidence
Walker <i>et al</i> 1992 ¹⁵⁰	166 black case subjects and age- and race-matched control subjects in South Africa	+	Prostate cancer risk
West <i>et al</i> , 1991 ¹⁵¹	358 case subjects, 679 age-matched population	+	Prostate cancer risk

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	control subjects, Utah		
Animal fat intake			
Gann <i>et al</i> , 1994 ¹⁴⁸	51 529 US men, 300 new cases, 126 advanced cases out of 47 855 initial participants, age- and energy-adjusted	+	Advanced prostate cancer
Giovannucci <i>et al</i> , 1993 ¹⁴⁹	Health Professionals follow-up, 51 529 US men, 300 patients including 126 advanced cases	+	Advanced prostate cancer intake
Walker <i>et al</i> , 1992 ¹⁵⁰	166 black case subjects and age- and race-matched control subjects in South Africa	+	Prostate cancer risk
Saturated fat intake			
Gann <i>et al</i> , 1994 ¹⁴⁸	51 529 US men, 300 new cases, 126 advanced cases from 47 855 initial participants, age- and energy-adjusted	+	Advanced prostate cancer risk
Giovannucci <i>et al</i> , 1993 ¹⁴⁹	Health Professionals follow-up, 51 529 US men, 300 patients including 126 advanced cases	+	Advanced prostate cancer
West <i>et al</i> , 1991 ¹⁵¹	358 case subjects, 679 age-matched population control subjects, Utah	+	Aggressive prostate cancer in the elderly
Monounsaturated fat intake			
Schuurman <i>et al</i> , 1999 ¹⁵²	58 279 men, 642 cases, Netherlands	+	OA and prostate cancer risk
Giovannucci <i>et al</i> , 1993 ¹⁴⁹	Health Professionals follow-up, 51 529 US men, 300 patients including 126 advanced cases	+	Advanced prostate cancer
Polyunsaturated fat intake			
Terry <i>et al</i> ¹⁵³	6272 Swedish men in prospective cohort	-	Fatty fish consumption and prostate cancer risk
Norrish <i>et al</i> , 1999 ¹⁵⁴	317 cases, 480 age-matched community controls, New Zealand	-	Prostate cancer risk reduced with dietary fish oils, EPA & DHA
Schuurman <i>et al</i> , 1999 ¹⁵²	58 279 men, 642 cases, Netherlands	-	LA and linolenic acid
Harvei <i>et al</i> , 1997 ¹⁵⁵	141 matched set of blood donors with and without prostate cancer, Norway	+	ALA and prostate cancer risk
Bakker <i>et al</i> , 1997 ¹⁵⁶	8 European countries and Israel	0	n-6 EFA and prostate cancer incidence
Gann <i>et al</i> , 1994 ¹⁴⁸	51 529 US men, 300 new cases, 126 advanced cases out of 47 855 initial participants	-	Prostate cancer risk
Giovannucci <i>et al</i> , 1993 ¹⁴⁹	Health Professionals follow-up, 51 529 US men, 300 patients including 126 advanced cases	+	ALA but not LA associated with advanced prostate cancer
West <i>et al</i> , 1991 ¹⁵¹	358 case subjects, 679 age-matched population control subjects, Utah	+	Prostate cancer risk
Kaul <i>et al</i> , 1987 ¹⁵⁷	55 black cases, 55 age- and race-matched hospital controls, Washington DC	-	LA intake and prostate cancer risk
Mishina <i>et al</i> , 1985 ¹⁵⁸	100 cases, 100 healthy age-matched controls	-	Seafood intake and prostate cancer risk
Trans fatty acids			
Bakker <i>et al</i> , 1997 ¹⁵⁶	8 European countries and Israel	+	Monounsaturated trans fatty acids

+ Positive association; - Negative association; 0 No association; *Non significant association

1.2.5.2 Animals:

Studies on the effect of dietary fat and individual fatty acids on prostatic carcinogenesis and prostate cancer progression have been hampered by the lack of a universally accepted animal model for human prostate cancer. In contrast to the frequent occurrence of prostate cancer in humans, it is much less common and rarely arises spontaneously in other mammals, especially in laboratory rodents¹⁵⁹. The dog is one of the few animals known to develop high grade PIN and adenocarcinomas spontaneously¹⁶⁰. Several similarities between human and canine prostate cancer make it a favourable model to study human disease. These similarities include the late age of onset, metastatic propensity, probable androgen independence of advanced disease and the heterogeneity of tumours. However, the high cost of maintaining dogs, the apparent low incidence of spontaneous disease and the inability to manipulate dogs genetically, limits its use as a human prostate cancer model¹⁶¹. Although little or no studies of EFAs on canine prostate tumours has been done, a combination of LA, ALA and vitamin E have been beneficial in reduction of multicentric lymphomas in dogs¹⁶². However, *in vitro* canine prostate cancer cell line CPA did not show significant inhibition by n-6 EFAs alone¹⁶³.

The rat is another animal in which spontaneous prostate adenocarcinomas can arise, but only after a long latency period and only in some strains. In addition, prostate cancer can be induced in rats by chemical carcinogens or stimulation by androgens or estrogens^{164,165}. Disadvantages of this model are great variations in reproducibility and reliability. Therefore, this model has not been used in testing the therapeutic effect of EFAs on prostate cancer. However, sublines of the Dunning R3327 rat model are of the most valuable rat models to study prostate cancer progression and metastasis. Future studies with EFAs on rat prostate cancer may prove to be useful^{159,166}.

Mice have limited success as models for human prostate cancer and several differences between human and murine prostates are responsible¹⁶¹. Apart from anatomical differences, the murine prostate atrophies with age, while the human

prostate hypertrophies. Furthermore, no spontaneous carcinomas have been reported in mice prostates indicating that the short life span of mice may reduce the effects of cumulative mutations that lead to cancer. Xenograft models have been established by inoculating human prostate cancer cell lines into severe combined immune-deficient or nude mice. Although the tumour cells and target organs are not from the same species and initiation of carcinogenesis is precluded, this model is valuable in studying the preventative and therapeutic use of compounds such as EFAs on prostate tumours. Several transgenic or genetically manipulated mouse and organ models have been established to cause spontaneous tumours in mice. Most investigators targeted SV40 T antigens to the mouse prostate where these oncoproteins functionally inactivate the tumour suppressor proteins p53 and Rb^{161,167}.

Most EFA studies on animals have been done on mice xenograft models. An elevation in the total intake of dietary fat increases the growth rate of human prostatic adenocarcinoma and PSA production in nude mice injected with LNCaP cells¹⁴². This is in keeping with total dietary fat intake in humans (Table 1. 2). However, a large gap in data still exists between animal and human studies, since the type of fat ingested may cause different responses in these two species¹⁶⁸. For example, an increase in dietary LA or n-6 EFA causes an increase in mice prostate tumour growth, while LA is negatively associated with some studies concerning human prostate cancer incidence¹⁴². In contrast, the long chain n-3 EFAs, which excludes ALA, decreased tumour growth in athymic nude mice injected with DU-145 cells^{169,170}. In men n-3 may increase or decrease prostatic tumour incidence (Table 1. 2). Since existing evidence does not explain the differences in mouse and human prostate cancer, more studies are needed. It is of medical importance to note that conjugated LA, but not LA itself, causes a drastic reduction in tumour growth and metastasis in SCID mice inoculated with DU-145 cells¹⁷¹.

1.2.5.3 *In vitro*:

The Wolmarans Laboratory, Department of Urology, found that EFAs and their derivatives, not only inhibit growth of the DU-145 prostate cancer cell line but also influence the expression of proteins in these cells¹⁷². At low EFA concentrations (<100 μ M) attainable through diet, it appears that EFAs have a proliferating effect on several prostate cancer cells such as PC-3, LNCaP and PNT2¹³¹. However, DU-145 cells do not show a significant increase in proliferation at low concentrations of EFAs¹⁷². At higher concentrations (200 – 400 μ M) that can only be attained by pharmacological doses, EFAs have an inhibitory effect on prostate cancer cells. At 200 μ M, DU-145 cells are killed¹⁷². It is interesting to note that when DU-145 cells are treated with a combination of EFAs, the effect is reduced¹⁴⁶.

1.2.6. Possible mechanisms of EFA action in cancer

Possible mechanisms of EFAs action are based on biological properties and functions of fatty acids, which include the following:

1.2.6.1 Eicosanoid regulation

Evidence exists that the eicosanoids, prostaglandins and leukotrienes can affect cell proliferation, immune response, tumour cell invasion and metastases of tumours¹⁷³. The prostaglandins and thromboxanes are derived from the n-6 EFAs AA *via* the cyclooxygenase (COX) enzyme and the leukotrienes *via* the lipoxygenase enzyme (Fig. 1. 11). Growth of both the prostate cancer cell lines DU-145 and PC-3 *in vitro* are inhibited by the cyclooxygenase and lipoxygenase inhibitors piroxicam and esculetin, respectively¹³¹. Furthermore, nonsteroidal anti-inflammatory drugs (NSAID) may be a novel target for cancer chemotherapy, since NSAID are known to inhibit both COX-1 and COX-2¹⁷⁴. Certain EFAs display similar functions to these drugs, since EPA and DHA inhibit prostaglandin synthesis by competitive inhibition of cyclooxygenase and inhibition of AA

metabolism, respectively. Therefore, these observations may provide a possible explanation for the tumour inhibiting effect of dietary fish oils.

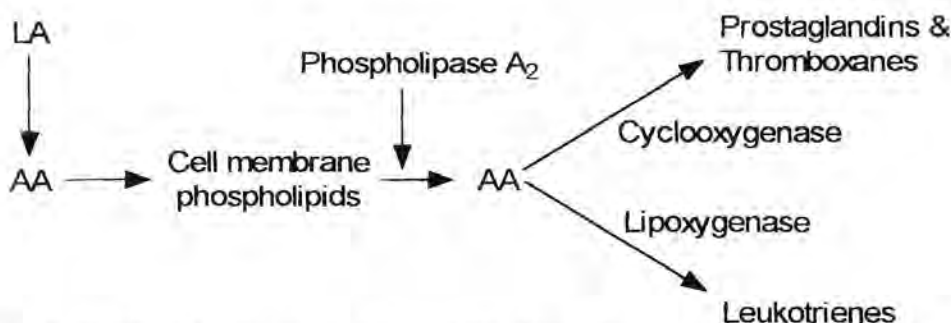


Fig. 1. 11 Production of eicosanoids from essential fatty acids.

1.2.6.2 Lipid peroxidation and free radical formation

Due to the presence of carbon double bonds, unsaturated fatty acids are very susceptible to lipid peroxidation. This process involves a cascade of chemical reactions, which produce reactive radicals such as superoxide, hydroxyl radical and a complex range of peroxydised and oxygenated lipids (Fig. 1. 12)¹⁷⁵. When a cell is unable to neutralise and control the reactive radicals, DNA damage occurs, which may initiate cancer¹⁷⁶. However, lipid peroxidation is reduced in dividing cells and therefore the reactive radicals may play a physiological role in the regulation of cell proliferation during progression of cancer. In rapid proliferating tissues, like aggressive malignant tumours, lipid peroxidation occurs at much lower levels than in slow proliferating tissues (Fig. 1. 12)¹⁷⁵. Mainly, two possible reasons exist for limited lipid peroxidation in malignant cells. Firstly, the presence of enhanced levels of antioxidants in tumour cells, such as OA and vitamin E, neutralize lipid peroxidation products¹⁷⁵. Secondly, tumour cells contain lower levels of the EFA metabolites, which act as substrate for lipid peroxidation¹⁷⁵. The reduction of EFA metabolites is probably caused by a deficiency in the 6-desaturation enzymes in malignant cells. Therefore, it is possible that the treatment of malignant cells with EFAs will increase the substrate for lipid peroxidation and in this way reduces cell proliferation. Furthermore, EFAs may be used in combination with lymphokines,

interferon, radiation and chemotherapeutic drugs to destroy malignant cells by the action of lipid peroxidation¹⁷⁵. Tumour cells may show resistance to the cytotoxic action of anticancer drugs alone *inter alia* by increased expression of antioxidant enzymes^{177,178}. Since ALA and EPA could reduce the amount of superoxide dismutase, catalase and glutathione, these EFAs may be used in treatment to increase lipid peroxidation and decrease cell proliferation¹⁷⁹.

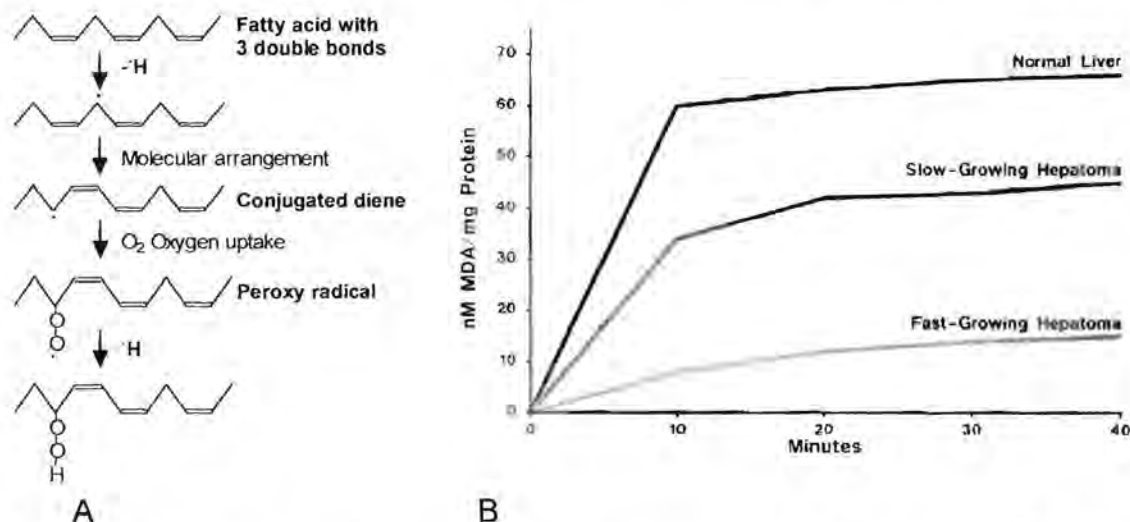


Fig. 1. 12 Lipid peroxidation. A: The initial reactions in the process of lipid peroxidation¹⁷⁵. B: The rate of liver peroxidation as indicated by MDA formation is highest in normal liver, lowest in a fast-growing hepatoma and intermediate in a slow growing hepatoma¹⁷⁵

1.2.6.3 Cell membrane integrity

Cell membranes are essential to the life of the cell, since membranes enclose cells and organelles, define their boundaries, maintain the differences between the intracellular and the extracellular environment and play an important role in cell communication. Changes in the dietary intake of fatty acids readily alter the fatty acid composition and structure of cell membranes. Alteration of the physical properties of the plasma membrane by EFAs may alter the environment of membrane proteins such as receptors, enzymes and transport proteins. In this way, EFAs could modulate their function and alter the cellular response to external stimuli such as growth factors and hormones. Treatment of malignant tumours with an EFA may alter membrane properties in such a way that when

combined with a cytotoxic drug, increased sensitivity to various cytotoxic drugs may be obtained¹⁸⁰.

1.2.6.4 Invasion and metastasis

In the process of invasion, tumour cells have to attach themselves to the target tissue and then invade or penetrate the matrix by proteolysis of the matrix components, before they can migrate through the basement membrane¹⁸¹. Therefore, proteolytic enzymes, such as urokinase-type plasminogen activator (uPA), play an important role in successful tumour metastasis. The EPA, GLA, decreases production of uPA in DU-145 cells and could therefore reduce prostate tumour metastasis¹⁷². In mammary tumours, EPA reduces metalloproteinase-9 expression and concurrently reduces formation of metastasis¹⁸².

1.2.6.5 Modulation of hormone messages

Modulation of a receptor/ligand system by various modulator compounds is critical to the outcome of the message transferred to the cell. A modulator substance acts at a precise location for a very short time in a reversible manner to modify the properties of a signal¹⁸³. Generally, the characteristics of modulators and messengers can be applied to fatty acids. Fatty acids can be delivered to the cell from extracellular sources and act directly on the cell membrane as first messengers or they are incorporated into membrane phospholipids from which they can be liberated by cellular phospholipases in response to other signals. Once liberated, the small pool of nonesterified fatty acids can propagate the primary message by serving as second messengers or they can modulate signals coming from other pathways, such as the binding of steroid hormones to their intracellular receptors. The steroid hormone and peptide factor signalling pathways are influenced by fatty acids obtained from nutrition or released from membranes in response to phenomena such as stress. Since these pathways lead to multiplication and differentiation, fatty acids play an important role in maintenance of normal health and development (Fig. 1. 13).

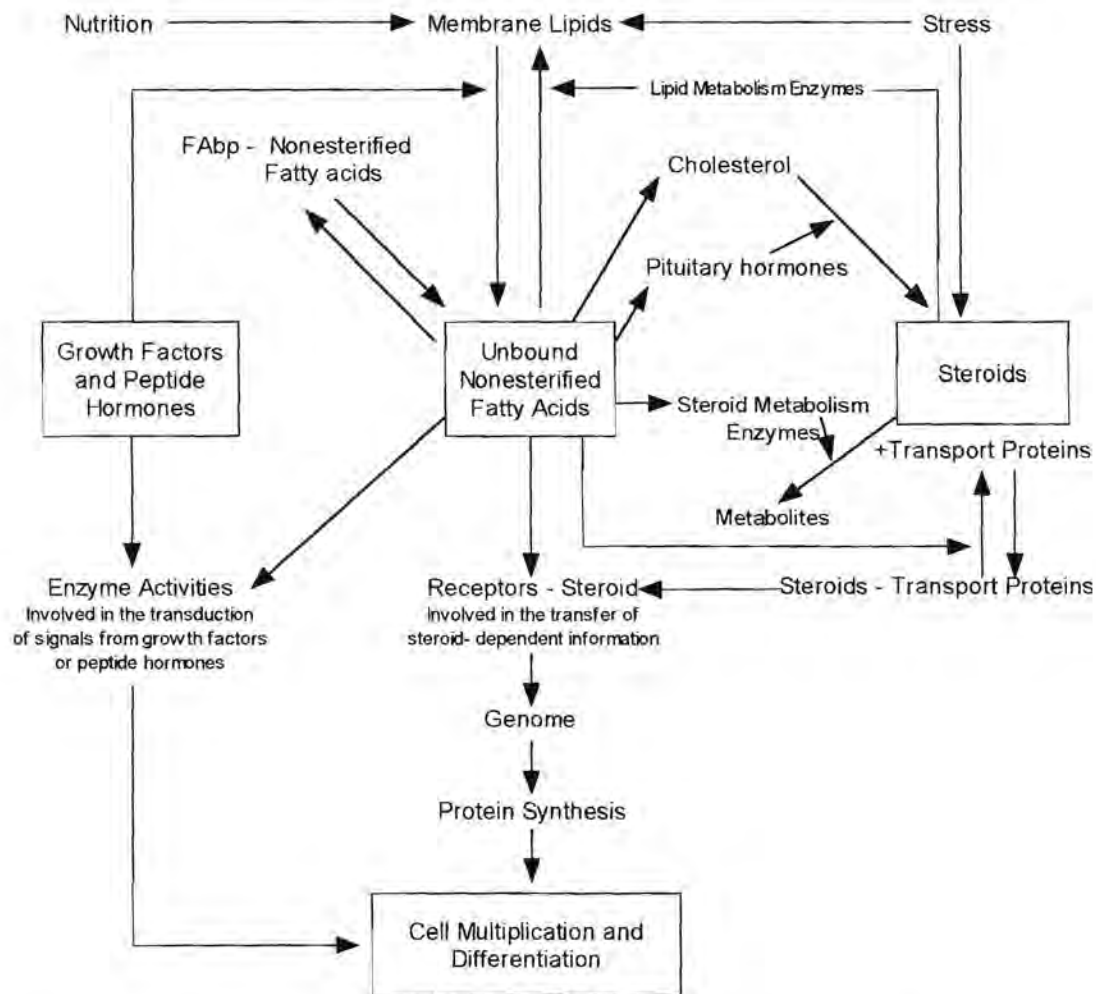


Fig. 1. 13 Overview of the role of fatty acids on the intertwined steroid and peptide pathways involved in cell multiplication and differentiation¹⁸³.

