PREDICTING PROGNOSIS IN PROSTATE CANCER

An evaluation of the role of serum prostate specific antigen and DNA flow cytometry

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A thesis submitted for the degree of Master of Surgery
University of London 1991

ABSTRACT

Prostate cancer is a disease characterised by a wide variation in its natural history. By accurate prediction of prognosis we can stratify clinical trials to answer the question, does our treatment work?. Furthermore the widespread application of a deferred treatment policy may not be in all patients' interest. Identifying those at risk of progression is a priority. Two recent developments, serum prostate specific antigen (PSA) and DNA flow cytometry promise better determination of prognosis but they have yet to be evaluated in a systematic way and in parallel to existing determinants. This work addresses that task. PSA is evaluated in various current patient and reference populations and in a group of prostate cancer patients recruited in 1984. The latter are also the subjects for the flow cytometry study using archival histological material. PSA has been shown to be of limited value in the initial diagnosis of prostate cancer. It has, however, been shown to be a powerful prognostic indicator and a useful adjunct to conventional staging procedures. This work also suggests that PSA has a role in identifying those patients for whom deferred treatment is an appropriate management policy. Multifactorial analysis indicates that serum PSA may be used as an alternative to isotope bone scanning in the detection of disease dissemination. DNA flow cytometry has been shown to be a weak predictor of progression and did not predict survival over the period studied. There was a significant correlation between histological grade and DNA flow cytometry. Multifactorial analysis showed that this

technique added no significant extra prognostic information once histological grade, the presence of bone metastases, age and haemoglobin were taken into account. In its present form it adds little to our prognostic armamentarium.

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List of abbreviations

BNI	Bladder neck incision
BPH	Benign prostatic hyperplasia
BSO	Bilateral subcapsular orchidectomy
CV	Coefficient of variation
Cr	Creatinine
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetracetate
eg	Exempli gratia (Latin) - for example
EORTC	European Organisation for Research into the Treatment of Cancer
FCM	Flow cytometry
Hb	Haemoglobin
ie	Id est (Latin) - that is to say
OPCS	Office of Population Census Surveys
PAP	Prostatic acid phosphatase (immunologically measured)
PSA	Prostate specific antigen
ROC	Receiver Operator Characteristics
RNA	Ribonucleic acid
TNM	Classification system for malignant tumours
	T The extent of the primary tumour
	N The absence or presence and extent of regional lymph node metastasis
	M The absence or presence of distant metastasis
TURP	Transurethral resection of the prostate
UICC	Union Internationale Contre le Cancer
VACURG	Veterans Administration Cooperative Urological Research Group
vs	versus

Acknowledgements

I wish to express may thanks to the large number of individuals whose help was invaluable. Mr P H Powell, Consultant Urologist, Freeman Hospital, Newcastle upon Tyne was generous both with his time and his continued support. He provided details of patients he used for another study in 1984 which were ideal for my purposes. Funding for my salary for one year was kindly provided by the Newcastle Health Authority Research Committee. Hybritech S A Liege Belgium kindly provided the PSA kits free of charge and Mr R R Hall, Consultant Urologist, Freeman Hospital was instrumental in this arrangement. Mr I Gibb, now Principle Biochemist, Royal Victoria Infirmary, Newcastle assisted in teaching the laboratory assay techniques and in performing about half the assays himself, the remainder I performed myself. Dr M C Robinson, Consultant Histopathologist at the Freeman Hospital spent many hours selecting cancer bearing chips and in reviewing and grading the histological material. Laboratory help was given by Mrs K Denton who cut, dewaxed and digested the sections for flow cytometry. Dr A Givan, Department of Surgery, The Medical School, University of Newcastle operated the flow cytometer. Further assistance in this regard was given by Dr B Shenton also at the Department of Surgery. Dr John Matthews, Reader in Medical Statistics, University of Newcastle upon Tyne provided statistical advice and performed the Cox's proportional hazards analysis. In addition I would like to thank Mr G Williams my supervisor for his guidance and support.

Finally I would like to acknowledge the debt I owe my family. The devotion of large amounts of time to this work involved sacrifices on the part of my wife and children. I thank them for their forbearance and encouragement.

Personal Statement

The work presented in this thesis was conceived, planned and performed by myself. I am also solely responsible for the analysis and interpretation of the results. I have made full acknowledgement of all assistance received.

DEDICATION

To my wife Sarah and our children James, Edward and Jennifer

INTRODUCTION

Prostate cancer - historical background

Incidence

Deferred treatment

Predicting prognosis

Histological grading

Tumour staging - T category

- Lymph node stage

- Metastases

Serum Biochemistry - Acid phosphatase

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- Performance status

- Ureteric obstruction

- Clinical features

New prognostic indicators

Prostate specific antigen

DNA flow cytometry

Multiple factor analysis

Prostate cancer - historical background

The word "prostate" is derived from the Greek "pro" and "states", literally "one that stands before". The development of early knowledge of the prostate and prostate cancer has been described by Murphy (1972). He states that the first detailed description of the prostate was by Nicolo Massa, a Venetian, in 1536 and it was illustrated in Versalius' Tabulare Anatomicae, 1538. With regard to the pathological enlargement of the prostate, according to Murphy (1972), Oribasius 325-403 AD described abnormal induration of the bladder neck presumably due to prostatic disease. John Hunter (1810) described lateral lobe enlargement, narrowing the lumen of the prostatic urethra and the projection of the enlarged middle lobe into the base of the bladder. Until the 19th century there remained confusion between prostatic hypertrophy and malignancy. Indeed in 1797 Baillie asserted that "the most common disease of the prostate gland is a scirrhous enlargement of it ". Benjamin Brodie, stated in 1849, "Malignant diseases of the prostate are of very rare occurrence". Despite this he later describes 2 cases of undoubted prostate cancer. The first was a man of 50 with urinary symptoms, weight loss and a large indurated gland in the groin, one of the earliest observations of lymphatic spread. The second a man of about 60 who developed symptoms of bladder outflow obstruction requiring catheterisation and bone pain of marked severity. Brodie commented that, "I have never seen a human being whose sufferings were more intense; and they were scarcely mitigated by the exhibition of very large doses of opium". This man later

became paraplegic and died shortly afterwards. Clearly he had disseminated bone disease and this represents one of the first descriptions of such a case. Von Recklinghausen described the typical osteoblastic bone metastases in 1891.

Albarran and Halle (1900) published a review of 100 cases of prostatism that had been treated surgically. The excised tissue was examined histologically and it was determined that 14 were in fact neoplastic not merely benign hyperplasias. They described the histological features of prostate cancer and how to distinguish it from benign hyperplasia. They also pointed out that whilst most men with prostate cancer died within a few months some patients diagnosed early following surgery for bladder outflow obstruction survived several years. These cancers were described by Albarran and Halle as "latent". More recently, latent carcinoma of the prostate is taken to mean that the disease was totally asymptomatic and therefore diagnosed only at post-mortem.

The first extensive review of the natural history of prostate cancer was carried out at the Mayo Clinic, in the USA, by Bumpus (1926). He examined the records of 1000 cases presenting to that institution with prostate cancer in the years prior to 1925. 650 patients presented with prostatism and 156 presented with bone pain. He observed that in the 485 cases in which no treatment was given the average duration of survival was 31 months. In contrast, he found that in the presence of metastases diagnosed on plain X-ray, 2/3 of the patients were dead within 3

months. This observation taken in conjunction with Albarran and Halle's regarding the better survival of incidentally diagnosed prostate cancers constituted the first steps in predicting prognosis in this disease. Bumpus, however, commented on the great difficulty in predicting the clinical course of an individual. He quotes an example of a man whose metastatic prostate cancer was diagnosed after a malignant inguinal lymph node was excised and who was still alive after 10 years with no treatment - " a vivid illustration of the fallacy of attributing to any form of treatment all the favourable results obtained". Despite the wisdom of this remark, large properly controlled trials of treatment modalities in prostate cancer were not undertaken until the 1960s.

With regard to treatment, the first radical surgical cure was attempted by Young in 1905 at the Johns Hopkins Hospital, Baltimore USA. He performed perineal prostatectomies on patients presenting with bladder outflow symptoms. He knew from Albarran and Halle's work that within such a population of patients there would be perhaps 10% with prostate cancer. He described 6 cases were he had performed the conventional enucleation procedure only to find that on histological examination the prostates were cancerous. He noted the all were hard and this led him to be suspicious of indurated glands. He reviewed the current literature and concluded that partial prostatectomy was associated with an unacceptable local recurrence rate and survival. He then developed an operation performed through a perineal approach which involved the removal of the entire prostate together with

the seminal vesicles and part of the bladder base. This was performed on 4 patients in whom the diagnosis of cancer was made intraoperatively on the basis of careful palpation. It was felt at that time that lymph node metastasis was a late event and therefore node excision was unnecessary.

At the same time across the Atlantic in England, a conservative approach was favoured. Suprapubic prostatectomy had recently been developed relying on the enucleation of the adenomatous tissue (Freyer 1901). The high recurrence rate after the attempted enucleation of what must have been fairly advanced tumours led Harrison (1903) to write, "They (prostate cancer patients) are not cases which should be submitted to prostatectomy. At all events, the prospect of recurrence should have full consideration". Indeed, Harrison favoured simple palliative procedures such as the fashioning of a suprapubic cystostomy. Oddly, the same surgeon performed an inguinal vasectomy on a patient with bladder outflow symptoms thought to be due to benign prostatic hyperplasia and achieved some relief for his patient. Possibly this was in fact due to infarction of the testicle (this had already occurred on the other side due to a previous varicocele operation). Yet it was only after the work of Huggins that hormone manipulation became commonplace (Huggins et al 1941). The different approach to prostate cancer, aggressive and radical in the USA, conservative in the UK persists to this day. The evolution of the various treatment options is discussed later but despite many

advances throughout this century prostate cancer remains common and still carries a high mortality.

Incidence

In England and Wales 7447 prostate cancer cases were registered in 1979 (Office of Population Census Surveys - OPCS - 1986). The mean age at registration was 73.7 years. In the Northern Region with an estimated male population of 1,503,300 there were 444 cases registered in 1982 (OPCS 1985), an annual incidence of 29.5 per 100 000 man years. With regard to mortality in England and Wales prostate cancer is the second commonest cause of death from cancer (Table 1). A similar situation with respect to mortality patterns pertains in the United States where an estimated 24,100 men die from the disease each year (Silverberg and Lubera 1983) making it second in the cancer death league as in England and Wales.

Table 1

1986 Male Cancer Deaths
England and Wales*

Trachea, bronchus and lung	25 235
Prostate	6 910
Stomach	5 937
Colon	4 937
Rectum, rectosigmoid junction and anus	3 161
Pancreas	2 950
All neoplasms	73 717

*OPCS 1988a

Deferred treatment

The concept of deferring treatment in prostate cancer patients is not new. Driven by the fear that treated patients may fare worse than those who are not, clinicians have inclined towards simple measures aimed at symptomatic relief rather than "curative" procedures. As already discussed, by the beginning of the 20th century there were divergent opinions as to the optimum management policy for prostate cancer. Bumpus' review (1926) contained a number of patients who were totally untreated and these died rapidly. However patients whose outflow obstruction was treated by simple suprapubic cystostomy appeared to enjoy better survival (37% at 5 years) than those treated with radiotherapy or surgery(approx 30%). For this reason Bumpus recommended a conservative approach to treatment. Since that time a further treatment modality has become available, namely hormone manipulation. The Veterans Administration Cooperative Urological Research Group (VACURG) studies which began in 1960 provided the first scientific justification for a deferred treatment policy. Study 1 (VACURG 1967) randomised 2017 patients with stage III and IV prostate cancer into four treatment arms, orchidectomy +/- placebo, oestrogen (5mg diethylstilboestrol), orchidectomy + oestrogens and placebo alone. 484 patients received placebo alone. The 5 year survival rates were no different from the other treatment arms and indeed those who had received oestrogens showed an increase of cardiovascular deaths. The authors suggested that "therapy by estrogen or orchiectomy should be withheld until the patient's

symptoms are so severe as to require relief". Further studies were undertaken. Study 2 was commenced in April 1967, this compared different doses of oestrogens, 0.2mg, 1mg, 5mg diethylstilboestrol with a placebo (Byar 1972). Survival amongst the placebo group was again comparable to that of the other groups. A review of patients taken off their assigned treatment showed that more patients receiving placebo were taken off study than those in other treatment groups (Hurst and Byar 1973). In stage III disease (locally advanced) 40% of the placebo patients were taken off study, whilst only 24% of those receiving other treatments were taken off. For stage IV disease (metastatic) the figures were 70% and 34% respectively. It could not be shown that hormone manipulation had lost its efficacy in the relief of symptoms as a consequence of deferring its use, and although more patients in the placebo group were taken off study to receive treatment, their median survival was longer so that overall it could not be shown that the placebo patients suffered any deleterious effect in terms of survival.

In a retrospective study, Parker et al (1985) compared the outcome of patients who had been treated at diagnosis with those who had been managed by a deferred treatment policy. They found no adverse effect on survival by delaying treatment and reported an overall 5 year survival of 50%. At the Freeman Hospital, Newcastle upon Tyne all asymptomatic patients are managed by a deferred treatment policy. In a retrospective review we found a 29% 5 year overall survival (Handley et al 1988). At first this

seemed low, but the overall VACURG 5 year survival was only 42% in younger patients. The Office of Population Census Surveys (1986) figures for 5 year survival of all cases of prostate cancer diagnosed in England and Wales in 1979 is 30% in line with our findings. Within 5 years 49% of our patients required treatment for prostate cancer (a similar figure to the placebo arm of the VACURG study), 18% died from intercurrent illness and 11% did not need treatment. 17% however died apparently from prostate cancer without receiving further treatment. A proportion of these deaths will have been wrongly ascribed to prostate cancer but it appeared that some patients progressed and died rapidly between follow-up visits.

Predicting Prognosis

If a deferred treatment policy is to be successfully applied it seems that patients at high risk of symptomatic progression should be identified and offered treatment or followed up more closely. In addition it should be possible to identify groups of patients at low risk of progression who could be reassured and seen less often. There are many ways in which prognosis in prostate cancer can be predicted. The existing established prognostic factors will be reviewed. The state of knowledge up to the start of this work relating to prostate specific antigen (PSA) and DNA flow cytometry will also be examined.

Histological grading

The concept that the microscopic appearance of a tumour,

its degree of differentiation, correlates with the clinical course of the disease was first introduced by Broders in 1920. In carcinoma of the lip he demonstrated that the tumour could be graded and that this determined the likely outcome for the patient. Although there had been a few limited previous attempts the first systematic grading of prostate cancer was undertaken by Evans et al (1942). They concluded that grading based largely on the architecture of the acini, the cell shape and the nuclear morphology was of value in predicting the survival of prostate cancer patients. In addition it appeared that metastatic disease was correlated with poor histological grade.

Since that time according to Mostofi et al (1987) over 30 grading schemes for prostate cancer have been proposed. Possibly the best known system is that developed by Gleason (1966). It is based on an assessment of glandular architecture made using low power microscopy. Nuclear morphology is not considered. The predominant pattern of glandular architecture is given a score ranging from 1 to 5, the secondary pattern is similarly scored. The two scores are usually added together (a homogeneous tumour would have its score doubled). The system cannot be used on cytological specimens such as Franzen needle biopsies. In a review of 1032 patients entered into the Veterans Administration studies first started in 1960, Gleason examined the correlation of his system with both stage and survival (Gleason and Mellinger 1974). With regard to stage it was found that 52% of stage IV (metastatic) tumours contained areas with a Gleason score of 4 or 5. In

contrast such areas were present in only 13% of patients with localised disease (stage I + II). However it is noteworthy that Gleason score 3 areas were found in a high proportion of tumours regardless of stage, 90% in stage IV, 85% in stages I + II. There was a stepwise increase in mortality with increasing Gleason score. Considering the predominant pattern only, the difference was greater than threefold, that is 0.09 vs. 0.3 deaths per patient year. There was only a small difference when the secondary pattern was considered alone, .15 to .21. Despite the poor correlation between the secondary pattern score and survival, according to the Gleason system this is added to the primary pattern score to produce a total score or Gleason grade. Although it was shown that for each increase in Gleason grade the mortality rose, 61% of patients had grades of 5 or 6. Conversely only 6.2% had grades of 2, 3 or 4. For practical purposes therefore the Gleason system although it contains 9 possible scores is usually aggregated to 3 or 4. Its value in predicting prognosis is obviously greatest for extreme cases and least in patients with moderate differentiation. Being a complex system doubts have been raised over its reproducibility and its reliability (Mostofi et al 1987). Indeed Mostofi himself an eminent urological pathologist states that he required a personal tutorial from Gleason to attain competence in using the system.

Several alternative systems have subsequently arisen. These include that described by Gaeta et al (1980). This considers both nuclear morphology and glandular differentiation attempting to produce a system that is

both simple and reliable. Each of these two features is given a score from 1 to 4. In both cases the worst area within the specimen is chosen and the worse score ie the greater is taken as the final grade. This therefore ranges from 1 to 4. Unfortunately only 3.5% of cases were grade 1, the percentages in grades 2-4 being 25.5, 49.2 and 21.8% respectively. Mortality in patients with a grade 4 was 9.6 times that of patients with grade 1 disease. Owing to the small proportion of patients with grade 1 disease a clinically more meaningful comparison would be the ratio of mortality of grade 2 and 4. This was only 3.5, similar to that obtained by the Gleason system.

In general the Gleason system has gained acceptance and is widely used (Murphy and Whitmore 1979). However, work by Schroeder et al (1985a) has demonstrated that focusing on one parameter, the glandular architecture as the Gleason system does, excludes other histological features which may have prognostic significance. It seems that the nuclear morphology is also an important factor and is to some extent independent of the glandular architecture. The same group have also shown that in a tumour containing areas with different histological characteristics,the worst area predicts prognosis. This supports the Gaeta method of scoring, taking the worst area as the final score. However, in poorly differentiated tumours the presence of well differentiated areas improved prognosis (Schroeder et al 1985b). Thus in such cases, the Gleason system, adding the scores from the predominant and the secondary patterns is more appropriate.

Throughout the UK most grading is performed on a personal ad hoc basis usually dividing tumours into well, moderate and poorly differentiated grades. Although this approach lacks sophistication and makes comparison between centres difficult it remains a powerful prognostic indicator. On review of routine reports using such a system in 278 patients we found for well, moderate and poor differentiation, 5 year survivals of 62, 24 and 7% respectively (Handley et al 1988).

Despite the undoubted power of histological grade as a prognostic indicator it has been pointed out that a proportion of patients with well differentiated disease may still progress and die (Kern 1978). Additional prognostic information is required.

Tumour staging

The first staging system in widespread use was that proposed by Whitmore in 1956 and later modified by Jewett (1975). A similar system was adopted by the Veterans Administration Co-operative Urology Research Group, the letters being replaced by Roman numerals. In this system the requirement necessary to exclude metastatic disease was no evidence of bone metastases on plain X-ray and a serum acid phosphatase of less than 1.0 King Armstrong Units. In this study the TNM system is used. This is favoured by most European Urologists, but it is a recent invention. The concept central to the TNM system was developed by Denoix in a number of articles dating from 1943. In 1954 the Research Commission of the Union

Internationale Contre le Cancer (UICC) set up a committee under the chairmanship of Denoix and in the following years the TNM concept was applied to tumours arising in various sites. Prostate cancer was not addressed until 1974 (UICC). It should be noted that the system was radically revised in 1987 (UICC) but the new version has yet to gain widespread acceptance amongst urologists (Schroeder et al 1988) so the earlier version has been used in this study. The details are tabulated below (Table 2). The Whitmore/Jewett system is also shown on the same table.

Table 2
Staging of prostate cancer
TNM

T0	No palpable tumour (incidentally found)
T1	Intracapsular tumour surrounded by palpably normal gland
T2	Tumour confined to gland but deforming its contour
T3	Tumour extending beyond the capsule
T4	Tumour fixed or invading neighbouring structures
N0	No lymph nodes involved
N1	Single regional node involved
N2	Multiple regional nodes involved
N3	Fixed mass of nodes on pelvic wall
N4	Involvement of juxta-regional nodes

(Without pelvic lymphadenectomy most patients are Nx)

Table 2 continued - TNM system

M0	No metastases
M1	Distant metastases

Whitmore/Jewett*

A	Impalpable disease
A1	Focal microscopic involvement (<5%)
A2	Diffuse or multifocal microscopic involvement
B	Palpable disease confined to the gland
B1	Single nodule ("classical Jewett nodule")
B2	Unilobar nodule occupying more than half one lobe
B3	Bilaterally palpable disease

(Some authors classify all unilateral involvement as B1 and bilateral as B2)

C	Extension into seminal vesicle(s) and/or extension outside the capsule
D	Metastatic disease
D1	Involvement of lymph nodes
D2	Bone or visceral metastases

The Veterans Administration Cooperative Urological research Group used essentially the same system but replaced the letters with Roman numerals I-IV.

*From Stamey et al 1989

T category

The local extent of the primary tumour correlates with prognosis. Jewett (1975) suggested that the incidentally

found tumours (stage A or T0) could be classified into two groups A1 and A2 on the basis of the microscopic extent of the disease. In a review of 117 patients with stage A prostate cancer, Cantrell et al (1981) analysed the proportion of prostate tissue that was replaced by tumour. They found that in patients with less than 5% of the tissue replaced by tumour progression was very rare (1/49). Whereas in patients with involvement of more than 50% progression was common (3/7). This is in accordance with previous work which had demonstrated that the survival of patients with a single focus of tumour, those with multiple foci and those with diffuse involvement was progressively worse (Byar and Mostofi 1972). The same workers demonstrated that further local progression, that is penetration into the capsule, was associated with a very much poorer 5 year survival, 49.7% versus 78.8%.

It was originally envisaged that T categorisation would be based on findings from digital rectal examination. The advent of transrectal ultrasonography has provided an alternative and more accurate method of determining the extent of a prostatic tumour, particularly whether it has spread beyond the capsule. It also raises the possibility of accurately determining the volume of the prostate allowing more objective assessment during follow-up.

Lymph node stage

The presence of lymph node involvement is a very powerful prognostic indicator. Due to deficiencies in the imaging of pelvic nodes reliable data is only obtained by surgery, usually prior to radical prostatectomy (Bruce et al 1977).

Many workers have shown that the presence of node metastases implies wide dissemination of the disease and that further progression is very likely (Prout et al 1980). The removal of involved lymph nodes does not reduce the chances of this occurring.

Metastases

The presence of metastatic disease which in the case of prostate cancer consists primarily of bone metastases has profound prognostic implications. In the first Veterans Administration study the 5 year survival of all patients with stage IV disease, that is raised acid phosphatase or X-ray evidence of metastatic disease was 25% (VACURG 1967). That for stage III was 50%. The treatment given, placebo, oestrogen, orchidectomy + placebo or orchidectomy + oestrogen made no difference to survival. In a subsequent and complex statistical analysis the presence of bone metastases has been shown to be of profound significance irrespective of the Gleason grade (Byar and Corle 1984). The diagnosis of metastatic disease has been greatly facilitated by the advent of isotope bone scans.

Serum Biochemistry

Acid phosphatase

The determination of serum acid phosphatase in prostate cancer patients was first performed by Gutman and Gutman in 1938. They had previously noted that acid phosphatase was present in both normal and carcinomatous prostate tissue and also in skeletal secondaries. Their method was

to add a substrate and adjust the pH to 4.9, the rate of reaction was measured and thus the enzyme activity could be quantified. An upper limit was set largely on clinical grounds such that from their 88 selected reference patients without prostate cancer only one had an elevated level. 11/15 patients with metastatic prostate cancer had elevated serum acid phosphatase. They felt the assay to be of limited value in the diagnosis of disseminated disease. There seemed to be no obvious correlation between the acid phosphatase level and the extent of the disease although there was, they felt, a tendency for high values to be associated with rapid disease progression.

Acid phosphatase is usually normal in patients with localised prostate cancer, limiting its value in diagnosis. Indeed, for this reason several North American workers have suggested that it may have a place in preoperative staging prior to radical prostatectomy. Whitesel et al (1984) demonstrated that of 25 patients found to have lymph node involvement at surgery, 15 (60%) had a raised acid phosphatase in contrast to 70/313 (22%) with no nodal involvement (X^2 17.9 $p<0.0005$). In addition a high proportion of those with a raised acid phosphatase and negative nodes developed bone metastases within 2 years. The year before, Wilson et al had suggested that acid phosphatase was of no value in staging (Wilson et al 1983). The data they present is incomplete, but it would appear that 10/36 (28%) lymph node positive patients and 6/73 (7.5%) with negative nodes had a raised acid phosphatase (X^2 8.41 $p<0.005$). Whilst it is disappointing that the assay only detected a little over a quarter of

patients with node involvement there clearly is a correlation with stage. Using sophisticated statistics Oesterling et al (1987) found a good correlation between serum acid phosphatase and capsular penetration ($p<0.003$) and seminal vesicle involvement ($p<0.01$) but for lymph node involvement it did not quite reach significant levels ($p=0.08$).

The advent of a more specific acid phosphatase assay based on antibodies directed against the prostatic acid phosphatase (PAP) was presented as a great advance (Foti et al 1977). It was claimed that serum PAP was raised in 67% of patients with localised prostate cancer (stages I+II). Unfortunately, using their cut-off level, 14% of patients with benign prostatic hypertrophy and 17% of patients with other cancers also had an elevated serum PAP. In an editorial relating to this study Gittes (1977) referring to the small proportion of prostate cancer patients who present with early and possibly "curable" disease, stated that " the clear implication of the accompanying report is that mass screening on the basis of a blood test alone can reverse this gloomy experience" This optimistic interpretation was mistaken. The reason is that a sensitivity of 67% does not mean 67% of all patients with an elevated PAP will have prostate cancer. The proportion of patients with an elevated PAP who also have prostate cancer is known as the positive predictive value. This concept was clearly described by Vecchio (1966). It can be calculated as follows:

Positive Predictive Value =

$$\frac{\text{Sensitivity X Prevalence}}{(\text{Sensitivity X Prevalence}) \text{ X } (1\text{-Specificity})(1\text{-Prevalence})}$$

Using data from Foti et al (1977) it has been calculated that the positive predictive value of a raised serum PAP would be 0.41%. That is one in every 244 positive tests would actually have prostate cancer. If those patients with a tumour that is palpable rectally are excluded leaving just the impalpable stage I, then the positive predictive value would be 0.19% or 1 in 526.

These mathematical predictions were confirmed when applied to patients (Fleischmann et al 1983). Amongst 295 patients who underwent a procedure that yielded prostate tissue for histological examination, 17 were found to have an elevated PAP and a clinically benign gland. Histology examination did not reveal cancer in any of these prostates and on follow-up (median time 11 months) no cancers became apparent.

It is salutary that the initial enthusiasm for the serum PAP assay led to reports in the lay press and many physicians requesting the test as a screen. In the USA urological referral for biopsy after a raised PAP was found had become common (Belville 1983). The parallel for prostate specific antigen is clear (see later).

Alkaline phosphatase

There is no doubt that a raised alkaline phosphatase has ominous implications for the patient. In one study of 357 patients with bone metastases, 85% had an elevated total

alkaline phosphatase largely due to elevation of the bone isoenzyme (Wajsman et al 1978). In addition, of 105 patients with multiple estimations a clear relationship between the level of alkaline phosphatase and disease progression was seen, those in whom progression occurred had significantly higher levels than those who remained stable or responded to treatment. The percentage decrease in alkaline phosphatase following therapy was also a predictor of response. Similar findings have been reported by others. Killian et al (1981) suggested a correlation between the extent of bone metastases and bone isoenzyme alkaline phosphatase. De Voogt et al (1989) showed alkaline phosphatase to be of strong predictive value especially in M1 patients.

Creatinine Kinase

The serum level of the isoenzyme CK-BB has been found to be elevated in some prostate cancer patients. The current situation has been reviewed by Pontes (1983). The elevation only occurs in a small proportion of patients and its prognostic significance is not clear.

Gamma seminoprotein

This substance is elevated in the serum of prostate cancer patients and there is a high degree of correlation with the PSA level (Siddall et al 1986b, Kuriyama et al 1986). Although with a molecular weight of 23 000 it is clearly different from prostate specific antigen whose molecular weight is 34 000, it appears to share many characteristics and seems very closely related.

Others

A wide variety of biochemical measurements have been evaluated in the prediction of prognosis in prostate cancer. These include erythrocyte polyamine, carcinoembryonic antigen (CEA), lactate dehydrogenase (LDH) isoenzymes and prostacyclin. Their value has been reviewed elsewhere, (Pontes 1983) none have been found of value in clinical practice.

Miscellaneous

Haemoglobin

Cox's proportional hazards analysis of the VACURG data demonstrated that for deaths from all causes a haemoglobin of less than 12g/dl was a prognostic factor (Byar and Corle 1984). Oddly when cancer deaths alone are considered this variable does not appear to be important. The authors conclude that it exerts a non-specific effect implying an increased likelihood of death from other causes. In contrast data from the European Organisation for Research into the Treatment of Cancer (EORTC) studies suggests that a low haemoglobin may indicate advanced disease probably with bone marrow involvement (de Voogt et al 1989). If this is the case then anaemia must be considered a prognostic factor.

Performance status

The same VACURG analysis demonstrated that for deaths from all causes performance status was a prognostic factor

(Byar and Corle 1984). This is born out by the EORTC (de Voogt et al 1989).

Ureteric obstruction

The finding of ureteric dilatation was found to be of prognostic significance in predicting death from prostate cancer in the VACURG studies (Byar and Corle 1984). In our own retrospective review an elevated serum creatinine was found to be associated with significantly shorter survival (Handley et al 1988).

Clinical features

In our review the length of history and the mode of presentation seemed to determine prognosis. Patients with a history of less than 6 months or who presented in acute retention experienced shorter survival times (Handley et al 1988).