1.2.6.6 Membrane signal transduction

The modulatory effect of various fatty acids and especially of AA on enzymes and proteins involved in membrane signal transduction has been studied¹⁸⁴. These enzymes include phospholipase A₂, phospholipase C, phospholipase D, adenylate cyclase, guanylate cyclase, G protein, protein kinase A and protein kinase C. In prostate cancer, protein kinase C (PKC) plays an important role in cell regulation and differentiation¹⁸⁵. Activation of this enzyme increases prostate cancer cell proliferation. Activators of PKC include LA and low levels of AA. However, high concentrations of AA inhibit PKC activity and could therefore reduce prostate tumour growth.

1.2.6.7 Transport proteins

Fatty acids regulate ion fluxes and Ca^{2+} mobilization directly by modulating ion channels, the Ca^{2+} pump and the Na-K-ATPase (sodium pump)¹⁸⁴.

1.2.6.8 Steroid hormone message

The EFAs may influence the steroid hormone message during synthesis and metabolism of steroid hormones. At concentrations less than 10 μ M, EFAs inhibit the 5α -reductase enzyme of human prostate cancer cells in culture in the following order: GLA > ALA > LA > OA¹⁸⁶. Furthermore, EFAs may affect the steroid hormone message during transport by plasma proteins to the target cells, transmembrane and intracellular transport, binding to cytoplasmic or nuclear receptors, DNA binding of the steroid receptor complex and transcriptional activity (Fig. 1. 14)^{183,187,188,189,190,191}.

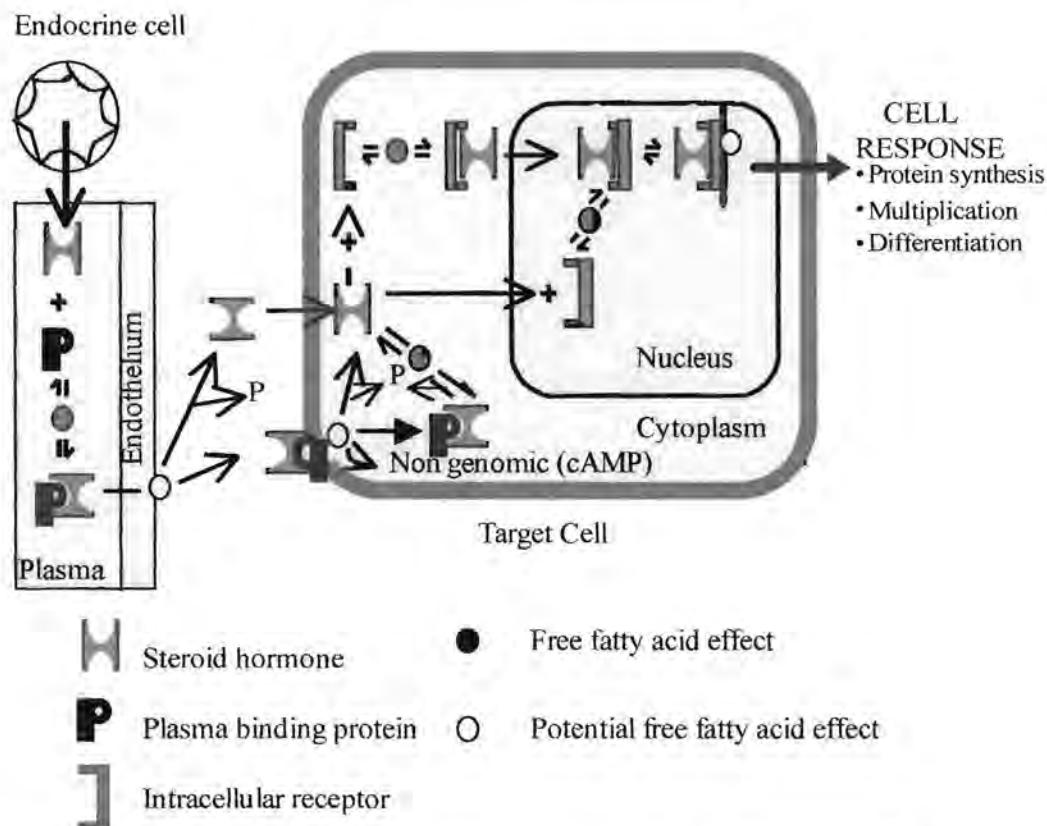


Fig. 1. 14 Free fatty acid effect on the steroid hormone message (P Phosphate)¹⁸³

Several researchers reported that EFAs affect the binding of the family of steroid hormones to their respective steroid hormone receptors (Table 1. 3). Most studies were done on AA in normal rat tissue. For example in the normal rat prostate, AA caused non-competitive inhibition of the AR. Since functional activity of steroid receptors are important in development, progression and treatment of prostate cancer, the influence of EFAs on the steroid hormone message in prostate tumours needs to be investigated for a possible beneficial role.

Table 1. 3 Modulation of steroid binding to steroid receptors by unsaturated free fatty acids¹⁸³

Receptor	Tissue	Species	Fatty Acid	Effect	Reference
Estrogen	Uterus	Rat	C22:6	Competitive Inhibition	191
			C22:6	Potentiation	191
	Uterus	Human	C20:4	Inhibition	192
			C20:4	Potentiation	192
	Uterus	Rat	C20:4	Non-competitive Inhibition	193
Antiandrogen binding sites	MCF-7 cells	Human	C22:6	Non-competitive Inhibition	183
	Liver	Rat	C20:4	Non-competitive Inhibition	194
Androgen	Prostate	Rat	C20:4	Non-competitive Inhibition	195
Progesterin	Uterus	Rat	C20:4	Non-competitive Inhibition	193
	Uterus	Human	C20:4	Inhibition	183
	Brain	Rat	C20:4	Non-competitive Inhibition	193
Glucocorticoid	Liver	Rat	C20:4	Competitive Inhibition	196
	Liver	Rat	C20:4	Mixed Non-competitive Inhibition	197
	Brain	Rat	C20:4	Non-competitive Inhibition	193

1.3. Hypothesis

Androgens play an active role in the development and progression of prostate cancer and androgen ablation therapy is used in the treatment of prostate cancer. Furthermore, estrogens are implicated in carcinogenesis even from the time in the womb, but are also used in treatment. Since binding to ER and AR exerts the action of estrogens and androgens respectively, the

modulation of binding is an important regulatory factor in prostate cancer. Essential fatty acids have been implicated in the modulation of steroid hormone binding to steroid hormone receptors in normal tissues. **Since EFAs inhibit prostate cancer cell growth, the hypothesis is that EFAs will modulate androgen and estrogen receptors by decreasing their binding affinity and capacity.** Furthermore, EFAs have been implicated in the reduction of the expression of various proteins including the ER protein in MCF-7 breast cancer cells¹⁹⁸. **Therefore it can be hypothesized that EFAs would decrease AR and ER mRNA and protein expression in the DU-145 prostate cancer cell line.**

1.4. Aims of the Study

1. To optimize conditions such as optimum incubation time and concentration of cold ligand for AR binding studies in DU-145 cells. Estrogen receptor and androgen receptor binding studies will be executed according to the Scatchard binding assay method applying standard working procedure as already practised in the Wolmarans laboratory.
2. To determine the optimal concentration of OA, ALA, EPA, LA and GLA with the largest effect on specific binding of testosterone and estradiol respectively to both AR and ER in DU-145 cells.
3. The results obtained in point 2 will be used in determining the effect of the EFAs, OA, ALA, EPA, LA and GLA, on the binding affinity and capacity of estradiol and testosterone respectively to both the AR and ER in DU-145 cells.
4. To determine the effect of EFAs on ER mRNA synthesis in DU-145 cells. Unfortunately a probe for AR was not commercially available at the time of this study and therefore the hypothesis could not be tested regarding AR mRNA.
5. To determine the effect of EFAs on AR and ER expression in DU-145 cells.

CHAPTER 2

2. KINETIC MODULATION OF ANDROGEN AND ESTROGEN RECEPTORS IN THE PRESENCE OF SINGLE ESSENTIAL FATTY ACIDS.

2.1. Introduction

Prostate cancer is a scary disease and made even scarier by the inadequate knowledge on the development and progression of this malignancy. Furthermore, some prostatic tumours grow slowly and silently and in some cases, malignancy is detected only when metastasis had already occurred, thereby complicating treatment. Fortunately, during recent years, there has been a stage shift in the diagnosis of prostate cancer and many cases are detected as soon as stage B (confined) or early stage C¹⁹⁹. Several intrinsic and extrinsic risk factors such as age, race, hereditary and environmental factors as well as diet have been implicated in the development and progression of prostate cancer¹. Extrinsic factors including diet can easily be manipulated to promote both prostate cancer prevention and treatment. Nutrients, such as saturated animal fat, red meats, eggs and dairy products, are associated with increased risk, while lycopene in tomatoes and tomato sauce, vitamin A, vitamin E, vitamin D, soy and certain essential fatty acids seem to have a protective effect^{2,200-209}. The EFAs and their metabolites may not only play a role in the prevention of prostate cancer, but also in the reduction of tumour growth, since several groups have reported the inhibitory effects of certain EFAs on malignant growth^{175,210,211,212,213}.

In addition to prostate and breast cancer, several other hormone-related disorders such as premenstrual syndrome and osteoporosis respond well to treatment with EFAs in preliminary studies^{214,215}. Therefore, it can be anticipated that EFAs do have an important effect on the endocrine system regarding the

steroid hormone message. However, the mechanism of this action has not been elucidated fully and further investigation is necessary to understand the role of EFAs in the prevention and treatment of hormone related disorders, especially breast and prostate cancer. Therefore, the endeavour of this study was to illuminate a possible mechanism of EFA action on estrogen and androgen receptors in prostate cancer.

In the Wolmarans laboratory several *in vitro* studies on the effect of both single and combined EFAs on DU-145 cells had been done and the presence of at least ER in whole DU-145 cells has been proved^{93,146, 172}. Therefore, it has been decided to continue investigating estrogen and androgen binding on whole human prostate DU-145 cells in monolayer. However, the conflicting results from the literature regarding the presence of androgen receptors (AR) and estrogen receptors (ER) in prostate tissue and in prostate DU-145 cell culture were taken into consideration^{13,58,71,93,98,101,104,216,217,218,219}. Some authors concluded that steroid receptors are absent in DU-145 cells, while other disagree^{71,93,220}. Since lysis of the cell may disrupt the receptor structure and renders it unable to bind ligand, whole DU-145 cells were used in this study⁹³. Nevertheless, without getting mixed up in a lively discussion on the presence of steroid receptors in the prostate tissue or cell cultures, our attention was further drawn to the importance of dietary fat on prostate and breast cancer²²¹. Since the widely used hormonal therapy attenuates or eliminates the hormone message in prostate and breast cancer, the question arose if polyunsaturated fatty acids (PUFAs) would affect ligand binding on whole prostate cancer cells in culture?

Though the cause of cancer is still unknown, some authors state that EFAs could play a significant role in the recovery of this disease^{132,145}. Therefore, the essential fatty acids linoleic (LA) and α -linolenic acid (ALA), the polyunsaturated γ -linolenic acid (GLA) and the super unsaturated eicosapentaenoic acid (EPA) were employed. Additionally, OA, a monounsaturated fatty acid, was used to represent the non-essential unsaturated fatty acids. These fatty acids were selected because elevated levels of OA are usually found in malignant tissues in contrast to the tumouricidal, lower levels of LA, GLA, ALA and EPA^{175,210,211,212}.

2.2. Materials And Methods

2.2.1. Materials

All reagents were of analytical grade. Ham's nutrient F-10 mixture was obtained from Flow Laboratories (Irvine, UK), while fetal calf serum was from Delta Bioproducts (Kempton Park, SA). BioWhitaker supplied trypsin (Walkerville, MD, USA). Bovine albumin and the unsaturated fatty acids: linoleic acid (LA), α -linolenic acid (ALA), γ -linolenic acid (GLA), arachidonic acid (AA), eicosopentaenoic acid (EPA) and oleic acid (OA) were obtained from Sigma-Aldrich (Atlasville, SA) as well as cold testosterone and DES. The [2,4,6,7- $^3\text{H}(\text{N})$]Estradiol-17 β (92 Ci/mmol) and [1,2,6,7- $^3\text{H}(\text{N})$]Testosterone (95 Ci/mmol) were obtained from DuPont NEN (Separation Scientific, SA. Boston, MA). The Bio-Rad Protein Assay kit was ordered from Bio-Rad Laboratories, Richmond, CA.

2.2.2. Cell culture

Human DU-145 prostate tumour cells were obtained from Sterilab (Johannesburg, SA) and maintained in Corning polystyrene flasks (Corning Glass Works, Corning, NY, USA) at 37°C, 5% CO₂ in Ham's F-10 nutrient mixture supplemented with 5% decomplimented fetal calf serum, gentamycin sulphate (0.004%), glucose (0.57%) and NaHCO₃ (0.12%). The growth medium was filtered through a Millipore Sterivex-GS filter (0.22 μm) fitted with an In-Line Prefilter (Millipore Filters AP 15 and 20) and stored at 4°C. The medium was changed every 2-3 days. Cells were harvested or passed by trypsinization. Immediately after trypsinization, F-10 medium with serum was added to the cell suspension and, following centrifugation (1000 x g for 5 min at room temperature), the cells were resuspended in F-10 medium with serum.

2.2.3. Specific binding studies

Whole cell E₂ and T uptake was done according to Turcotte *et al*²²² and Viljoen *et al*⁹³. The DU-145 cells (8 x 10⁵) were inoculated into 25 cm² Corning polystyrene culture flasks and 5 ml F-10 medium containing serum was added. After plating for 24 hours at 37°C, 5% CO₂ the medium was aspirated and changed on the second day. The cells were then incubated at 37°C for another 24 hours in fresh medium with serum. In order to increase binding capacity for [³H]-E₂ and [³H]-T the medium was changed to serum free conditions on the third day. After another 24 hours incubation under serum free conditions, on the fourth day, the cells were washed twice with cold saline solution (SS) consisting of 0.154 M NaCl supplemented with 1.2 mM CaCl₂ x 2H₂O, 0.5 mM MgCl₂ x 6H₂O, and 0.4 mM MgSO₄ x 7H₂O. Serum-free medium (2 ml) was added to the cells as well as radioactive steroids. The concentrations used for [³H]-E₂ and [³H]-T were 0.8 nM and 12 nM, respectively, as determined in separate assays (Fig. 2. 3)⁹³. The non-specific bindings were eliminated with a 200- and 400-fold excess of unlabelled DES and T. Next, the EFAs were added in duplicate at different concentrations (0 - 200 μM) to the monolayer of cells. The final ethanol concentration in the experimental medium without serum was 0.2%. Since the maximum specific binding for E₂ and T was time-dependent, equilibrium was reached after 45 minutes for E₂ and 2 hours for T at 37°C. Therefore, the E₂ and T containing flasks were respectively incubated for 45 minutes and 2 h, respectively. Afterwards, the cells were solubilized by the addition of 1.0 ml of 0.3 N NaOH-EtOH (4:1; v/v) solution. The solubilized cell mixtures (500 μl) were transferred to scintillation vials, and 4 ml of Ready Protein+ cocktail added. Radioactivity was determined in a Beckman LS 5000 CE liquid scintillator (Beckman Instruments, Palo Alto, CA).

2.2.4. Steroid binding capacity of whole cells

Titration of E₂ and T binding sites were primarily done as described under the previous 'Specific binding studies' (par. 2.2.3). The ER and AR levels were

measured in intact DU-145 cells (8×10^5), which were inoculated in 25 cm² Corning flasks containing 5 ml F-10 medium with serum. On the fourth day, after the cells had been washed twice with SS, they were incubated at 37°C in 2 ml serum-free culture medium in the presence of [³H] E₂ or [³H]-T with or without a 200-fold or 400-fold excess of DES or T. Once more, the incubation times for the E₂ and T binding assays were 45 minutes and 2 h, respectively, in the presence or absence (control) of 10 μM single FA. As explained before, the final ethanol concentration in the experimental medium without serum was 0.2%. After incubation the medium was removed, and the monolayers were washed 5 times with cold SS in order to remove free steroids. The cells were solubilized by the addition of 1.0 ml of 0.3 N NaOH-EtOH (4:1; v/v) solution. The solubilized cell mixtures (500 μl) were transferred to scintillation vials and 4 ml Ready Protein⁺ cocktail were added. Radioactivity of the solubilized cell mixture was determined in the Beckman LS 5000 CE liquid scintillator. These experiments for both steroids were repeated 3 times in duplicate. The [³H]-E₂ and [³H]-T levels for the individual multipoint titrations ranged between 0.15 - 2.0 nM and 6 - 16 nM, respectively. Protein concentration of the solubilized cells was determined by means of the Bio-Rad Protein Assay kit, with bovine albumin as standard. Absorbance was measured in a Hitachi 150-20 spectrophotometer (Tokyo, Japan) at 595 nm. Results were analyzed according to the Scatchard analysis²²³.

2.2.5. Statistical analysis

Statistical analysis was performed on the GraphPad Prism Version 3.0 computer program. The control and the different fatty acids were compared with respect to the K_d and B_{max} values. The student's t-test was employed to compute the significance of comparisons between the respective fatty acids and control with respect to the response variables.

2.3. Results

2.3.1. Optimization of [³H]-T binding

Since steroids easily attach themselves to cellular components other than their receptors, it is necessary to distinguish between specific binding and non-specific binding in a radioactive assay. This is accomplished by reading radioactivity in the presence and absence of an excess amount of unlabelled steroid. By subtracting the non-specific counts from the total counts, the specific binding of a labelled steroid to its receptor can be obtained. Therefore, an experiment was conducted to determine the optimum amount of unlabelled steroid to be used for [³H]-T binding in whole DU-145 cells. From Fig. 2. 1 it can be concluded that maximum specific binding was reached in the presence of 400 times unlabelled T after 2 hours at 37°C. For [³H]-E binding, the optimum amount of unlabelled DES was taken from previously recorded data (Viljoen), which was 200 times⁹³.