New Prognostic Indicators

Two new means of determining prognosis have recently emerged. They are prostate specific antigen and DNA flow cytometry. These are discussed in detail below and together form the basis of this study.

Prostate Specific Antigen

Early History

Background

Assay Systems - Pros-check
- Tandem-R
- Assay system comparisons

Tissue Specificity and Histological Localisation

Immunoperoxidase Staining of Histological Material

Histological diagnosis of prostate cancer and prognosis

Molecular Structure and Physiological Function

Mechanism of release into the serum and subsequent clearance

Clinical Applications of Serum PSA

Reference Populations and "Normal Levels"

Elevation in Benign Disease

Diagnosis and Screening

Predicting Prognosis

Monitoring disease

Prostate specific antigen

Early History

Prostate specific antigen (PSA) was first described by Wang and his co-workers at Roswell Park Memorial Institute, Buffalo, New York (Wang et al 1979). The antigen was isolated from homogenised prostate tissue. Female rabbits were inoculated with a crude extract of human prostate and the antisera that was raised was then used in the purification of a single antigen from the homogenised prostate tissue. Finally a pure preparation was produced and shown to be a single entity on immunoelectrophoresis against the rabbit antisera, to be tissue specific and to have no acid phosphatase activity. The antigen was present in homogenates of both benign and malignant prostate tissue. It was described as "prostate antigen" (PA) although subsequently it is now almost universally known as prostate specific antigen or PSA.

The measurement of serum PSA in patients was first carried out at Roswell Park. Papsidero et al (1980) using a rather insensitive rocket immunoelectrophoresis assay showed that of 219 patients with prostate cancer, 17 (8%) had elevated serum PSA. None of the controls comprising both "normal" individuals and patients with other cancers had elevated PSA levels. Later the same year, Kuriyama et al (1980) reported an enzyme linked immunoassay that was considerably more sensitive.

With regard to the clinical use of serum PSA three roles have been suggested: diagnosis (Kuriyama et al 1980), predicting prognosis and monitoring treatment (Kuriyama et

al 1981). These roles emerged following early work at Roswell Park and subsequent research has focused on the same areas.

Background

Assay Systems

Although the early development of serum PSA assays was pioneered in Roswell Park, commercially available immunoassays have allowed many other centres to contribute further observations. The majority of workers have used either the Tandem-R assay from Hybritech or the Pros-check assay from Yang Laboratories. A third from Diagnostic Products Corporation, DPC is also available in this country but little published work has been presented using this system. The Tandem-R and Pros-check assays differ both in their basic design and in the values they produce. Before continuing to examine the further work on PSA it is therefore important to describe these 2 assays in more detail.

Pros-check

The original technique described by Wang et al (1979) has been used to prepare a polyclonal antibody raised in rabbits directed against PSA. This assay kit uses a conventional radio-immunoassay technique involving a second precipitating antibody and labelled PSA.

Hybritech Tandem-R

This is an immunoradiometric assay using two monoclonal antibodies directed against PSA. The first is bound to a

plastic bead, the second is radio-labelled and binds to a different antigenic site on the PSA molecule. Serum is incubated with the bead and the tracer antibody. PSA in the serum is bound to the bead and then in turn binds the tracer antibody forming an antibody-antigen-antibody sandwich. The excess tracer is washed off and the bead's radioactivity counted. The radioactivity is proportional to the amount of PSA in the serum.

Assay system comparisons

When work for this thesis commenced, in February 1987, no published comparisons were available. There are no international standard reference preparations of PSA, consequently the values obtained using different assays are unlikely to be identical. For this reason many of the samples were processed using all three kits available in the UK, although the Hybritech system was adopted as the principal assay.

Tissue specificity + histological localisation

Shortly after its discovery immunohistochemical techniques were used to determine the histological localisation of PSA. Nadji et al (1981) used anti-PSA antibody raised in female rabbits to stain histological material using standard immunoperoxidase methodology. Positive staining was found in normal acinar and ductal epithelium as well as in the secretions they contained. Periurethral glands, seminal vesicles, vasa defferentia, transitional cell epithelium, the glandular lining of von Brunn's nests and areas of cystitis glandularis were all negative. In

addition other normal tissues including stomach, small and large intestine,pancreas, salivary gland, liver, kidney, lung, testis and breast were negative. 73 primary prostate cancers and 49 metastatic deposits all stained positively, although the authors comment on the patchiness of staining in some tumours. None of 78 tumours of non-prostatic origin stained positive. It is clear that PSA is indeed a tissue specific antigen, as it is found in normal, hyperplastic and neoplastic prostate and that it is resistant to fixation and paraffin embedding.

Histological diagnosis of prostate cancer and prognosis

In 1983 Allhoff et al showed the value of this PSA staining technique in determining the tissue of origin of anaplastic tumour deposits. These findings have been confirmed by others although the extent to which prostate tumours are PSA positive varies. For instance, Stein et al (1984) found positive staining for PSA in 75% of prostate tumours at autopsy and 78% at biopsy, contrasting with Papsidero et al (1985) who, using a different antibody found positive staining in 100% of metastatic prostate cancers. Epstein and Eggleston (1983) in a review of 19 patients suggested that those tumours with areas of weak or negative PSA staining may have a poorer prognosis. This suggestion was supported by Papsidero et al (1983) who found that in an analysis of tissue from 25 prostate cancers that the poorer Gleason grades demonstrated less intense staining for PSA overall and greater heterogeneity of staining in addition. Later work by Sinha et al (1986) showed that PSA staining intensity was not uniformly

related to tumour grade but they did confirm that in poorly differentiated disease there is a wider variation in staining. In summary, at present PSA seems the best tissue marker in the determination of the histogenesis of a poorly differentiated tumour but whether the quality of PSA staining can be used to predict prognosis remains uncertain.

Molecular structure and physiological function

At an ultrastructural level PSA has been identified in the endoplasmic reticulum, cytoplasmic vesicles and vacuoles and within the lumina of prostatic glands (Warhol and Longtime 1985). It is suggested that the antigen is synthesised in the rough endoplasmic reticulum, then stored in vesicles and vacuoles prior to secretion by exocytosis. When secreted into the seminal fluid its function is thought to be related to the liquefaction of semen. Watt et al (1986) have determined the precise amino acid sequence of the molecule. It is a single polypeptide chain of 240 amino acids with a calculated molecular weight of 26,496. Carbohydrate side chains make this up to the 33-34,000 described by Wang et al in 1979. It is a serine protease with close homology to the kallikrein family of proteases and is thought to perform its physiological role by means of its hydrolytic activity.

Mechanism of release into the serum and subsequent clearance

Physiologically it seems that PSA is secreted in prostatic fluid but how it makes its way into the serum is unknown.

Wang et al (1982) have shown that PSA in serum has a molecular weight of 90-100 000, possibly indicating that PSA binds either to itself or to plasma proteins. There is no evidence that prostate cancers leak more PSA into the serum than benign hyperplasia (BPH), although this has been suggested (Takeuchi et al 1983). Workers from Roswell Park have demonstrated that PSA levels in localised prostate cancer and BPH are often indistinguishable (Kuriyama et al 1980) and that the tissue concentration of PSA is similar in both conditions. To date no data relating to the half-life or "pharmacokinetics" of PSA have been published. It is not known how concomitant disorders such as hepatic or renal failure may affect the handling of PSA and therefore the serum level.

Clinical applications of serum PSA

Reference populations and "normal" levels

The finding of an elevated serum PSA in patients with prostate cancer has led to attempts to define a "normal" or reference range. The first quantitative assay was reported by Kuriyama et al (1980) from Roswell Park. Using an enzyme labelled sandwich type assay serum PSA was measured in 68 "healthy volunteers". The range was 0.1 - 2.6 ng/ml. 17 of these volunteers were female and none had detectable PSA in their serum. However, of the 51 men, 17 also had no measurable PSA (ie <0.1 ng/ml), possibly indicating a low sensitivity of the assay. The mean PSA amongst the men was 0.47 ng/ml with a standard deviation of 0.661 ng/ml. The fact that the mean minus the standard

deviation is less than zero demonstrates that the distribution of PSA in "healthy volunteers" is not Gaussian but heavily skewed to the left. Despite this "normal limits" were set at the mean + 2 standard deviations, ie 1.8 ng/ml. Owing to the skewness of the distribution a high number of values were above this limit, 5 out of 51 or 9.8%

The importance of setting a cut-off level is that it affects the apparent diagnostic value of serum PSA estimation. This will be reviewed later. However, it is interesting to note at this point that the same group of investigators have used 7.5 ng/ml (the mean + 2 standard deviations of their BPH patients) and 2.5 ng/ml (the mean + 3 standard deviations of the healthy volunteers) in different papers in the same year (Kuriyama et al 1982a+b). Pontes et al (1982) also from Roswell Park evaluated the correlation between serum PSA and the clinical course of localised prostate cancer. They found that the then established cut-off level 1.7 ng/ml was of no value and adopted 10 ng/ml as it appeared clinically more useful. The following year Takeuchi et al (1983) using an assay based on antibody from Roswell Park published a report of PSA levels in urological patients in Japan. They set their cut-off at 2.5 ng/ml, the mean + 3 standard deviations of 51 American healthy volunteers.

The advent of commercially available PSA assay kits has led to contributions from other centres. Liedtke and Batjer (1984) using the Pros-check kit from Yang Laboratories tested serum from 276 apparently healthy men.

They also found that the distribution of PSA values was not symmetrical about the mean and for this reason set an upper limit of normal at the 99th centile, a statistically more correct approach. This limit was 2.7 ng/ml. In 1985 Killian et al using the same kit set a cut-off level at 2.2 ng/ml based on the mean + 2 standard deviations of 127 apparently healthy males. Using an assay from Clinical Assays, Cambridge, Mass. Stromme et al (1986) compared the diagnostic efficiency of serum markers for prostate cancer. They fixed a "discrimination limit" chosen so that 5% of their BPH group would fall above this level. For PSA this was 46 ng/ml. Siddall et al (1986a) using the Tandem-R kit suggested 10ng/ml as an "operational cut-off limit" as this seemed clinically the most useful (E H Cooper 1987 Personal communication). They did not analyse a large reference population.

In summary, up to February 1987, the upper limit of normal for serum PSA has been quoted as 1.7, 2.2, 2.5, 2.7, 7.5, 10 and 46 ng/ml. The likely explanation is the differing composition of the reference groups used. The proportion of patients with BPH is of crucial importance and for this reason all subjects within a reference group should be adequately assessed for prostatic disease including symptom assessment, physical examination, transrectal ultrasonography and histological evaluation where possible.

Elevation in benign disease

Many have demonstrated moderate elevations of PSA in BPH (Kuriyama et al 1980, 1982a+b, Liedtke and Batjer 1984,

Stromme et al 1986). One might expect that the elevation would correlate with the volume of prostatic tissue ie. the size of the gland. However, Takeuchi et al (1983) found no correlation with the size of the gland enucleated at open operation. In contrast, Ryan et al (1988) demonstrated a good correlation between the log of the volume of the gland measured by transrectal ultrasound and the log of the serum PSA. If confirmed this raises the possibility of allowing for the size of the gland in setting normal limits so that a given PSA is interpreted as normal or elevated for the size of the patient's prostate.

Little is known regarding the influence of other factors on serum PSA in benign disease. Some workers have measured modest elevations of PSA in prostatitis (Takeuchi et al 1983) although this condition is difficult to diagnose precisely and no worker has stated his diagnostic criteria. It has also been suggested that PSA is unexpectedly high in patients with acute retention due to BPH (Newling 1987 personal communication). Rectal examination, catheterisation and operative procedures may all cause elevation of PSA. If moderate rises in PSA are to be clinically useful clearly these factors require investigation.

Diagnosis and screening

Following the development of a quantitative assay for serum PSA (Kuriyama et al 1980) it was suggested that PSA measurement may be of diagnostic value. It was found that

a statistically significant difference in serum PSA existed between their 19 cases of BPH and both 56 stage C cancers ($p<0.01$ student t test) and 344 stage D cancers ($p<0.001$). However they failed to demonstrate any significant difference between PSA in BPH and localised cancers, stages A and B (n=8+34 respectively). Although the usefulness of a serum marker to detect early prostate cancer was discussed by the authors they felt that further trials were necessary to determine if PSA was such a marker.

Two years later the same workers reported the use of PSA in combination with immunologically measured prostatic acid phosphatase (PAP) in the diagnosis of prostate cancer (Kuriyama et al 1982a). This study involved 192 patients with prostate cancer at various stages, 22 healthy males and 29 patients with BPH. Taking an elevation of PSA or PAP or both as a positive test they claimed that for stages A, B, C, and D the sensitivity in the diagnosis of prostate cancer was 58%, 58%, 68%, and 92% respectively. The cut-off levels for both PSA and PAP were set so that 90% of the BPH patients would fall below this value, consequently 10% of these would be false positives. The authors point out that better sensitivity would be obtained by using cut-off levels related to healthy males, however 41% of the BPH patients would be above this level. Despite this limitation the authors suggest a possible role for serological markers in what they term the "immunodiagnosis" of prostate cancer.

Takeuchi et al (1983) concluded that serum PSA was "of

great value in the serologic diagnosis of prostate cancer". In their study 27 patients with prostate cancer are described but unfortunately no details of staging or previous treatment were given. Only 57% had elevated PSA levels using a cut-off derived from the earlier work at Roswell Park. 7% of their BPH patients (n=27) had elevated PSA. The work of Kuriyama (1980) had clearly shown large variation in the incidence of PSA elevation in the different stages of prostate cancer. Without details of staging this work remains difficult to evaluate, but it would seem that a sensitivity of 57% does not support the assertion of the great value of PSA in diagnosis.

Liedtke and Batjer (1984) reported the use of the newly produced Pros-check assay from Yang Laboratories. They first derived an upper reference limit based on 276 male patients. No clinical data is available, the patients were merely described as undergoing routine physical examination. A low reference level, 2.7 ng/ml was set. In 12 out of 14 cases of BPH the serum PSA was above this level and in 5 cases above the 7.5 ng/ml level set by Kuriyama et al (1982a) from a group of BPH patients. (Liedtke and Batjer actually quote Kuriyama's level as 8.5 ng/ml, this is a mistake). It is surprising that 5/12 were above this limit set by Kuriyama at the 90% level. It suggests that not all BPH patients share similar PSA levels and that Kuriyama et al selected a reference group in which the PSA tended to be particularly low. Analysing 205 specimens from 183 patients suspected of having prostate disease in that clinicians had ordered acid phosphatase estimation, 103 that is, 50% had an elevated

PSA by their definition. No follow-up data was given but it would seem that only one patient was diagnosed as having prostate cancer. The authors commented that the high incidence of raised PSA was related to the number of patients with BPH in their group. None the less the authors imply that there may be a role for PSA in the early detection of prostate cancer.

In a systematic and rigorous analysis Stromme et al (1986) evaluated the diagnostic efficiency of PSA and 11 other possible tumour markers. They studied a total of 264 patients, 66 with non-prostatic urological disease, 106 with benign hyperplasia, 59 with untreated prostate cancer and 33 with treated prostate cancer. The T category and M status of all the untreated patients was documented although N staging was omitted as it was not performed routinely. In order to compare diagnostic efficiency, cut-off levels were set using the BPH patients as a reference population. For each tumour marker the discriminant level was set so that only 5% of their reference BPH population would have values above this limit. For PSA this was 46 ng/ml (Clinical Assays kit, Cambridge, Mass.). Predictably the sensitivity was lowest amongst the T0-2 M0 patients 6/11 (54%), higher in the T3-4 M0 patients 11/14 (78%) and best in the M1 patients 18/21 (86%). There were only 3/59 (5%) false positives amongst the non-prostatic urological disease patients (PSA was apparently measured in only 59 of the 66 patients with non-prostatic urological diseases). Whilst this study showed PSA to be the most efficient diagnostic marker it seemed to lack the

sensitivity required to detect early prostate cancer.

In summary, a number of workers have suggested or implied that PSA is of value in the diagnosis of prostate cancer. This possibility is of particular interest currently as many clinicians feel that earlier detection offers a better outlook to the patient. The use of PSA in this context requires examination in three areas.

Firstly, that the choice of an appropriate reference population is crucial. Using an inappropriate reference group such as healthy young men a low cut-off can be set and an impressive diagnostic sensitivity can be claimed. In practice the clinician will need to discriminate between benign disease and cancer predominantly in patients with symptoms of bladder outflow obstruction. Should screening of asymptomatic elderly men be contemplated, it remains vital to use an appropriate reference group, matched for age, urinary symptoms and preferably prostatic volume. In the evaluation of claims regarding the diagnostic worth of PSA the details regarding stage of disease in the cancer patients must be quoted as the presence of large numbers of advanced stage disease patients will improve the apparent performance of the test. From a clinical standpoint the detection of early stage disease would be the most valuable. PSA as a diagnostic test should be evaluated primarily in such patients.

Secondly, it is important that we appreciate how other factors can affect serum PSA. It is well established that BPH causes moderate PSA elevation but nothing is known

with regard to the effect of presentation with acute retention, subsequent catheterisation or rectal examination.

Thirdly, it may be possible to improve the diagnostic accuracy of serum PSA by taking the size of the prostate into account. Should the relationship between prostatic volume and PSA (Ryan et al 1988) be confirmed, it may be possible to define reference values for given prostate volumes and thus improve the diagnostic accuracy.

Predicting Prognosis

In the first report of a sensitive assay for serum PSA, Kuriyama et al (1980) showed clearly that PSA levels correlated with stage. The mean serum PSA for stages A, B, C, and D were 4.8, 5.0, 10.1, 24.2 ng/ml. It follows therefore that as disease stage is a powerful determinant of outcome, PSA is of value in predicting prognosis. Whether it would give additional prognostic information and be able to determine the outlook for individuals within the same stage is not established.

The same workers analysed the outcome of 96 patients with advanced disease already treated by hormone manipulation who were entered into chemotherapy trials (Kuriyama et al 1981). There was a definite correlation between PSA levels and survival. Patients were assigned arbitrarily into one of three groups on the basis of their PSA, below 3.6 ng/ml n=16, 3.7 - 9.0 ng/ml n=26 and above 9.1 ng/ml n=54. The median survival times were 44, 25 and 20 months respectively, $p<0.01$. No details are given regarding the

treatments each patient received but presuming they were randomly allocated the effects of treatment are unlikely to explain the survival differences observed.

A further study in 1985 used complex statistical methods in the evaluation of the prognostic importance of PSA (Killian et al 1985). Sera collected over a 6 year period from 70 patients with "regionally confined" prostate cancer, stages B2 to D1 were analysed. A highly significant association was found between PSA and disease free survival time (p=0.00002 Cox's proportional hazards model). Of 26 patients who experienced disease progression 24 had elevated PSA levels in the preceding 12 months. It was said that if the PSA was markedly elevated the positive predictive value was 100%. However, they also found that 9 out of 44 patients who did not progress had elevated PSA levels within the 12 months preceding their last sample date. The predictive value could be enhanced by adjusting the cut-off level as in diagnosis. Whilst very high levels almost certainly mean recurrence, at moderate levels there remains a considerable overlap making the prediction of prognosis more difficult. Regrettably the authors did not make use of their no doubt extensive data to extend the Cox's proportional hazards model to other prognostic indicators especially alternative serum markers such as PAP. It is possible to assess the impact of a new test to predict prognosis in addition to those already done routinely by the use of such statistical models, but oddly this has not been done.

In England, Siddall et al (1986a) have reported the

prognostic significance of PSA in 86 patients who were M0 at diagnosis. 16 patients developed metastases within 3 years, 15 had elevated PSAs whilst 7 had elevated PAPs. The apparent advantage of PSA over PAP in predicting prognosis amongst M0 patients may not be as large as it seems. At the time of diagnosis, only 16 had an elevated PAP, but 35 had elevated PSAs. The sensitivity of PSA in predicting metastases was 94%, for PAP it was 44%. However the specificity of PSA was 71%, but 87% for PAP. Adjusting the cut-off level for each test to produce the same level of specificity would considerably reduce this difference. The same group confirmed the relationship between stage and serum PSA described earlier (Siddall et al 1986a+b). They also demonstrated that tumour grade is correlated with PSA; the poorer the grade the higher the PSA. However the patients were not stratified for grade and stage so this apparent correlation may simply reflect the fact that poorly differentiated tumours are often advanced whilst well differentiated ones usually are not.

Monitoring

The use of serum PSA in the monitoring of patients with prostate cancer was first envisaged at the time that a sensitive assay was developed (Kuriyama et al 1980). The following year workers from Roswell Park published a study specifically addressing this subject (Kuriyama et al 1981). Serial measurements were made from 10 patients with stage D2 prostate cancer prior to treatment with chemotherapy and from 32 patients undergoing " curative therapies for localised prostate cancer". In general terms

the patients in the former group either responded to chemotherapy or showed no signs of further progression at least in the short term and PSA levels were said to mirror this. In the latter group 5 patients developed metastases after an average follow-up of 56 weeks (range 12 - 114 weeks). The authors state that all 5 cases "exhibited increasingly elevated PA (PSA) levels". In fact on examination of their data, it seems that in only 3 cases did the PSA rise in association with progression, in the remaining 2 patients the PSA was unchanged but had always been elevated (above their upper limit 1.8 ng/ml which is rather low). Of the 3 that demonstrated a rise in PSA in only 1 did this precede the detection of progression, in the other 2 cases the PSA rose at the time of clinical progression. No data was given relating to the patients who did not progress. Thus, although PSA showed promise in the monitoring of prostate cancer patients its value had not been proved.

Pontes et al (1982) studied a total of 47 patients with apparently localised prostate cancer who were entered into National Prostate Cancer Project trials. All were staged by pelvic lymphadenectomy and then treated by radical prostatectomy or external beam radiotherapy. 18 patients underwent radical prostatectomy and 4 developed metastases. Following surgery these were staged as two stage D1, and one stage C and one stage B2. All were said to have an elevated serum PSA greater than 10 ng/ml in association with their disease progression. The lag between changes in PSA and the clinical detection of

progression was not discussed. In 2 cases, both stage D1, there was a persistent elevation of PSA but no evidence of progression after follow-up of 24 - 124 weeks (no mean or median given). 29 patients were treated by external beam radiotherapy. 5 of these developed metastases, but in only 2 cases was this said to be associated with a rise in PSA above their 10 ng/ml cut-off. Oddly between 6 and 36 weeks 10 patients showed elevations of PSA after their radiotherapy including the 3 who later progressed . It was suggested that the necrosis of tumour cells following this treatment may be the explanation. In all but 1 of the 7 patients who did not later progress the PSA fell to "normal" levels.

Killian et al (1985) performed a further evaluation of PSA in monitoring prostate cancer. This study has already been discussed in relation to predicting prognosis (page 57). It included 70 patients with "regionally confined prostate cancer", staged by lymphadenectomy. Treatment given consisted of radical prostatectomy, cryosurgery or radiotherapy and in addition some patients received long term adjuvant chemotherapy. They were recruited over a 6 year period. A total of 602 specimens were assayed for PSA and complex statistical analyses were used in an effort to interpret the resulting data. As the patients had undergone a variety of different treatments a Cox's proportional hazards model analysis was used to determine the relative importance of PSA, the stage of disease and types of treatment. It showed that PSA predicted progression and that the type of treatment and disease stage amongst this selected population was relatively

unimportant. Only 43 patients were used for this analysis. The predictive value of a single PSA measurement was also evaluated. They found that taking the highest PSA value before recurrence was detected, in 24 out of 26 cases who recurred the PSA was elevated (above 2.2 ng/ml). However amongst the 44 who did not recur, 9 were above their cut-off. The authors commented that 5 times their upper limit, that is 11 ng/ml was a clinically useful threshold as above this 72% of patients recurred. This is in agreement with Siddall's limit of 10 ng/ml yet the authors still favour their own very low upper limit. The lead time from elevation of PSA to disease progression depended on the degree of elevation, the higher the PSA the shorter the lead time. Conversely the predictive value of a single PSA estimation is directly related to the threshold used.

A theoretical advantage of serial measurements is that the patient can act as his own reference and changes from the previous results may be significant even if they do not cross an arbitrary limit. Regrettably this question is not adequately addressed. The trends of PSA with respect to time are presented for the 26 patients who recurred. Although 4 different patterns were identified the number of patients in each group was not defined. The 2 "typical" patterns were that the PSA fell to a low level following treatment and then rose prior to recurrence or that the level remained moderately elevated and did not fall, but slowly rose until recurrence occurred. No data was presented with respect to the time trends of those patients who did not recur.

Thus we cannot determine whether the 9 elevations not associated with progression could be identified as false positives by virtue of their relationship to time. It might be possible to exclude these if, for instance, the general trend was downwards with one aberrant result.

Killian et al (1986) produced a further analysis based on a larger number of similar patients (79) using a Cox's proportional hazards model once more. The value of PSA in predicting prognosis was compared to other serum markers, namely acid phosphatase (enzyme activity), total alkaline phosphatase, bone alkaline phosphatase and PAP. It seemed superior in that the relative risk coefficient was highest for PSA and furthermore once the PSA was taken into account measurement of the additional markers seemed to add no further prognostic information. PSA was found to predict progression 6 months before it was detected by other means. A group of 51 D2 patients, ie with bone metastases was also studied. Interestingly, the relative value of PSA was less marked and prediction of progression 6 months in advance was not possible. No analysis of changes in serum PSA with time were made.

Siddall et al (1986a) studied 51 patients managed by a deferred treatment policy. These men were monitored after diagnosis and received no treatment unless progression occurred. 14 men showed signs of progression after 1-10 years follow-up (no median given, nor stage and grade). All had elevated PSAs but there was an overlap with those who did no progress. 19 patients who did not progress were

studied in detail with multiple PSA measurements. Although their median PSA was low, 3.15 ng/ml (range 0.3 - 16.4 ng/ml) they all showed a steady increase in PSA with the passage of time, suggesting that it would be difficult to identify patients who were about to progress by means of the pattern of change of their PSA with respect to time.

It is well established that serum PSA correlates with stage and prognosis. A major limitation is the moderate elevations seen in benign disease. In theory serial measurements on the same individual should increase the predictive value of PSA and should provide an easy means of staging the disease at each out-patient visit. Further studies particularly in untreated patients are required.

DNA Flow Cytometry

Static Cytometry

Flow Cytometry

- Principle
- DNA fluorochromes
- Cell suspensions
- Interpretation
- Application to prostate cancer

Multiple factor analysis

Static cytometry

The amount of DNA in the nuclei of tumour cells was first systematically investigated by Leuchtenberger et al (1954). Prior to that time attempts to determine the quantity of nuclear DNA relied on biochemical assays applied to tissue homogenates. Leuchtenberger used the Feulgen reaction to stain DNA and a microspectrophotometer to measure the light absorbance due to the presence of DNA within a single nucleus. They found that within the nuclei of many normal human tissues the DNA content was relatively similar. At this time it was not appreciated that DNA contained the genetic code. They also demonstrated that many tumours contained increased amounts of DNA and often these amounts were multiples of the normal nuclear DNA compliment. Knowing that chromosomal counts and nuclear DNA content in different species were correlated the authors inferred that the nuclei of tumour cells carried multiples of the normal diploid chromosome compliment or were "polyploid".

Tavares et al (1966) performed the first analysis of nuclear DNA content in prostate cancer that was correlated with survival. They studied paraffin embedded histological material from 35 patients with good follow up data available. The slides were stained using the Feulgen method and the nuclear DNA content of about 30 cancer nuclei was measured by means of a microdensitometer, an instrument for measuring light absorbance. Two groups of patients emerged, those who appeared to have triploid and hexaploid cell lines who fared badly and those with

diploid and tetraploid cell lines who fared better. Although the median survival times were markedly different, 4.2 and 9.2 years respectively, the authors did not feel that a reliable statistical analysis could be performed as only 9 patients were in the former group. Nevertheless it seemed established that a relationship did exist between nuclear DNA content and survival time in prostate cancer.

Sprenger et al (1974) found that nuclei from prostate cancer cells contained significantly more DNA than the diploid nuclei found in BPH and "chronic prostatitis". They felt that tumour cell populations could be distinguished from benign cells. This view was over optimistic in that many subsequent studies have shown that tumour cells may contain entirely normal amounts of DNA and therefore are considered diploid (Fordham et al 1986, Frankfurt al 1985, Tribukait et al 1983).

Zetterberg and Eposti (1976) conducted a survey of nuclear DNA content in prostate cancer. Again they used cytophotometric measurements of individual cancer nuclei stained by the Feulgen method and identified under the microscope. Of 24 cases diagnosed to have prostate cancer all had a higher proportion of "hyperdiploid" cell nuclei than the 3 samples from patients with BPH that constituted their control group. Higher percentages of hyperdiploid cells were associated with poor cytological grade. It was suggested that the addition of DNA measurements may contribute to the prediction of prognosis, although no follow up data was presented. The same workers later

published a review of 43 highly selected prostate cancer patients followed up for at least 5 years (Zetterberg and Esposti 1980). 21 of these patients were selected because they showed a good response to hormone treatment. In general these showed a lower proportion of hyperdiploid cells and those that were observed were for the most part tetraploid. Only 3 of these patients were found to have more than 80% hyperdiploid nuclei. 22 patients were selected as they had responded poorly to hormone manipulation. In 15 cases (68%) more than 80% of the nuclei were found to be hyperdiploid. It was concluded that the nuclear DNA content may reflect the degree of malignancy of a tumour. Unfortunately as the patients were selected for study on the basis of their treatment response no firm conclusion regarding the use of nuclear DNA measurements in predicting prognosis can be made.

Bibbo et al (1985) reported a small study of histological specimens from 24 prostates. Abnormal nuclear DNA content was noted to correlate with histological grading (Gleason), clinical staging and nuclear atypia. The authors point out that more cases were required to establish the statistical significance of the trends they had observed.