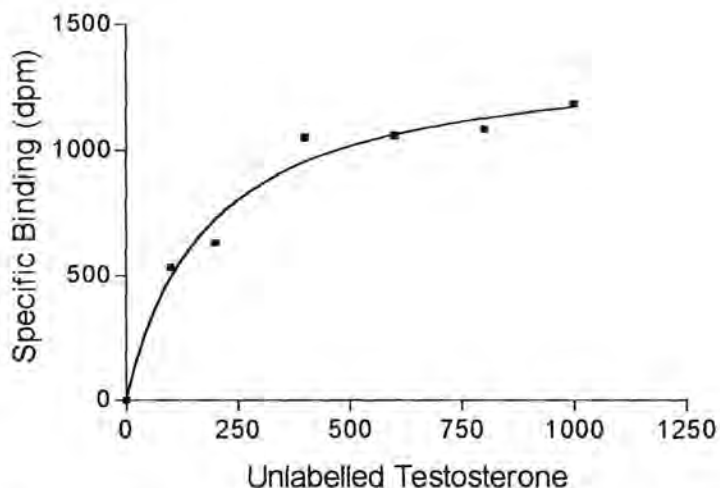


Fig. 2. 1 Specific binding of [³H]-Testosterone (4 nM) with excess unlabelled testosterone (100 to 1000 times).

All cells readily take up steroid hormones, but only target cells with receptors, retain these hormones. Uptake is defined as the initial rate of movement by steroids into the cells, while retention is defined as the amount of steroid found in the cells under equilibrium conditions. The Scatchard analysis is performed at equilibrium conditions and therefore an experiment was conducted to establish the optimum incubation time for [³H]-T to reach maximum specific binding in intact whole DU-145 cells. Results in Fig. 2. 2 depicted 2 hours as the optimum binding time for equilibrium to be established. Optimum time studies for [³H]-E₂ binding has previously been performed by Viljoen *et al* and found to be 45 minutes⁹³.

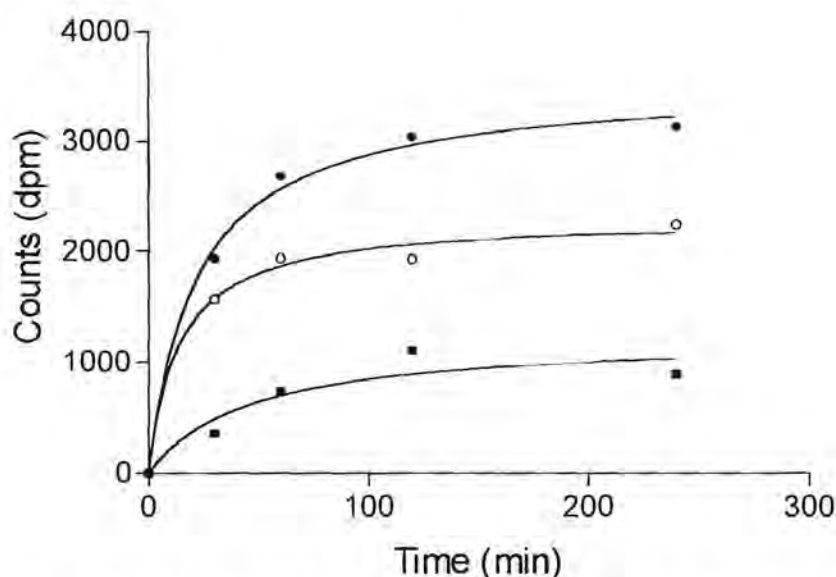


Fig. 2. 2 Binding time kinetics of [³H]-Testosterone uptake in DU-145 cells in the presence and absence of a 400 times unlabelled testosterone (● Total Binding; ○ Non-specific Binding; ■ Specific Binding).

Two of the characteristics of receptors are that receptors must have finite binding capacities and high affinities for the ligands²²⁴. Employing multipoint titrations a gradual increase for both total binding and non-specific binding were observed, while the calculated specific binding reached a maximum (Fig. 2. 3). In order to determine the maximum amount of binding or binding capacity as well as the binding affinity of AR and ER in intact whole DU-145 cells, the mathematical

method of Scatchard was employed to analyze the multipoint titration assays. The binding affinity of T and E₂ was calculated as 12.12 ± 4.68 nM and 0.6982 ± 0.2105 nM respectively, while the maximum binding capacity was 210.3 ± 20.0 fmol/mg protein and 54.1 ± 6.5 fmol/ mg protein respectively (Fig. 2. 4). These experiments were carried out three times in duplicate for the controls and each EFA.

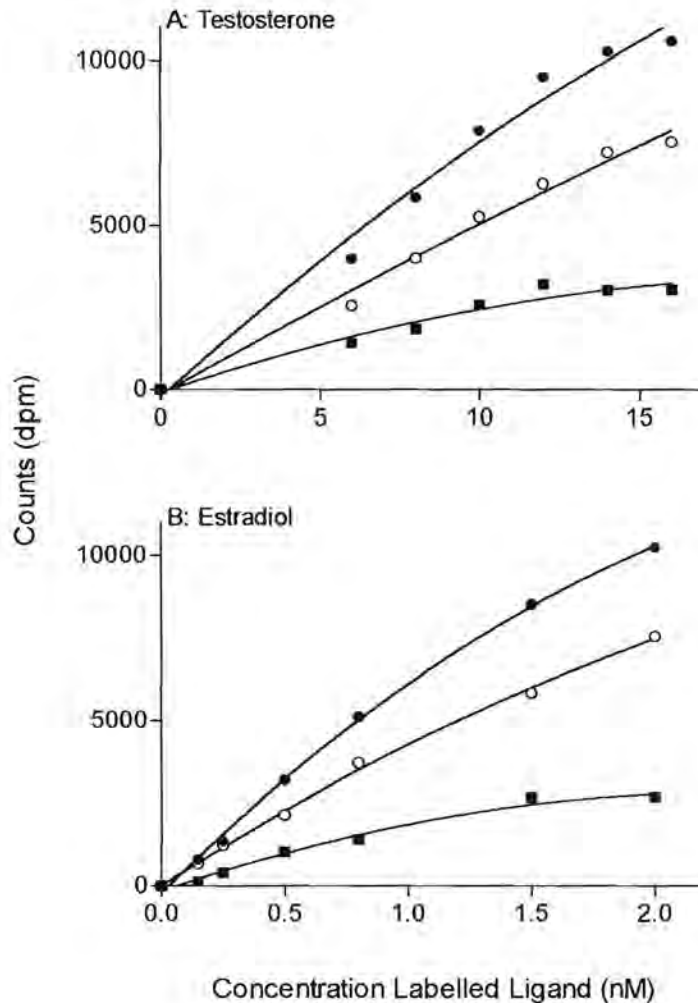


Fig. 2. 3. Uptake of labelled ligand by whole DU-145 cells growing in monolayer culture. A: Various concentrations of labelled T (0 – 16 nM) were incubated at 37°C for 120 minutes in the presence and absence of a 400-fold unlabelled T. B: Various concentrations of labelled E₂ (0 – 2.0 nM) were incubated at 37°C for 45 minutes in the presence and absence of a 200-fold unlabelled DES (● Total Binding; ○ Non-specific Binding; ■ Specific Binding).

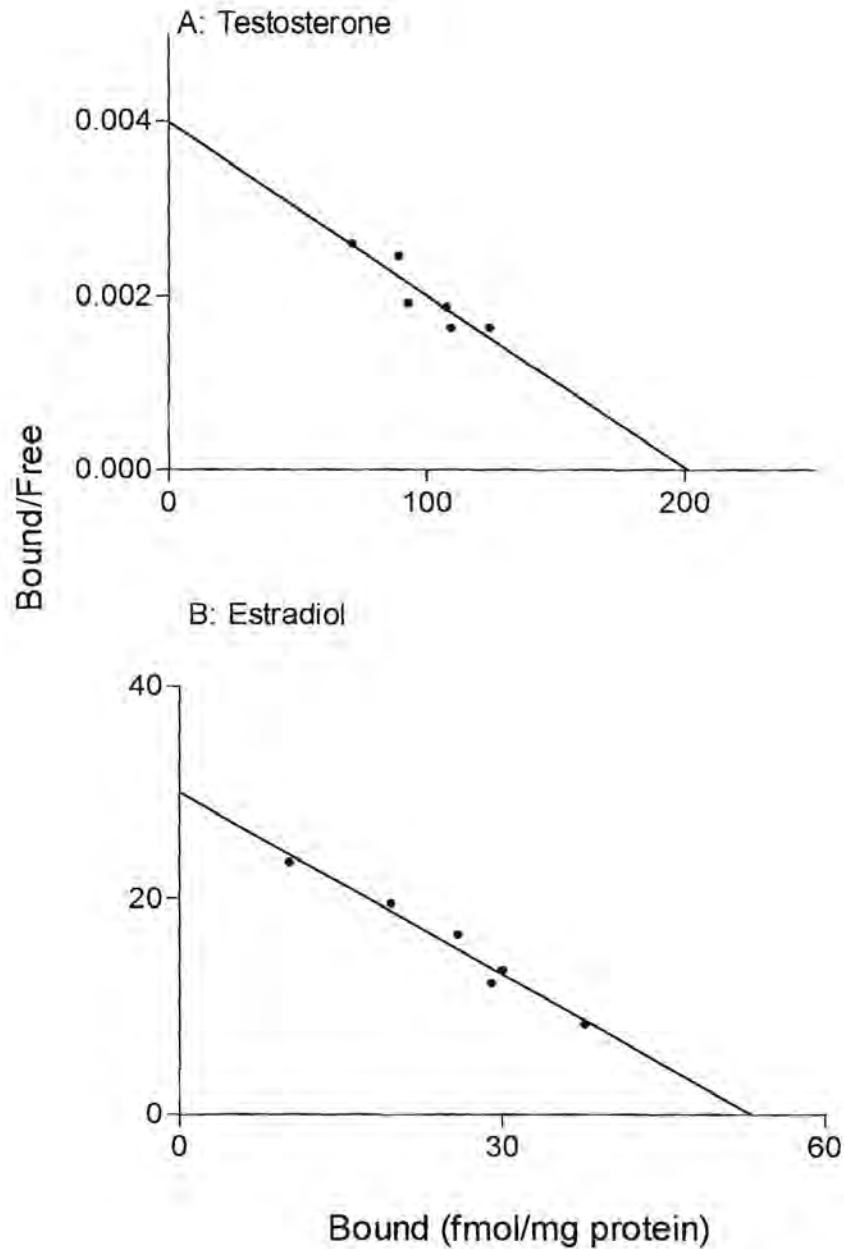


Fig. 2. 4 Scatchard analysis in whole DU-145 cells growing in monolayer culture. A: Specific binding of labelled T, incubated for 120 minutes at 37°C; B: Specific binding of labelled E₂ incubated for 45 minutes at 37°C.

2.3.2. Essential fatty acids

The effect of essential fatty acids on specific binding of 12 nM and 0.8 nM labelled testosterone and estradiol respectively, at different concentrations of EFAs were determined (Fig. 2. 5). From this experiment an EFA concentration were chosen to use in the Scatchard analysis. Since an effect was observed for both steroids at low concentrations of EFAs, it was decided to use a concentration of 10 μM for all the EFAs under investigation. At this concentration cell death does not occur¹⁷².

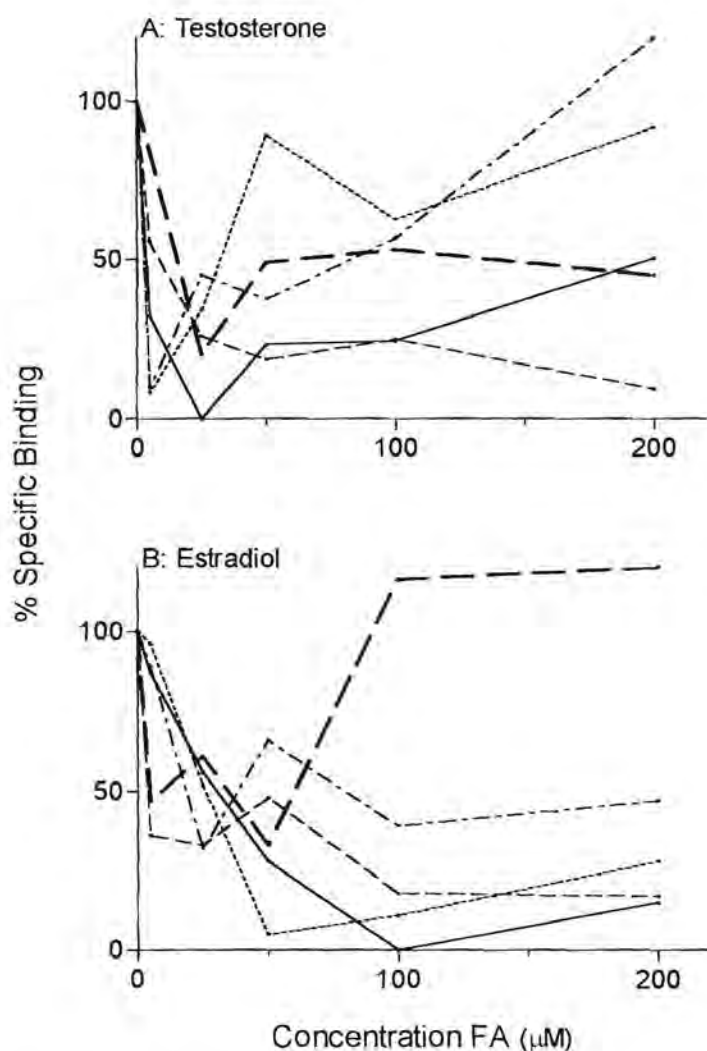


Fig. 2. 5 Specific binding of labelled testosterone (12 nM) and labelled estradiol (0.8 nM) in the presence of fatty acids. — ALA; EPA; - - - LA; - · - · GLA; - - - OA.

In order to supply an indication of the type of effect the EFAs confer on the SH–SHR interaction, the Scatchard analysis was carried out. The analysis showed that the binding of T to whole DU-145 cells was competitively inhibited by LA (n-6), uncompetitively inhibited by EPA (n-3) and noncompetitively inhibited by ALA, GLA and OA (Fig. 2. 6). This was not the case with E₂ binding where stimulation was observed (Fig. 2. 7). Table 2. 1 illustrates the binding capacities and affinities of AR and ER. Except for LA, with no effect, OA (P<0.05), ALA (P<0.05), EPA (P<0.001) and GLA (P<0.01) reduced the binding capacities (B_{max}) for T binding. In contrast, n-3 EFAs, ALA (P<0.001) and EPA (P<0.01) as well as the n-6 EFAs, LA (P<0.05) and GLA (P<0.01) increased binding capacity for E₂ binding. The monounsaturated FA, OA had no effect on binding capacity of ER. Shifting our attention to binding affinity (K_d), OA, ALA, LA and GLA had no significant effect on AR affinity, while the n-3 EFA, EPA (P<0.05) significantly increased the affinity for AR. Pertaining to the ER, only the n-3 EFAs, ALA (P<0.01) and EPA (P<0.05) significantly decreased the binding affinity for ER. The n-6 EFAs, LA and GLA, as well as the n-9 monounsaturated FA, OA had no significant effect on ER binding affinity.

2.3.2. Essential fatty acids

The effect of essential fatty acids on specific binding of 12 nM and 0.8 nM labelled testosterone and estradiol respectively, at different concentrations of EFAs were determined (Fig. 2. 5). From this experiment an EFA concentration were chosen to use in the Scatchard analysis. Since an effect was observed for both steroids at low concentrations of EFAs, it was decided to use a concentration of 10 μ M for all the EFAs under investigation. At this concentration cell death does not occur¹⁷².

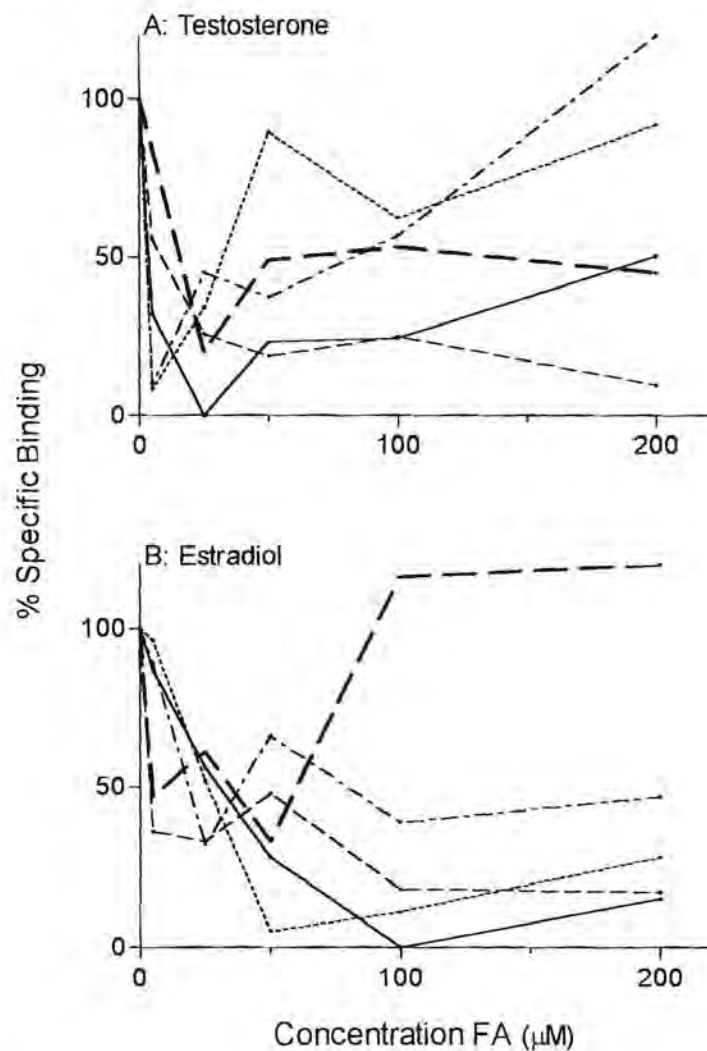


Fig. 2. 5 Specific binding of labelled testosterone (12 nM) and labelled estradiol (0.8 nM) in the presence of fatty acids. — ALA; EPA; --- LA; - - - GLA; - - - OA.

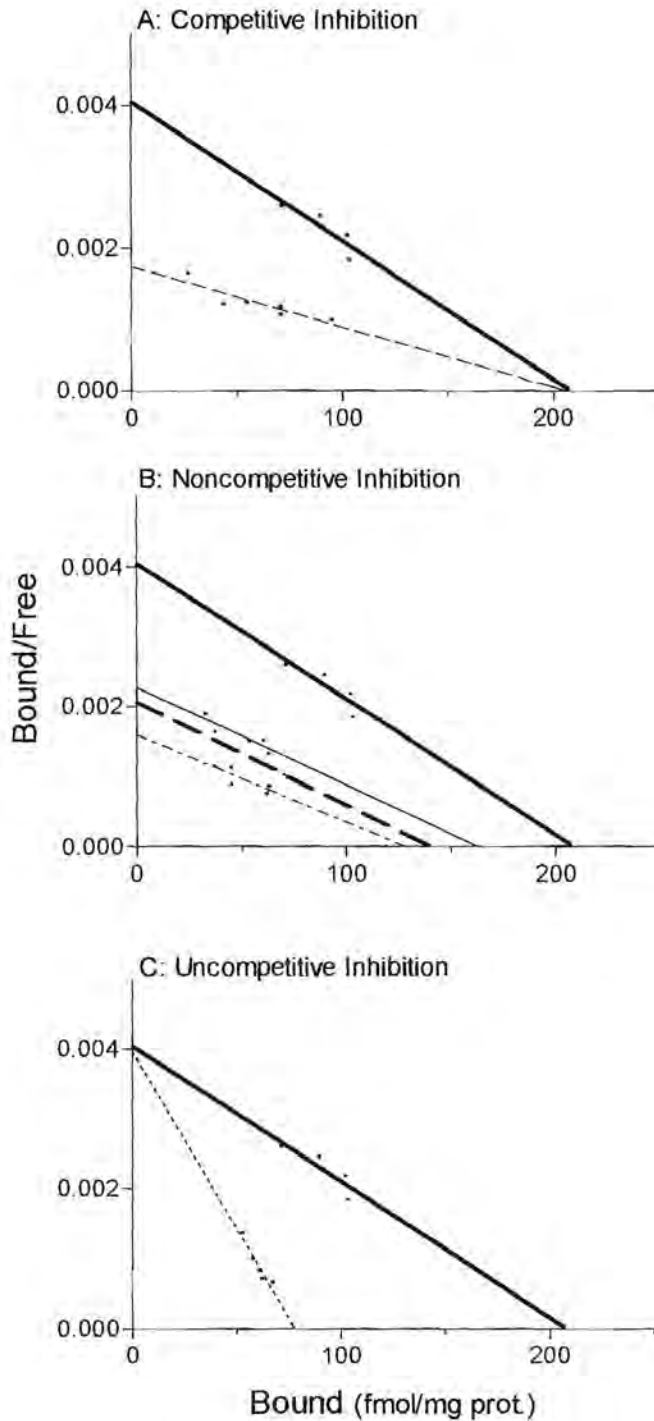


Fig. 2. 6 Inhibition of testosterone binding to whole DU-145 cells in the presence of EFAs (10 μ M). — ALA; EPA; - - - LA; - · - · GLA; - - - OA.

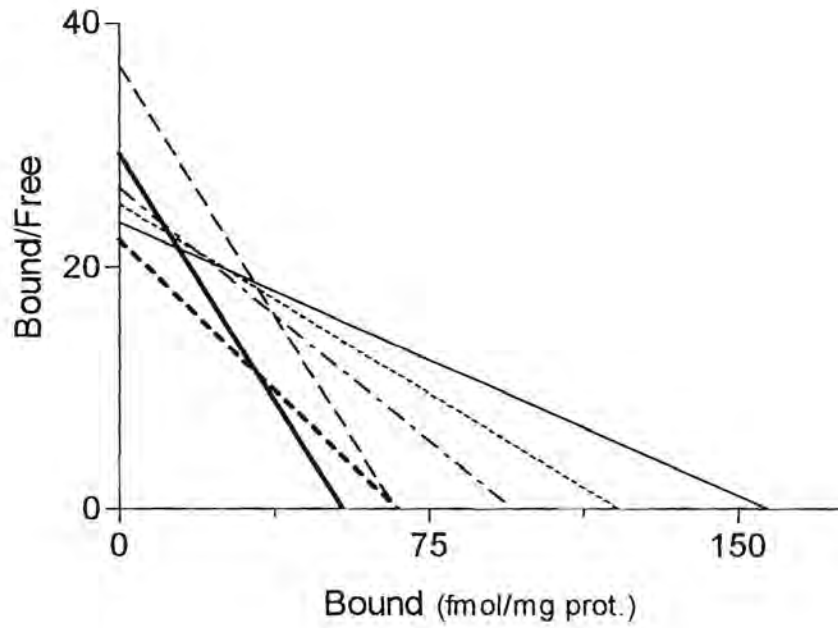


Fig. 2.7 Stimulation of estradiol binding to whole DU-145 cells in the presence of EFAs (10 μM).

— ALA;EPA, - - - - LA, - · - · - GLA, - - - - OA.

Table 2. 1 The influence of fatty acids on estradiol and testosterone binding to estrogen and androgen receptors of whole human DU-145 cells: The P-values were calculated with the Student's t-test. NS = not significant.

FA (10 μ M)	ER				AR			
	B_{max} (fmol/mg)	P<	K_d (nM)	P<	B_{max} (fmol/mg)	P<	K_d (nM)	P<
Control	54.1 \pm 6.5		0.6982 \pm 0.2105		210.3 \pm 20.0		12.12 \pm 4.68	
OA	67.3 \pm 6.5	NS	1.104 \pm 0.1828	NS	153.0 \pm 21.56	0.05	13.20 \pm 4.07	NS
ALA	157.1 \pm 8.5	0.001	2.5221 \pm 0.5069	0.01	166.0 \pm 6.3	0.05	12.48 \pm 3.63	NS
EPA	120.9 \pm 14.0	0.01	1.83 \pm 0.54	0.05	70.5 \pm 14.6	0.001	4.05 \pm 0.98	0.05
LA	66.9 \pm 2.6	0.05	0.7249 \pm 0.0932	NS	197.7 \pm 10.2	NS	17.32 \pm 1.05	NS
GLA	95.2 \pm 8.1	0.01	1.0898 \pm 0.2770	NS	125.1 \pm 11.24	0.01	12.43 \pm 0.39	NS

2.4. Discussion

Since the biological response to steroid hormones is a saturable phenomenon, which depends on the formation of hormone-receptor complexes, binding of a ligand to a receptor must display a finite binding capacity²²⁴. Furthermore, steroid receptors should possess a high affinity for their respective hormones, because the circulating levels of steroid hormone are usually 10^{-10} to 10^{-8} M. The finite binding capacities obtained for both T (B_{max} , 210.3 ± 20.0 fmol/mg protein) and E_2 (B_{max} , 54.1 ± 6.5 fmol/mg protein) binding to whole DU-14 cells indicated that AR and ER or AR- and ER-like receptors were present in DU-145 cells. Furthermore, binding of E_2 revealed a higher affinity (K_d , 0.6982 ± 0.2105 nM) than binding of T (K_d , 12.12 ± 4.68 nM).

Binding parameters of E_2 correlated well with results previously obtained by Viljoen *et al* with DU-145 cells (B_{max} , 44.7 ± 4.0 fmol/ mg protein; K_d , 0.6 ± 0.6 nM) and with other ER positive cell cultures⁹³. The average B_{max} and K_d values for human breast cancer MCF-7 cells are 63 – 210 fmol/mg protein and 0.68 – 2.5 nM respectively^{225,226}. Ogawa *et al*²²⁷ found that the K_d for $ER\alpha$ and $ER\beta$ are 0.2 nM and 0.6 nM respectively in COS-7 cells transfected with either $ER\alpha$ or $ER\beta$. Since both $ER\alpha$ and $ER\beta$ may have a K_d value of approximately 0.6 nM it is difficult to distinguish between $ER\alpha$ and $ER\beta$ applying radiolabelled binding assays. Therefore, northern blotting, immunocytochemistry and western blotting will be employed in the following chapters to establish the presence of $ER\alpha$ and $ER\beta$ in DU-145 cells.

In contrast to autoradiographic, cytochemical and immunocytochemical techniques, which stained positively for AR in DU-145 cells, radiolabelled binding assays did not prove the presence of AR in these cells, previously^{98,228}. A possible reason for this phenomenon is that the cells were homogenized, which may disrupt the AR activity. Therefore, it was decided to use whole DU-145 cells in this study to determine AR binding. The binding parameters of T obtained in this study seemed to correspond with type II binding sites as reported on human and rat prostates: Castagnetta *et al*²²⁹ showed that both type I (K_d , 0.1 - 0.9 nM)

and type II (K_d , 1.3 – 9.2 nM) androgen binding sites are present in BPH and prostate cancer tissues. In this case, B_{max} values could not be compared since receptor concentrations were expressed as fmol/mg DNA. Generally type I and type II binding sites display high affinity with low capacity and low affinity with high capacity, respectively²²⁴. Lamarre *et al*²³⁰ found that the specific binding of a synthetic androgen, R1881, to a prostatic cytosol fraction is the highest between 10 and 15 nM. Once again, the latter values are consistent with the maximum specific binding (± 12 nM testosterone) in this study (Fig. 2. 3). To summarize, the AR in DU-145 cells is probably a type II binding site, since the same ratio of the human prostatic AR was found namely a low binding affinity and a high capacity.

A point of criticism on the results presented in this thesis may be that T was used in this study and not DHT. Dihydrotestosterone is the major hormone in the prostate. In a way, the use of T complicates the experiment, because DU-145 cells contain the 5α -reductase enzyme and can rapidly convert T to DHT²³¹. However, DHT itself is rapidly metabolized in the prostate²³². An advantage of using T in this study is the fact that DU-145 cells retain high levels of unconverted T, which could bind to receptors and GLA may inhibit 5α -reductase^{186,233}. Furthermore, Gann *et al*²³⁴ observed that circulating T and not DHT, correlates with prostate cancer incidence. Metabolism of T and DHT may explain the relative high non-specific binding observed in this and in other studies²²². Most scientists choose to use, the synthetic androgen, methyltrienolone (R1881) that is not metabolized during binding assays in DU-145 cells.

The binding of T to whole DU-145 cells was competitively inhibited by LA (n-6), uncompetitively inhibited by EPA (n-3) and noncompetitively inhibited by ALA, GLA and OA (Fig. 2. 6). Previously arachidonic acid (20:n-4) inhibited AR noncompetitively in the rat prostate¹⁹⁵. The different types of inhibition and models for competitive inhibition are displayed in Fig. 2. 8 and Fig. 2. 9 respectively. When these models are applied to the EFAs under investigation,

LA, would bind only to the receptor, OA, GLA and ALA to the receptor and the ligand-receptor complex, and EPA only to the ligand-receptor complex.

In the case of E₂, binding was stimulated in the presence of all the EFAs under investigation, while OA had no effect (Fig. 2. 7). Previously, both potentiation and inhibition of ER was found in human and rat uteri with arachidonic acid (20:n-4) and docosahexanoic acid (22:n-6)^{191,192,235}.

From Table 2. 1 it can be seen that except for LA (B_{max}, 197.7 fmol/mg protein), which had no effect on the maximum AR binding (B_{max}, 210.3 fmol/mg protein), the AR capacity decreased significantly with OA, ALA, EPA and GLA (B_{max}, 70.5 - 166.0 fmol/mg protein). The opposite effect was found with the ER capacities, namely significantly increased capacities were presented with ALA, EPA, LA and GLA (B_{max}, 66.9 - 157.1 fmol/mg protein), but no significant difference between the control (B_{max}, 54.1 fmol/mg protein) and cells treated with monounsaturated OA (B_{max}, 67.3 fmol/mg protein). Although the AR dissociation constants (K_d) stayed the same between the control cells (K_d, 12.12 nM) and the cells treated with OA, ALA, LA and GLA (K_d, 12.43 - 17.32 nM), a significant increase in affinity was obtained in the presence of EPA (K_d, 4.05 nM). However, once again this was not the case with the affinities calculated for the ER. The OA, LA and GLA (K_d, 0.725 - 1.104 nM) had no significant effect on the ER affinity (K_d, 0.698 nM) in contrast to the n-3 fatty acids ALA and EPA, which decreased the ER affinity significantly (K_d, 1.83 - 2.52 nM).

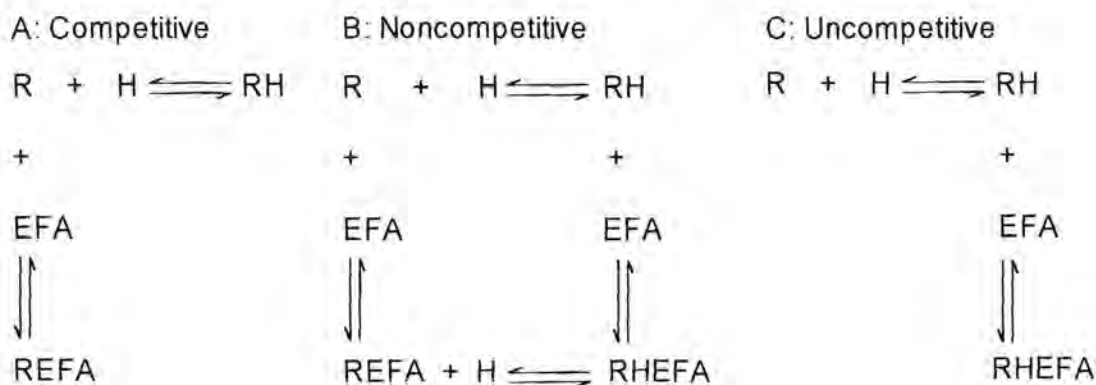


Fig. 2. 8 Types of inhibition: A Competitive Inhibition, B: Noncompetitive Inhibition, C: Uncompetitive Inhibition²³⁶

The ER binding parameters were most affected by the position of the double bond, the n-3 EFAs, ALA and EPA, having the greater effect on binding affinity and capacity than the n-6 EFAs (Table 2. 1). In contrast, binding to the AR was not affected by the position of the double bond but by the chain length, the longer the EFA, the greater the effect on both binding affinity and capacity. The twenty-carbon chain EPA had a larger effect on the AR affinity and capacity than the other eighteen-carbon atom chain EFAs. The number of double bonds did not play a significant role, except at the AR affinity, which increased with increasing number of double bonds (LA < GLA = ALA < EPA).

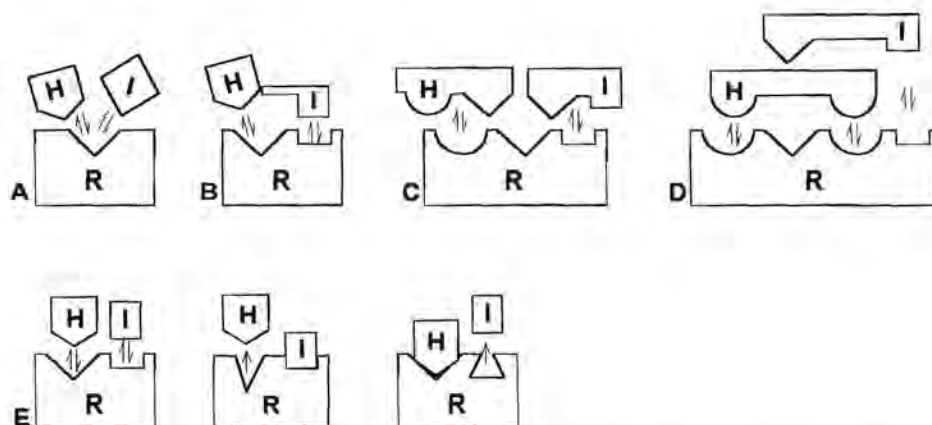


Fig. 2. 9 Models of competitive inhibition. A: H and I compete for the same binding site. B: H and I are mutually exclusive because of steric hindrance. C: H and I share a common binding group on the enzyme. D: The binding sites for H and I are distinct, but overlapping. E: The binding of I to a distinct inhibitor site causes a conformational change in the receptor that distorts or masks the hormone-binding site (and *vice versa*)²³⁶.

Since antiandrogens and estrogens are used in treatment to reduce androgen action in the malignant prostate, the use of EFAs may benefit the prostate cancer patient by reducing the T and increasing the E₂ action. It could be valuable to measure the effect of EFAs in combination with antiandrogens, estrogens and adrenal androgens to establish a possible synergistic role during

hormone treatment. In addition to the results obtained in this thesis, EFAs may have an inhibitory effect on prostate cancer since it is known that GLA inhibits 5α -reductase activity and the formation of DHT¹⁸⁶.

Fatty acids may alter the physical properties of the plasma membrane, and thus the permeability of the cell¹⁸⁰. This may explain the changes in the steroid receptor capacities of the whole DU-145 cells in the presence of PUFAs by way of an increased and decreased membrane permeability for estrogens and androgens, respectively. Another possible explanation for the DU-145 steroid receptor capacity alterations in the presence of FAs, may be found in the latest report that ER and AR are present on MCF-7 and T cells plasma membranes, respectively and both receptors are present on osteoblast plasma membranes^{237,238,239}. These receptors are similar, but not identical to its intracellular counter parts since they have a non-genomic function instead of a genomic function. If membrane receptors would be present in DU-145 cells, FAs may bind directly to the DU-145 plasma membrane steroid receptors affecting the capacities. Another possibility is that changes in the plasma membrane could cause alterations in the hormone signalling of membrane receptors, which could affect the ensuing nuclear mechanisms that change the expression of various genes. However, further research is still required to clear these mentioned probabilities.

To conclude, this study confirmed that environmental predisposing factors, such as the diet and in particular, dietary fat, might play an important role in the development and treatment of prostate cancer by means of modulation of the steroid hormone binding to cells. The fact that EFAs inhibit the androgen binding reminds of the use of antiandrogens, while high doses of E_2 reduce prostate growth directly and this effect may be enhanced by the addition of EFAs²⁴⁰.

CHAPTER 3

3. METHODOLOGIES AND METHODOLOGICAL PROBLEMS ENCOUNTERED IN A STUDY OF ESTROGEN RECEPTOR mRNA EXPRESSION IN DU-145 PROSTATE CANCER CELLS

3.1. Introduction

Since epithelial cells are constantly replicating, they have the highest risk for malignant transformation and about 90% of all human cancers, which include tumours of the prostate and breast, develop from these cells²⁴¹. Cancer cell characteristics include increased growth rates, loss of differentiation, escape from cell death pathways, evasion of anti-proliferative signals, a decreased reliance on exogenous growth factors and escape from replicative senescence²⁴². Malignant cells acquire these features by impairment of normal cellular growth control, which involves inappropriate regulation of gene expression²⁴³. Gene expression can be influenced by a vast amount of factors, including mutations, hypermethylation of the gene's promoter, inappropriate deacetylation of regulatory chromatin elements, inactivation of tumour suppresser genes, re-expression of telomerase genes, aberrant chromatin formation, DNA helicase deficiencies and other transcription factor abnormalities^{242,244,245,246,247}. Steroid hormones may regulate these genomic enzymes, since it was recently demonstrated that telomerase activity is upregulated by androgen ablation therapy in rhesus monkeys²⁴⁸. On the other hand, some of the above genetic and epigenetic events have been associated with steroid hormone receptor expression for example, hypermethylation of the AR causes downregulation of AR in prostate cancer²⁴⁴. Since AR and ER act as ligand-activated transcription factors, any factor regulating AR and ER expression may modify the effect of androgens and estrogens in normal reproductive and malignant cells.