In 1986 using a microscope photometer for single cell fluorescence cytometry, Seppelt et al (1986) analysed 100 nuclei in smears made from fine needle biopsies of the prostate. 85 preparations from prostate cancers and 20 from BPH were examined. Histograms were used to express the results, plotting the frequency of observation against

the DNA measured for each nucleus. A variety of characteristics of each histogram were determined mathematically and analysed by complex statistical methods. It was found that 85% of prostate cancers could be diagnosed by this technique with 10.6% false negatives and 4.3% false positives. Grading could not be performed reliably using DNA histograms. No attempt was made to correlate the DNA cytometry findings with clinical stage or the patients' clinical course.

The principle disadvantage of static cytometry, measuring light absorbance of a single stained nucleus is that it is very time consuming. Individual nuclei have to be inspected under the microscope and the measurements made. Usually between 30 and 300 nuclei are examined for each case. The advent of flow cytometry (FCM) offers an automated system that allows the examination of many thousands of nuclei.

Flow Cytometry

Principle

The technique of quantitative measurement of cell fluorescence using a flow system was first described by van Dilla et al (1969). Cells held in aqueous suspension were passed by means of a laminar flow fine nozzle through a beam of laser light. The fluorescence arising from each particle as it passed through the laser was measured electronically and stored on a computer. A histogram of the fluorescence intensities of large numbers of cells, typically 50,000 could then be generated. This system has

become the standard basic design of flow cytometers. The information obtained can be enhanced in two ways. Firstly the use of selective optical filters allows the detection of light of specific wavelengths and therefore, the simultaneous measurement of fluorescence due to different fluorochromes. Thus fluorescein labelled antibodies can be used with propidium iodide so that oncoproteins or other antigens can be measured in addition to DNA in a large number of cells (Crissman and Steinkamp 1973, Watson et al 1985). Secondly the spatial distribution of the light detectors (photomultipliers) can be arranged to provide extra information. In the Becton Dickenson FACS 420 used in this project photomultipliers are situated to detect both forward scatter and right angle scatter of light. The forward scatter photomultiplier is directly in line with the laser. Obstructing the direct passage of light from the laser to the photomultiplier is a metal bar. As particles pass through the laser beam, light is scattered above and below this bar and is detected. The degree to which this occurs depends to some extent on the size of the particles involved (Benson et al 1984). The light scattered at right angles is frequently used in studies involving lymphocytes and is said to relate to the granularity of the cell. Also situated at right angles to the laser beam are the fluorescence detecting photomultipliers which detect light due to fluorescence of specific dyes only by the use of selective filters. There are two further requirements for flow cytometry, a cell suspension and a suitable fluorochrome to stain DNA.

DNA Fluorochromes

A variety of fluorochromes have been used to stain DNA for flow cytometry. These include propidium iodide (PI), ethidium bromide which is chemically similar, mithramycin and DAPI (4',6-diamidino-2-phenylindol-hydrochloride). The latter stains DNA specifically but requires an ultraviolet light source not available to us at the start of this project. Similarly the wavelength necessary for the excitation of mithramycin, 395 nm cannot easily be produced by our argon laser. Of the others, propidium iodide was chosen as this agent has been used by the greatest number of investigators working with prostate cancer and it is suitable for use in our flow cytometer.

Propidium iodide binds to DNA by intercalation (Wilson et al 1986). Unbound, its optimum absorption and emission wavelengths are 482 and 608 nm respectively and 540 and 608 nm when bound to DNA (Tas and Westerneng 1981). The degree of PI binding is proportional to the amount of DNA present ie the binding is stochiometric (Tas and Westerneng 1981, Boer 1975 - using the closely related ethidium bromide). The same workers have shown that propidium iodide does not bind to other biological macromolecules except double stranded RNA. The binding to RNA is potentially misleading , but it can easily be abolished by the addition of RNAase which will specifically degrade RNA whilst leaving DNA intact (Vindelov 1977).

Cell Suspensions

The preparation of a cell suspension from a solid tumour has been attempted in several ways.

Mechanical

- Fine needle aspiration

This technique has been principally used by workers from the Karolinska Institute, Stockholm. It seems to produce sufficient cells for flow cytometry although some extra disaggregation is necessary. Tribukait et al (1980) used pepsin and Whelan et al (1986) ethylene-diamine tetracetate (EDTA). One problem with this approach is that the sampling error is potentially large because a very small biopsy is taken and this is performed usually without any localisation technique. The principal advantage is that the test may be repeated without an anaesthetic.

- Fine mincing/cutting/ultrasonic agitation

A variety of mechanical disaggregation techniques have been used. Schultz et al (1985) minced their fresh prostate samples and then passed the crude suspension repeatedly through a 19 gauge needle before filtering through a fine mesh. Charig (Institute of Urology, London - Personal communication 1987) used a purpose built device not unlike a small kitchen blender apparently with good effect. Lawry et al (1987) used an ultrasonic bath in addition to enzyme digestion for breast tumours, which are similar in texture to prostate.

Detergent - eg Nonidet

Vindelov (1977) pioneered the use of detergents in the production of nuclear suspensions from fresh Franzen needle aspirations. The detergent was added to the sample together with the propidium iodide. It causes the lysis of cell membranes and therefore allows access to the nuclei for staining.

Chelating agent - eg EDTA

Whelan et al (1986) reported the production of a cell suspension from a needle biopsy core after it had been cut into 1mm lengths and placed in 0.2% EDTA. It is known that calcium ions play a part in the function of desmosomes which stick cells together (Seglen 1976). The chelation of this ion therefore can cause a degree of cellular disaggregation.

Enzyme

Enzymes such as pepsin (Tribukait et al 1980) and trypsin have been used with good effect (Engelholm et al 1985) usually in combination with mechanical methods.

Cell suspensions from paraffin embedded material

In contrast to fresh tissue one technique predominates in the preparation of cell suspensions from paraffin embedded material, that of Hedley et al (1983). A thick section is cut from the paraffin block. The tissue is dewaxed with xylene and then rehydrated through serial washes with 100, 95, 70 and 50% ethanol and then finally distilled water. The tissue is then digested for 10 minutes with 0.5%

pepsin in normal saline with the pH adjusted to 1.5 by the addition of hydrochloric acid. A suspension of cells, or more accurately of nuclei with some adherent cytoplasm, is produced. Although the tissue used was ovarian the technique works well with many other tissues. Indeed the same workers have also studied carcinoma of the breast and adenocarcinoma of unknown primary site (Hedley et al 1985). They felt that the use of embedded material several years old had the great advantage that a retrospective analysis could be performed and the flow cytometry compared with the known outcome of the patients. However they also point out that the use of paraffin embedded material results in a loss of resolution of the DNA histogram with wider peaks and higher coefficients of variation (CVs).

Interpretation

There is no universally accepted method of interpreting DNA flow cytometry. Most authors interpret DNA histograms in terms of the cell cycle. A histogram derived from a suspension of normal cells contains a main peak corresponding to the diploid quotient of DNA and a smaller peak containing exactly twice the diploid quotient of DNA. The first peak is often referred to as the G0/1 peak and the second the G2/M peak. Between these two peaks is a plateau comprising cells in the S phase. Abnormal histograms may show nuclei which contain a clearly pathological amount of DNA or abnormal numbers of nuclei in the S phase or G2/M phase. There is experimental evidence which seems to support the accuracy of flow

cytometry in determining the proportions of cells in the various phases of the cell cycle (Fried et al 1976). However, Tribukait et al (1986) have found that the use of the suffix - ploid (eg diploid) may not be justified. They found that whilst cells in the diploid and tetraploid regions on flow cytometry did contain roughly a normal chromosome or a double chromosome complement respectively, "aneuploid" cells seemed to contain more DNA on flow cytometry than would be expected from chromosome counting. It is probably inappropriate therefore to refer to a group of nuclei with an abnormal DNA content as aneuploid unless chromosome studies have been performed. In 1984 a number of workers, principally American, published a convention on nomenclature for DNA cytometry (Hiddemann et al 1984). They recognised the terminology problem and suggested that any abnormal cells on DNA cytometry should be termed either an abnormal DNA stemline or DNA aneuploid. In practice this is largely ignored and authors tend to define their own terminology and interpretation protocol. They also favoured the calculation of the DNA index whereby the position of a second peak can be defined in terms of a multiple of the normal G0/1 peak DNA content. Thus a tetraploid peak or a G2/M peak would have a DNA index of 2 as it corresponds to twice the DNA content of the normal G0/1 peak.

Application to Prostate Cancer

Following Zetterberg's work with static cytometry, Bichel et al (1977) from Denmark, applied the concept of measurement of nuclear DNA content using a flow cytometer

to prostate cancer. Fresh aspirated cells from 50 patients were suspended in buffer at 7.4 pH and stained with ethidium bromide in the presence of sodium citrate, sodium chloride and a detergent Nonidet-P40 (method described by Vindelov 1977). Using this technique 50,000 cells were analysed. In all cases examined a diploid peak corresponding to that of human lymphocytes was seen. Only 4 out of 29 patients with BPH displayed any discrete second peak and these were in the tetraploid region. They then analysed cells from well, moderately and poorly differentiated prostate cancers and found an increased proportion of abnormal DNA histograms, the highest amongst the poorly differentiated tumours. No data with respect to survival was given.

Roters et al (1980) analysed cell suspensions from 31 prostate cancers. Fresh cells were obtained by transrectal aspiration and were stained with ethidium bromide and mithramycin after disaggregation of any cell clumps with pepsin. Using a mathematical model to correct for "background" (presumably due to cell and nuclear fragments) and for aggregation of cells they claimed that all of their 7 BPH cases had greater than 95% of cells in the G0/1 or diploid peak. All carcinomas had less than 95% of cells in this peak. 64% were described as diploid; presumably the authors meant that their main peak was in the diploid position and 36% were tetraploid. Amongst all the prostate cancers analysed there was no non-tetraploid aneuploidy. For theoretical reasons these workers felt that the results obtained by Bichel et al

(1977) were not due to different nuclear DNA content in the nuclei of tumours with the same grade but due to the sampling problem inherent in needle aspiration biopsy which are small and essentially taken blind. The problem is that a variable number of normal cells will be aspirated with the tumour cells, diluting the effect of the tumour cells on the DNA histogram. No experimental evidence was given to support this argument. Roters et al (1980) also failed to demonstrate any correlation between histological grade and nuclear DNA content. They envisaged the role of flow cytometry in the diagnosis of cancer rather than predicting prognosis.

Contemporaneous work from the Karolinska Institute, Stockholm contradicts this view. Tribukait et al (1980) analysed cells from 54 prostate cancers, again using fresh cells obtained by needle aspiration. These were fixed in ethanol, disaggregated with pepsin, resuspended in EDTA and finally stained with ethidium bromide prior to flow cytometry. DNA histograms were divided into diploid and aneuploid on the basis of the percentage of tetraploid cells. The vast majority of aneuploid peaks were in the tetraploid region although no precise limits were set to define the extent of this region. 62 samples from patients with BPH were studied. The mean % of cells in the G2 or second peak was 2.4 with a standard deviation of 1.75. Despite the distribution being slightly skewed an upper limit of 5.9, that is the mean + 2 standard deviations, was set. Regrettably we do not know what proportion of benign samples fell outside this limit as it is not stated. There was a definite preponderance of aneuploid

DNA histograms in poorly differentiated tumours and in addition the more obviously abnormal traces predominated amongst this tumour grade.

Later work (Lammel et al 1981) suggested that there was indeed some relationship between DNA flow cytometry and histological grade in that it was often impossible to distinguish between well differentiated prostate cancer and BPH.

Ronstrom et al (1981) analysed some 500 aspiration samples from the prostate. 301 were cytologically defined as benign, 33 suspicious of malignancy and 166 definite carcinomas. In the benign samples the mean percentage of cells in the second or G2/M peak was 4.2% with a standard deviation of 1.4%. Using the mean + 2 standard deviations a cut-off of 7% was set. Of the 166 cancers 45 (27%) had diploid traces, 75 (45%) had tetraploid traces ie a large second peak in the G2/M position and 46 (28%) were non-tetraploid aneuploid having second or multiple peaks in abnormal positions. The correlation between ploidy and histological grade was marked. Later work, with the number of prostate cancer samples increased to 301, produced very similar results (Tribukait et al 1983). Poorly differentiated tumours had a higher proportion of abnormal traces of all types, tetraploid, simple non tetraploid and those with multiple peaks.

Frankfurt et al (1984), undertook a large study using DNA flow cytometry in a variety of tumours from different anatomical sites. Included amongst these were 18 prostate

cancer cases. Again a close correlation was found with histological grade and with clinical staging. Interestingly, this correlation could only be demonstrated in prostate and bladder cancer. Further work using material from 45 prostate cancer cases (Frankfurt et al 1985 + 1986) again showed correlation with Gleason grade and the degree of tumour dissemination. Thirty four of the specimens were acquired from radical prostatectomies performed for what was presumed to be localised disease. In addition 11 needle biopsies were taken from patients with distant metastases (stage D_2), 8 from the primary tumour and 3 from a metastatic deposit. All patients with no capsular penetration or lymph node involvement had diploid tumours (n=11). 15 had capsular penetration but no lymph node involvement (stage C), 47% of these had aneuploid flow cytometry. Of the 8 patients found to have pelvic lymph node involvement (stage D_1), 62.5% had aneuploid flow cytometry. The proportion of aneuploid DNA histograms rose to 73% amongst the 11 patients with distant metastases (stage D_2). Although both glandular differentiation as shown by the Gleason score and DNA flow cytometry correlated with clinical stage, the authors felt that the combination of these two appeared to predict staging more accurately then either taken alone. Specifically the combination appeared to enhance the detection of patients with clinically localised disease. Of 14 patients with low Gleason scores (5-6) and diploid flow cytometry, 10 (71%) had localised disease. Of 8 patients with Gleason scores also of 5-6 but aneuploid flow cytometry, none had localised disease. Flow cytometry

in patients with poorly differentiated tumours ie Gleason score 7-10 was of no additional value. In the presentation of this aspect of their data the authors exclude 5 cases, including 2 with diploid flow cytometry and Gleason scores of 3 and 4. The latter case was stage B, the former was D_2. The exclusion of a patient who had disseminated disease, a low Gleason score and diploid flow cytometry is difficult to justify. Also excluded were 3 patients whose biopsies were taken from metastases who could not be classified by Gleason's system. One of these had diploid flow cytometry. These workers were the first to suggest that DNA flow cytometry when added to conventional grading could enhance our ability to detect patients likely to have localised disease and therefore a favourable prognosis.

The widespread use of paraffin embedded archival pathological material following the work of Hedley et al (1983) led to further work relating ploidy to grade and prognosis. De Vere-White et al (1984) using fresh human prostate cancer cells noted a correlation between aneuploidy and a high Gleason score, however amongst the intermediate histological grades there was a wide variation in the DNA flow cytometry indicating that this may possibly be of additional prognostic value. They report a comparison of the Dunning rat prostatic carcinoma both fresh and after formalin fixation and paraffin embedding. The embedded material gave better DNA histograms with higher peaks and smaller coefficients of variation.

In the UK, Fordham et al (1986) reported a retrospective study of 72 patients. They had undergone surgery for relief of bladder outflow obstruction and were found to have prostate cancer. The majority of patients received treatment, either hormone manipulation, radiotherapy or both, but it is not clear whether this was given electively at the time of diagnosis or held in reserve until symptomatic progression occurred. Paraffin embedded histological material was used. 35 tumours were said to be diploid, the remaining 37 were aneuploid. No significant difference could be found between the Gleason scores of the aneuploid and diploid tumours. Both the Gleason score and the presence of aneuploidy correlated with survival. Thus these two factors appeared to give independent prognostic information and in combination produced a more accurate prediction of outcome.

In summary DNA flow cytometry has been found to be related to clinical staging and to prognosis. Some have suggested that it provides additional prognostic information over and above conventional histology.

Multiple factor analysis.

A key question in evaluating a technique for the prediction of prognosis is whether this method generates new information or merely information already available to the clinician by another means. For instance it is possible that DNA FCM may be closely related to histological grading. When this fact is taken into account there may be little to be gained by performing DNA FCM in addition to routine histology even though taken in

isolation it is a prognostic indicator.

The problem of analysing multiple and perhaps interlinked prognostic factors in prostate cancer has been tackled by Byar and Corle (1984). Using data from 4,000 patients entered into the VACURG studies they performed an analysis to determine the relative importance of each factor. They then selected a number of variables that were useful to determine prognosis and calculated a prognostic score. For localised disease the factors were size of the primary, Gleason score, serum acid phosphatase and whether endocrine treatment had been given. For locally extensive and disseminated disease (stages III and IV) these were as above with the addition of the presence of metastases and ureteric obstruction. The statistical technique they used, Cox's proportional hazards model is frequently used in similar situations (eg Stephenson et al 1987 - DNA flow cytometry discussion).

PATIENTS

Prospective Study

Men without prostate cancer

Reference

Elective surgery for BPH

Acute retention due to BPH

Men with prostate cancer

Retrospective Study

Prostate cancer patients

Benign controls for DNA FCM

Subsidiary studies

Patients

Patients were recruited prospectively and taken from a previous study commenced in 1984, the latter forming a retrospective study group.

Prospective Study

Men without prostate cancer

Reference group

50 men admitted to the urology wards for surgery unrelated to bladder outflow obstruction or to prostate cancer. Age matching was achieved by selecting patients of 49 or over. Those with a history of prostatic disease, those being investigated for bladder outflow symptoms or being considered for prostatic surgery were excluded. Urinary symptoms were assessed by means of a symptom scoring system (Frimodt-Moller et al 1984). Patients underwent digital rectal examination and transrectal ultrasonography to exclude prostatic disease and to measure prostate volume.

Elective surgery for BPH

51 men admitted for elective surgery for BPH. Patients with evidence of prostate cancer preoperatively on digital rectal examination or transrectal ultrasonography were excluded. In 9 cases a bladder neck incision alone was performed and therefore histology was not available. In the remaining 42 cases histological examination confirmed the presence of BPH alone. 5 patients were considered preoperatively to have benign disease and therefore were provisionally included. However they were reclassified

postoperatively as their histology revealed an unsuspected cancer (ie To). These men were reallocated to the prostate cancer group described below and are not included in the group of 51 patients described here.

Emergency admissions for acute retention due to BPH

44 men admitted with acute retention. 34 subsequently underwent TURP and were found to have BPH on histological examination of the resected material. 10 patients admitted with retention did not undergo TURP, 9 had no operation and were either managed with their catheter in situ or successfully passed urine after its removal, the remaining patient underwent a urethrotomy. In none of these was prostate cancer suspected. A further 7 patients who presented with retention were later shown to have prostate cancer and were allocated to the prostate cancer group.

Men with prostate cancer

95 patients with recently diagnosed prostate cancer. All were untreated apart from their initial TURP. Staging was by digital rectal examination, transrectal ultrasound and isotope bone scan. 42 patients had cancer apparently confined to the prostate, 28 locally advanced disease and 25 metastatic disease. Lymph node staging was not attempted.

Retrospective Study

Prostate cancer patients

61 prostate cancer patients recruited in 1984 by Mr P H Powell for a study relating to serum markers in prostate cancer (Gibb et al 1986). These represent a consecutive unselected population of untreated patients that presented

to urologists at the Freeman Hospital. Assessment comprised digital rectal examination, T staging and prostate volume estimation by transrectal ultrasonography and isotope bone scanning. Blood was taken for numerous assays in relation to the original project and these included PSA (Hybritech Tandem-R kit). Patients were followed up in the prostate cancer clinic at the Freeman Hospital. This was at intervals of not less than 6 months. At each visit symptoms were assessed and the above assessment procedure repeated. A deferred treatment policy was applied, that is hormone manipulation was reserved for those who developed symptomatic progression. The recruiting and initial assessment of these patients was performed by Mr Powell, subsequent follow-up data was obtained by myself.

Benign controls for DNA FCM

48 consecutive patients who underwent prostatectomy either by TURP or by open operation in 1984 were identified by reference to records held in the histopathology laboratory, Freeman Hospital. All histology was reviewed and only those with BPH were selected.

Subsidiary studies

Diurnal variation

6 inpatients with serum PSA values covering a broad range.

Surgery - pilot study

4 inpatients undergoing bladder outflow surgery.

Rectal examination

20 consecutive patients attending the prostate cancer clinic.

Women

10 women patients each over the age of 50 admitted for urological surgery or investigation.

Diagnoses - 3 superficial bladder tumours, ureteric colic, recurrent urinary tract infections, carcinoma of the rectum with urinary symptoms, clear cell carcinoma of the kidney, mucous retention after caecocystoplasty, ileal conduit stenosis, pelvi-ureteric junction obstruction, investigation of high residual bladder volume after colposuspension.

Radical cystoprostatectomy patients

7 men who had previously undergone the total excision of their prostate in conjunction with a radical cystectomy for bladder cancer.

METHODS 1

Prostate Specific Antigen

Prospective and retrospective studies

Patient assessment

Prospective study

Symptoms
Transrectal ultrasound
Blood investigations
Isotope bone scan

Retrospective study

PSA

Sample collection and handling
Assay

Ethics

Statistical methods - see Methods 2 page 194

Appendix Symptom scoring questionnaire

Patient assessment

Prospective study

Symptom assessment

This was performed using a standard questionnaire which focuses on obstructive and irritative bladder symptoms. (adapted from Frimodt-Moller et al 1984 -see appendix page 93)

Transrectal Ultrasound

Transrectal ultrasonography was performed by a single operator(TC) on all patients studied prospectively. The aim was to exclude any clinically unsuspected pathology in the prostate, to measure the prostatic volume and in the case of cancers to determine the T category. All ultrasound scans in the main retrospective group were performed by a then Senior Registrar in Urology (Mr P H Powell - PHP); in this group staging was the main aim, only relatively crude estimates of prostatic volume were obtained. A Kretz Combison 320 machine was used (Kretztechnik (UK) Ltd, Spindle Way, Crawley, Sussex RH10 1TG) with a rotating 6 MHz ultrasound probe which produces images in the transverse plane.

Volume determination was by the planimetric method described by Hastak et al 1982 and by Bartsch et al 1982 using the software incorporated into the ultrasound machine. The rectal probe is clamped onto a specially designed jig which holds the probe steady and allows it to be withdrawn in 5mm increments. The probe is aligned so that a true transverse image is obtained rather than an oblique one. The instrument is inserted until the most

proximal part of the prostate is seen. The image is frozen on the screen and the area of the prostate is measured by drawing around it with a light pen. The probe is then withdrawn 5mm and the area measurement repeated and so on until the apex of the prostate is reached. The computer in the machine calculates the volume from all the area measurements (Volume = (A1xA2x...Ay)1 where A=area, y areas are measured and 1 = distance between area measurements). It is well established that digital rectal examination gives a poor estimate of prostatic size (Meyhoff and Hald 1978) and it has certainly been demonstrated in experimental conditions that ultrasound volume determination is accurate (Peeling et al 1987).

Transrectal ultrasound was preferred to digital rectal examination in the determination of T category. Many studies relating to the pathological examination of radical prostatectomy specimens have shown that digital rectal examination underestimates the T category (Pontes et al 1985, Fujino and Scardino 1985).

Blood Tests

Blood was taken for a variety of investigations. These included haemoglobin, creatinine and alkaline phosphatase which were measured in the routine laboratory. Prostatic acid phosphatase was measured using the Hybritech Tandem E radio-immunometric assay (Hybritech, Minerva House, Spaniel Row, Nottingham). The upper limit of normal quoted by the hospital for this assay was 4 ng/ml. However in order to compare this assay with PSA no limit was set for this study. A limit was set using the same reference

population as for PSA, the assays being performed in parallel. The measurement of PSA is discussed below.

Isotope bone scans

These were all performed in the Medical Physics Department of the Freeman Hospital. A standard protocol has been in operation during the period of this study.

The patients were not prepared in a specific way. A dose of 555 MBq of technetium 99 labelled methylene diphosphonic acid was injected. After 2-3 hrs the patients were imaged using a wide aperture gamma camera. Three views were taken with the patient supine and three in the prone position in order to obtain anterior and posterior views. The scans were reported by a consultant radiologist in conjunction with the senior medical physicist. Equivocal scans were reviewed with the same radiologist with plain X-rays if appropriate. Scans were considered positive ie showing bone metastases or negative.

Retrospective study

The initial assessment of these patients was performed by Mr P H Powell. All patients underwent symptom review, clinical examination, transrectal ultrasonography and a variety of blood investigations including PSA. Bone scans were performed at diagnosis and at each follow-up visit. This was at 6 monthly intervals or more often if clinically indicated.

Prostate Specific Antigen (PSA)

Sample collection and handling

Blood for serum PSA was taken prior to rectal examination,

transrectal ultrasound or in the case of acute retention cases prior to catheterisation. The blood was allowed to clot in plain glass tubes and the serum was then separated and frozen in small aliquots at -18°C. Typically the time between venesection and separation was 2 - 4 hours. Studies to confirm the stability of PSA in unseparated blood at room temperature and its stability in the freezer were undertaken (see page 127).

Assay

Three testing kits were used, but the Hybritech PSA Tandem R kit (Hybritech UK Ltd, Minerva House, Spaniel Row, Nottingham) was taken as the standard. Unless otherwise specified PSA values quoted are from this assay. The other assays, Yang Pros-check (Baxter Travenol) and DPC-PSA (Diagnostic Products Limited) were used to allow comparison of different systems. The Hybritech Tandem-R is a radio-immunometric assay, using two monoclonal antibodies. The first is bound to a plastic bead and to this serum or calibrant is added. A second radio-labelled antibody solution is added to the tubes containing serum/calibrant and beads. This antibody binds to a separate antigenic site on the PSA molecule. After vortex mixing the tubes are incubated at room temperature on a horizontal agitator for 2 hours. Any PSA present will bind to the first antibody and the labelled second antibody then binds to the PSA forming a sandwich. The beads are washed and placed in a gamma counter, the amount of radioactivity measured is proportional to the concentration of PSA in the sample. Calibrant solutions are supplied with the kit. The 5 point calibration curve

that results is not quite a straight line but is slightly curved. Thus a polygonal interpolation program was used to derive the PSA values from the radioactivity counts (software written by I Gibb, Principal Biochemist, Freeman Hospital, Newcastle upon Tyne).

The performance of the assay was checked by both internal controls made up from pooled sera and commercial quality control material. A full evaluation of the technical performance of the Hybritech assay and that of the other two can be found as an appendix to the prospective study results and discussion section (page 133).

Ethics

Patients in this study were managed by a deferred treatment policy which is the routine in the Freeman Hospital. For this reason they were carefully followed up and frequently investigated. No additional radiology or isotope scans were ordered for research purposes and the amount or frequency of blood sampling was not changed. In the prospective study patients were asked to submit to transrectal ultrasonography which was not a routine part of their treatment. Verbal consent was obtained. In addition a few patients were asked to give extra blood samples. The work described here was approved by the Freeman Hospital Ethical Committee.

Appendix to methods

PROSTATE STUDY:SYMPTOM QUESTIONNAIRE

Name Study no

Hospital No

Date Age DoB

Length of symptoms

IRRITATIVE SYMPTOMS

Frequency

Hours	Points	Score
>3	0	
2-3	1	
1-2	2	
<1	3	

Nocturia

No/night	Points
0-1	0
2	1
3-4	3
>4	4

Enuresis

No	0
Yes	1

Urgency

None	0
Mild	1
Moderate	2
Incontin	3

Score

OBSTRUCTIVE SYMPTOMS

Stream Score

Normal	0
Variable	2
Weak	3
Dribbling	4

Hesitancy

No	0
Yes	1

Intermittency

No	0
Yes	1

Terminal Dribbling

No	0
Yes	1

Bladder Emptying

Normal	0
Variable	1
Incomplete	2
Retention	4

Straining

No	0
Yes	1

Score

Total Score_____

Adapted from Frimodt-Moller et al 1984

RESULTS AND DISCUSSION -1

PSA - prospective study

RESULTS

Men without prostate cancer

Group characteristics

- reference
- BPH elective admissions
- BPH acute retention

Follow-up of extreme values

Distributions and cut-off levels

Prostate cancer patients

Patient characteristics
PSA and tumour volume/stage

PSA in diagnosis

ROC analysis

Comparison with PAP

Further data from prospective study

Transrectal ultrasound
- volume measurement
- diagnosis
- staging

Assays
- performance of Hybritech
- comparison with Yang and DPC

DISCUSSION

Men without prostate cancer

Follow-up of extreme values

Distributions and cut-off levels

Diagnosis of prostate cancer

ROC curve analysis

Transrectal ultrasound
- volume measurement
- detection of prostate cancer
- staging

Conclusions

Men without prostate cancer

Three groups of men without prostate cancer were studied (see patients and methods).

Reference group

The characteristics of this group with respect to age, prostate volume by ultrasound, serum PSA and PAP and symptoms are given below (table 3).