The n-3 and n-6 EFAs have been implicated not only in posttranslational mechanisms such as signal transduction, but evidence exists that n-3 and n-6 EFAs are involved in regulation of gene expression²⁴⁹. These EFAs may regulate the amount of proteins *via* interference with the translation of mRNA or an alteration in the amount of mRNA encoding the protein. To substantiate the possibility that EFAs may regulate steroid hormone receptor expression, EFA regulation of fatty acid synthase (FAS) expression is reviewed briefly. Usually FAS is elevated in many neoplasms including prostate cancer²⁵⁰. In rats, dietary PUFAs regulate the synthesis of hepatocyte FAS and S14 primarily *via* a reduction in the amount of mRNA encoding these proteins^{251,252}. This effect is not due to a long term process of incorporating fatty acids into the cell membrane, since both FAS and S14 gene transcription are initiated within 90 minutes after ingesting triolein oil containing oleic acid²⁵³. When the diet is changed to menhaden oil, which contains n-3 EFAs, mRNA levels of FAS and S14 are reduced more than 50% and reach a nadir after consumption of the second meal. When menhaden oil is removed from the diet, the reducing effect reverses within 3 hours. Transcription rates of β -actin, PEP- carboxykinase and tyrosine aminotransferase are unchanged, indicating that this treatment is gene specific for FAS and S14^{251,253}.

The inhibitory potency of a dietary fat depends on the concentration of the fatty acid constituents of the dietary lipid, as well as the structure thereof. To inhibit the expression of genes encoding lipogenic proteins, a dietary fatty acid has to contain a minimum of 18 carbons and possessed at least 2 conjugated double bonds located at the 9 and 12 position²⁴⁹. Therefore, LA (18:2n-6), GLA (18:3n-6), ALA (18:3n-3) and EPA (20:5n-3) were chosen in this study, with OA (18:n-9) as control to study gene expression.

Low levels of EFAs may be the reason why elevated FAS expression occurs in cancer cells. In breast cancer FAS is associated with ER status and an increase in the rate of transcription of the FAS gene resulted in higher levels of FAS mRNA^{254,255}. Like FAS, AR and ER levels are increased in many prostate and breast neoplasm's respectively and therefore the hypothesis was that EFAs

might influence transcription of AR and ER mRNA in DU-145 prostate cancer cells, as well^{256,257}. However, since AR and ER β probes for northern blotting were not commercially available at the time of study, our focus returned to the ER α . For convenience, the labelling reaction, the labelling efficiency (spot blotting) and mock hybridization procedures are illustrated in Fig. 3. 1

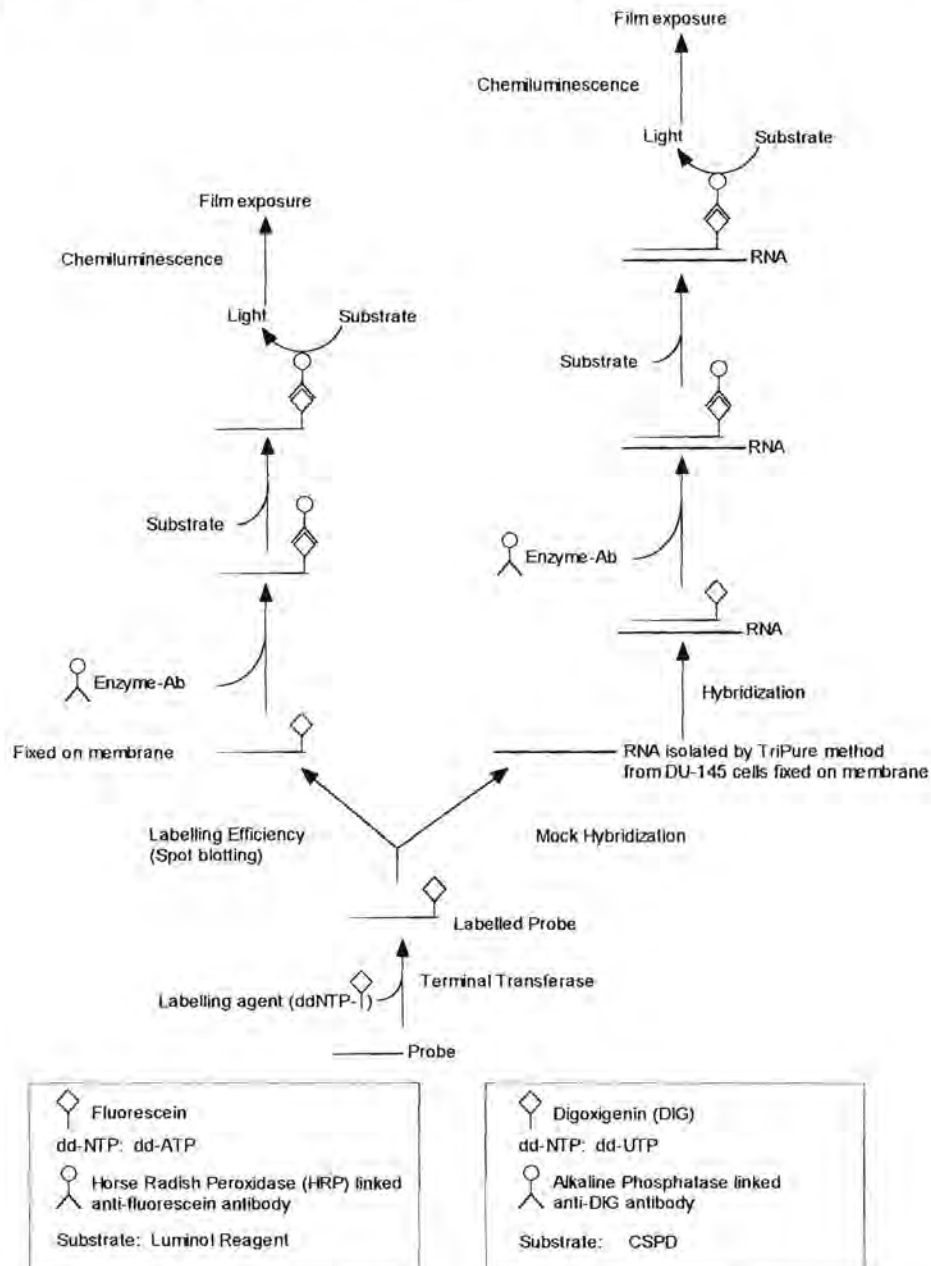


Fig. 3. 1. Illustration of spot blotting and mock hybridization procedures. The fluorescein and DIG-labelling systems are depicted in each box.

3.2. Materials And Methods

3.2.1. Materials

The QuickPrep Total RNA Extraction kit was obtained from Amersham Pharmacia Biotech and the 3'-End Labelling Fluorescein kit from NEN Life Sciences. TriPure RNA isolation reagent, DIG nucleic acid labelling and detection kits, the washing, blocking, maleic acid and detection buffers, as well as positively charged nylon membranes for northern blotting were obtained from Roche Diagnostics (Roche Diagnostics, SA). Sigma Aldrich (Kelvin, SA) supplied DEPC. The ER and GAPDH probes were obtained from Oncogene Products (Cambridge, MA, USA) and the G3PDH and β -actin probes were obtained from Clontech Laboratories (Palo Alto, CA).

3.2.2 Cell cultures

Human DU-145 prostate tumour cells were obtained from Sterilab (Johannesburg, SA) and maintained in Corning polystyrene flasks (Corning Glass Works, Corning, NY, USA) at 37°C, 5% CO₂ in Ham's F-10 nutrient mixture supplemented with 5% decomplicated fetal calf serum, gentamycin sulphate (0.004%), glucose (0.57%) and NaHCO₃ (0.12%). The growth medium was filtered through a Millipore Sterivex-GS filter (0.22 μ m) fitted with an In-Line Prefilter (Millipore Filters AP 15 and 20) and stored at 4°C. The medium was changed every 2 to 3 days. Cells were harvested or passed by trypsinization. Immediately after trypsinization, F-10 medium with serum was added to the cell suspension and, following centrifugation (1000 x g for 5 minutes at room temperature), the cells were resuspended in F-10 medium with serum.

3.2.3 RNA isolation with the QuickPrep Total RNA Extraction kit

The QuickPrep Total RNA extraction kit is based on a procedure that combines the disruptive and protective properties of guanidinium thiocyanate

with selective precipitation and isopycnic centrifugation using lithium chloride and cesium trifluoroacetate (CsTFA)²⁵⁸. Guanidinium thiocyanate inhibits endogenous RNase activity. All solutions and chemicals reserved for RNA work should be kept RNase free²⁵⁹. Alpha Q water and buffers were treated overnight with 0.1% DEPC and autoclaved to remove the DEPC. Cells grown in 25 cm² Corning cell culture flasks were trypsinized, washed with PBS and centrifuged for 5 minutes at 500 x g. The cell pellets were resuspended in PBS at a concentration of 5 x 10⁶ cells/ml and placed on ice. After spinning for another 5 minutes at 500 x g, lithium chloride solution was added to the cell pellet, mixed and ground in a Wheaton dounce tissue grinder. Before completely homogenizing the cell extract, β-mercaptoethanol and extraction buffer from the kit, which contained guanidinium thiocyanate and N-lauroyl sarcosine, was added to the cell extract. Then the cell extract was placed on ice, mixed with CsTFA and left on ice for 10 minutes. After centrifugation at room temperature for 15 minutes at 12 000 x g, the pellet contained the RNA while the top protein coat and DNA liquid phase was removed by vacuum aspiration. The RNA pellet was washed with addition of extraction buffer, lithium chloride solution, CsTFA and centrifugation at 12 000 x g for 5 minutes at room temperature. After 70% ethanol was added, centrifugation at 12 000 x g for 5 minutes and air-drying of the pellet for 10 minutes at room temperature followed. The pellet was dissolved in DEPC treated RNase free water by placing the samples on ice for 30 minutes, vortexing and heating at 65°C for 10 minutes. The RNA was quantified in a GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Buckinghamshire, UK) and stored at -70°C.

3.2.4 RNA isolation with TriPure Isolation kit

Total RNA was extracted by a guanidinium isothiocyanate-phenol method with TriPure Isolation Reagent^{260,261}. RNA was isolated from confluent, monolayer DU-145 and MCF-7 cell cultures, which were grown in 25 cm² flasks. The medium was decanted and 3 ml of TriPure solution was added. After

passing the cell lysate through a pipette several times, the cell extract was incubated for 5 minutes at room temperature to ensure the complete dissociation of nucleoprotein complexes. Chloroform (0.6 ml per flask) was added and the mixture was shaken vigorously for 15 seconds after which the cell extract was incubated for 15 minutes at room temperature. The centrifugation of $12\ 000 \times g$ at 4°C for 15 minutes allowed the separation of the mixture into three phases. The clear, top phase containing the RNA was aspirated to a clean tube and 1.5 ml isopropanol was added to precipitate the RNA. After the mixture was inverted several times and incubated for 10 minutes at room temperature, the samples were centrifuged at $12\ 000 \times g$ for 10 minutes at 4°C . The RNA pellets were washed with 3 ml 75% ethanol by vortexing, followed by centrifugation at $7500 \times g$ for 5 minutes at 4°C . After discarding the supernatant, the excess ethanol was removed from the RNA pellets by air-drying for 10 minutes. The RNA pellets were resuspended in DEPC treated RNase free water and left at room temperature for 10 minutes, followed by incubation at $55 - 60^{\circ}\text{C}$. The RNA was dissolved completely by passing the solution through a pipette several times. To measure the total RNA concentration, $2\ \mu\text{l}$ of each sample was diluted with $998\ \mu\text{l}$ TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and measured with a GeneQuant analyzer. The A_{260}/A_{280} ratio was greater than 1.8 for each sample and suitable for northern blotting.

3.2.5 Probe labelling with 3'-End Labelling Fluorescein kit

The ER and housekeeping probes were labelled with the '3-end labelling fluorescein kit. The reaction is based on the terminal transferase enzyme, which links Fluorescein- N^6 -ddATP to the 3'-end of an oligonucleotide probe. The following solutions were mixed in an RNase free tube: $12.5\ \mu\text{l}$ reaction buffer, $2.5\ \mu\text{l}$ CoCl_2 solution, $4.0\ \mu\text{l}$ Fluorescein- N^6 -ddATP solution, $10.0\ \mu\text{l}$ of the $2.5\ \text{pmol}/\mu\text{l}$ ER or GAPDH probe solution ($2.5\ \text{pmol}/\mu\text{l}$), $4.0\ \mu\text{l}$ of DEPC treated water and $2\ \mu\text{l}$ terminal transferase solution. This reaction mixture was incubated at 37°C for 15 minutes and then placed on ice and stored at -70°C .

The efficiency of the labelling reaction was estimated by comparing the fluorescein incorporation to the control fluoresceinated oligonucleotide supplied in the kit. In short, the control probes as well as the ER and housekeeping probes were diluted into several dilutions between 0.02 pmol/ μ l and 0.0004 pmol/ μ l. One μ l of each dilution was spotted onto a Duralon^{UV} membrane and fixed by UV crosslinking for 20 seconds. Detection was carried out as described in par. 3.2.6.

3.2.6 Chemiluminescent detection of the fluorescein labelled probes

Detection was based on fluorescein recognition by an antifuorescein antibody, which was conjugated with the horseradish peroxidase enzyme. The membrane with the fixed probe or RNA was immersed and vigorously agitated in Buffer 1, consisting of phosphate buffered saline (PBS), pH 7.4 and 0.05% Tween 20. After gentle agitation in Buffer 2 (PBS, pH 7.4, 0.05% Tween 20, and 0.5% Blocking agent) for 1 hour at room temperature, the membrane was placed in the conjugate solution (1:1000 antifuorescein-HRP conjugate in Buffer 2) for 1 hour with gentle agitation. Next, the membrane was vigorously washed four times for 5 minutes in Buffer 1 and once for 5 minutes in Buffer 3 (0.10 M Tris-HCl, 0.15 M NaCl). Only during labelling efficiency reactions, 0.1% SDS was added to Buffer 1, Buffer 2 and the conjugate solution. After transferring the membrane to a clean container, the chemiluminescent substrate, which consisted of equal amounts of the Enhanced Luminol Reagent and the Oxidizing Reagent, was poured onto the membrane and incubated with gentle agitation for one minute²⁶². The membrane was placed between two plastic sheets and exposed to autoradiographic film, which was developed by hand.

3.2.7 Hybridization and stringency washes for fluorescein labelled probes.

Membranes with fixed RNA were placed in 5x SSC solution and then transferred to the prehybridization buffer, which contained 5x SSC, 0.1% SDS, 0.5% Blocking Agent and, 5% dextran sulphate, for 1 hour at 53°C in a Hybaid

oven. The prehybridization buffer was discarded and replaced with the hybridization buffer, which included the fluorescein labelled probes at a final concentration of 10 pmol/ml for the GAPDH probe. Hybridization was carried out overnight and the next day all unhybridized probes were removed by washing the membrane in a salt/detergent mixture. First, the membrane was washed once with a 2x SSC, 0.1% SDS buffer for 10 minutes at 53°C, then once in 0.2x SSC solution for 10 minutes at 68°C. Lastly, the membrane was washed twice with Buffer 1 without added SDS for 5 minutes at room temperature. Next the detection procedure for the fluorescein 3'-end labelled probes was followed (par. 3.2.6).

3.2.8 Probe labelling with DIG 3'-end labelling kit

The ER and GAPDH probes were labelled with the DIG 3'-end labelling kit from Roche Diagnostics²⁶³. The labelling reaction was based on the terminal transferase enzyme, which links a single digoxigenin-labelled dideoxyuridine triphosphate (DIG-ddUTP) molecule to the 3'-end of an oligonucleotide probe. This method enables the determination of DNA or RNA sequences without the use of radioactive probes. The following solutions were mixed in an RNase free tube: 8 µl reaction buffer, 8 µl CoCl₂ solution, 10 µl of the 2.5 pmol/µl ER or GAPDH probe solution, 2 µl DIG-ddUTP solution, 2 µl terminal transferase solution (100 units) and 10 µl of DEPC treated water. The reaction mixture was incubated at 37°C for 15 minutes and placed on ice. To terminate the labelling reaction, 2 µl of a 200 mM EDTA (pH 8.0) was added and then the labelled probe was stored at -70°C till needed.

Next the labelling efficiency of the reaction was estimated with the "spot blotting test". The DIG labelled control oligonucleotide from the DIG 3'-endlabelling kit, the DIG-labelled experimental control probe as well as the ER and GAPDH oligonucleotide probes, was diluted to concentrations of 50 fmol/µl, 10 fmol/µl, 2 fmol/µl, 0.4 fmol/µl and 0.08 fmol/µl. One µl spots of each dilution were applied to the positively charged nylon membrane and fixed by baking at

120°C for 15 minutes. Thereafter the spots were detected by chemiluminescence (3.2.9) and the spot intensities were compared to the control probe intensity.

3.2.9 Chemiluminescent detection for DIG labelled probes

After fixing the total RNA or oligonucleotide probes to the membrane, the membrane was washed for 5 minutes in a 1x washing buffer, followed by blocking for 30 minutes in a 1x blocking solution, which was prepared in maleic acid buffer. The antibody solution (1:10 000) was prepared in 1x blocking solution immediately before use and applied to the membrane with at least 20 ml per 100 cm². After 30 minutes of incubation in the antibody solution, the membrane was washed twice for 15 minutes in washing solution, preceded by a quick rinse. To equilibrate the membrane for detection, the membrane was quickly rinsed followed by a 2 minute equilibration in the same buffer. The CSPD substrate solution supplied in the kit (diluted 1:100 in 1x detection buffer) was applied to the membrane and incubated for 5 minutes at room temperature before the sealed plastic bag with membrane was incubated for 26 minutes at 37°C. Finally, the membrane was exposed to Kodak X-OMAT AR film, whereafter the film was processed by hand in developer and fixer solutions.

3.2.10 Mock hybridization for optimization of probe solutions

In order to optimize the concentration of the probe solutions during hybridization, mock hybridizations were carried out with the G3PDH, β -actin, GAPDH and ER probes. The RNA samples were diluted with an equal volume RNA dilution buffer (DEPC treated water: 20x SSC: formaldehyde in ratio of 5:3:2). The 20x SSC buffer contained 3 mM sodium chloride and 0.3 mM tri-sodium citrate at pH 7.0. One μ l of RNA in RNA dilution buffer was spotted on positively charged nylon membranes, air-dried and baked for 30 minutes or crosslinked with UV light for 20 seconds. The previous methods were performed to fix the RNA to the membrane. Next, the membranes were incubated for 1 hour in preheated prehybridization solution at 53°C in a 6 well plate. A serial dilution

(1:10) was set up for each probe followed by heating (53°C) and overnight incubation at 53°C. The following day, the membranes were rinsed once and washed twice for 10 minutes with 2 x washing solution at room temperature followed by two washes for 20 minutes with 0.1x washing solution (68°C). Afterwards, the membrane pieces were transferred to clean plates for chemiluminescent detection as described in 3.2.9.

3.2.11 Hybridization and stringency washing for DIG-labelled probes

Prehybridization and hybridization were performed at 53°C in DIG-Easy Hyb for 1 hour and overnight respectively. The membrane was washed two times for 10 minutes in 2x washing solution at room temperature and twice for 20 minutes in 0.1x washing solution at 68°C. Post hybridization washing buffers (0.1x) consisted of 0.1x SSC and 0.1% SDS. The SSC buffer contained 15 mM sodium chloride and 1.5 mM tri-sodium citrate pH 7.0. After the washing steps, chemiluminescence was carried out as described under paragraph 3.2.9. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeper.