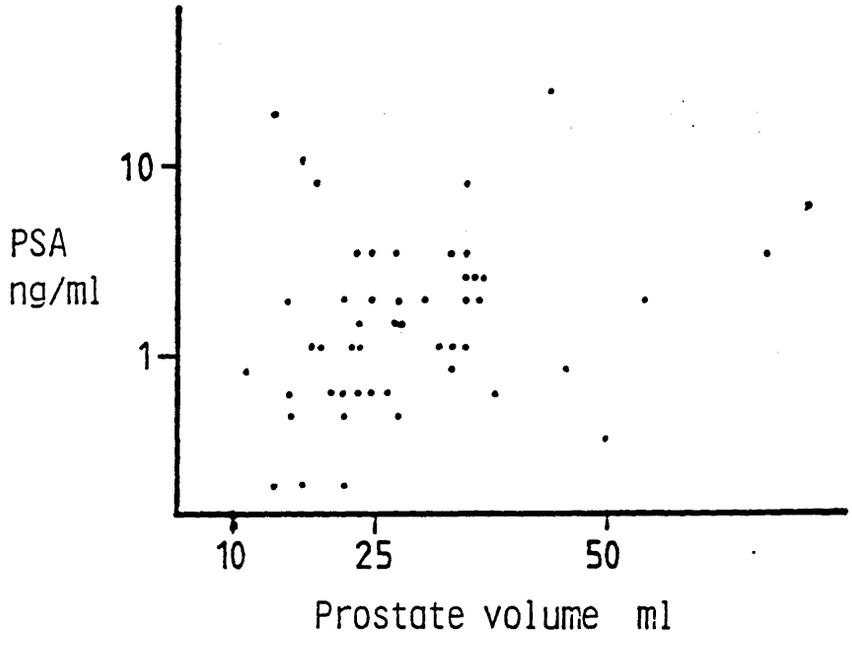
Table 3
Reference group

n=50	Mean	S. D.	Range	Median
Age (yr.)	67	8.63	49-83	66
US vol (ml)	29.5	13.1	11.5-74.1	26.6
PSA (ng/ml)	3.05	5.09	0.2-27.0	1.6
PAP (ng/ml)	1.47	0.87	0.5-5.3	1.17
Symptoms	4.3	4.7	0-18	2.0

There was no relationship between PSA (or logPSA) and prostatic volume, age and symptoms in this group (figure 1 a-d). There was correlation between PSA and PAP but this was not particularly close ($r=0.327$, $p<0.05>0.02$). The exclusion of patients with enlarged prostates, volumes greater than 30mls or with more than mild urinary symptoms, symptom score greater than 5 made little difference to mean, median or the shape of the distribution.

Figure 1a+b

Men without prostate cancer - reference group
PSA vs prostate volume



PSA vs age

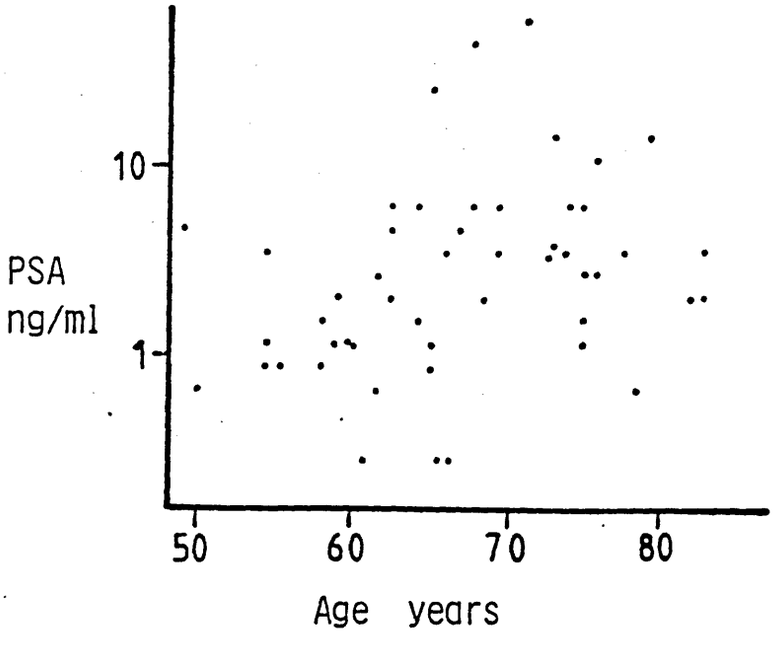
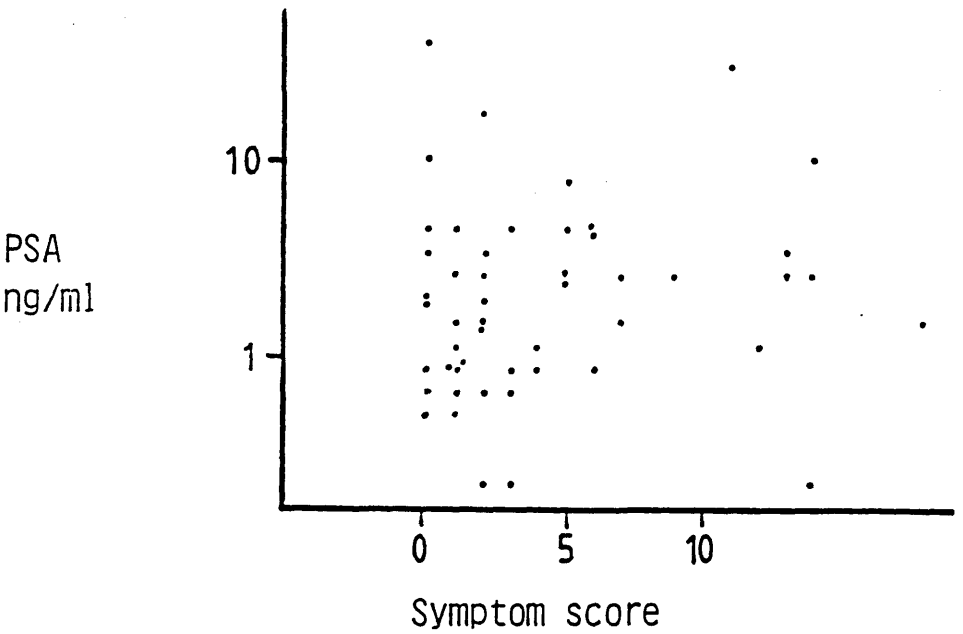
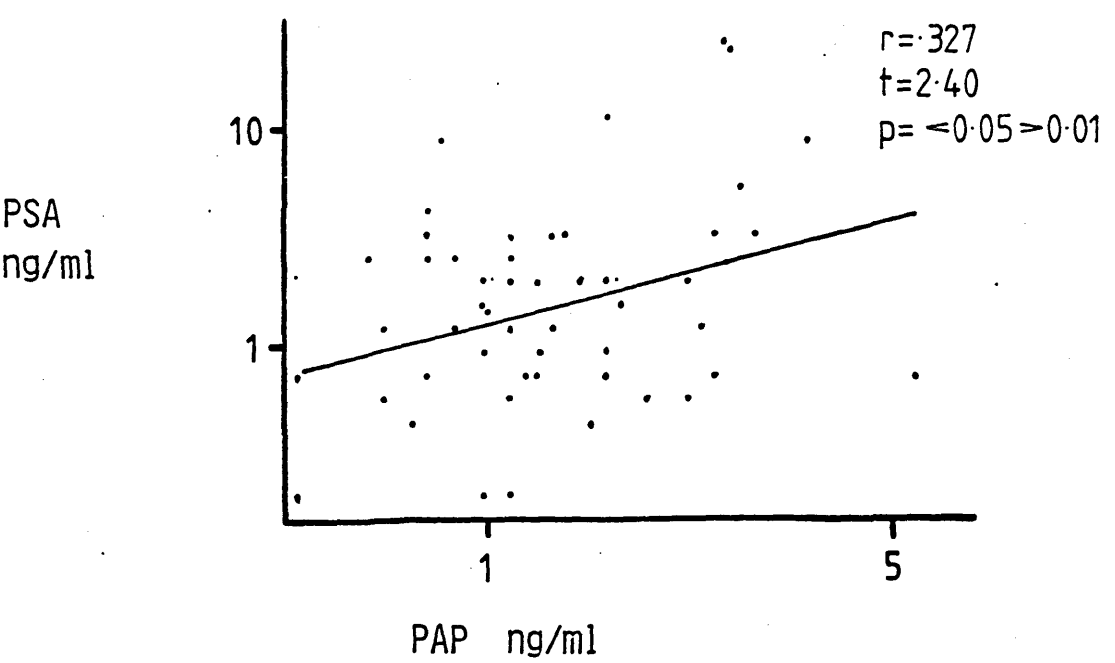


Figure 1c+d

Men without prostate cancer - reference group
PSA vs symptom score



PSA vs PAP



Benign Prostatic Hyperplasia

Elective group

Data from this group is summarised in table 4. In 42 patients the presence of BPH was confirmed histologically after examination of prostate chips from transurethral resection. The remaining 9 were treated by bladder neck incision alone and no histology is available. Data relating to these two subgroups of patients is presented in table 5.

Table 4
Benign prostatic hyperplasia - elective group
All patients

n=51*	Mean	S. D.	Range	Median
Age (yr.)	69.1	7.48	53-84	70
US vol (ml)	53.0	28.6	15.1-126	47.4
PSA (ng/ml)	6.99	9.79	0.5-66.5	4.7
PAP (ng/ml)	2.99	3.25	0-20.6	2.2

* Histology available on 42, 9 patients underwent bladder neck incision with no biopsy

Table 5
BPH-Elective group
TURP and histologically confirmed

n=42	Mean	S. D.	Range	Median
Age (yr.)	70.0	6.89	57-84	70
US vol (ml)	58.6	28.5	15.1-126	53.9
PSA (ng/ml)	8.13	10.4	0.5-66.5	5.65
PAP (ng/ml)	3.32	3.48	0-20.6	2.55

Table 5 continued

BNI-no histology

n=9	Mean	S. D.	Range	Median
Age (yr.)	65.2	9.23	53-78	65
US vol (ml)	27.0	5.84	18.6-35.5	25.2
PSA (ng/ml)	1.71	1.09	0.6-3.2	1.2
PAP (ng/ml)	1.41	0.76	0-2.7	1.5

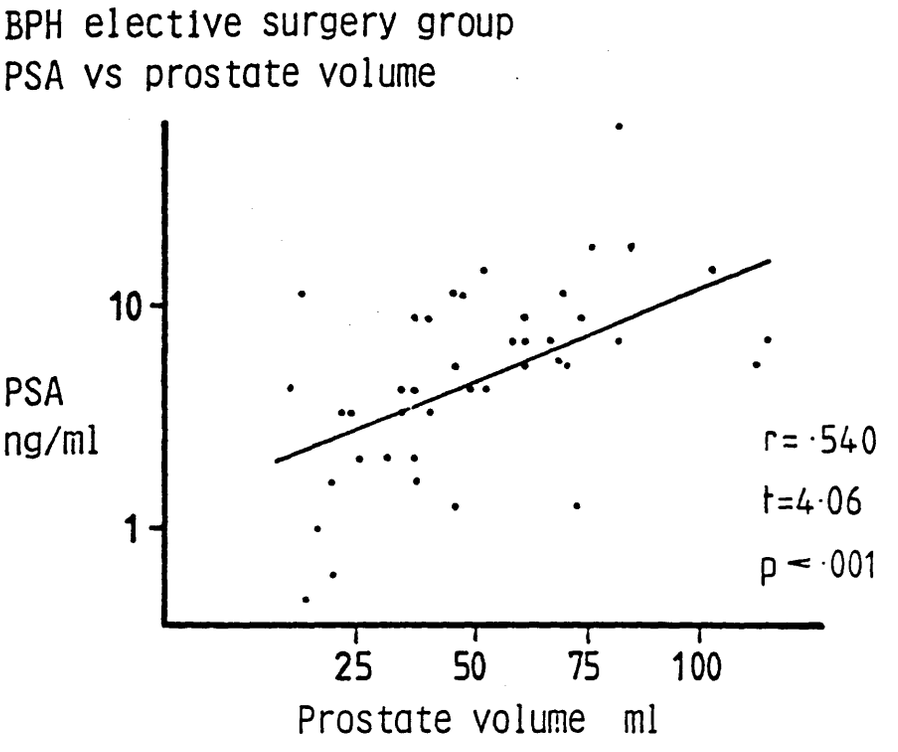
The bladder neck incision group had smaller glands with lower PSA and PAP in addition. These were significant differences, $p=0.002$, $p<0.001$, $p<0.001$ prostate volume, PSA and PAP respectively (unpaired T test, after log transformation in the case of PSA and PAP). Patients in the BNI group tended to be younger with a mean age of 65.2 years compared with 70.0 years for those treated by TURP but this difference did not quite reach statistical significance ($p=0.08$).

For both the whole elective surgery group (n=51) and those 42 with histological confirmation there was a correlation between the serum PSA and the prostate size. This is most clearly seen after taking logs, $r=.622$ $t=5.56$ $p<0.001$ for all cases, n=51 and $r=.540$ $t=4.06$ $p<0.001$ for histologically confirmed cases, n=42 (figure 2a). There was no correlation between either PSA or PAP and age, but there was good correlation between PSA and PAP ($r=.564$ $t=4.21$ $p<0.001$) (figures 2b+c).

Benign Prostatic Hyperplasia
Acute retention group

The data for these patients is seen in table 6. Of the 44

Figure 2a+b


BPH elective surgery group
PSA vs prostate volume
10
1
PSA
ng/ml
r=·540
t=4·06
p<·001
25
50
75
100
Prostate volume ml

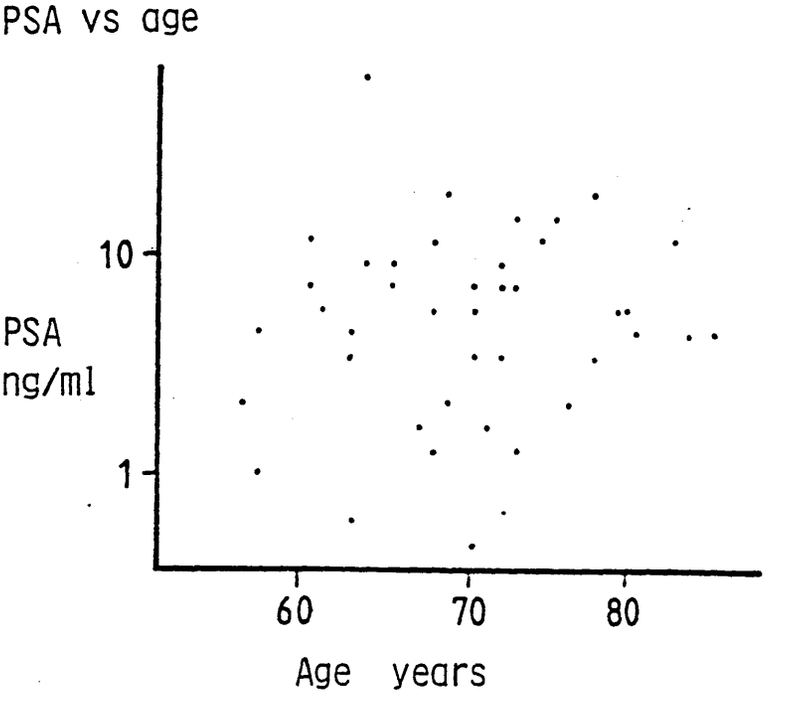
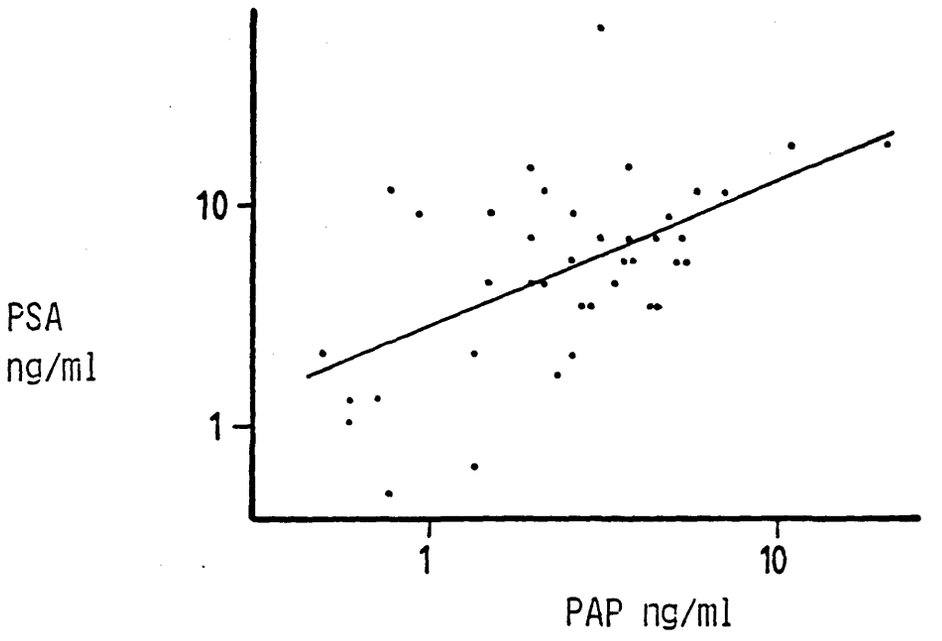

PSA vs age
10
1
PSA
ng/ml
60
70
80
Age years

Figure 2c

BPH elective surgery group
PSA vs PAP



r = ·564

t = 4·21

p < ·001

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patients who presented in acute retention, only 34 underwent a TURP. In 9 cases no operation was undertaken and in 1 a urethrotomy alone was performed. For these patients there was no histological confirmation of the benign nature of their prostates. They were of comparable age to the patients who underwent a TURP. Their prostatic volumes were significantly lower p=0.001 (t test). PSA and PAP also tended to be lower in the non-operative group but this difference was not significant. (table 7)

Table 6
BPH-Acute retention group
All patients

n=44*	Mean	S. D.	Range	Median
Age (yr.)	72.3	7.75	52-88	74
US vol (ml)	70.2	34.3	14.7-163	71.9
PSA (ng/ml)	19.8	25.0	0.4-115	10.8
PAP (ng/ml)	14.0	19.3	1.1-91.8	5.83

* All patients, 10 without histological confirmation, 9 did not undergo surgery and 1 was treated by urethrotomy alone.

Table 7
Acute retention - TURP and histologically confirmed

n=34	Mean	S. D.	Range	Median
Age (yr.)	72.2	7.10	52-84	73
US vol (ml)	76.0	34.5	23.2-163	73.9
PSA (ng/ml)	21.6	27.0	0.4-115	11.7
PAP (ng/ml)	16.6	21.3	1.1-91.8	7.97

Table 7 continued
Acute retention - no TURP, no histology

n=10	Mean	S. D.	Range	Median
Age (yr.)	72.7	13.3	52-88	77
US vol (ml)	43.3	16.5	14.7-61.5	49.1
PSA (ng/ml)	13.5	16.1	2.1-55.2	6.55
PAP (ng/ml)	5.37	3.30	1.8-13.1	5.15

In all subsequent analyses involving the elective and acute retention group only patients with histologically confirmed BPH will be used.

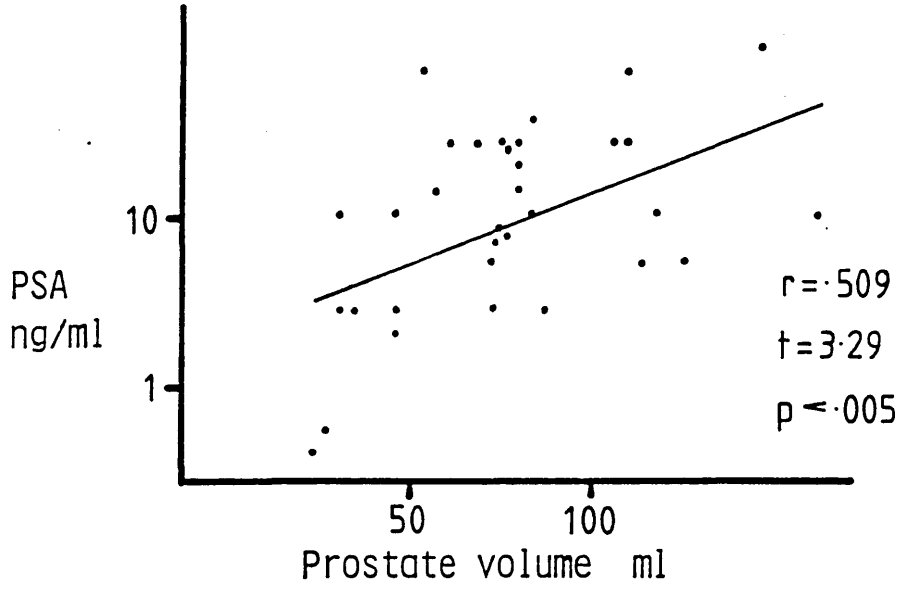
The mean age of acute retention patients was slightly greater than that of the elective group, however this difference is not statistically significant. Retention patients in general had larger prostates and higher PSA and PAP levels $p=0.023$, 0.011 and <0.001 respectively (students t test). There was a correlation between log PSA and prostatic volume, $r=.509$ $t=3.29$ $p<.005$ (figure 3a). If the regression lines for log PSA against prostatic volume for both elective and retention patients are plotted together it can be seen that they do not coincide. In other words, the PSA per unit prostatic volume is higher in acute retention cases and the observed difference between the two groups seems to be not entirely explained by the difference in prostatic volumes. The difference however between the two regression lines does not quite reach statistical significance.

As in the case of the elective surgery patients there was no correlation with age and fairly close correlation with

Figure 3 a+b

BPH Acute retention group

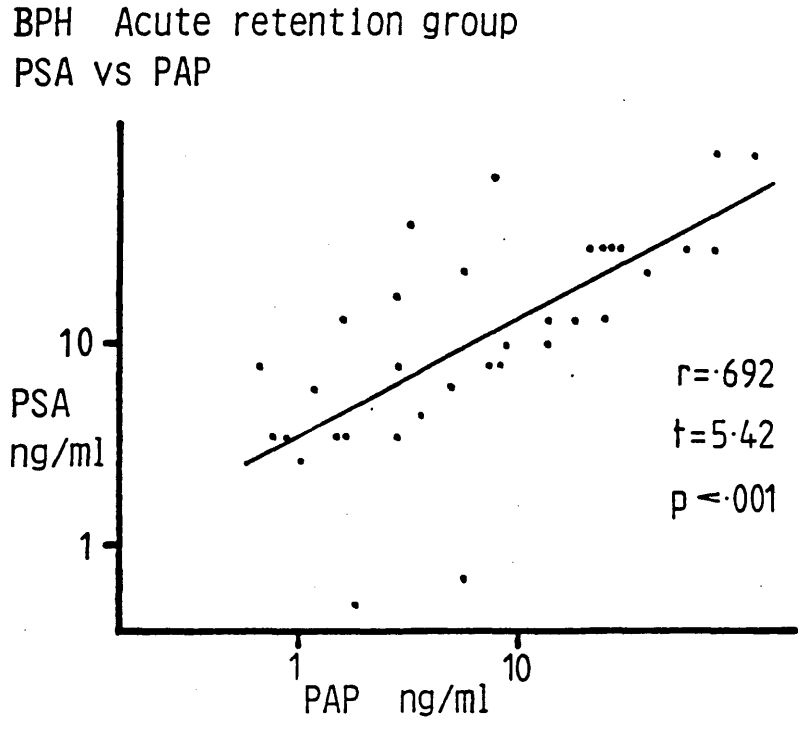
PSA vs prostate volume



PSA vs age



Figure 3c


BPH Acute retention group
PSA vs PAP
10
PSA
ng/ml
1
r=·692
t=5·42
p <·001
1
10
PAP ng/ml

PAP (figure 3b+c).

Follow-up of extreme values

Within the elective and the acute retention groups further samples were taken from some patients found to have significantly elevated PSA levels. These have been tabulated (table 8). In all cases after prostatic resection the PSA levels taken some months after operation had fallen significantly.

Table 8
Follow-up of high PSA levels

	Pre-op PSA (ng/ml)	Follow-up PSA (ng/ml)	Interval (months)
Elective	66.5	2.7	12
Group	21.6	4.2	12
n=3	8.1	3.4	12
Acute	115	4.2	10
Retention	76	0.5	6
Group	75	0.3	13
n=5	41	0.4	12
	37	0.5	6

Distributions and cut-off levels

PSA in all 3 study groups without prostate cancer, that is reference, elective and acute retention cases shows a markedly skewed distribution. This can be clearly seen in the histograms (figure 4) and by inspection of the mean and median values. In a normal or Gaussian distribution these should coincide. The standard practice of taking the mean and adding a multiple of the standard deviation to set a cut-off level is only valid if the distribution is Gaussian. If as in the case of PSA it is skewed to the left then the cut-off will be set lower than envisaged.

Figure 4

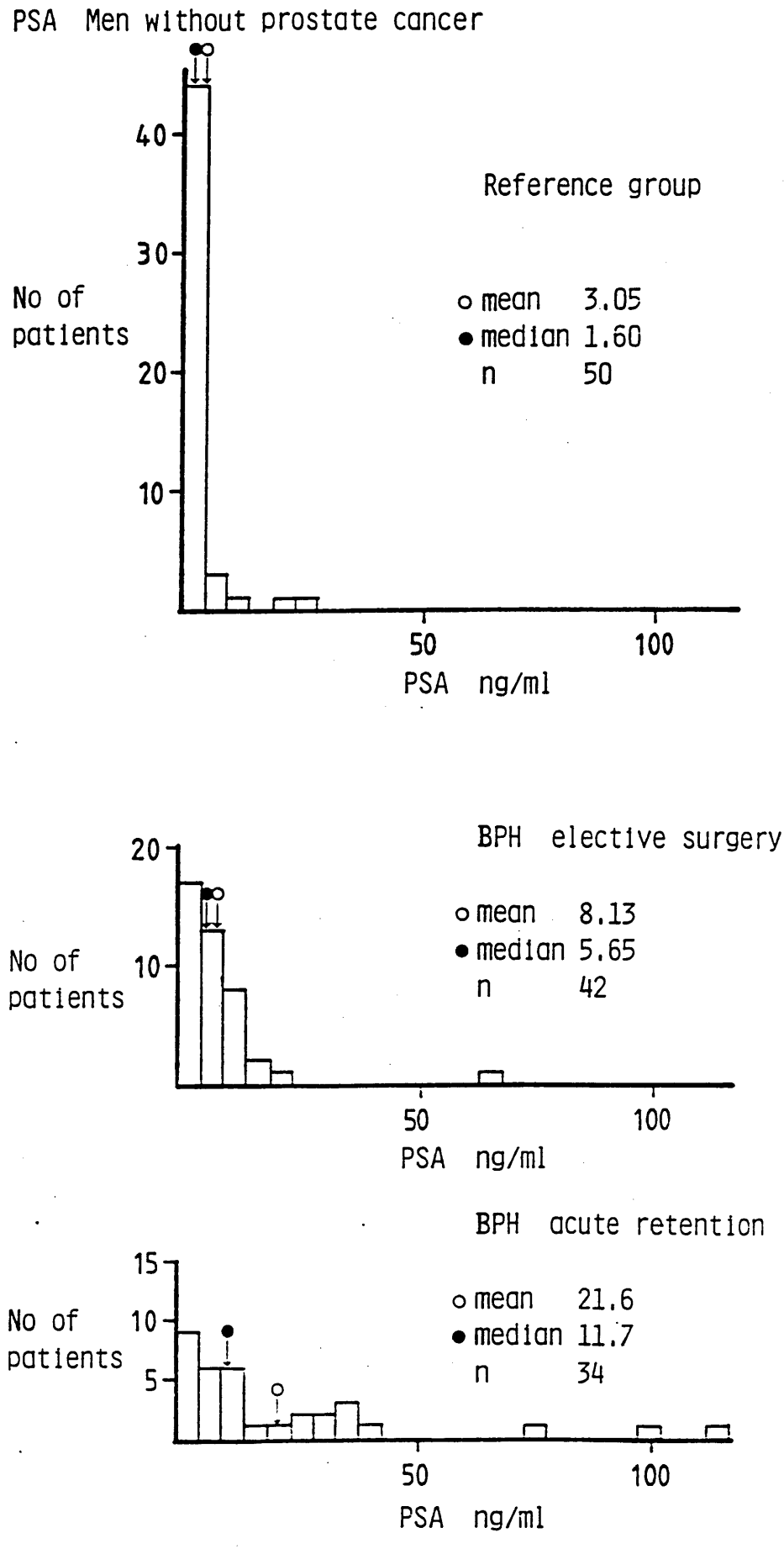
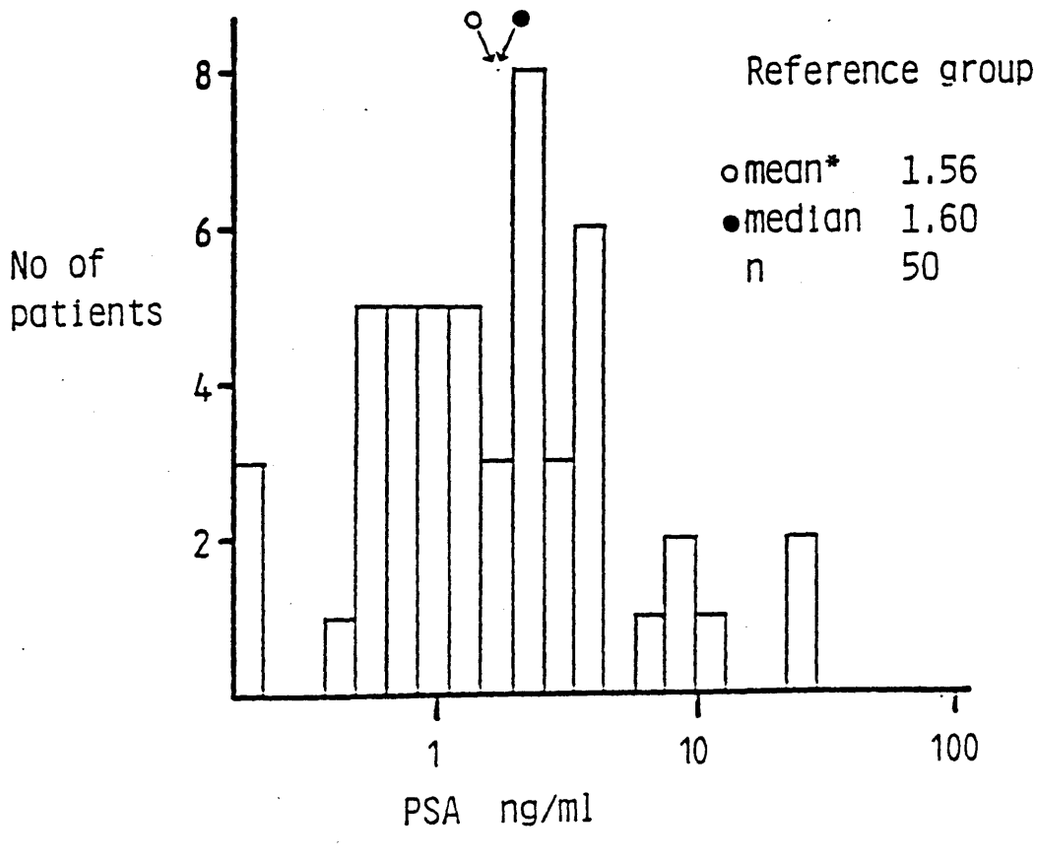

PSA Men without prostate cancer
Reference group
No of patients
40
30
20
10
o mean 3.05
● median 1.60
n 50
50
100
PSA ng/ml
BPH elective surgery
20
10
No of patients
o mean 8.13
● median 5.65
n 42
50
100
PSA ng/ml
BPH acute retention
15
10
5
No of patients
o mean 21.6
● median 11.7
n 34
50
100
PSA ng/ml

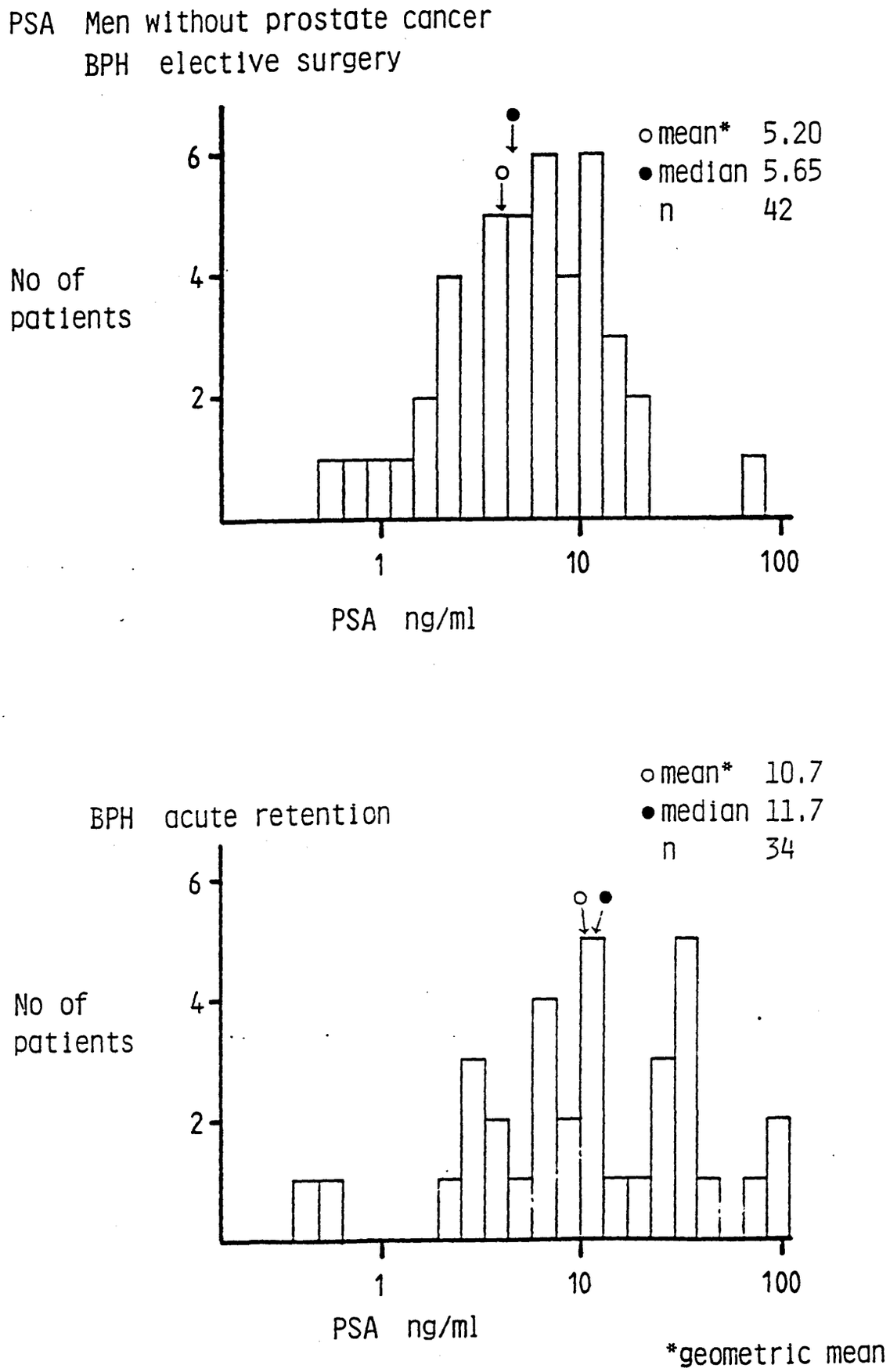
Figure 5a

PSA Men without prostate cancer



*geometric mean

Figure 5b+c


PSA Men without prostate cancer
BPH elective surgery
mean* 5.20
median 5.65
n 42
No of patients
6
4
2
1
10
100
PSA ng/ml
BPH acute retention
mean* 10.7
median 11.7
n 34
No of patients
6
4
2
1
10
100
PSA ng/ml
*geometric mean

After taking logs the distribution becomes approximately Gaussian with the median and the antilog of the mean of the log data (the geometric mean) coinciding (figure 5a-c). Using this data a cut off may be set. In order that 95% of values in the study group are below the cut-off, this may be set by taking the mean + 1.64 standard deviations (see table 9). (1.64 is the factor for a single tail 5%, the more familiar 1.96 applies to both tails, ie 2.5% in each) It is also possible to calculate the standard error for this estimated 95% cut-off level.

Table 9
95% cut-off levels
PSA (ng/ml)

Patient Groups	n	cut-off level	+/- SE
Reference	50	9.3	7.3 - 11.8
Elective	42	25.3	20.1 - 31.8
Acute Retention	34	94.5	66.3 - 135

Cancer patients

95 patients with untreated prostate cancer were studied. Their characteristics have been summarised in table 10. In 42 patients the disease was clinically localised, locally advanced in 28 and metastatic in 25.

Table 10
Prostate cancer - all stages $T_{0-4}\ N_X\ M_{0-1}$

n=95	Mean	S. D.	Range	Median
Age (yr)	72.9	7.45	57-88	73
PSA (ng/ml)	196	481	0.2-2695	31.9
PAP (ng/ml)	23.3	49.2	0.3-308	5.1

Prostate cancer and tumour volume/stage

The data for prostate cancer patients broken down by stages is shown in table 11.

Table 11
Prostate cancer

- locally confined $T_{0-2}\ N_X\ M_0$

n=42	Mean	S. D.	Range	Median
Age (yr.)	74.2	7.41	61-88	76
US vol (ml)*	37.9	22.3	9.4-113	33.7
PSA (ng/ml)	38.8	60.1	0.2-256	10.2
PAP (ng/ml)	6.2	9.99	0.3-55.7	6.5

* Total gland size including unknown amount of benign tissue

- locally advanced $T_{3-4}\ N_X\ M_0$

n=28	Mean	S. D.	Range	Median
Age (yr.)	74.3	6.24	63-87	79
US vol (ml)	Not measurable			
PSA (ng/ml)	91.1	141	0.2-526	27.5
PAP (ng/ml)	12.5	20.5	0.3-98	4.55

Table 11 continued

- metastatic T_{0-4} N_X M_1

n=25	Mean	S. D.	Range	Median
Age (yr.)	69.1	7.74	57-84	67
US vol (ml)	Not measurable in all cases			
PSA (ng/ml)	577	820	0.6-2695	245
PAP (ng/ml)	64.2	80.3	0.8-308	30

This data is also presented as a dot plot (figure 6). It can be seen that whilst there is a clear increase in PSA levels corresponding to disease stage, there remains an appreciable overlap. The data can be further analysed in terms of histological grade (figure 7). The correlation between tumour volume and PSA is more difficult to evaluate. The absence of a clear boundary echo on transrectal ultrasound of locally advanced disease means that it is not possible to determine the tumour volume. Within a prostate containing a localised cancer it may be very difficult to define the precise limits of the tumour and hence one can only measure the total gland size which will include an unknown proportion of benign tissue.

PSA in the diagnosis of prostate cancer

The PSA levels obtained from the 3 groups of patients without prostate cancer can be compared with those from the prostate cancer patients and the performance of the assay as a diagnostic test can be evaluated.

The simplest evaluation is to analyse the performance of PSA using the cut-off levels defined earlier, set by

Figure 6 PSA Men with prostate cancer by stage

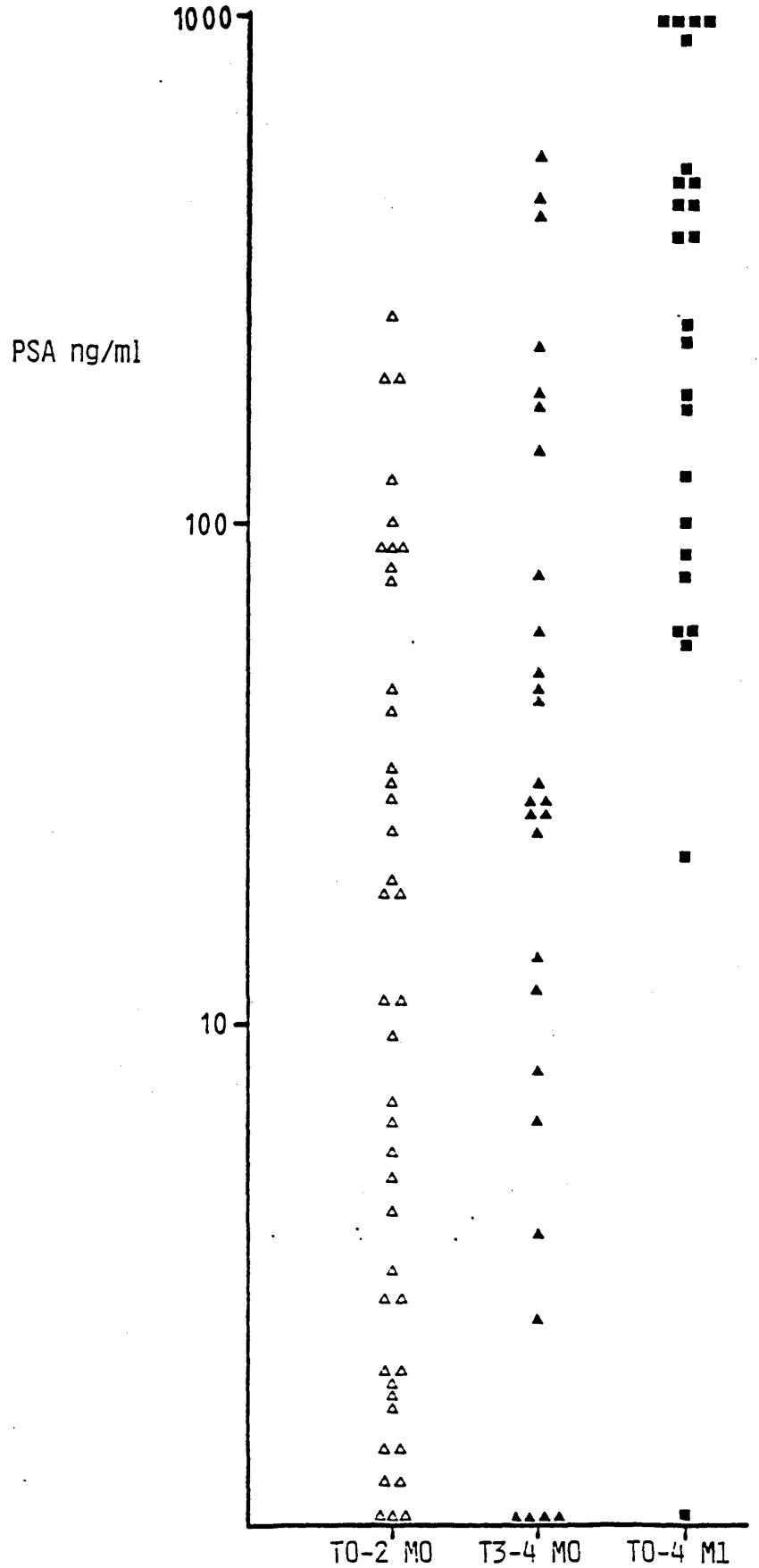
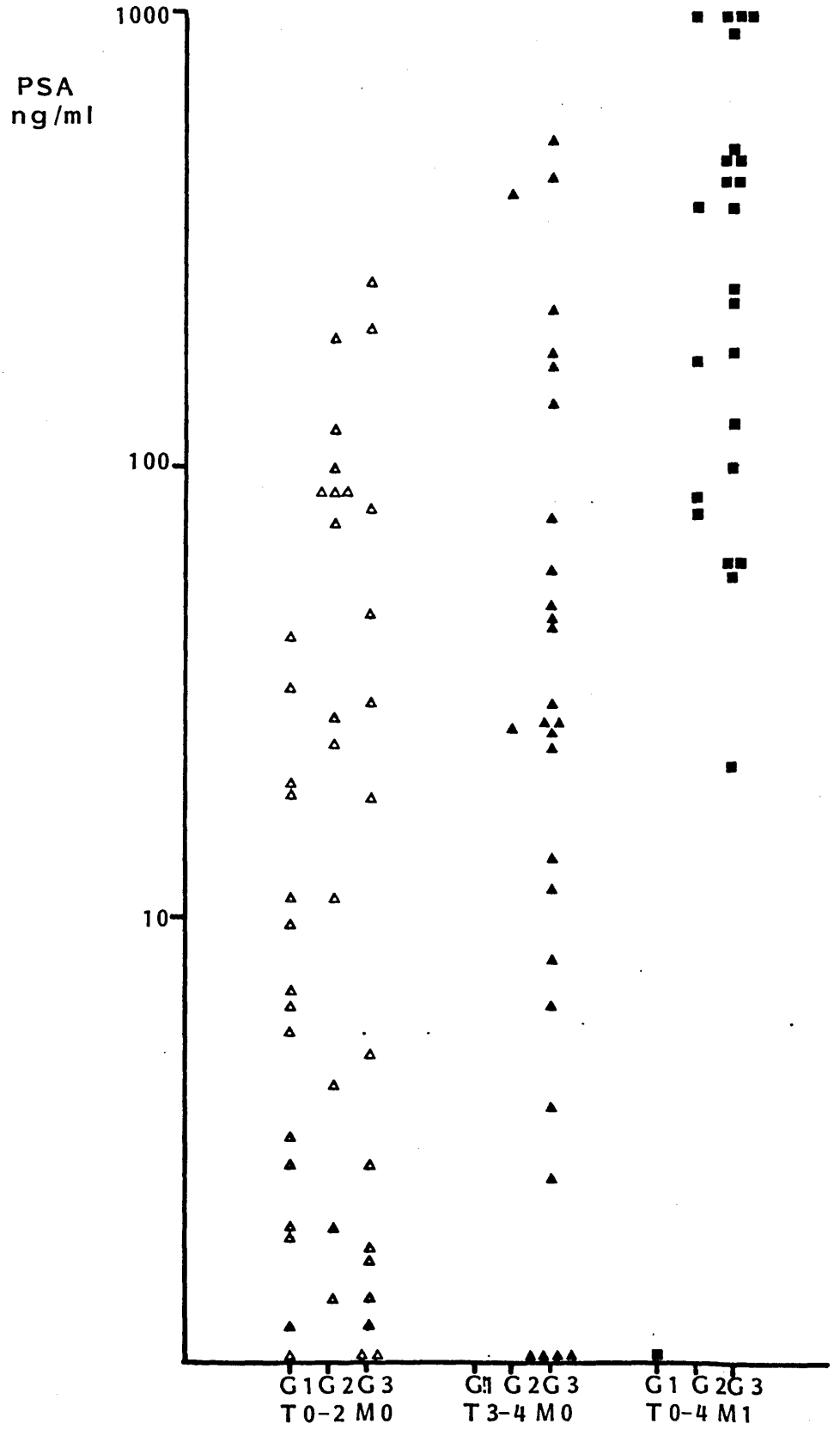


Figure 7

PSA Men with prostate cancer by stage and grade



definition to produce 95% specificity. 5 clinical situations have been reviewed.

1 Diagnosis of all stages of prostate cancer

Study population to be detected - Prostate cancer patients all stages n=95

Reference population from which these are to be found - Reference group n=50

Cut-off 9.3 ng/ml

Sensitivity	**69%**
Estimated positive predictive value* (Vecchio 1966)	**1.4%**

95% Specificity

* Assumes a disease prevalence of 100/100 000 (OPCS 1988b) - an approximation for the 60-70 age group (incidence 60.4/100 000 for 60-64 and 138.2/100 000 for 65-69).

2 Diagnosis of localised prostate cancer

Study population to be detected - Localised prostate cancer patients n=42

Reference population from which these are to be found - Reference group n=50

Cut-off 9.3 ng/ml

Sensitivity	**52%**
Estimated positive predictive value*	**1.0%**

95% Specificity

* Assumes a disease prevalence of 100/100 000

3 Diagnosis of T0, clinically undetected prostate cancer

Study population to be detected - T0 prostate cancer patients n=18

Reference population from which these are to be found - Reference group n=50

Cut-off 9.3 ng/ml

Sensitivity **44%**
Estimated positive predictive value* **0.9%**

95% Specificity

* Assumes a disease prevalence of 100/100 000

4 Diagnosis of localised prostate cancer from elective admissions for TURP for BPH

Study population to be detected - localised prostate cancer patients n=42

Reference population from which these are to be found - Elective group n=42

Cut-off 25.3 ng/ml

Sensitivity **36%**
Estimated positive predictive value* **43%**

95% Specificity

* Assumes a disease prevalence of 9.5% (Neal et al 1989)

5 Diagnosis of localised prostate cancer from admissions with acute retention due to BPH

Study population to be detected - localised prostate cancer patients n=42

Reference population from which these are to be found - Acute retention group n=34

Cut-off 94.5 ng/ml

Sensitivity **12%**
Estimated positive predictive value* **20%**

95% Specificity

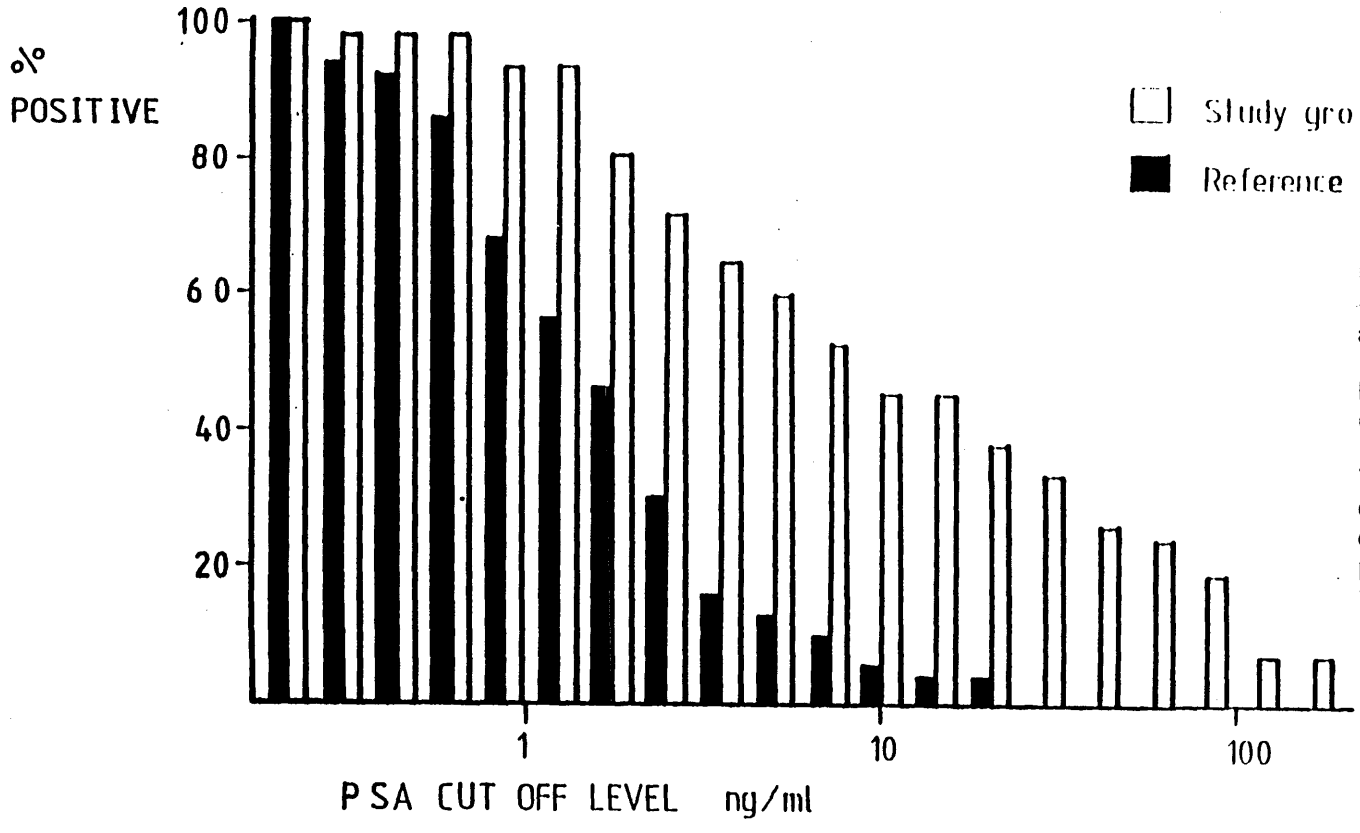
* Assumes a disease prevalence of 9.5% (Neal et al 1989, assuming the prevalence of cancer is the same amongst elective and acute retention patients)

Receiver Operator Characteristic curve analysis

The apparent performance of a test will depend critically on the cut off level used. One way to eliminate this effect and allow more meaningful comparisons with existing alternatives is the use of Receiver Operating Characteristic curves (Robertson and Zweig 1981). The percentage of true and false positives over a range of cut-off or decision levels is calculated. Starting with low cut-off levels many test values in the reference group will be positive, false positives. As the cut-off level is moved upwards the number of false positives will decrease. Ideally there should be little loss of true positives, that is test levels taken from patients with the disease should remain above the cut-off. It may be possible to reach a level where few of the reference values, but most of those from diseased patients are above the cut-off. The degree to which this is possible is easily appreciated when this data is plotted, % true positive against % false positive. This plot is termed a Receiver Operating Characteristic (ROC) curve, a term that arose from its first inception in the interpretation of radar images. Acting as a concise summary of a test's performance it allows easy comparison as the curves from a number of tests can be displayed on the same plot. It should be noted that the % true positive is equivalent to the sensitivity and the % false positive is the reciprocal of the specificity (figures 8a-c).

ROC curves for PSA have been constructed corresponding to the 5 clinical situations described earlier. The derivation of these plots is more easily appreciated if

THE CONSTRUCTION OF A RECEIVER OPERATOR CHARACTERISTIC CURVE
REFERENCE GROUP vs ALL PROSTATE CANCER STAGES

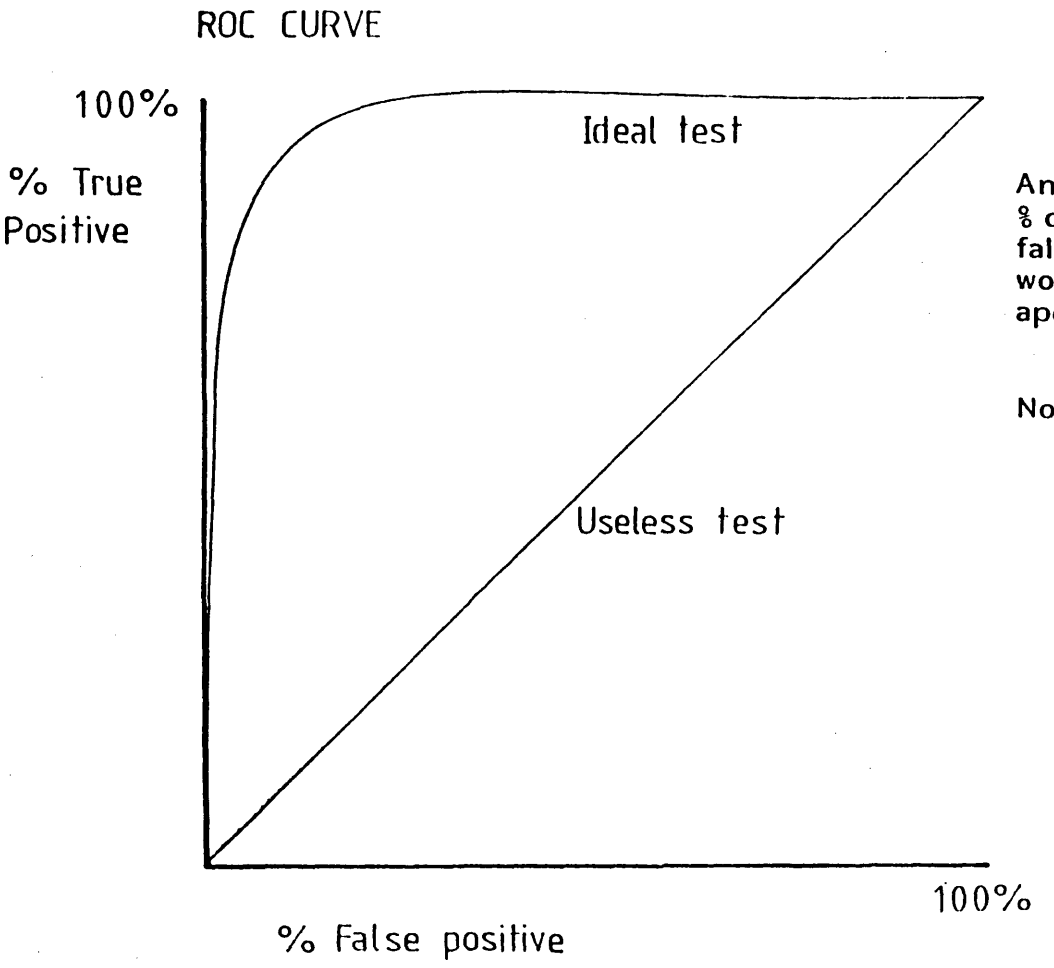


Each positive from the study group - those whom we wish to identify is a true positive

Each positive from the reference group is a false positive

Thus the % of true and false positive over a range of PSA cut-off values can be plotted one against the other producing a ROC curve - see following figure

Figure 8a



An ideal test would have a high % of true positives and very few false positives. Thus the curve would be sharply angulated with its apex in the top left hand corner

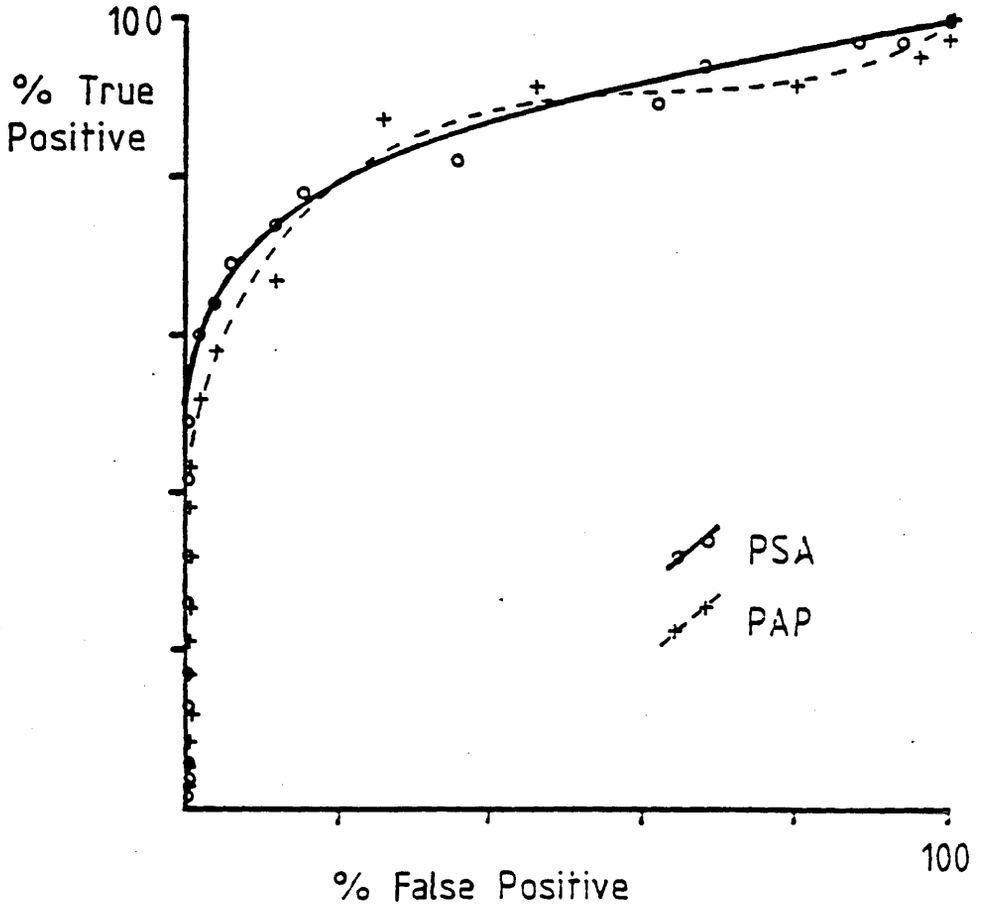
Note: % true positive = sensitivity
% false positive = 100 - specificity

Figure 8b

Figure 8c

ROC Curve
Prostate cancer all stages vs reference group

The diagnosis of all stages of prostate cancer from a reference population



the % true and false positives are also plotted as histograms, see figure 8a relating to the diagnosis of all stages of prostate cancer from asymptomatic men, for clarity the ROC curves alone are given for the remaining 4 clinical situations described earlier (figures 9a+d). The plot for localised cancer vs metastatic disease is also given (figure 10).