3.2.12 Agarose gel electrophoresis and capillary transfer

Total RNA was prepared for agarose electrophoresis and diluted 1:2 to 1:5 with sample buffer (62.5% formamide, 20.75% formaldehyde, 1x MOPS buffer, 10% Bromophenol Blue, 10% Xylene Cynole and 0.025% ethidium bromide)²⁶⁴. The samples were incubated for 10 minutes at 60 - 65°C and quick cooled on ice. Each well was loaded with 10 µg RNA and subjected to 1% agarose gel electrophoresis containing 2.2 M formaldehyde and 1x MOPS buffer. A 1x MOPS buffer was used as the running buffer. After 3 hours electrophoresis at 110 V, the gel was pre-soaked in water five times for 1 minute, followed by 30 minutes in 50 mM NaOH solution and twice in 100 mM Tris-HCl for 15 minutes. This process partially degraded the RNA for easier transfer to the membrane. Next the gel was equilibrated twice in 20x SSC solution for 15 minutes before the RNA

was transferred to a positively charged nylon membrane by capillary blotting overnight with 10x SSC. The next day the membrane was fixed with heat (120°C, 15 min) on the nylon membrane. The membrane was subjected to prehybridization, hybridization and detection procedure as discussed in paragraphs 3.2.11 and 3.2.9 respectively.

3.3. Results

Table 3. 1. Labelling efficiency of fluorescein labelled probes (spot blotting).

[Probe] pmol/ μ l	Standard probe	Control probe	ER Probe Amersham	GAPDH probe Amersham	G3PDH probe Clontech	β -actin probe Clontech
0.020	+++++	+++++	++++	++++	+++++	+++++
0.012	++++	+++	+++	+++	+++++	++++
0.00625	+++	++	++	++	+++++	++++
0.00156	++	+	+	+	++++	++++
0.0004	+	-	-	-	++++	+++

Table 3. 2. Mock hybridization with fluorescein labelled probes.

Control target DNA pmol/ μ l	Intensity of control probe	Intensity of G3PDH probe (Clontech)	Intensity of β -actin probe (Clontech)
0.1	+++++		
0.025	++++		
0.00625	+++		
0.00156	++	-	-
0.0004	+	-	-

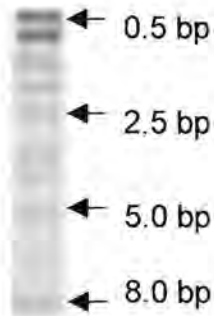


Fig. 3. 2. Northern blot showing the molecular markers (from top to bottom: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 kb).

Table 3. 3. Labelling efficiency of DIG labelled probes

[Probe] fmol/ μ l	Standard Probe	Control Probe	ER Probe	GAPDH Probe
50	+++++	+++	++++	++++
10	+	+	++	++
2	-	-	+	+
0.4	-	-	-	-
0.08	-	-	-	-

Table 3. 4. Mock hybridization with DIG labelled probes

[Probe] pmol/ml	GAPDH probe	ER Probe
3	+++	-
1.5	++	-
0.75	+	-
0.375	++	-
0.1375	+	-
0.0675	+	-



Fig. 3. 3. Northern blot applying the GAPDH DIG 3'-Endlabelling housekeeping probe (10 pmol/3ml). RNA was isolated from DU-145 (lanes B, D) and MCF-7 (lanes A, C) cells. Exposure times were 1 hour (lanes A, B) and 2 hours (lanes C, D).

3.4. Discussion

Total RNA was isolated from DU-145 cells with the QuickPrep Total RNA Extraction kit. Since the A_{260}/A_{280} RNA/DNA ratio obtained was less than 1.6, this method was not suitable for RNA extraction with DU-145 cells. Therefore, it was decided to use the TriPure RNA isolation kit, which gave an A_{260}/A_{280} RNA/DNA ratio of greater than 1.8 that was suitable for northern blotting. In conclusion, although the TriPure RNA isolation reagent contained more hazardous chemicals such as phenol, the TriPure isolation reagent gave a higher purity of RNA compared to the QuickPrep RNA isolation reagent. A possible reason for this is that with the QuickPrep RNA isolation reagent DNA from the tube walls may

contaminate the RNA pellets more easily than in the TriPure method where the RNA is removed from the tube first.

The housekeeping (GAPDH, G3PDH, β -actin) and ER probes were labelled with fluorescein as described in paragraph 3.2.5 and the labelling efficiencies were compared with the standard and control probes supplied in the labelling kit (Table 3. 1). According to the intensities of the spot blots, the G3PDH and β -actin probes were labelled most efficiently. The labelling efficiencies for the fluorescein (Table 3. 1) as well as the DIG labelled probes (Table 3. 3) compared well with the standard labelled probes and the control labelled probes supplied in the kits. Therefore, the labelling of both the fluorescein and the DIG labelled probes were successful.

During spot blotting different concentrations of the labelled probe are spotted and fixed to the membrane (Fig. 3. 1). In contrast, during mock hybridizations equal concentrations of RNA spots are fixed to the membrane followed by hybridization with different concentrations of probe. The mock hybridization step was included to optimize hybridization conditions. Therefore, hybridization of the probe to RNA isolated from DU-145 cells was investigated using mock hybridizations. Mock hybridizations with the G3PDH and β -actin fluorescein labelled probes were only successful with the supplied control DNA and not with the RNA (Table 3. 2). A possible reason is that a high enough concentration could not be attained and therefore it was decided to carry on the investigation with GAPDH cDNA probe as the housekeeper.

Since the possibility existed that the RNA fixing with UV translinking was inadequate, the fixing process was investigated. Mock hybridizations with the GAPDH probe employing 12, 30 and 60 seconds of UV translinking did not show any signal. However, baking in an oven (120°C) for 15 or 30 minutes revealed a slight signal on Duralon but not Boehringer Mannheim membranes with both yeast and DU-145 RNA (results not shown). Translinking the RNA to the membranes did not work properly and a possible reason is that the UV bulbs may have exceeded their lifetime. Since it was cheaper and more effective, the

baking method was decided on in an effort to fix the RNA on Duralon membranes.

Next, RNA was separated on a 1% agarose gel (3.2.12), transferred to a Duralon membrane by capillary action, prehybridized and hybridized with the fluorescein labelled GAPDH probes (10 pmol/ml) (3.2.7), washed and detected with the fluorescein detection method (3.2.6). Since no signal except the molecular markers (Fig. 3. 2) was obtained with the northern blot it was decided to use the more expensive DIG 3'-end labelling kit.

Once again the ER and GAPDH probes were labeled, but with the DIG label (3.2.8) and the labelling efficiencies determined (Table 3. 3). The labelling efficiencies of the GAPDH and ER probes were better than the control probes.

To optimize the GAPDH and ER probe concentrations to be used in northern blotting, mock hybridizations were performed (Table 3. 4). However, only the DIG-labelled GAPDH probe and not the DIG-labelled ER probe successfully produced signals after mock hybridization.

Next, RNA was separated on a 1% agarose gel (3.2.12), transferred to a Boehringer Mannheim membrane by capillary action, prehybridized, hybridized with the GAPDH and ER probes (10 pmol/ml) (3.2.11), washed and the DIG detection method was applied (3.2.9). The northern blot of the GAPDH probe was successful (Fig. 3. 3). Unfortunately, no bands could be detected with the DIG labelled ER probe in the presence or absence of EFAs despite the presence of the 18S and 28S RNA bands on the agarose gel before capillary transfer.

A possible reason for the inability to detect ER mRNA is that there is no ER α in DU-145 cells. However, this statement may not be correct, since no ER α bands could be seen in MCF-7 cells, which do contain ER α . However, the question remains why the manufacturer discontinued the ER probe from the market. Therefore, the effect of EFAs on ER mRNA quantity in DU-145 cells could not be established and compared with protein expression in the previous chapters. The next objectives were to prove the presence of ER α in prostatic DU-145 cells by employing immunocytochemistry and western blotting.



Other methods to detect ER mRNA include the reverse transcriptase polymerase chain reaction. This method is based on the reverse transcriptase enzyme, which produce cDNA from mRNA in the presence of a set of probes, which recognise the specific mRNA. Then the cDNA is amplified with the PCR reaction. A benefit of this method is that the mRNA is converted to a cDNA product, which is not rapidly degraded by RNase and can be easily visualised on an agarose gel stained with ethidium bromide. Another new technique is *in situ* hybridization where mRNA can be detected in the cell.

CHAPTER 4

4. IMMUNOCYTOCHEMICAL LOCALIZATION OF ESTROGEN RECEPTOR α IN DU-145 CELLS

4.1. Introduction

Since clinicians use receptor content in diagnosis of prostate and breast cancer, the accuracy of receptor assays is important. In most biochemical approaches for the determination of receptor content, cells and tissues must be homogenized during preparation of the assay²⁶⁵. A disadvantage of homogenization is that the receptor may be disrupted, causing conformational changes of ER and/or binding to type II and III sites, which would lead to misrepresentations of the actual receptor concentration²⁶⁶. Furthermore, since tumours are often heterogenous in receptor content, homogenization would fail to reveal a high receptor content in individual cells and tissues that comprise a small proportion of the tissue on which they are performed. Therefore, morphological techniques, such as immunohistochemistry, which define the distribution of receptor containing cells within the tumour mass, provide important information regarding tumour heterogeneity both before and after treatment.

In breast carcinomas, cytosolic ER concentrations are used to predict the outcome of endocrine therapy. In most cases ER positive tumours are treated with antiestrogens, although approximately 60% of these tumours do not respond to this treatment²⁶⁵. In contrast to breast carcinomas, the presence of ER in prostatic DU-145 cells could not be confirmed with northern blotting (chapter 3), although positive Scatchard analyses were illustrated in chapter 2 with these cells. Therefore it was decided to turn to immunocytochemistry.

4.2. Materials And Methods

4.2.1 Materials

Primary Antibodies for ER α were obtained from Novocastra (NCL-ER-6F11/2). The detection system LSAB[®]2 (K0675) and 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen (K3466) was supplied by DAKO.

4.2.2 Immunocytochemistry

DU-145 and MCF-7 cells were harvested by trypsinization and fixed to the slide by spraying with 95% ethanol and dried. The control paraffin cuts of breast tissue were deparaffinized in xylene to remove embedded media and hydrated with distilled water. Antigen demasking was exercised in 10 mM citrate buffer (pH 6.0) at 700 W in a microwave oven for a total of 13 minutes (citrate buffer was filled up every 5 minutes), followed by endogen peroxidase blocking in 3% H₂O₂ (15 minutes). The slides were incubated for 90 minutes at room temperature in an ER α antibody solution (NCL-ER-6F11/2), which was reconstituted with 0.1 ml of sterile distilled water as indicated on vial and diluted (1:100) in NGS. The detection method of DAKO, the LSAB[®]2 kit was used according to the manufacturer's specifications. In short, the slide was rinsed and the specimen was covered with the bridging Link Antibody. After 10 minutes at room temperature, the slides were rinsed and the specimen covered with Streptavidin reagent (10 minutes), followed by the DAB chromogen incubation (5 minutes) and rinsing. Hematoxylin counterstain for 2 minutes was performed, whereafter the slides were rinsed once more in water, dipped ten times in 37 mM ammonia water, rinsed in distilled water for 5 times, dehydrated and mounted in Canada Balsam.

4.3. Results

Immunocytochemical staining of breast cancer tissue or MCF-7 cells containing ER α was applied as controls to determine the ER α status of human prostatic DU-145 cells²⁶⁷.

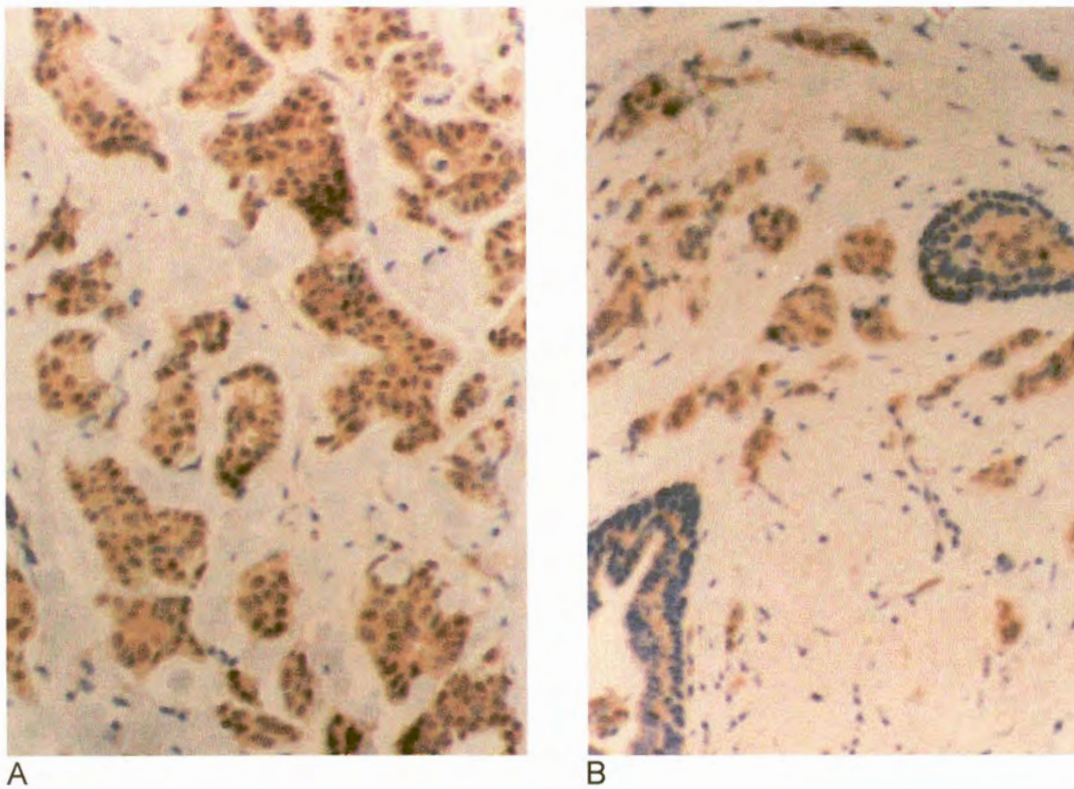


Fig. 4. 1. Immunohistochemical staining of estrogen receptor α (ER α) in breast cancer tissue using the NCL-ER-6F11/2 antibody and the DAB chromogen. A: Brown nuclei indicated the presence of ER α in the breast cancer tissue. B: Blue nuclei indicated the absence of ER α in breast cancer tissue.

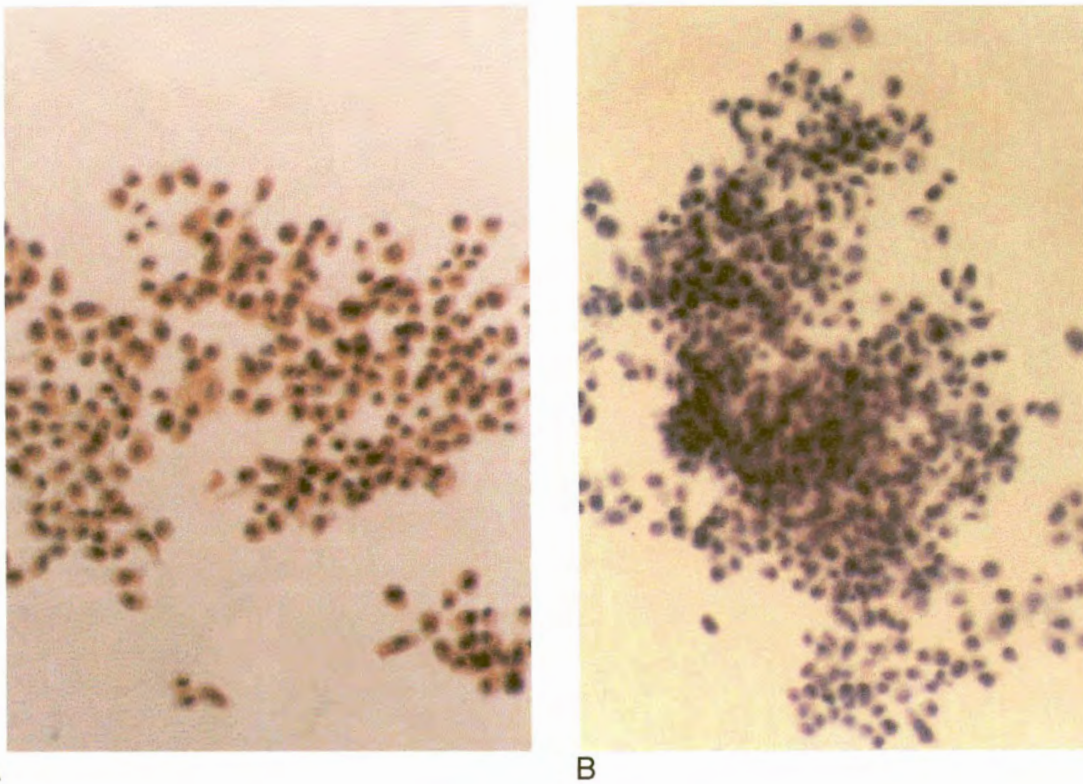


Fig. 4. 2. Immunocytochemical staining of estrogen receptor α (ER α) in MCF-7 cells using the NCL-ER-6F11/2 antibody and the DAB chromogen. A: Brown nuclei indicated the presence of ER α in MCF-7 cells. B: Nuclei of MCF-7 cells stained blue when the NCL-ER-6F11/2 antibody was omitted.