Comparison with prostatic acid phosphatase

The use of ROC curves allows comparison with other tests. Any bias introduced by the setting of a single cut-off point is eliminated as both tests are compared over a wide range of cut-offs rather than just one. For this reason data for PAP has been added to the ROC curves (figures 8c, 9a-d + 10).

ROC Curve

Localised prostate cancer (T0-2 M0) vs reference group

The detection of localised prostate cancer from a reference population

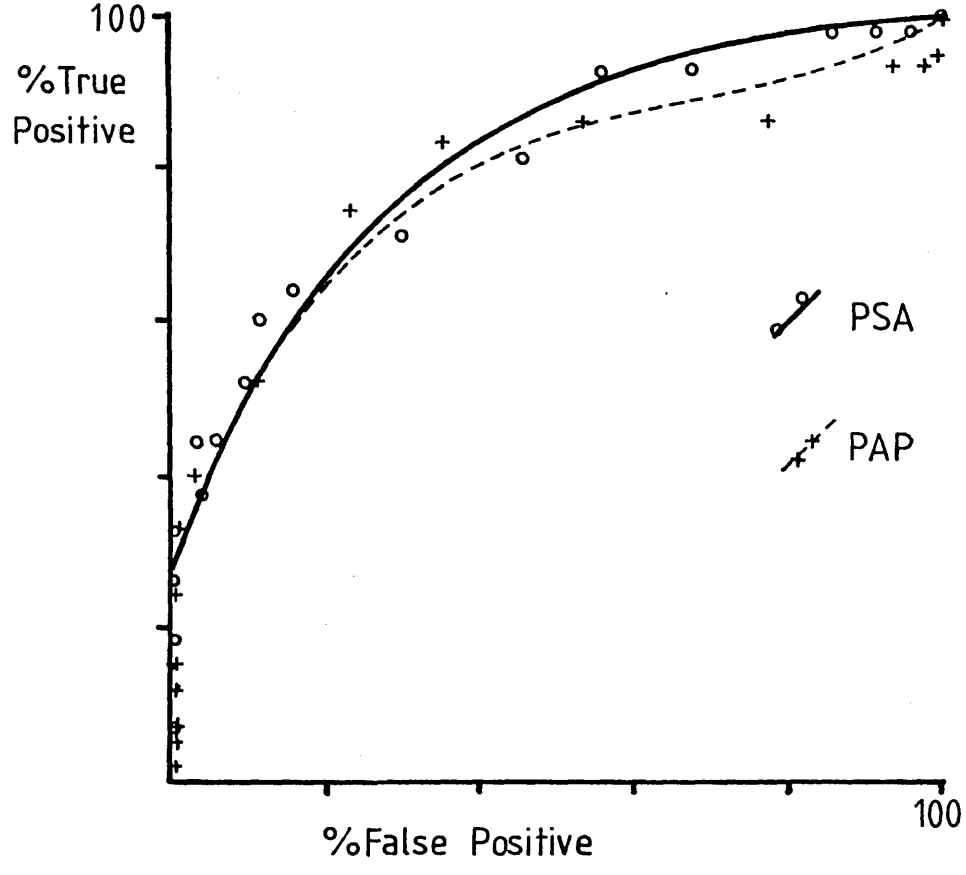
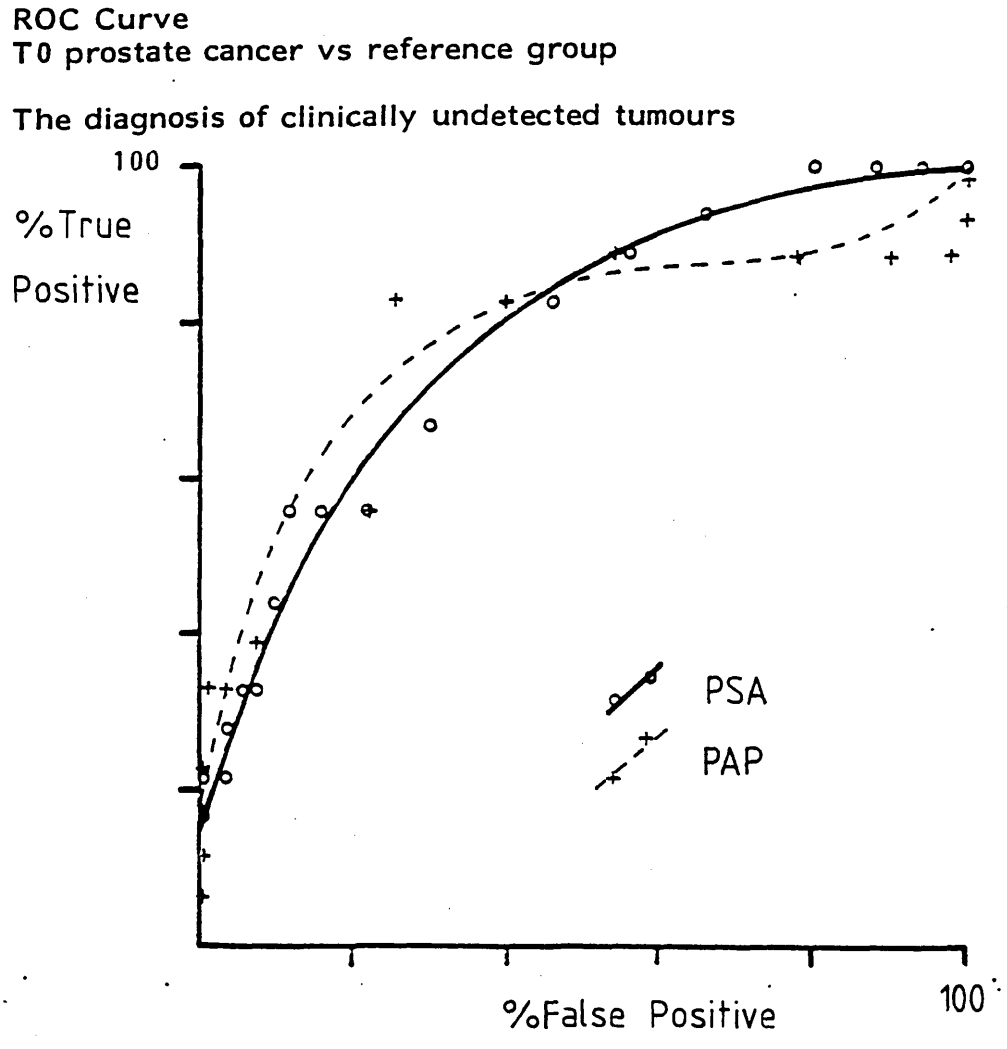


Figure 9a

Figure 9b


ROC Curve
T0 prostate cancer vs reference group
The diagnosis of clinically undetected tumours
100
%True
Positive
PSA
PAP
%False Positive
100

ROC Curve
Localised prostate cancer (T0-2 M0) vs BPH elective surgery

The diagnosis of localised prostate cancer from patients with BPH

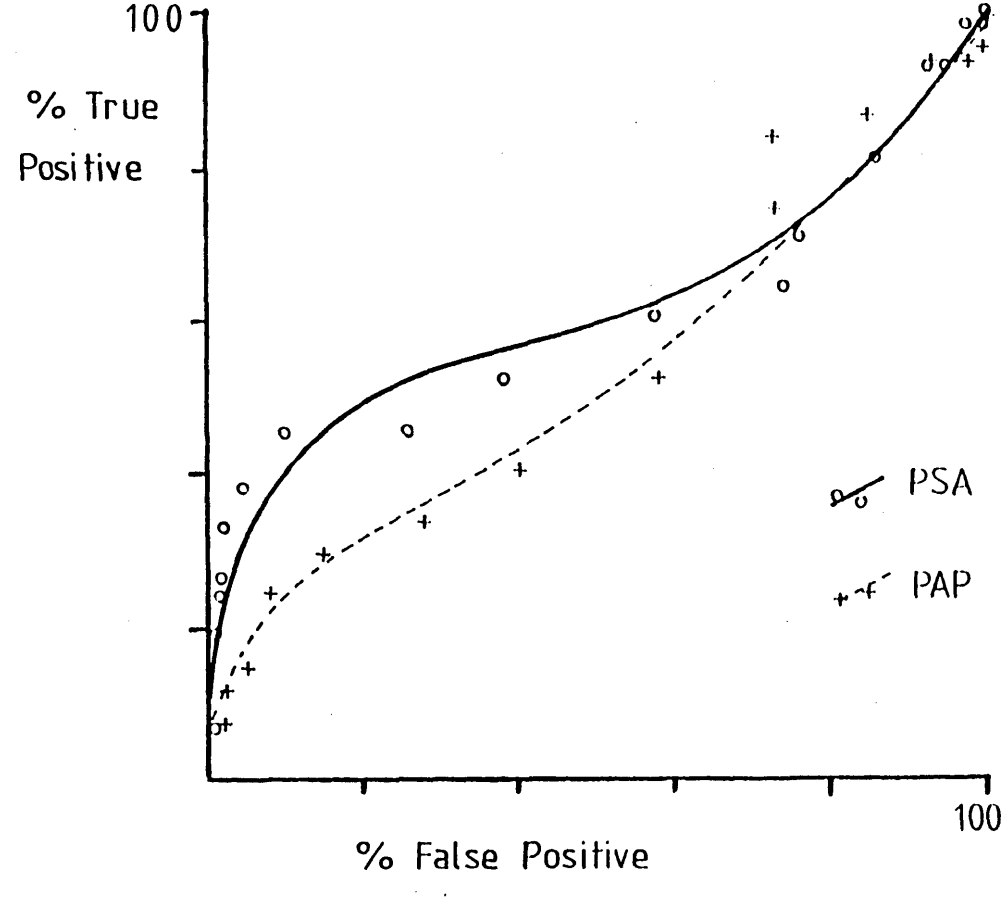


Figure 9c

Figure 9d
ROC Curve
Localised prostate cancer (T0-2) vs BPH acute retention

The diagnosis of localised prostate cancer from patients with BPH and acute retention

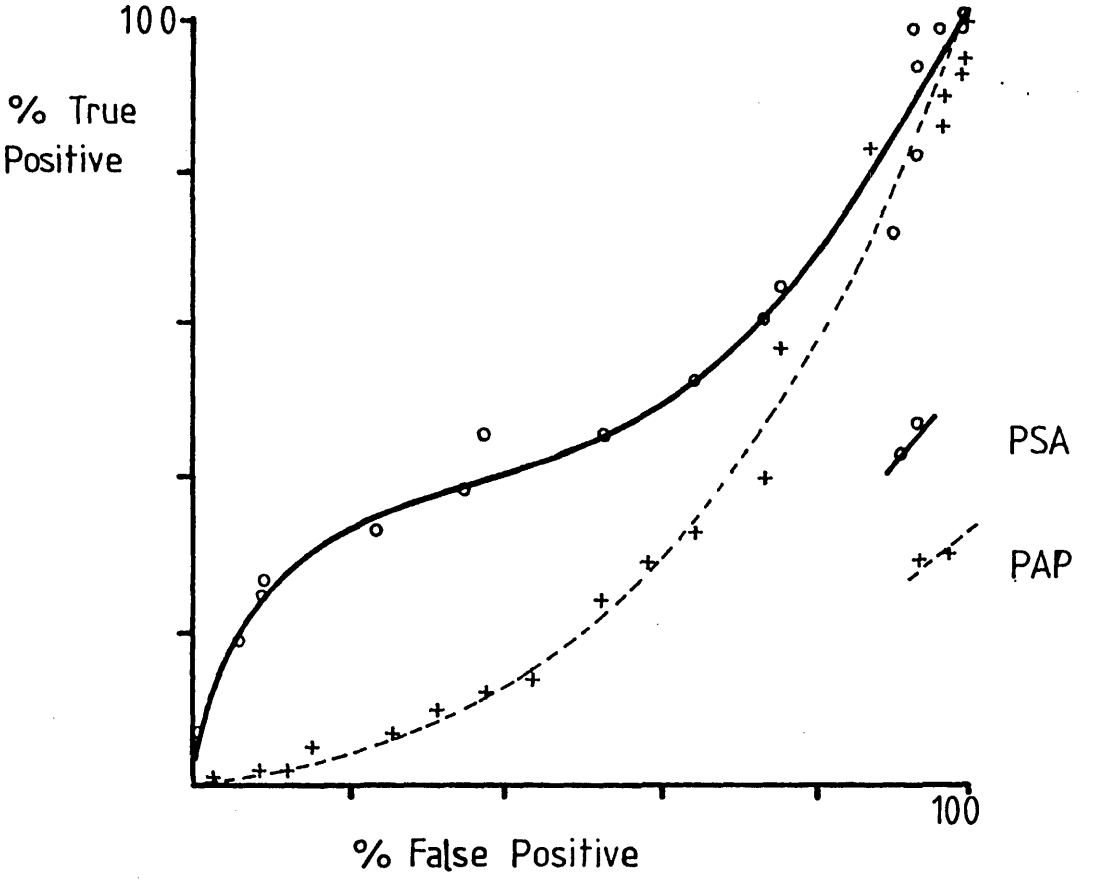
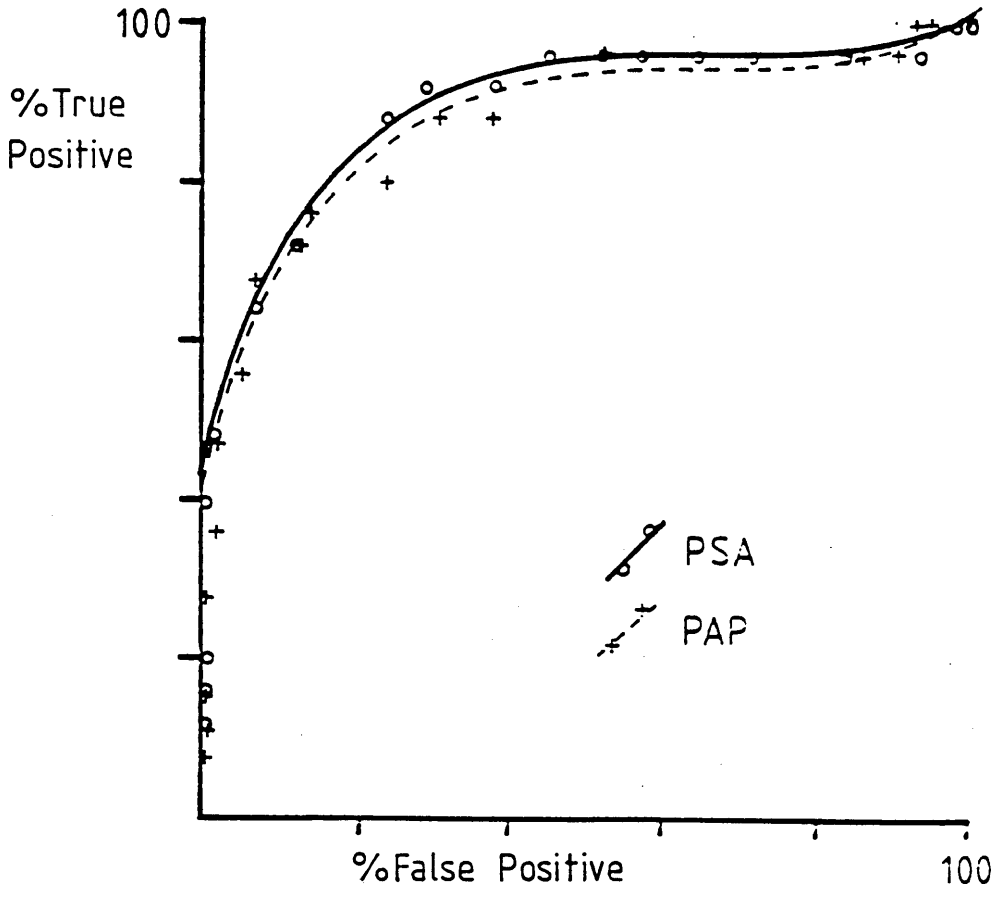


Figure 9d

Figure 10

ROC Curve
Localised vs metastatic prostate cancer

The diagnosis of disease dissemination



Further data from prospective study

Women

Sera from 10 women of approximately similar ages was tested. In no case was PSA detected.

Standing time

Samples of unseparated clotted blood from 13 patients were left at room temperature for various time intervals extending up to 72 hours.

No significant change in PSA levels occurred. In this study serum was separated on the morning or afternoon it was taken and then frozen.

Stability of 10 samples in the freezer over 5 months was confirmed.

Effect of catheterisation

Sera was available on 19 patients after catheterisation for acute retention. Small elevations of serum PSA occurred in some cases, the magnitude of these variations was not of clinical significance and certainly did not reach statistical significance (Wilcoxon signed rank test) (table 12).

Table 12
Effect of catheterisation on the PSA of patients presenting with acute retention

n=19	Pre catheterisation PSA ng/ml	Post catheterisation PSA ng/ml
	37	33
	4.3	5.9
	14.1	14.3
	75	79
	3.7	4.2
	2.1	2.3
	17.2	18.1
	0.4	0.4
	12.8	11.2
	189*	174
	199	177
	36.5	29.9
	18.9	20.2
	115	88.6
	1.5	2.8
	6.3	7.6
	3.4	2.9
	11.4	9.6
	22.1	22.0

* This patient had prostate cancer and was excluded from the acute retention group. He is included here as he presented in retention and was catheterised

Wilcoxon signed rank test - not significant

Effect of rectal examination

20 patients underwent a second venesection for serum PSA 20-30 minutes after rectal examination and transrectal ultrasonography. Again no significant changes in PSA levels occurred (table 13).

Table 13
Effect of rectal examination on serum PSA

Before rectal exam PSA ng/ml	After rectal exam PSA ng/ml
0	0
0	0
0	0
0	0
0.25	0.19
0.77	0.68
1.3	1.4
1.5	1.6
5	5.2
12	11.8
2.3	2.7
13.8	14.8
19.1	20.1
16	14.8
22.5	23.7
23.4	24.7
5.9	7.4
77.1	86.6
60.7	77.7

Wilcoxon signed rank test - Not significant

Effect of surgery

4 patients had serum PSA estimations before, during and after prostatic surgery. Significant rises in PSA occurred and these did not fall to the preoperative levels within the patients hospital stay. The measurement of PSA immediately after TURP perhaps in a patient with newly diagnosed prostate cancer is therefore of minimal value. The limited number of patients studied did not allow any parameters such as the half life to be determined. The large number of assays needed for a more thorough investigation were not available to us.

Diurnal variation

6 patients had 5 serum samples taken at 4 hourly intervals over a 24 hour period with an 8 hour gap overnight. No

significant variation occurred in all patients the CVs for their serum PSA were comparable to that of the assay itself.

Transrectal ultrasound

Volume measurement

Using a balloon filled with known amounts of water suspended in a water bath it was possible to determine the accuracy of the ultrasound planimetry technique to measure volume. The results are tabulated below (table 14).

Table 14
Volume measurement by transrectal ultrasound

Balloon Volume (ml)	Mean Ultrasound Volume n=5 (ml)	CV
20	20.1	1.8
30	28.6	2.1
50	49.3	3.6
75	72.7	2.2
100	99.2	0.8

The correlation between the actual volume of the balloon and the ultrasound measurement is shown in figure 11.

Diagnosis of prostate cancer

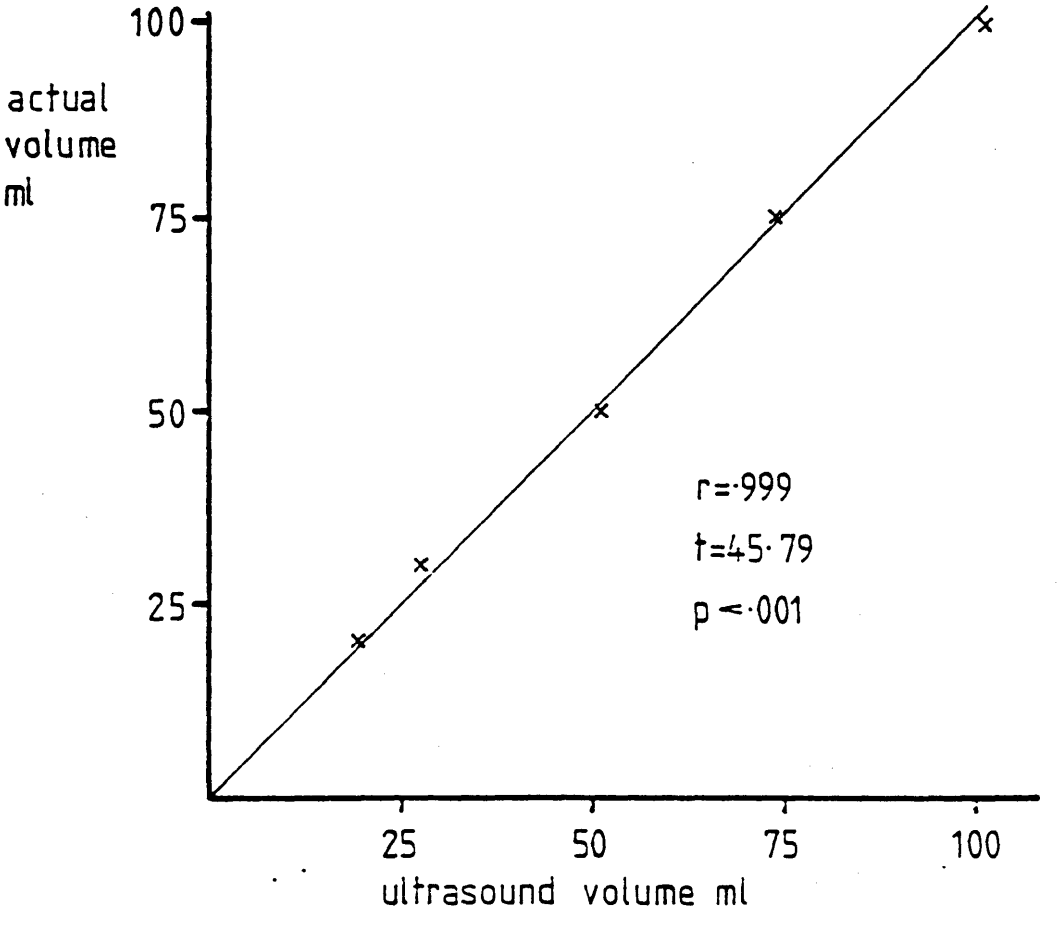
9 patients were found to have prostate cancer which preoperatively was clinically unsuspected. Thus the incidence of T0 disease was 10%. Ultrasound diagnosed 7 of these but there were 12 false positives. Thus its sensitivity was 78%, specificity 84% and positive predictive value 37%.

Staging

In the absence of any radical prostatectomy specimens it was not possible in this study to assess the accuracy of transrectal ultrasound in this context.

Figure 11

Volume measurement by ultrasound



APPENDIX

Analytical performance of Hybritech Tandem R kit used to measure PSA in this study + comparison with 2 other assays

Study Details

* Standard kits used according to manufactures instructions

* Quality control sera - commercial "level A-C"
  - pooled sera "pool 1-3"

* Parameters evaluated

- Within batch imprecision n=10 (multiple assays of control sera within the same batch)

- Between batch imprecision n=12 (multiple assays of control sera in different batches)

- Precision profile - 5 replicates at each of 6 levels using pooled sera

- Sensitivity - 2 standard deviations for the zero standard for the kit

- Parallelism - a sample with elevated PSA was serially diluted with the kit zero standard. 4 replicates for each dilution

- Calibration characteristics - the slope and the intercept of the calibration curves for 12 assays

- Cost - based on weekly half kit use with full calibration and 2 quality controls. Allowances made for repeats with dilution for samples over the standard range (Hybritech 16%, DPC 30%, Yang 29%)

* Study population

- Reference - 50 age matched men with no previous history of prostate disease

- BPH - 42 men with histologically confirmed benign prostatic hyperplasia (BPH) - not in retention

- BPH + AR - 34 men with BPH presenting in acute retention

- Prostate cancer - 151 men with localised disease (n=53), locally advanced (penetration of the prostatic capsule on transrectal ultrasound) (n=54) and metastatic prostate cancer (n=44). Includes patients who were treated, these were not included in the remainder of the study but they provide increased numbers for this assessment relating only to the biochemical performance of the assays.

HYBRITECH, TANDEM-R

* Solid phase (plastic bead) two site radio-immunometric assay
* Two mouse monoclonal antibodies directed at different sites
* 2 hours incubation with rotation (or overnight)
* Beads washed to remove unbound labelled antibody
* Bound activity measured
* 6 point calibration (0-100 ng/ml)

RESULTS HYBRITECH TANDEM-R

* Within batch imprecision: n=10 in each case

	Level A	Level B	Level C
Mean ng/ml	1.5	3.4	6.9
CV%	5.3	5.3	1.5

* Between batch imprecision: n=12 in each case

	Level A	Level B	Level C
Manufacturer's target (+ or -)	1.7(0.4)	3.5(1.0)	7.1(1.7)
Mean ng/ml	1.6	3.3	6.6
CV%	4.0	3.9	2.0
	Pool 1	Pool 2	Pool 3
Mean ng/ml	4.2	35.9	76.9
CV%	8.3	7.2	6.0

* Precision profile: n=5 at each level

Mean ng/ml	0.8	3.4	6.9	12.9	38.0	82.1
CV%	6.6	5.0	1.6	3.7	3.1	2.6

* Sensitivity: 0.08 ng/ml

In post cystoprostatectomy patients mean PSA 0.2 ng/ml (all 0.2 ng/ml) n=7

In females mean PSA 0.2 ng/ml (0.2-0.3) n=10

* Parallelism: n=4 at each level

Measured PSA ng/ml	80.0	40.6	20.7	10.7	5.3	2.8
Dilution	-	1/2	1/4	1/8	1/16	1/32
Corrected %	100	101	103	107	106	112

* Calibration characteristics: 9 assays over 6 weeks

	Mean	CV
Slope	12.1	9.7
Intercept	35.1	10.4

* Cost (Ex labour) Singleton assay £3.26

STUDY GROUPS

		PSA ng/ml		
Group	n	Mean	Median	Range
Reference	50	3.0	1.6	0.2-27
BPH	42	8.1	5.6	0.5-66.5
BPH+AR	34	21.6	11.7	0.4-115
Prostate cancer	151	153.7	23.8	0.2-2695

CORRELATIONS

		Hybritech vs DPC		Hybritech vs Yang	
Group	n	Slope	r	Slope	r
Reference	50	.83	.96	.56	.99
BPH	42	.80	.95	.68	.98
BPH + AR	34	.31	.82	.58	.98
Prostate Cancer	151	.70	.98	.60	.99

DIAGNOSTIC PRODUCTS LTD, DPC-PSA

* Double antibody radio-immunoassay
* Rabbit first, goat anti-rabbit second
* Overnight incubation
* Second antibody to precipitate then centrifugation and decantation
* Bound radioactivity in pellet after centrifugation measured
* 7 point calibration (0-50 ng/ml)

RESULTS DIAGNOSTIC PRODUCTS LTD DPC-PSA

* Within batch imprecision: n=10 in each case

	Level A	Level B	Level C
Mean ng/ml	3.8	7.8	14.7
CV%	8.8	4.6	13.2

* Between batch imprecision: n=12 in each case

	Level A	Level B	Level C
Manufacturer's target (+ or -)	1.9(0.4)	4.4(1.0)	9.3(1.9)
Mean ng/ml	3.4	7.8	12.8
CV%	22.3	24.2	27.5
	Pool 1	Pool 2	Pool 3
Mean ng/ml	6.8	60.6	100.9
CV%	23.2	22.9	23.2

* Precision profile: n=5 at each level

Mean ng/ml	3.8	7.9	14.7	25.6	48.3
CV%	8.8	4.2	13.2	14.1	16.8

* Sensitivity: 1.8 ng/ml

In post cystoprostatectomy patients mean PSA 2.6 ng/ml (2.1-3.1 ng/ml) n=7

In females mean PSA 2.8 ng/ml (2.3-4.1) n=10

* Parallelism: n=4 at each level

Measured PSA ng/ml	32.5	15.3	7.6	4.2	2.5
Dilution	-	1/2	1/4	1/8	1/16
Corrected %	100	94	94	103	123

* Calibration characteristics: 9 assays over 6 weeks

	Mean	CV
Slope	1.88	9.9
Intercept	0.85	36.6

* Cost (Ex labour) Singleton assay £5.08

STUDY GROUPS

		PSA ng/ml		
Group	n	Mean	Median	Range
Reference	50	5.0	2.7	1.1-29.3
BPH	42	13.7	11.0	2.3-73.6
BPH+AR	34	41.0	23.0	1.1-398
Prostate cancer	151	208.6	22.0	0.3-4350

CORRELATIONS

		DPC vs Hybritech		DPC vs Yang	
Group	n	Slope	r	Slope	r
Reference	50	1.16	.96	.64	.97
BPH	42	1.13	.95	.80	.97
BPH + AR	34	2.13	.82	1.37	.89
Prostate Cancer	151	1.39	.98	.86	.99

YANG LABORATORIES INC, PROS-CHECK PSA (UK Agents BAXTER)

* Double antibody radio-immunoassay
* Rabbit first, goat anti-rabbit second
* Overnight incubation (4 hours possible)
* Second antibody to precipitate then centrifugation and decantation
* Bound radioactivity in pellet after centrifugation measured
* 7 point calibration (0-50 ng/ml)

RESULTS YANG LABORATORIES INC PROS-CHECK (BAXTER)

* Within batch imprecision: n=10 in each case

	Level A	Level B	Level C
Mean ng/ml	2.4	5.6	10.8
CV%	11.0	10.5	6.6

* Between batch imprecision: n=12 in each case

	Level A	Level B	Level C
Manufacturer's target (+ or -)	3.5(0.7)	7.3(1.5)	15.8(3.2)
Mean ng/ml	2.7	5.8	13.0
CV%	12.7	8.8	12.9
	Pool 1	Pool 2	Pool 3
Mean ng/ml	6.7	58.6	123.5
CV%	9.2	6.2	10.0

* Precision profile: n=5 at each level

Mean ng/ml	2.6	5.7	10.8	21.0	45.4
CV%	8.4	7.4	5.9	9.3	12.1

* Sensitivity: 0.3 ng/ml

In post cystoprostatectomy patients mean PSA 0.5 ng/ml (0.3-0.7 ng/ml) n=7

In females mean PSA 0.5 ng/ml (0.3-0.8) n=10

* Parallelism: n=4 at each level

Measured PSA ng/ml	71.0	34.0	17.0	7.8	3.8
Dilution	-	1/2	1/4	1/8	1/16
Corrected %	100	96	97	89	87

* Calibration characteristics: 9 assays over 6 weeks

	Mean	CV
Slope	-2.16	4.0
Intercept	1.31	10.6

* Cost (Ex labour) £7.78 or £3.89 (half volumes of reagents)

STUDY GROUPS

		PSA ng/ml		
Group	n	Mean	Median	Range
Reference	50	5.1	2.0	0.4-46.3
BPH	42	12.9	9.5	0.9-88.8
BPH+AR	34	37.6	23.0	0.6-194
Prostate cancer	151	238.3	35.0	0.3-5180

CORRELATIONS

		Yang vs Hybritech		Yang vs DPC	
Group	n	Slope	r	Slope	r
Reference	50	1.72	.99	1.46	.97
BPH	42	1.41	.98	1.17	.97
BPH + AR	34	1.65	.98	.57	.89
Prostate Cancer	151	1.62	.99	1.15	.99

ASSAY COMPARISON - CONCLUSIONS

* In our hands the DPC assay was unsatisfactory in terms of imprecision and accuracy (when compared with commercial quality control material), in addition the precision profile, sensitivity and calibration were poor

* The Hybritech and Yang assays were broadly similar, although the Hybritech assay did show some advantages

* Cost was comparable

* There was a close correlation between the Hybritech and the Yang assays, the results however are not interchangeable

* The values obtained with the Yang assay were consistently higher than those from the Hybritech

Discussion - PSA Prospective Study

The aim of this work is to evaluate the role of PSA in prostate cancer. In order to do this it is important to examine what factors influence the level of PSA in the serum thus enabling PSA values in cancer patients to be interpreted in a meaningful way.

Patients without prostate cancer

The apparent performance of any test will depend on the group of subjects chosen to represent the disease free or "negative" population. Other workers have chosen younger men and excluded those with any prostatic enlargement (eg Killian et al 1985, Stamey et al 1987). This results in a reference population with low PSA values and therefore a low reference range and cut-off level. It is important to consider the clinical question which it is hoped will be answered by measuring PSA. For example if one hopes to screen the male population over 50 the negative group must contain men of that age group. If on the other hand one wishes to detect those patients on a long waiting list for TURP who in fact have a prostate cancer that is not clinically apparent then a negative group must comprise patients listed for TURP and shown by later histology to have benign disease only. For this reason 3 groups of men were chosen to act as negatives in different situations. The extent to which these groups represent valid negative populations is discussed below.

Reference group

These men were age matched and represent a group attending for reasons other than prostatic disease. Patients with

previous prostatic surgery and those with suspicious glands on digital rectal examination or transrectal ultrasound were excluded. They could not be taken to represent a random sample of men over 50, however they do represent the "negative" hospital population with which a Urologist would come into contact.

Elective admissions for TURP for BPH

These patients are representative of the patients treated at the Freeman Hospital for BPH. Their characteristics are comparable with patients from a larger study performed at the same hospital (Neal et al 1989). It should be noted that Urologists in this hospital favour a bladder neck incision in cases were the prostate is small or it is felt that the bladder neck rather than the prostate is causing the obstruction. Biopsies are not routinely taken and therefore histology is not available. As one might expect these patients were younger and had smaller glands and therefore lower PSA levels. The group with proven benign histology following TURP has been taken for subsequent analysis, this obviously excludes younger patients with smaller glands that elsewhere might have undergone a TURP.

Emergency admission for acute retention

In a similar way a proportion of these patients were the subject of a successful trial without catheter and therefore avoided operation. These individuals were admitted to the study as it was felt necessary to take blood for PSA before catheterisation. Again as one would expect these patients had smaller prostate glands and lower PSA values. Those without histological confirmation

of the benign nature of their prostate were excluded. It is possible that other Urologists may have offered these patients a TURP.

Follow-up of extreme data

It was not possible to trace all patients towards the extreme end of the distribution curve, however a representative number were found. In all cases after TURP the PSA fell dramatically and below a level that would be considered by most workers to be "normal" (highest value 4.2 ng/ml). One would expect that the persistence of an elevated PSA is indicative of a carcinoma possibly with further growth within the prostate or distant/nodal spread which remains unaffected by a TURP. It therefore seems the case that the high levels observed were due to the presence of BPH alone. A histological review of these patients did not demonstrate any consistent features such as inflammation or infarction that might explain the PSA elevation, subjectively these features were present in the same proportion as in specimens not associated with such high PSA levels (M C Robinson 1988, Consultant Histopathologist, Freeman Hospital, Newcastle - personal communication).

Distributions and cut-off levels

The consistent finding of a log normal or skewed distribution of serum PSA is in line with many other studies although few authors have commented on it. The great significance of this finding is in setting cut-off levels. If these are to be set by taking multiples of the standard deviation then this only has real meaning if the

distribution is Gaussian. The % of PSA levels above the cut-off can then be determined from first principles depending on the factor used to multiply the standard deviation. Using this technique after log transformation it has been found that the cut-off for the reference group should be 9.3 ng/ml in line with the work of Siddall et al (1986) who adopted the level of 10 ng/ml. The cut-offs for the elective TURP and the acute retention groups are surprisingly high indicating the limited value of measuring PSA in such patients. A further fact to emerge is that the standard error of this estimation that is the 5% cut-off is large and that the need to log the data amplifies this effect. This in part explains the difference in levels used by different workers. It must be admitted however that some appear to take an overly optimistic view and set the cut-off levels extremely low (Stamey et al 1987). The comparison of one test with another on the basis of a single often arbitrarily chosen cut-off is not valid. The use of ROC curves in this context has much to commend it.

Diagnosis of prostate cancer

The findings of this study do not support a role for PSA as a screening test for prostate cancer. Using the reference group as the negative population the positive predictive value of the test would be only 1.4%. Using it to detect treatable, ie localised disease is worse, the positive predictive value being 1.0%. The situation is still worse looking at patients whose cancer is not clinically apparent, T0 cases, positive predictive value

0.9%. Furthermore given that one hundred normal patients must be further investigated to find one cancer, the sensitivity was only 44% so half the cancers screened would be missed. Reducing the cut-off level to enhance the sensitivity would result in further increasing the false positive rate. For reasons alluded to earlier, the PSA levels in a screened population of men with no urological symptoms may be lower than the reference group used here. However it would seem inconceivable that any such difference or any type 2 error relating to the relatively small numbers in this study could be of sufficient magnitude to obscure any supposed value of PSA in this role.

The value of PSA in the diagnosis of prostate cancer in populations at higher risk is also dubious. Amongst patients admitted for elective TURP the positive predictive value is 43%. Although this may appear a great improvement the sensitivity is low at 36% hence 2 out of 3 cancers would be missed. The performance of PSA in this role is still worse amongst patients presenting in acute retention.

The literature remains contradictory on this subject. Cooner et al (1987) imply that serum PSA may be used as a screen in conjunction with transrectal ultrasound. Of 12 patients with an impalpable lesion proven to be cancer on biopsy 5(42%) had a PSA > 10 ng/m (Hybritech Tandem R). Unfortunately the number of elevated PSA levels with negative biopsies are not given. Barak et al (1989) suggest that PSA is a "valuable laboratory adjunct to the

diagnosis of prostate cancer". They base this assertion on a group of prostate cancer patients without staging information. Many may have advanced disease. The control group is made up of younger healthy men. The calculation of sensitivity and specificity is therefore of limited value, and the more informative positive predictive value has not been determined. In contrast Williams (1988) in an editorial review highlights the difficulty in diagnosing early disease with PSA and states that it is not a useful general screen.

Staging

A review of the ROC curves (figures 8c, 9a-d + 10) shows that PSA behaves most like an ideal test in the diagnosis of disseminated disease. It seems likely that PSA may have a place as an adjunct to conventional staging procedures prior to radical prostatectomy. Stamey et al (1989) report their experience of 102 such patients who were evaluated preoperatively. Those with a PSA of greater than 50 ng/ml (Yang assay) had microscopic lymph node metastases in 2/3 of cases and 90% had seminal vesicle involvement. Conversely no patient with a PSA of less than 10 ng/ml had lymph node metastases. There clearly is a large intermediate range where interpretation of the PSA level is problematical. Lange et al (1989) also found preoperative PSA valuable. Using the Hybritech Tandem R assay they found that the cut-off of 10 ng/ml gave a positive predictive value of 78% and a negative predictive value of 61% in the detection of extracapsular spread. (Note: 10 ng/ml by the Yang assay is not equivalent to 10 ng/ml by the Hybritech assay)

The role of PSA in predicting prognosis is addressed in the next chapter.

ROC Curve Analysis

ROC curve analysis shows that PSA performs best in the diagnosis of disseminated disease (figure 10). This is the chief reason for its apparent good performance in the diagnosis of all stages of prostate cancer (figure 8c). When localised disease alone is analysed, (figure 9) the test performs much less well.

This method of analysis lends itself to the comparison of two assays. For this reason the curves for PAP using the same patient populations have also been drawn. Comparison over a wide range of cut-off or decision levels is possible. It can be seen that contrary to earlier enthusiastic reports regarding the enhanced sensitivity and specificity of serum PSA its performance in the clinical situations examined is remarkably similar to PAP. One suspects that the longer experience with PAP has led to more realistic cut-off levels than is the case for PSA. When compared on an equal footing the difference is difficult to see.

Recent work by Drago et al (1989) lends some support to this view. They reviewed the value of PSA and PAP amongst 193 patients, of whom 48 had prostate cancer. In the diagnosis of prostate cancer they found that PSA was much more sensitive 73% vs 31%. However their analysis included a calculation of the positive predictive value and the situation was reversed in favour of PAP, 88% for PAP, %85

for PSA.

Two groups of workers using ROC curve analysis have shown that immunologically measured prostatic acid phosphatase is little different from the standard enzyme assay (Carson et al 1985, Gibb et al 1986). It is regrettable it has not been possible to include a comparison between PSA and the enzyme assay for acid phosphatase in this work.

Subsidiary studies

Data regarding the effects of leaving the blood sample unseparated, catheterisation, operation and rectal examination in addition to levels in women and men who have undergone cystoprostatectomy have already been presented and do not merit extensive discussion. Since the beginning of this work others have reported with regard to the minimal diurnal variation in serum PSA (Dejter et al 1988, Maatman 1989, Schifman et al 1987). In addition the stability of PSA in serum refrigerated at $4^{0}C$ and frozen at $-20^{0}C$ has been confirmed (Schifman et al 1987).

Analytical performance

A number of workers have reported similar assay performance using the Hybritech Tandem R PSA kit (Schifman et al 1987, Maatman T 1989). Others have used the Yang Pros-Check kit in comparative work similar to that described in the appendix to the preceding chapter (Chan et al 1987, Hortin et al 1988). Again their findings were in line with those described here.

Transrectal ultrasound

This technique was employed for 3 reasons, firstly to measure the volume of the prostate as others had shown this correlates with serum PSA (Peeling et al 1987), secondly to diagnose localised prostate cancer and thereby exclude these from the negative groups, and thirdly to more accurately stage the disease.

Volume measurement

The use of a water filled balloon shows that the algorithm programmed into the ultrasound machine produces an accurate measure of the volume of a spherical object. In clinical use there are 3 areas of difficulty, the prostate is not spherical, the distance between the planes may not be exactly 5 mm if the prostate moves downwards as the probe is withdrawn and finally and most importantly the boundary echo is not always clear. When 2 measurements were taken from the same patient they were always in close accordance, however in this case the same assumptions about where the edge of the prostate lies on the ultrasound image will remain the same. The ideal, to withdraw and re-insert the probe repeatedly on the same patient could not be ethically justified. The volumes quoted appear large in comparison to the volume of tissue a Urologist is used to resecting. This is because the boundary echo occurs at the interface between the prostatic capsule and the periprostatic fat. The weight of tissue resected is about two thirds that measured by ultrasound (Neal et al 1989). Other workers have shown that the planimetry method for determining prostatic volume is accurate

(Hastak et al 1982, Bartsch et al 1982, Peeling et al 1987), although the former also found that the weight of tissue resected was less than the ultrasound volume. A further possible explanation is the observation that prostatic tissue if carefully dried with nylon foam weighs less after resection or scalpel dissection (Rasmussen 1975) than it does intact. It was suggested that the loss of acinar fluid caused the discrepancy.

Diagnosis

The performance of transrectal ultrasound in the detection of prostate cancer was in some respects disappointing, although it is in line with the experience of others. Chodak et al(1986) reported a sensitivity of 86%, a specificity of 41% and a positive predictive value of 36%. The positive predictive value found in this study, 37% would mean that only one in three of those patients suspected of having prostate cancer actually had the disease. However the negative predictive value was very high, 97%. Consequently the chance that a negative ultrasound is a missed cancer is 3%. This is further reassurance that the reference group is unlikely to contain men with cancer.

Staging

It is generally appreciated that digital rectal examination tends to understage prostate cancer. In particular there is a tendency to miss capsular penetration. Fujino and Scardino (1985) found that 44% of their patients thought to have localised cancer had evidence of extracapsular extension or seminal vesicle

involvement. These patients were treated by bilateral pelvic lymphadenectomy and radioactive gold seed implantation. Palpation of the prostate intraoperatively confirmed the accuracy of ultrasound, detecting a total of 15/16 tumours that had spread beyond the capsule compared with 7/16 for digital rectal examination alone. In an evaluation based on radical prostatectomy specimens Pontes et al (1985) detected 5/7 cases of capsular penetration. They comment that intracapsular spread, that is into the capsule but not beyond it, could not be diagnosed by ultrasound. However, it performed well in the diagnosis of seminal vesicle involvement. Hardeman et al (1989) report a detection rate of 13/24 cases of extracapsular spread with 8 false positives. Whilst this is better than 8/24 for digital rectal examination there remains inappropriate staging in about half such cases. Without the use of open surgical techniques it has not been possible in this study to evaluate the accuracy of ultrasound in staging. It seems likely that whilst better than rectal examination alone it is far from perfect. To some extent therefore the large overlap in PSA levels from patients with different disease stages may be due to the inadequacy of existing staging techniques.

PSA Prospective Study
Conclusions

Serum PSA in men without prostate cancer shows a log normal distribution

Levels of PSA amongst such patients especially those with BPH and those presenting in acute retention are often above upper limits proposed by other workers 95% cut-off or decision levels may be set after taking logs

The diagnostic value of serum PSA in a variety of clinical situations is poor

When compared on an equal footing PSA is comparable with PAP in a diagnostic role

PSA is closely related to the volume of prostatic tissue, to the prostate volume itself in BPH and localised prostate cancer and to stage in more advanced disease

PSA is organ specific and is therefore unmeasuable in women and men whose prostates have been entirely removed

PSA is elevated in patients with acute retention and following prostatic surgery. The milder trauma of rectal examination and catheterisation produce little change in PSA levels

RESULTS AND DISCUSSION - 2

PSA - retrospective study

RESULTS

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PSA
Retrospective Study

61 patients with carcinoma of the prostate recruited in 1984 were followed up until 31 January 1988. (See Patients page 84-5). The median follow-up time was 40 months (range 32-46 months). The characteristics of these patients are shown in table 15.

Table 15
Retrospective study - patient characteristics

n=61	Mean	SD	Range
Age	71.3	7.96	55-85
PSA	168.9	479	0.4-2885
PAP	10.0	19.9	0.5-95

M status and grade

M_0 n=52		M_1 n=9	
G_1	22	G_1	1
G_2	15	G_2	3
G_3	15	G_3	5

T category

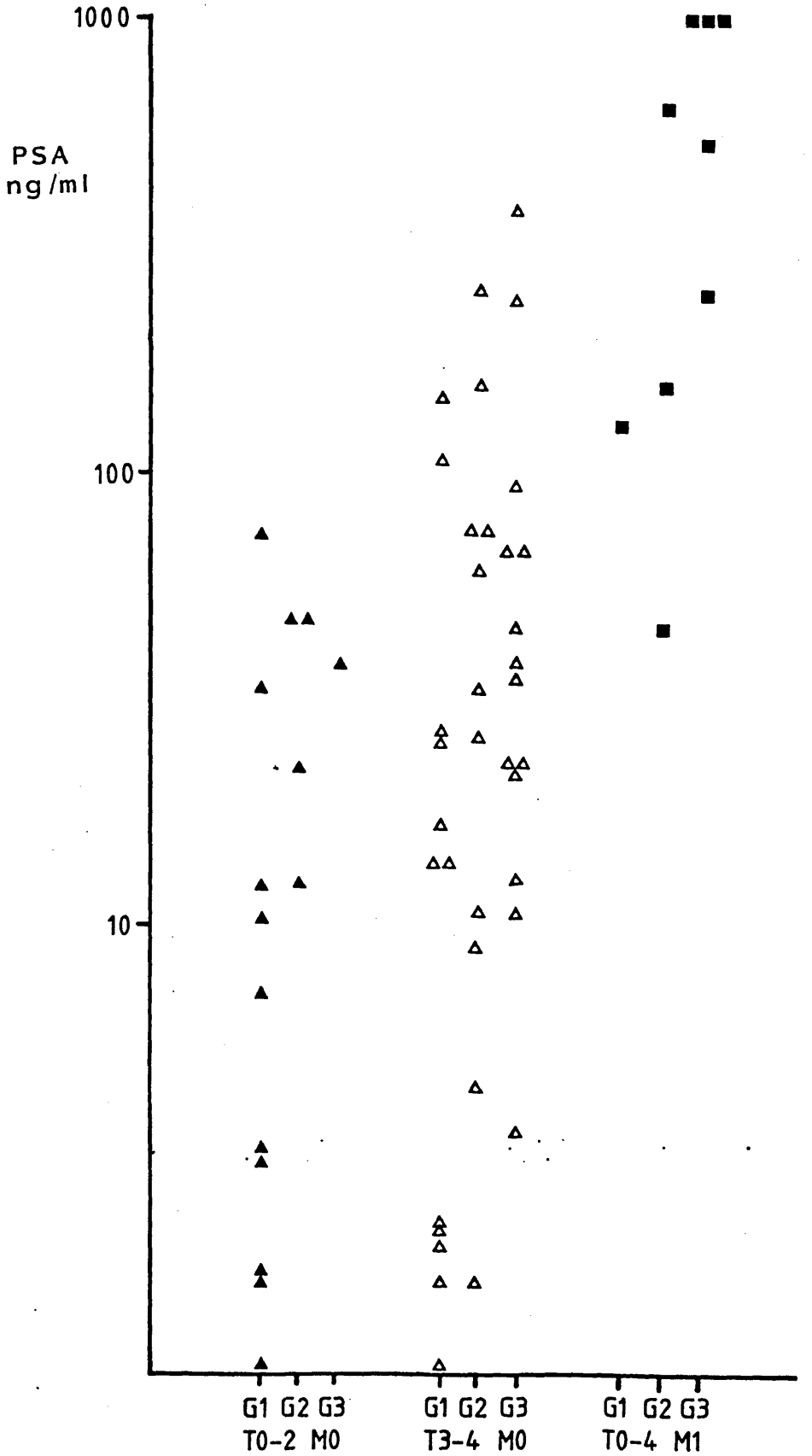
M_0		M_1	
T_0	7	T_0	0
T_{1+2}	8	T_{1+2}	0
T_{3+4}	37	T_{3+4}	9

The distribution of serum PSA levels by staging and also by grade are shown in figure 12. As noted in the prospective study there is a clear correlation between the stage of the disease and the PSA, although there remains considerable overlap between stages.

Clinical outcome

Of the 61 patients 9 had bone metastases at the time of diagnosis. All of these patients were eventually treated

Figure 12
PSA Retrospective study PSA vs stage and grade



by hormone manipulation, a bilateral subcapsular orchidectomy (BSO) in 8 cases, and cyproterone acetate in the remaining case. It can be seen from table 16 that 6/9 patients with bone metastases became symptomatic and were treated within 2 months, however a minority 3/9 were initially symptom free but required treatment at 14, 21 and 23 months. In all cases PSA and PAP were elevated at initial entry into the study (using the upper limits derived from the prospective study 9.3 ng/ml for PSA and 2.93 ng/ml for PAP).

Table 16
Patients with bone metastases Tx Nx M_1

Time to progression requiring treatment (months)	Time to death -from prostate cancer (months)	PSA ng/ml at diagnosis	PAP ng/ml
1	2	2885	95
2	8	249	33.7
1	9	1000	26.6
2	14	525	74.5
1	15	2240	48
23	27	157	1.9
1	39 Alive	640	76
14	40 Alive	126	4.7
21	40	46.3	4.3

7 patients died from prostate cancer within the study period, 4 within 14 months. No patients died without treatment.

Amongst the 52 M_0 patients, 6 died from prostate cancer including one man who was not treated with hormone manipulation. He had advanced local disease with perineal discomfort. His bone scan was negative and due to doubts regarding the aetiology of his pain, hormone manipulation was deferred. He died at home 2 months after recruitment and before his next outpatient review. A post mortem was

not held, his GP had noted a generalised deterioration in health, had ascribed this to prostate cancer and had not felt any further action to be necessary. This man was the only patient who died apparently from prostate cancer without being treated.

Nine patients died from other causes. These are shown in table 17. Only patients with clear evidence that the cause of death was other than from prostate cancer were included in this category. 8 received no further treatment for prostate cancer. One patient was treated for symptomatic local progression (persistent haematuria after TURP) but died shortly afterwards from disseminated large bowel cancer. Liver metastases were biopsied and after immunohistochemical staining these were found to have originated in the colon rather than the prostate.

Table 17
Patients who died from other causes - all T_{0-4} Nx M_0

Cause of death	n=9
Mesenteric infarction	
Myocardial infarction	
Myocardial infarction + dysrhythmia	
Myocardial infarction	
* Adenocarcinoma of colon - BSO for persistent haematuria post TURP. Liver metastases histology definite colonic origin	
Adenocarcinoma - disseminated unknown primary, histology mucin secreting, PSA staining negative	
Acute exacerbation of chronic bronchitis	
Myocardial infarction	
Myocardial infarction + left ventricular failure	

* Only one case treated for progression of prostate cancer

A total of 34 patients progressed and were treated. The patient who died from colonic cancer shortly after BSO has been excluded as a censored observation, follow-up being impossible (see table 17*). Thus 33 remain. Of these 9 were M_1 patients and 24 were M_0. 13 patients were treated for local progression alone, 11 for metastases and 9 for both. In 28 cases this consisted of bilateral orchidectomy sometimes combined with other treatments (table 18). 13 patients died from prostate cancer within the study period, that is 7 of the M_1 patients and 6 of the 52 Mo patients.

Table 18
Patients who were treated for symptomatic progression

Type of treatment	
Bilateral subcapsular orchidectomy	23
Bilateral subcapsular orchidectomy + cyproterone acetate	3
Bilateral orchidectomy	2
Depot goserelin (Zoladex)	2
Stilboestrol	2
Cyproterone acetate	1
	n=33

PSA in predicting prognosis

The performance of PSA in the prediction of prognosis is reviewed in a number of ways. It is compared with PAP throughout. The simplest method of analysis is to use a single cut-off level. Those derived from the reference group were used, 9.3 ng/ml for PSA and 2.93 ng/ml for PAP. These values were set at the 95% level by adding 1.64 X

standard deviation to the mean after log transformation - see Results 1 page 110. The results for the M_0 patients are shown in table 19.

Taking death from prostate cancer as an end point 6/6 patients had an elevated PSA whilst for PAP only 3/6 showed elevated levels. With regard to progression requiring treatment, 17/18 patients had a raised PSA whilst only 7/18 had a raised PAP.

Table 19
Prediction of prognosis M_0 patients

	Cancer Deaths	Other* Deaths	Progress -ion+	Alive+no progression	n
PSA					
Elevated PSA	6	6	17	8	37
Normal PSA	0	3	2	10	15
PAP					
Elevated PAP	3	1	7	1	12
Normal PAP	3	8	12	17	40
Total	6	9	19	18	61

PSA cut-off 9.3 ng/ml PAP 2.93 ng/ml

* Only definite deaths from others causes included - listed in table 17.
+ A total of 25 patients were treated for symptomatic progression, all by hormone manipulation, predominantly orchidectomy. One patient who did not receive treatment had extensive T_4 non-metastatic disease and died at home. No post mortem was performed and therefore his death was classified as from cancer. One further patient was treated for symptomatic local progression but died from disseminated adenocarcinoma which arose from the large bowel. Biopsied liver metastases demonstrated unequivocal evidence of bowel origin and stained negative for PSA. So 1 patient who died from other causes was treated for progression whilst another who died from prostate cancer was untreated. The remaining 5 patients who died from prostate cancer were all treated.

The results for the 9 M_1 patients are tabulated below (table 20).

Table 20
Prediction of prognosis M_1 patients

	Cancer Deaths	Other Deaths	Alive	n
PSA				
Elevated PSA	7	0	2	9
Normal PSA	0	0	0	0
PAP				
Elevated PAP	6	0	2	8
Normal PAP	1	0	0	1
Total	7	0	2	9

Cut-off PSA 9.3 ng/ml PAP 2.93 ng/ml

All patients known to have metastases were treated only when symptoms developed. For the majority but not all patients, this was shortly after the diagnosis of bone metastases. Those with a positive bone scan were not automatically treated. Consequently the time to treatment is equivalent to the time to symptomatic progression. The PSA and PAP levels have been tabulated against the time to treatment for this group table 21. The PSA values have been ranked in order of magnitude so that the correlation between PSA and time to treatment may be seen.

As in previous analyses (See results 1 page 117) the data can be reviewed using ROC curves. Patients with progression requiring treatment constitute the positive group that the test aims to identify and those who do not progress are the negatives.

Table 21
Time to treatment - M_1 patients

Time (months)	PSA (ng/ml)	PAP (ng/ml)
21	46.3	4.3
14	126	4.7
23	157	1.9
2	249	33.7
2	525	74.5
1	640	76
1	1000	26.6
1	2240	48
1	2885	95

n=9

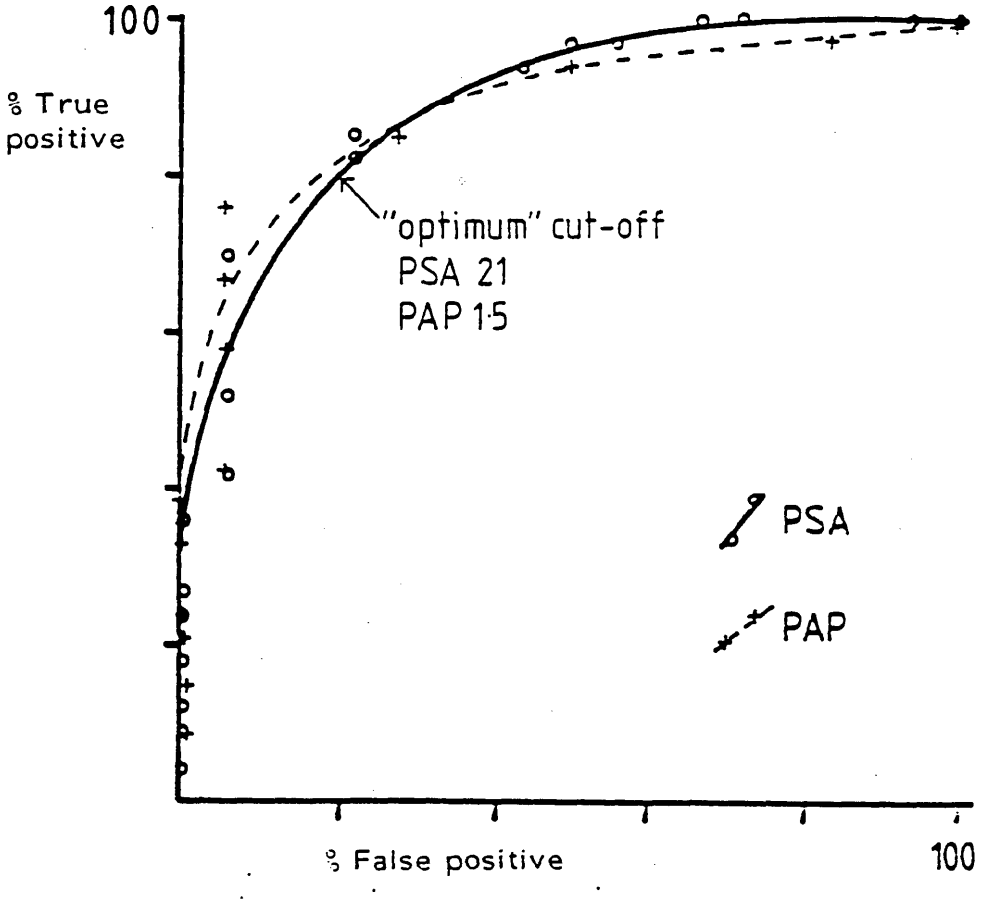
ROC curve analysis

The 33 patients who were treated for symptomatic disease progression were taken as the positive group. The negative was derived from the 18 patients who did not require treatment. The 9 patients who died from other causes were excluded. The patient who died apparently from prostate cancer was also excluded. Comparison of the curves demonstrates that they are in fact remarkably similar (figure 13). This appears contrary to the findings presented earlier (tables 19+20) which suggested that PSA was markedly superior in the prediction of progression. It seems that choice of cut-off level may profoundly affect the apparent performance of a new test.