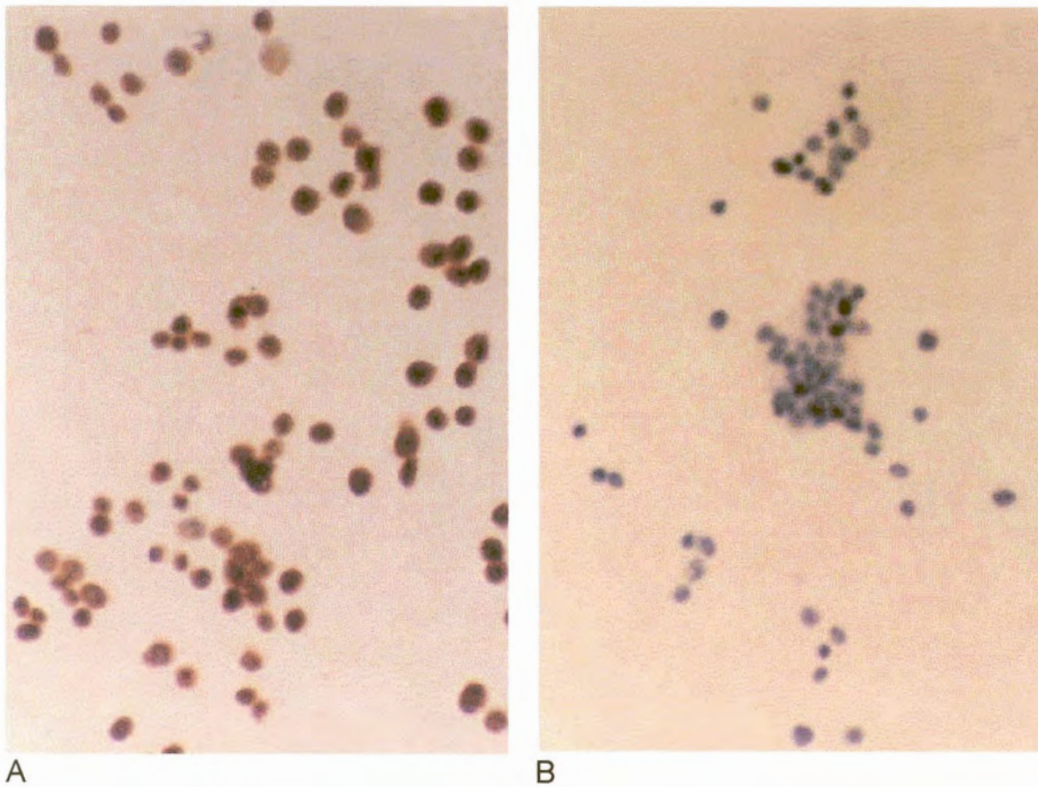


Fig. 4. 3. Immunocytochemical staining of estrogen receptor α ($ER\alpha$) in DU-145 cells using the NCL-ER-6F11/2 antibody and the DAB chromogen. A: Brown nuclei indicated the presence of $ER\alpha$ in DU-145 cells. B: Nuclei of DU-145 cells stained blue when the NCL-ER-6F11/2 antibody was omitted.

4.4. Discussion

Compared to the MCF-7 cells (99%), less DU-145 (70%) cells were ER α positive. Generally all DU-145 cells in clusters stained ER α positive. Some exceptions were found within single cells (not shown). This heterogeneity of the staining pattern is reported previously with MCF-7 cells as well²⁶⁶. From Fig. 4. 2 and Fig. 4. 3 it can be observed that both nuclei and cytoplasm of MCF-7 and DU-145 cells stained ER α positive²⁶⁸. A possible explanation is that except for the limited estrogen content in the FCS, no additional estrogens were added to the media. Additional exogenous estrogen would cause translocation of all the ER α to the nuclei where it would bind to the HRE on the DNA²⁶⁹. Unbound receptors remain in the cytoplasm where it is detected by the ER α antibody.

Previously, ER α was not detected with immunocytochemical staining of DU-145 cells⁹⁸. A possible reason for this phenomenon is that the cells were not treated with trypsin as in this thesis. The action of the trypsin could render the cell membrane more permeable to large molecules such as antibodies. Trypsin is often used in immunohistochemistry for revealing overfixed antigenic sites or to loosen the tissue structure and allow penetration of macromolecules^{270,271}.

In conclusion, the results in Fig. 4. 3 confirmed that ER α is present in DU-145 cells, which is consistent with the Scatchard analysis of ER in chapter 2. It also indicated that the failure to detect ER α mRNA with northern blots (chapter 3) was not due to the absence of ER α mRNA in DU-145 cells.

CHAPTER 5

5. AR AND ER PROTEIN EXPRESSION OF DU-145 PROSTATE CANCER CELLS IN THE PRESENCE AND ABSENCE OF EFA

5.1. Introduction

Within the last 11 years, an exciting age has dawned upon medical research with the development of gene therapy and the first clinical trial in 1990²⁷². Although several trials are in process, gene therapy for prostate cancer remains in its infancy and many future challenges lie ahead^{272,273}. Major medical breakthroughs, which will transform the medical world, are expected in thirty year's time²⁷⁴. In the mean time, the identification of ways other than gene therapy in regulating the expression of target genes are valuable in the search for better treatment of prostate cancer. New, target genes or proteins may be identified applying the new field of proteomics, which aims to discover new molecular targets for therapy, biomarkers of early detection as well as new endpoints for therapeutic efficacy and toxicity^{275,276}. Recently, after genes in normal prostatic and cancer tissues were compared using microarrays, 200 genes from 10 000 genes were identified as possible target molecules²⁷⁷. Although the discovery of new target genes and proteins is essential, the development of new treatments or supplements targeting well-known molecules, remain an option. An example is the popularity of different hormonal treatments for prostate and breast cancer, which target the AR and ER directly and/or indirectly.

Since steroid hormones have profound effects on prostate tumour development and treatment, it is important to identify and define steroid receptor expression in prostate tissues. As already mentioned, certain prostate tumours display AR gene amplification and overexpression of the amplified AR genes, which are associated with acquired resistance to prostate cancer treatment⁴⁹. In

contrast to AR, which is up regulated, ER α and ER β mRNA are down regulated in many hormone-refractory prostate tumours^{4,98,273,278}. Therefore, abnormalities in not only AR expression, but in ER α and ER β expression may influence prostatic cancer development and modulate the growth response to hormone treatment. Since the concentration of a functional steroid receptor is central to the mediation of hormonal effects, regulation or control of steroid receptor gene expression is essential. Although the regulation of steroid hormone receptor expression is still unclear, it is known that steroid hormones, antihormones, growth factors and transcription factors up regulate or down regulate steroid hormone receptor expression^{279,280,281,282,283,284}. For example, both wild type and mutant AR protein were rapidly degraded at a nuclear location during treatment with bicalutamide, a non-steroidal antiandrogen²⁸⁵. Down regulation or elimination of AR, ER α , or ER β can be accomplished by promoter hypermethylation, which prevent the gene being transcribed^{286,287}.

Gene expression can be regulated by EFAs in the adipose cell as well as in tumour cells of the prostate and breast^{172,198,288}. In DU-145 prostate cancer cells, EFAs decreased protein levels of urokinase-type plasminogen activator, which is involved in metastasis¹⁷². In PC-3 prostate cancer cells, the n-6 EFA, AA, up regulated the levels of c-fos and COX-2²⁸⁹. In cultured rat hepatocytes, AA suppressed gene expression of fatty acid synthase (FAS), which is elevated in many human cancers, including prostate cancer^{290,291}. In breast cancer, LA increases the amount of BRCA1²⁹¹. Lastly, in ER positive human breast xenografts, ER expression is reduced when female nude mice are treated with GLA¹⁹⁸. In addition to this effect, the antiestrogen, tamoxifen, and GLA synergistically reduced ER expression. Since EFAs inhibit cell proliferation of DU-145 cells together with T and E₂ binding, which was inhibited and stimulated respectively, the aim of this study was to establish whether EFAs might modify the amount of AR and/or ER in prostate cancer cells. Since controversy in the literature exists regarding the presence of AR and ER α in DU-145 cells, western blots will be employed to investigate the receptors' presence to confirm results obtained in chapter 2 and 4.

5.2. Materials And Methods

5.2.1 Materials

TriPure isolation reagent was obtained from Roche Diagnostics (Boehringer Mannheim Roche Diagnostics SA). The protease inhibitors PMSF, aprotinin and leupeptin were obtained from Sigma-Aldrich. (Atlasville, SA). The Bio-Rad Protein Assay kit was ordered from Bio-Rad Laboratories, Richmond, CA. The ExcelGel 7.5 and 12.5 homogenous PAGE gels as well as the Rainbow™ coloured protein molecular weight markers were manufactured by Amersham Pharmacia Biotech and supplied by Separations Scientific (Honeydew, SA). Immobilon P membranes were obtained from Microsep (Bramley, SA). Primary rabbit antihuman antibodies for ER α and ER β , as well as the secondary monoclonal antirabbit immunoglobulins conjugated with Horseradish peroxidase, were supplied by Sigma-Aldrich (Atlasville, SA). The primary mouse antihuman antibody for AR (NCL-AR-318) was from Novo Castra (Southern Cross Biotechnology, Johannesburg, SA) and the secondary antibody F(ab')₂ goat antimouse IgG was conjugated with Horseradish peroxidase (Serotec, Separations, Randburg, SA). The Super Signal West Pico chemiluminescence Substrate was obtained from Pierce, Rockford, Illinois, USA and the Kodak Scientific imaging film, X-OMAT™AR, was from AEC-Amersham (Kelvin, SA). Film developer and fixer solutions were obtained from Tec Med Imaging (Halfway House, SA).

5.2.2 Cell cultures

Cell cultures were maintained as described under 2.2.2. The MCF-7 breast cancer cell line was maintained in Corning polystyrene flasks (Corning Glass Works, Corning, NY, USA) at 37°C, 5% CO₂ in EMEM nutrient mixture supplemented with 5% decomplemented fetal calf serum, gentamycin sulphate (0.004%), and NaHCO₃ (0.10%). To optimise western blotting conditions, DU-145 and MCF-7 cells were grown in 25 cm² flasks before proteins were extracted. For

EFAs studies, DU-145 cells were plated (1×10^6) in 25 cm² Corning cell culture flasks on the first day. On the third and fifth day, 10 μ M OA, ALA, EPA, LA and GLA were added and incubated for 48 hours and 2 hours respectively. Ethanol was added to the control flask. The ethanol concentration was 0.05%. The EFAs was made up in 100% ethanol with a concentration of 0.095 M. After incubation for 2 hours or 2 days in the presence of 10 μ M at 37°C and 5% CO₂, protein was isolated according to the following methods.

5.2.3 Protein isolation

Three different types of protein isolation methods were evaluated:

5.2.3.1 TriPure method

The TriPure method is designed to isolate RNA, DNA and proteins from a sample. Monolayer DU-145 and MCF-7 cell cultures were grown in 25 cm² flasks till confluent and after the medium was decanted, 3 ml of TriPure solution was added to each flask. After passing the cell lysate through a pipette several times, the cell extract was incubated for 5 minutes at room temperature to ensure the complete dissociation of nucleoprotein complexes. Chloroform (0.6 ml per flask) was added and the mixture was shaken vigorously for 15 seconds. The cell extract was incubated for 15 minutes at room temperature. Centrifugation (12 000 x g) at 4°C for 15 minutes allowed the separation of the mixture into three phases. The clear, top phase contained RNA while the white interphase and the red bottom phase contained the DNA and protein. After the top phase was removed, the DNA was precipitated with 0.9 ml 100% ethanol, inversioned to mix thoroughly, incubated for 3 minutes at room temperature and centrifuged at 2000 x g for 5 minutes at 4°C. The supernatant containing proteins was precipitated with 4.5 ml isopropanol, mixed, incubated at room temperature (10 minutes) and centrifuged (12 000 x g, 10 minutes, at 4°C). The protein pellets were resuspended with 6 ml 0.3 M guanidine hydrochloride in 95% ethanol and incubated at room temperature for 20 minutes, followed by centrifugation at

7500 x g for 5 minutes at 4°C. This washing step with guanidine hydrochloride/ethanol was repeated another two times. A final washing step was performed with 100% ethanol alone and after vortexing, was incubated at room temperature for 20 minutes and centrifuged (7500 x g, 5 minutes, 4°C). The supernatant was discarded and the excess ethanol was removed by air-drying. The protein pellets were dissolved in 1% SDS with repeated passing the solution through a pipette at 50°C. Insoluble material was resuspended by centrifugation (10 000 x g, 10 minutes, 4°C) and the supernatant was stored at -15°C or directly used on a western blot.

5.2.3.2 Water method

The cells were incubated in serum free conditions for 24 hours before they were trypsinized. After treatment with 0.25% trypsin, the action of the trypsin was inhibited with the addition of medium with fetal calf serum. The cells were pelleted by centrifugation (1000 x g, 5 minutes, 4°C), whereafter it was washed with TBS and centrifuged (1000 x g, 5 minutes, 4°C). After the third centrifugation, Alpha Q water was added to the pellets and mixed. Protein concentrations were determined on the GeneQuant reader.

5.2.3.3 RIPA buffer with protease inhibitors

DU-145 and MCF-7 cells were lysed with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 1.0% IGEPAL, 0.1% SDS) containing 1 mM DTT and protease inhibitors (100 µg/ml PMSF, 1 µg/ml Aprotinin, 2 µg/ml Leupeptin) on ice with shaking for 30 minutes²⁹². The lysed cells were scraped from the bottom, passed through a 21 gauge needle and centrifuged (10 500 x g, 10 minutes, 4°C). The supernatant was transferred to a clean tube and the protein concentration was determined *via* the BioRad micro assay. Bovine albumin was used to set up the standard curve.

5.2.4 Dot blots

Dot blots were carried out in order to obtain optimum conditions for western blotting regarding the primary and secondary concentrations, the percentage CasBlock and exposure times. Cell extracts from DU-145 and MCF-7 cells were diluted 1:2 with sample buffer (0.0675 M Tris-HCl pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 0.125% bromophenol blue), boiled for 3 minutes and immediately placed on ice. One microliter of each sample was spotted onto an Immobilon P membrane. The membrane was blocked for 1 hour at room temperature or overnight at 4°C in Cas-Block and treated with the primary AR, ER α and ER β and secondary antibodies for 1 hour each in Tris buffered saline-Tween 20. The secondary antibody contained Horseradish peroxidase. Next, the membrane was incubated in Super Signal West Pico chemiluminescence substrate for 5 minutes and exposed to Autoradiography X-ray film (Kodak) in a dark room. The film was developed by hand (5 minutes), rinsed in running water, fixed (5 minutes) and rinsed again.

5.2.5 Western blots

Proteins of DU-145 and MCF-7 cells, as well as the molecular weight markers were diluted with sample buffer (1:2), boiled for 3 minutes and immediately placed on ice. Cell extract proteins were separated with electrophoresis on homogeneous 7.5% or 12.5% ExcelGel SDS gels, with a Pharmacia Multiphor II (200 V) system. After electrophoresis of 1 hour at 110 V, the gels were equilibrated in transfer buffer for 20 minutes. Proteins were electrotransferred to Immobulin-P membrane with the discontinuous Tris base transfer buffer system (pH 8.1 - 8.4, 25 mM) or with the continuous Towbin transfer buffer system (25 mM Tris, 193 mM glycine, 20% methanol, 0.1% SDS, pH 8.2 - 8.4). The membrane was blocked for 1 hour at room temperature or overnight at 4°C in 1% CasBlock diluted with TBS-T to eliminate non-specific binding of antibodies. After exposure to the primary antibody (diluted 1:1000 in 1% CasBlock TBS-T) for 1 hour, the membrane was washed 6 x 5 minutes in

TBS-T. The washing procedure was repeated after exposure to the secondary antibody (1:10 000 in 1% CasBlock TBS-T) for 1 hour. The SuperSignal West Pico substrate containing luminol and a stable peroxide solution was mixed, applied to the membrane and incubated for 5 minutes at room temperature. Next, the membrane was exposed to X-OMAT™AR Kodak scientific imaging film in a dark room and developed by hand for (5 minutes), rinsed in running water, fixed (5 minutes) and rinsed again.

5.3. Results

5.3.1 Optimization of conditions

Table 5. 1 Optimizing the conditions of DU-145 and MCF-7 cell cultures for use in western blotting in the presence of primary antibodies for ER and AR.

Condition	Cell line			
	DU-145 cells		MCF-7 cells	
	AR	ER	AR	ER
+PR	+++	+++	+++	++
-PR*	++	++	+++	++
+FCS	++	+++	++	++
-FCS	++	++	++	++

*EMEM without PR (Ham's F10 without PR was not commercially available)

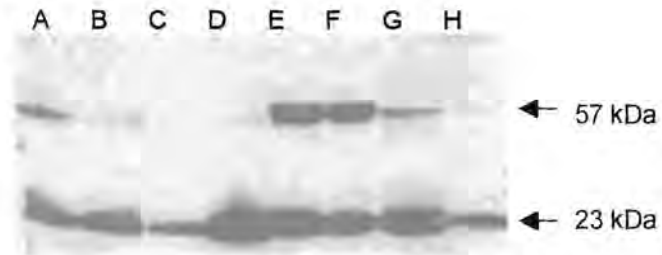


Fig. 5. 1. Western blot of TriPure extracted cell proteins. Cells from DU-145 (lanes C, D, G, H) and MCF-7 (lanes A, B, E, F) cultures were extracted. A western blot with AR (lanes A to D) and ER α (lanes E to H) antibodies were applied to extracts of cells grown in the absence (A, C, E, G) and presence (B, D, F, H) of FCS.

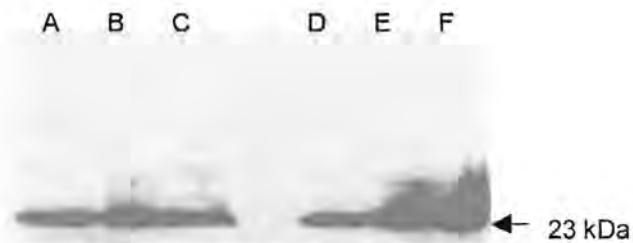


Fig. 5. 2. Western blot of water extraction. Cells from DU-145 (lanes C, F) and MCF-7 (lanes A, B, D, E) cultures were extracted. A western blot with AR (lanes A to C) and ER α (lanes D to F) antibodies were applied to extracts of cells that were grown in the presence (A, C, D, F) and absence (B, E) of FCS.

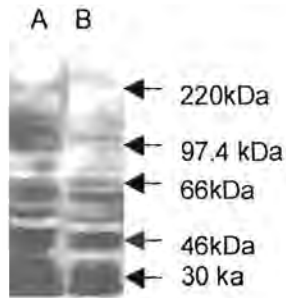


Fig. 5. 3. Western blot of RIPA buffer extraction. Cells from DU-145 (lane A) and MCF-7 (lane B) cultures were grown in the presence of FCS. Antibodies against ER α were used. The arrows indicate the position of the molecular markers.

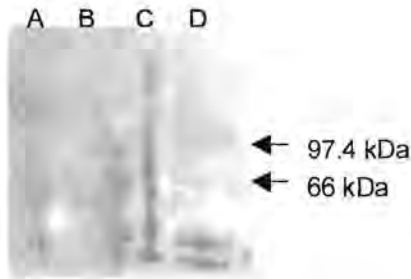


Fig. 5. 4. The optimum amount of Tween 20 in the western blotting buffers was investigated. A western blot of a MCF-7 RIPA cell extract were targeted with ER β antibody under conditions of 0.1% (lanes A and B) and 0.05% (lanes C and D) Tween 20 with 5% CasBlock.



Fig. 5. 5. The optimum amount of CasBlock in the blocking buffer was investigated. A western blot of MCF-7 (lane A) and DU-145 (lane B) RIPA cell extract targeted with ER β antibody in the presence of 1% CasBlock solution.

5.3.2 Effect of EFAs on AR, ER α and ER β expression in DU-145 cells



Fig. 5. 6. Western blot of AR in DU-145 cells after 2 hours incubation with 10 μ M EFAs. OA (lane B), ALA (lane C), LA (lane D), EPA (lane E) and GLA (lane F). Control (lane A) contained 0.05% ethanol. The AR antibody was diluted 1:1000, the secondary antibody 1: 10 000, with 1% CasBlock and 0.05% Tween 20. Electrophoresis was carried out on a 7.5% PAGE gel.



Fig. 5. 7. Western blot of AR in DU-145 cells after 2 days incubation with 10 μ M EFAs. OA (lane B), ALA (lane C), EPA (lane D), LA (lane E) and GLA (lane F). Control (lane A) contained 0.05% ethanol. The AR antibody was diluted 1:1000, the secondary antibody 1: 10 000, with 1% CasBlock and 0.05% Tween 20. . Electrophoresis was carried out on a 7.5% PAGE gel.

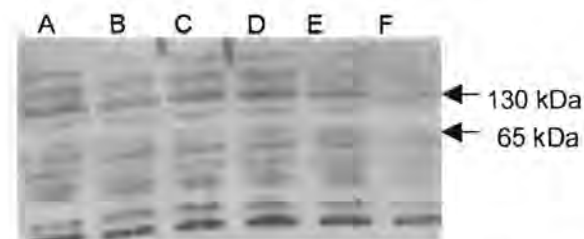


Fig. 5. 8. Western blot of ER α in DU-145 cells after 2 hours incubation with 10 μ M EFAs. OA (lane B), ALA (lane C), EPA (lane D), LA (lane E) and GLA (lane F). Control (lane A) contained 0.05% ethanol. The ER α antibody was diluted 1:1000, the secondary antibody 1: 10 000 with 1% CasBlock and 0.05% Tween 20. Electrophoresis was carried out on a 12.5% PAGE gel.

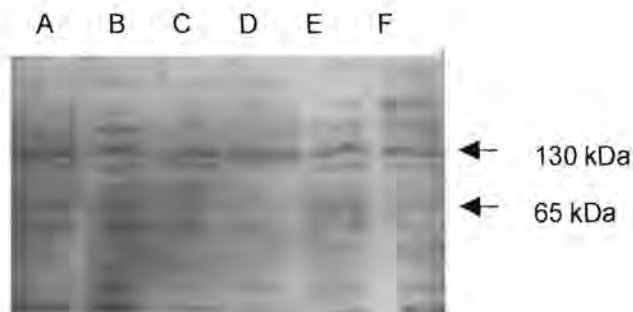


Fig. 5. 9. Western blot of ER α in DU-145 cells after 2 days incubation with EFAs. OA (lane B), ALA (lane C), EPA (lane D), LA (lane E) and GLA (lane F). Control (lane A) contained 0.05% ethanol. The ER α antibody was diluted 1:1000, the secondary antibody 1: 10 000 with 1% CasBlock and 0.05% Tween 20. Electrophoresis was carried out on a 12.5% PAGE gel

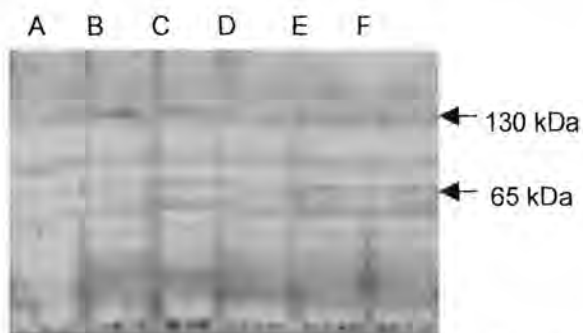


Fig. 5. 10. Western blot of ER β in DU-145 cells after 2 hours incubation with EFAs. OA (lane B), ALA (lane C), EPA (lane D), LA (lane E) and GLA (lane F). Control (lane A) contained 0.05% ethanol. The ER α antibody was diluted 1:1000, the secondary antibody 1: 10 000 with 1% CasBlock and 0.05% Tween 20. Electrophoresis was carried out on a 12.5% PAGE gel

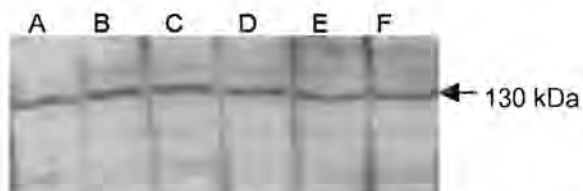


Fig. 5. 11. Western blot of ER β in DU-145 cells after 2 days incubation with EFAs. OA (lane B), ALA (lane C), EPA (lane D), LA (lane E) and GLA (lane F). Control (lane A) contained 0.05% ethanol. The ER α antibody was diluted 1:1000, the secondary antibody 1: 10 000 with 1% CasBlock and 0.05% Tween 20. Electrophoresis was carried out on a 12.5% PAGE gel

5.4. Discussion

Several substances such as hormones, growth factors, serum proteins and estrogenic substances like phenol red (PR) are present in medium containing serum. Since these compounds may influence steroid hormone expression, ER and AR levels were determined in the presence and absence of PR and FCS (Table 5. 1). However, the dot blots in Table 5. 1 indicated that the intensity of recognition with both AR and ER α antibodies between cell extracts from cells grown with or without serum were not different from each other. No difference was found between the dots with and without phenol red although phenol red was believed to contain estrogenic activity²⁹³. However, recently it was demonstrated that the concentration of phenol red in medium is not sufficient to cause estrogenic effects²⁹⁴.

In contrast with the TriPure (Fig. 5. 1) and water (Fig. 5. 2) extractions, the extraction applying the RIPA buffer (Fig. 5. 3) did not lead to degradation of the protein to a pronounced 23 kDa band, possibly due to the presence of protease inhibitors. Therefore, it was decided to use the RIPA buffer extraction method with proteinase inhibitors for subsequent electrophoresis studies. Furthermore, better ER α and ER β results were obtained with the 12.5% PAGE gel than with the 7.5% PAGE gel used (not shown).

To reduce non-specific binding and high background Tween 20 can be added to the blocking and antibody solutions. Therefore the optimum amount of Tween 20 to add was investigated (Fig. 5. 4). Bands were more visible in the presence of 0.05% Tween 20 than in the 0.1% Tween 20 buffer. This may be due to the fact that high concentrations of Tween 20 can elute the proteins from the membrane. Since bands were not clearly visible in the presence of 5% CasBlock, it was decided to decrease the CasBlock blocking agent from 5% to 1% CasBlock in the blocking and antibody solutions (Fig. 5. 5).

Western blots with the AR antibody revealed a band at 110 kDa^{12,13}. However, no significant visual effects were seen with the AR expression after 2 hours or two days of exposure with OA, ALA, EPA, LA, or GLA.

Different protein bands (130 kDa, 65 kDa, 61 kDa, 49 kDa, 24 kDa) were seen (Fig. 5. 9, Fig. 5. 10) when using the ER α antibody and compared with several variants of ER α as reported previously^{295,296}. The possible dimer (130 kDa) was more pronounced than the single band at 65 kDa after both exposure times to EFAs, which may indicate that the receptors were in the nuclear state^{17,18}. This was also the case with ER β . No significant visual effects were seen with ER α and ER β expression after 2 hours or two days of exposure with OA, ALA, EPA, LA, or GLA, indicating that the EFAs in this study did not affect the expression of the receptor proteins.

To conclude, western blotting confirmed the presence of AR, ER α and ER β in DU-145 cells. Furthermore, EFAs did not affect the total expression of AR, ER α and ER β in this prostatic cancer cell line, since they were visually similar. A possible reason for this observation may be that the EFAs was prevented from exercising an effect on the receptor concentration, since they were bound to the FCS albumine²⁹⁷. This avenue needs to be investigated further.

CHAPTER 6

6. CONCLUDING DISCUSSION

Over recent years, the importance of a healthy diet and lifestyle has become more evident in the prevention of prostate cancer. Therefore, the objective of this study was to investigate the hypothesis that EFAs modulate ER and AR via the binding parameters or amount of ER and AR in the prostate cell. However, the results showed that binding was affected and not ER and AR protein expression.

Since the presence of AR and ER α receptors in prostate cancer is controversial, their presence in DU-145 cells was investigated applying whole cell radiolabelled binding assays to measure specific binding of labelled T and E₂ to AR and ER receptors in the cells. The binding affinity (0.698 ± 0.210 nM) and capacity (54.1 ± 6.5 fmol/mg protein) for ER correlated well with other studies indicating a type I binding site for ER α and/or ER β . Since activation of ER α and ER β may have different cellular responses, it may be useful in future studies to separate the effects of EFAs on these two receptors. Here, ER negative cells could be transfected with either ER α or ER β , whereafter Scatchard binding studies could be employed in the presence of EFAs. The binding affinity (12.12 ± 4.68 nM) and capacity (210.3 ± 20.0 fmol/mg protein) for the AR compared with type II binding sites in the literature and the importance of these binding sites in human DU-145 cells is still unclear.

From the EFAs under investigation EPA (n-3) and LA (n-6) inhibited T binding to AR uncompetitively and competitively respectively, while OA (n-9), ALA (n-3) and GLA (n-6) inhibited binding of T non-competitively. Compared to the control, EPA demonstrated the largest decrease in the binding capacity (Control = LA > OA = ALA > GLA > EPA) and largest increase in affinity of AR for T (Control = LA = OA = ALA = GLA < EPA). The decrease in capacity and increase in affinity indicated that EPA caused less T to bind to AR, but more tightly. Chain length of the EFAs seemed to influence the T binding the most,

since EPA had the longest chain length of the EFAs under investigation. These results are in accordance with the hypothesis that EFAs would inhibit AR binding in DU-145 cells.

In contrast to the AR, the EFAs generally stimulated binding to the ER, since the Scatchard diagram lines were to the right of the control and OA lines. The different EFAs increased and reduced the ER binding capacity (Control = OA < LA < GLA < EPA < ALA) and affinity (Control = LA = OA = GLA > EPA > ALA) respectively. Oleic acid had no significant effect, while the n-3 EFAs, EPA and ALA showed the largest effects on both binding capacity and affinity. This indicated that n-3 EFAs increased the number of binding sites for E₂, which was bound more loosely. Furthermore, the position of the double bonds is important, since the n-3 EFAs had a greater effect than the n-6 EFAs. Although the stimulation of ER binding is contrary to the hypothesis that EFAs would inhibit ER binding, a possible beneficial role will become clear in the next paragraph.

Since low doses of estrogen are used in prostate cancer treatment, the addition of the n-3 EFAs may enhance the binding to the steroid receptor with a positive effect on the prostate cancer patient. The growing interest in antitumour molecules synthesized by plants has led to the discovering of the phytoestrogens as anticancer agents. Therefore, the EPA and ALA may enhance the beneficial effect of these phytoestrogens on prostate cancer, especially since side effects may be reduced. Furthermore, since the phytoestrogen, genistein may act as an antiandrogen and EPA inhibited T binding to DU-145 cells, a possible synergistic effect of these two naturally occurring compounds need to be investigated¹³⁰. These investigations may be focused more towards the ER β , since phytoestrogens have a higher affinity for ER β than for ER α .

Since certain EFAs altered the binding capacities of the receptors, the question arose whether this phenomenon was due to a direct effect on the number of binding sites on each receptor, or indirectly due to an alteration in the number of receptor molecules. The literature indicates the presence of AR and ER β in DU-145 cells, but not ER α . Unfortunately, the presence of ER α mRNA could not be confirmed in DU-145 cells, since the ER α probe was discontinued

from the market and probes for AR and ER β were not yet commercially available at the time of this study. Immunocytochemistry confirmed that ER α was present in DU-145 cells and that the failure to indicate ER α was not due to the absence of ER α mRNA. Western blotting proved the presence of ER α , ER β as well as AR in DU-145 cells. However, EFAs generated no effect on the protein expression of these receptors. Therefore it was concluded that EFAs caused a direct effect on receptor binding capacity and not indirectly by protein expression of AR, ER α and ER β . Further studies may be necessary, since binding of EFAs to serum proteins in the medium could possibly explain the inability of EFAs to inhibit or stimulate the expression of the receptor proteins²⁹⁷.

Although, the EFAs may inhibit or stimulate the nuclear ER and AR directly, another possibility is that the EFAs may alter the physical properties of the plasma membrane. The EFAs may increase the fluidity and thus the permeability of the membrane for steroid hormones. The classic description of an estrogen receptor is that of a nuclear or cytoplasmic receptor, but in the last decade it has been proposed that steroid hormone receptors or steroid binding proteins are also expressed on membranes such as the plasma membrane and microsomal membranes. Nuclear and membrane steroid receptors have a genomic and non-genomic function respectively. Therefore, the alteration in plasma membrane properties may affect the binding characteristics of the membrane steroid hormone receptors as well. The membrane androgen receptor may play a role in the indirect effects of androgens on genes that do not contain AREs in the nuclei²⁹⁸.

Changes in the composition of the nuclear membrane may influence translocation of steroid hormones across the nuclear membrane by altering the lateral pressure profile of this membrane. The lateral pressure profile will likely interfere with the structural arrangements of the protein-translocating machinery. Therefore, it may be interesting to separate cytoplasmic and nuclear fractions to investigate a possible role for EFAs in translocation of the AR and ER across the nuclear membrane²⁹⁹.

Binding of T and E₂ to AR and ER respectively initiates specific transcription activation events, resulting in the transcription and expression of T or E₂ responsive genes. The expression of these genes could be measured as an endpoint for the effect of EFAs on the steroid hormone message. However, the quantification of T or E₂ responsive genes may be laborious and difficult. Therefore, the introduction of artificial, ER-regulated reporter gene constructs, such as the luciferase gene into MCF-7 cells may be employed³⁰⁰. Likewise, the effect of EFAs on the AR endpoint may be investigated by the transfection of CV-1 cells with a stable human AR containing a promoter construct with a luciferase reporter gene. Luciferase activity can easily be detected in a cell lysate.

To conclude, the results of this study confirmed that men of all races could benefit by including EFAs rich foods in their diets, especially the n-3 EFAs, EPA and ALA, which occur in seafoods. The mechanism of action of these EFAs may include the modulation of the estrogenic and androgenic messages in the normal and diseased prostate. Although more research and clinical studies are needed to evaluate the conditionally dispensability and indispensability of EFAs in prostate cancer, certain EFAs may delay the onset of this common disease for a decade or two.

CHAPTER 7

7. FUTURE PROSPECTS

A better understanding of steroid hormone receptor function in the normal prostate, as well as in hormone-responsive and hormone-unresponsive prostate cancers may provide a starting point for development of better treatment methods for prostate cancers. This study has shown that EFAs may alter ER and AR binding parameters and therefore alter the estrogen and androgen messages in prostate cancer cells, respectively. These properties may be applied to benefit the health of men from all races. Since lipid and fatty acid biochemistry has previously been neglected in many respects compared to protein and nucleic acid research, it is likely that more clinical advances in the 21st century may come from manipulating lipids and fatty acids.

For example, the results of this study may apply to other hormone-related disorders such as PMS and osteoporosis in women. In some women suffering from premenstrual irritability and dysphoria, serum testosterone levels are higher than in controls³⁰¹. Since EFAs inhibit testosterone binding, the administration of oils rich in EFAs may decrease the action of testosterone and in this way ease some of the symptoms associated with PMS. Premenstrual syndrome can successfully be treated with estradiol^{302,303}. Therefore, EFA administration may stimulate the binding of endogenous estrogens to the ER and relieve some of the symptoms without having to apply estradiol treatment, which may have dangerous, long lasting effects. This combined effect of inhibition and stimulation of androgen and estrogen binding, respectively, may explain the success in some women treated for PMS with EFAs³⁰⁴. However, steroids such as dehydroepiandrosterone, pregnenolone and alloprenenolone, which can be classified as the neuroactive steroids, may too be influenced by EFAs³⁰⁵. These steroids play a role in mood disorders, such as PMS and bind to membrane

receptors. Therefore it could be valuable to investigate the effects of EFAs on these membrane receptors. Lastly, in women suffering from PMS, LA and the n-3 EFA levels were elevated in contrast to GLA levels which was low, probably due to a deficiency of Δ -6 desaturase³⁰⁶. Therefore, oils with high levels of GLA, such as evening primrose oil can be considered in the treatment of PMS.

The concept of ER stimulation may be applied in osteoporosis as well, since bone density decreases with estrogen deficiency and increases with the administration of estrogens or EFAs^{307,308}. When estrogen is used in combination with a diester consisting of GLA and EPA, bone density increased synergistically. This synergistic effect may partly be explained by the results obtained in this study where EFAs increased estradiol binding to ER. Another disease in which estrogen deficiency plays a role and EFA intake may benefit the patient is Alzheimer's disease³⁰⁹. Further research on EFAs regarding the ageing person could be of extreme value especially since man is expected to live longer due to medical advances. Interestingly, certain EFAs may prevent the age-related decrease in the number of spermatozoa in chickens³¹⁰.

The result of steroid hormone action in the cell includes proliferation, metabolism, biosynthesis and secretion. Since these are complex processes, it may be difficult to explain how a steroid could manage all these functions *via* chromatin-directed signals alone. Therefore, it has been postulated that there may be different mechanisms involved, which include cross-talk between steroid hormone and growth factor pathways as well as signalling *via* non-genomic membrane proteins. Most investigators agree that changes in all kinds of hormones and their receptors occur along with malignant transformation of normal prostatic epithelial cells³¹¹. It is hypothesized that no receptor – ligand system in the body is completely isolated from another and that the disruption of the normal prostate with its intertwined system of different ligands, receptors and co-factors, leads to prostatic cancer. To solve this complexity, the need for application of computational techniques is imperative. Computational techniques have already been used in data base construction, to assign patient symptoms to disease category and to predict medical outcome of treatment³¹². In prostate

cancer, these artificial neural networks are computer-based statistical models and have been used in diagnosis, predicting response to therapy and recurrence³¹³. The sensitivity of the network can be 81% to a 100%³¹⁴. Other computer programs exist which can simulate multiple interactions between cells to produce a visual interpretation that represents tissue growth and differentiation^{315,316}. These programs are user friendly and enable the researcher with almost no computer experience to design complex models of cell interactions on the World Wide Web and it can also be an excellent resource for teaching³¹⁵.

In the new information technology era, the growing application of the computer is inevitable. However, all the high-tech in the world would come to no avail, if man does not put the theories into practice and change his ways. It may be easy to change a diet to higher levels of healthy EFA consumption, but increasing pollutants in our food, water and environment are a major concern³¹⁷. In some EFA studies, which show an increase in cancer risk with the consumption of EFAs, environmental pollutant chemicals are not taken in consideration. Chemical pollutants usually accumulate in the fat stores of animals and humans, causing a greater health risk in obese patients with an increased body burden of chemical pollutants, especially during weight loss^{318,319,320}. Although controversies exist, clear associations of environmental pollutants in adipose tissue with breast cancer risk have been demonstrated³²¹ and correlations with prostate cancer risk may soon follow. Therefore, the question could be asked whether EFAs may play a role in protecting the body from harmful chemicals by neutralizing the binding of endocrine disrupter chemicals to hormone receptors? Although, the possibility exists that the environmental chemicals may react with the EFAs to increase the toxicity of the pollutants³²², EFAs may enhance total-antioxidant status to provide increasing protection against destructive man-made chemicals³²³. All these avenues need to be explored to maintain general health of the population.

Although more laboratory research, clinical trials and computer programs, would bring us closer to winning the battle against diseases like prostate cancer,

cancer is increasing in this modern, restless and hurried society, urging man to re-evaluate and alter his lifestyle. Personal changes may be small and even cheap, but could create an immense difference in the quality of life. However, global changes is more difficult to attain and a lot more expensive and complex, testing the resolve of researchers, health workers, doctors and governments across the globe in the fight against cancer and other illnesses.

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