The point of theoretical maximum test efficiency is the top left hand corner, that is 100% true positives and 0% false positives. The point on the curve nearest to this ideal can be seen and the corresponding cut-off level can be estimated, 21 ng/ml for PSA and 1.5 ng/ml for PAP.

Figure 13

Predicting Progression
ROC curve analysis



Correlation between PSA and PAP

The relationship between PSA and PAP is shown in figure 14. There is a close correlation r=.799, t=10.2, p<0.001. It can be seen that the spread of PSA values is greater in the lower ranges than is the case for PAP. For this reason one might suppose that amongst those patients with limited disease that PSA may perform better. The numbers available in this study do not allow this hypothesis to be tested.

PSA and time to progression

For all 33 patients who progressed and were treated there was a significant correlation between PSA and time to progression, r=-.628, t=4.5, p<0.001, n=33 (figure 15). This correlation persists when M_0 and M_1 patients are analysed separately, r=-.427, t=2.21, p<0.05, n=24 for M_0 cases, r=-812, t=3.68, p<0.02, n=9.

Within the 33 patients who progressed there persists a strong correlation between PSA and PAP, r=.757, t= 6.45, p<0.001, n=33. As one would expect therefore the level of PAP also correlates with time to progression, r=-.523, t=3.42, p< 0.01, n=33 for all cases. However this is less strong in the M_0 patients r=-.208, t= 1.0, p>0.2, n=24 although the correlation remains when M_1 cases are examined, r=-.948, t=7.85, p<0.001, n=9.

Taking the optimum cut-off levels derived from ROC curve analysis (figure 13) actuarial curves for risk of progression can be plotted (figure 16a+b). This form of analysis takes no account of other prognostic factors; this aspect is addressed in Results and Discussion 4.

Figure 14

PSA Retrospective study

PSA vs PAP

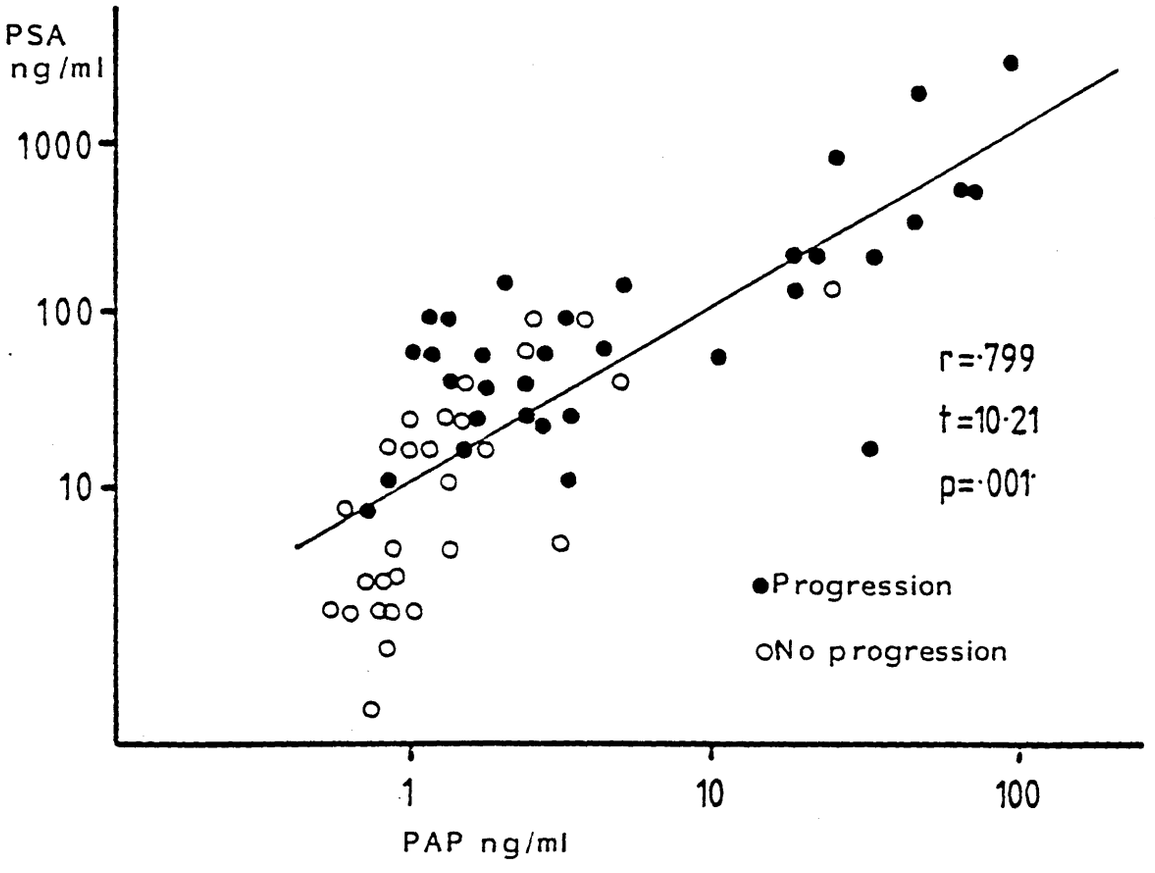


Figure 15

PSA and PAP vs time to progression

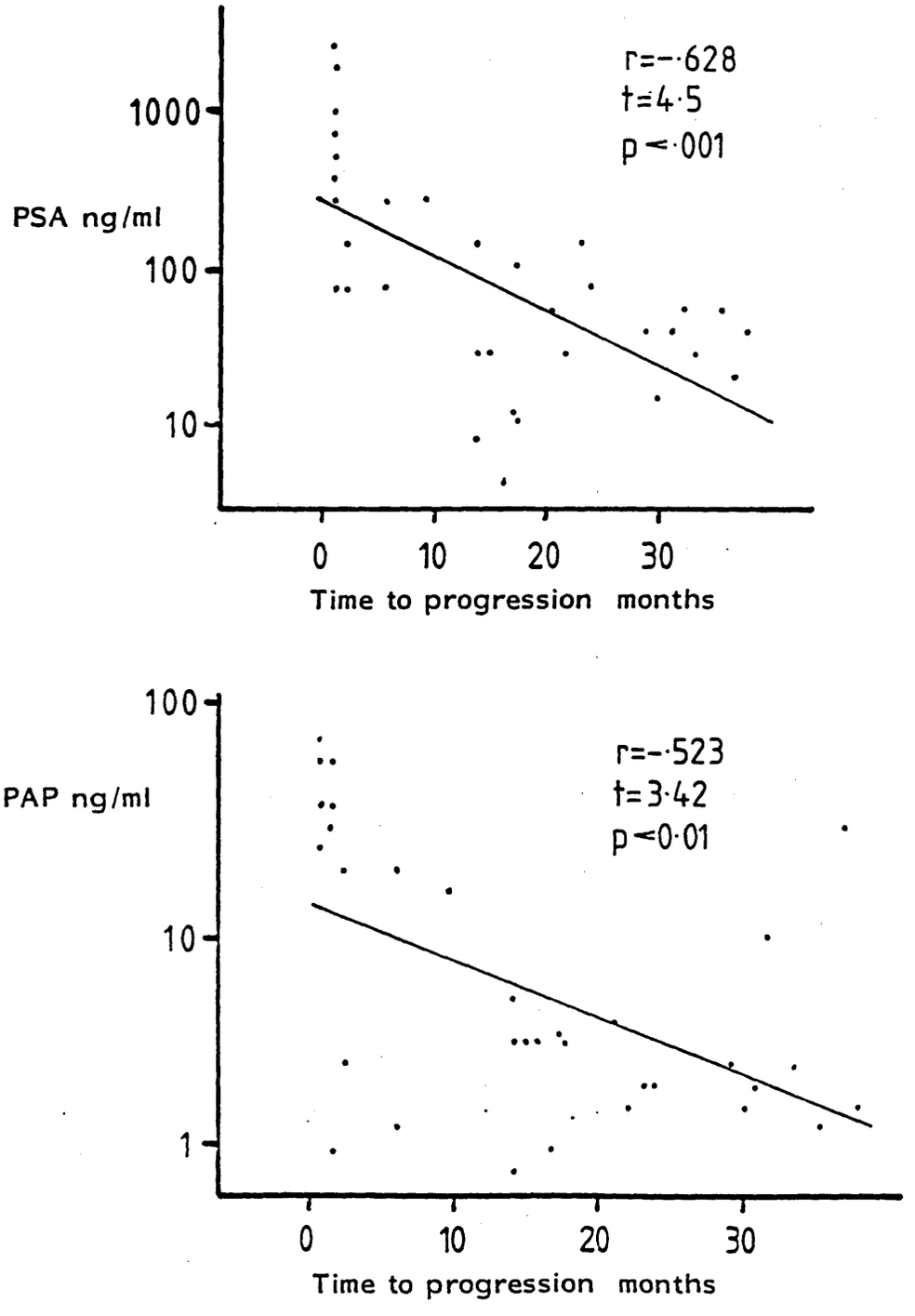


Figure 16a

Probability of progression

PSA Life Table Analysis

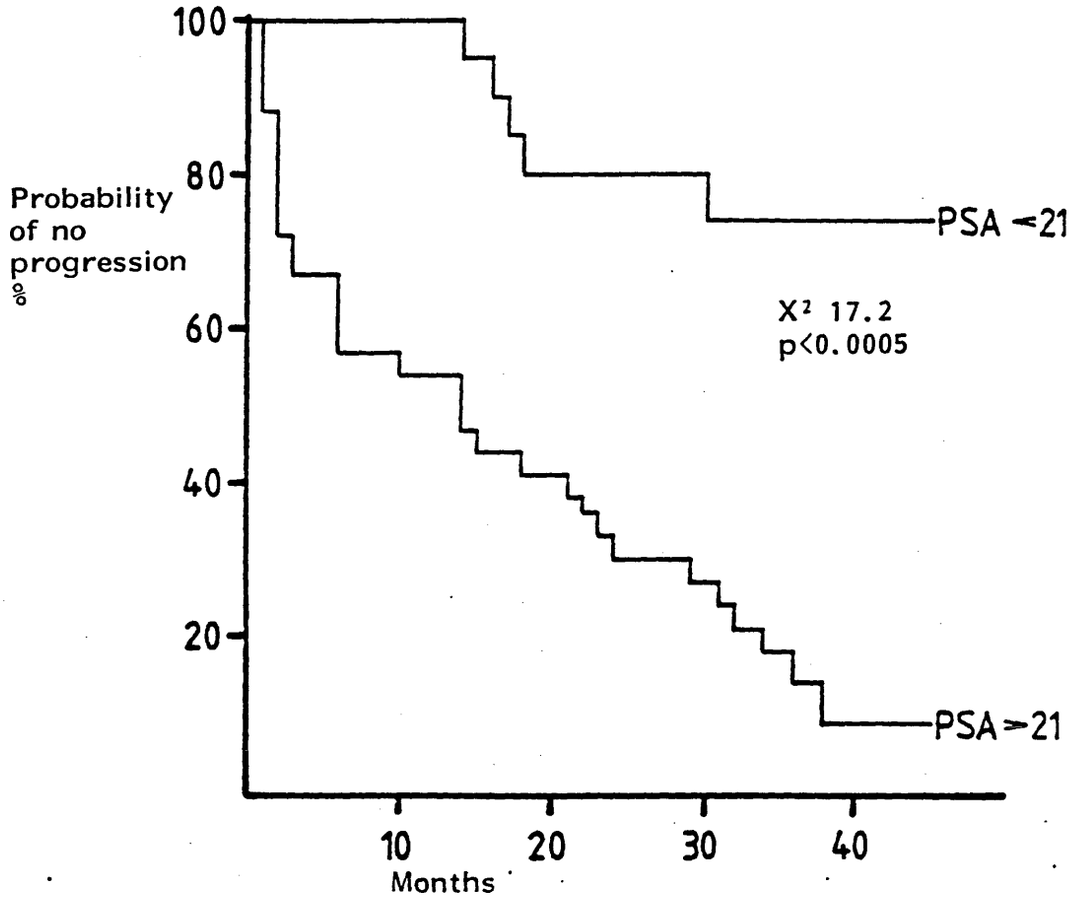
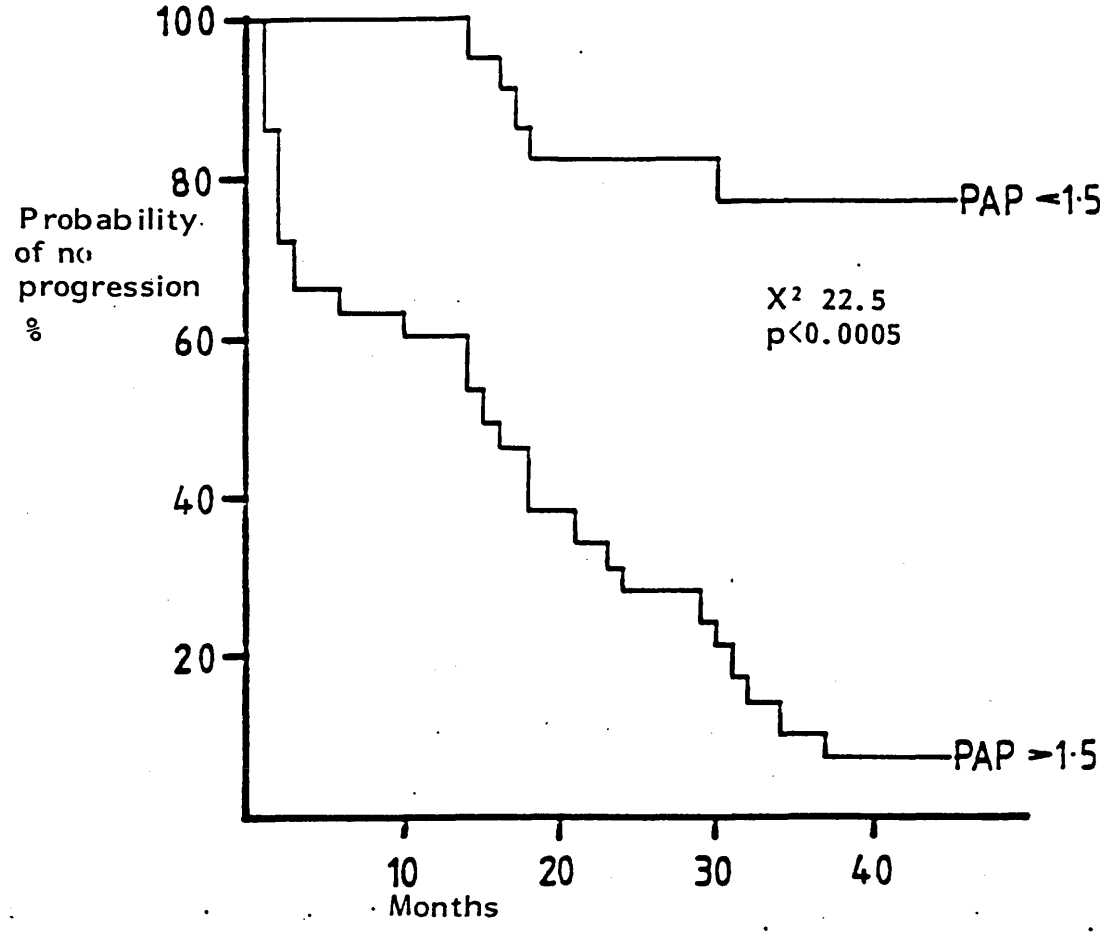


Figure 16b

Probability of progression

PAP Life Table Analysis



PSA and survival

13 patients died from prostate cancer within the study period. Life table survival curves for death from prostate cancer have been plotted (figure 17a+b), suggesting a predictive capability for PSA, X^2 6.80 $p<0.005$ but much less so in the case of PAP, X^2 2.22 p=not significant.

Figure 17a

Survival curve

PSA death from prostate cancer

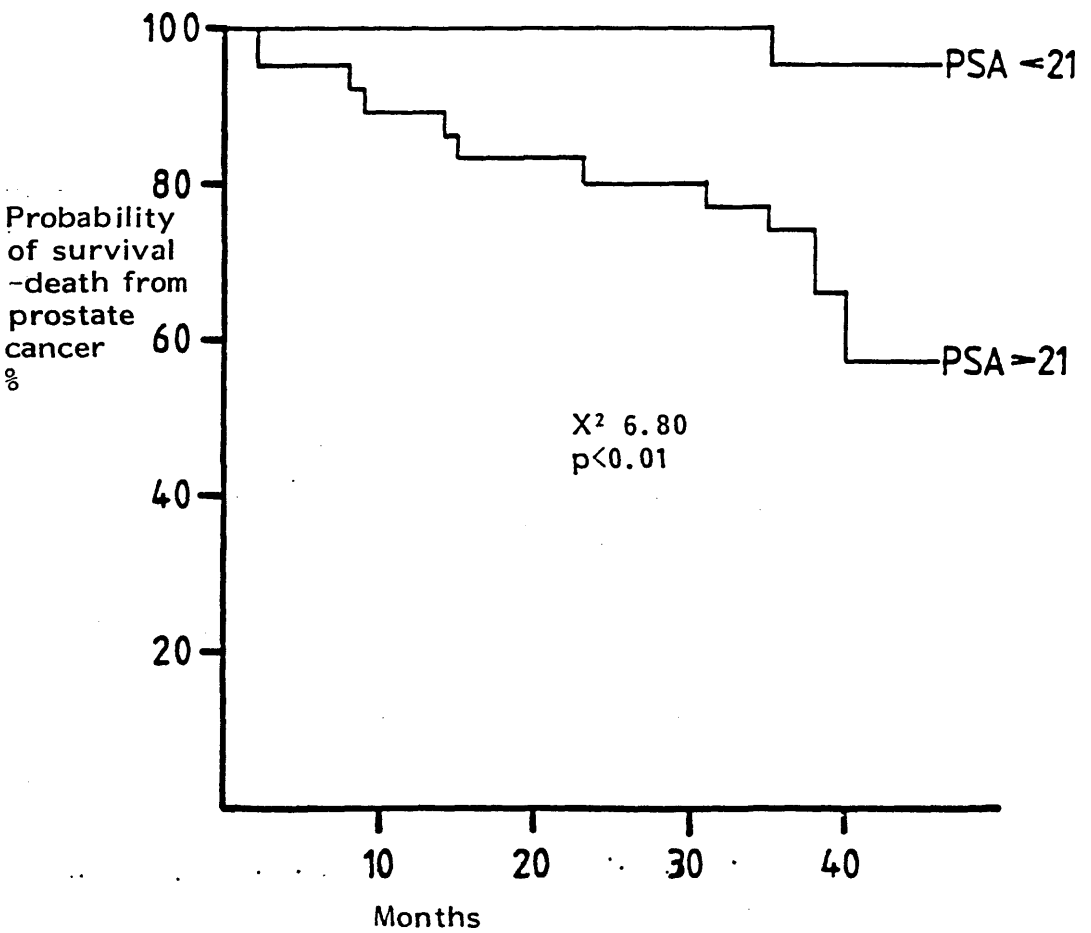
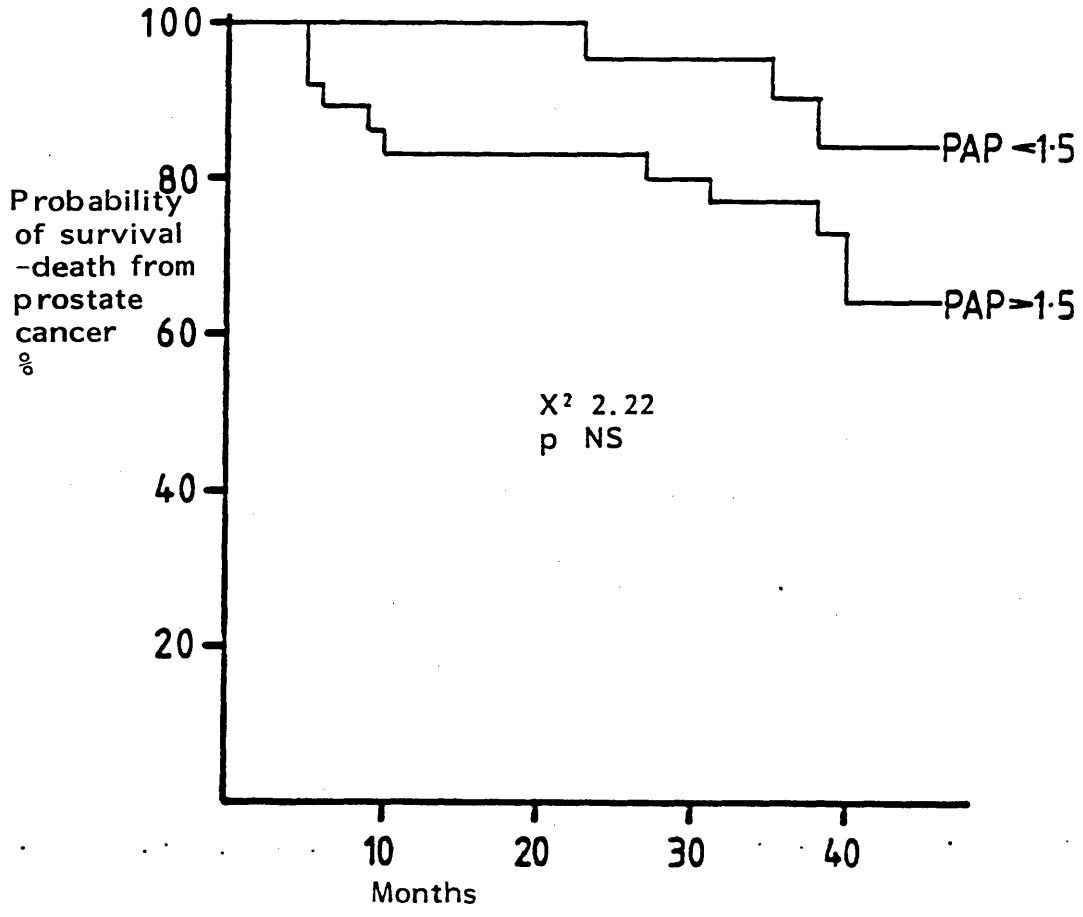


Figure 17b

Survival curve

PAP death from prostate cancer



Discussion - Retrospective PSA study

PSA and stage

As expected PSA correlated well with disease stage and to some extent grade. Tumours that are poorly differentiated tend overwhelmingly to be advanced so the relationship between PSA and grade may not be as good as at first appears. It is noteworthy that the overlap between PSA values of different stage patients is considerable. These observations are entirely in line with those of others and with the prospective study.

Cause of death

It is well recognised that studies of prostate cancer are beset by the problem that as the population is elderly, a number will die for reasons unrelated to their prostate cancer. In the VACURG studies this constituted about 1/3 of all deaths. In a relatively small study it is necessary to attempt to exclude these patients and use just cancer deaths as an end point. The selection of these patients has been rigorous and well documented deaths from other causes have been accepted into that category. Where doubt persists I have classified such cases as cancer deaths. The end point of cancer death is not satisfactory for another reason. The follow up was short in this study. Only 13 out of 61 patients died from prostate cancer within the study period. Whilst for patients with bone metastases these deaths 7/9 accounted for 78%, amongst patients in the Mo group there were only 6/52 cancer deaths, 12%, making this a less than ideal end point.

Symptomatic progression requiring treatment occurred in 24/52 Mo patients and this is perhaps a more informative end point. Although one could argue that the decision to treat a patient is subjective it is clinically very important to identify those patients who are likely to need treatment. We are then in a position to follow them up more closely or perhaps treat them whilst asymptomatic. Conversely, those at low risk can be seen less often. It should be noted that the clinician was not aware of either the PSA or the PAP levels.

The choice of end point and its precise delineation is a problem in prostate cancer. Ultimately the gold standard is overall survival in a large lengthy study. However it is valid in the context of a deferred treatment policy to evaluate the relevance of PSA in the prediction of progression. This is an important question as it may be that some patients are not suitable for this approach and these need to be identified. For example, the patients with a positive bone scan at diagnosis (ie M1) and a high PSA (>200 ng/ml) all developed symptoms and required treatment within 2 months. One could argue that there is little to be gained in deferring treatment in such patients.

Prediction of prognosis

PSA in the prediction of prognosis is best examined with reference to the Mo patients. All M_1 patients had elevated PSA levels although it is interesting that there remained a correlation between PSA and the time to treatment for

symptomatic progression. Analysis of the data relating to Mo patients showed that a raised PSA is associated with a high risk of disease progression. Furthermore the magnitude of the PSA elevation correlates with the time to progression. Patients with a high PSA progressed earlier. Reference to the ROC curve analysis shows little difference between PSA and PAP in this role. As noted in the prospective study looking primarily at diagnosis, the fixing of the cut off level is crucial to the apparent performance of a test. Lewenhaupt et al (1988) in a study of a variety of serum markers found that in predicting prognosis the cut-off levels quoted by the manufacturers were inappropriate, tending to be too low. He makes the point that the "discrimination limits should be selected with regard to the type of information looked for". ROC curve analysis with relevant study and reference populations seems the most logical means to determine how these levels are set. An all-purpose upper limit of normal is illogical and may be misleading.

The plot of PSA vs PAP is interesting (figure 14 page 164). It shows close correlation between PSA and PAP for higher values but towards the lower end of the range the spread of PSA values is much greater than for PAP. This implies at these lower levels that PSA provides a better discrimination between those at risk of progression and those who are not. The numbers available in this subgroup prevent meaningful statistical analysis. This pattern of relationship of PSA and PAP, a straight line with a curve at the left hand end towards zero (hockey stick shape) has been observed by Seamond et al (1986) although he does not

specifically comment upon it.

This study suggests that there is little difference between PSA and PAP and therefore there is little to be gained by using both assays. With regard to the prediction of prognosis, there is some evidence that PSA offers better discrimination at lower levels. For this reason it would seem the logical preference.

PSA Retrospective Study - Conclusions

PSA correlates well with stage and grade (as noted in the prospective study)

ROC curve analysis shows that PSA and PAP are almost of equivalent value in the prediction of progression and that different cut-off levels (ie not the routine upper limit of normal) are appropriate

PSA identifies those at high risk of progression and death

PSA correlates with time to progression

PSA may be slightly superior to PAP in this role particularly in early disease when marker levels are low

DNA flow cytometry

Preparation of cell suspension

Principles of flow cytometry

Calibration and setting up

Technical aspects
- Amplifiers + electronic offset
- Fluorescence of material other than propidium iodide
- Concentration of propidium iodide
- Effects of formalin fixation
- Operator factors

Interpretation of results

Statistical methods

DNA Flow Cytometry

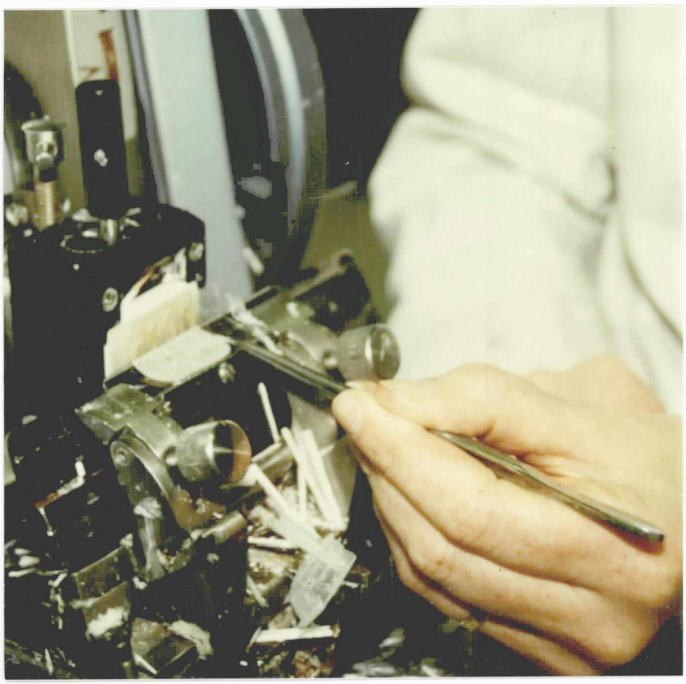
Preparation of cell suspensions

The first requirement for flow cytometry is to make a suspension of single cells from the tissue to be examined. In this study flow cytometry was largely performed on paraffin embedded material dating from 1984. At that time and indeed up to the present moment, the fixation and embedding of prostate chips has been performed in a standard way. Firstly the chips are fixed in 10% formol saline overnight. Then the fixative is changed to formalin with mercury for 30 mins. Finally the chips are embedded. Each paraffin block contains approximately 5g of tissue. Routinely 4 blocks are embedded ie about 20g. If the weight of prostate tissue resected is below 20g then all the tissue is embedded. If the weight is above 50g or if there is strong clinical suspicion of prostate cancer then more than 4 blocks are be prepared. During the processing of the specimen the pathologist selects out the chips that look macroscopically suspicious of carcinoma. The chances of overlooking a cancer using this system are said to be 1 - 9.7% (Rismyhr et al 1980, Garborg and Eide 1985). For routine histological assessment 6 um sections are cut and stained with haematoxylin and eosin (H&E). These slides were reviewed by a consultant histopathologist (Dr M C Robinson - MCR) and the two containing the most poorly differentiated tumour affecting the greatest number of chips were selected. The tumour bearing chips were mapped out on the slide with a felt-tip pen (photograph page 177). A 50 um section was then cut from the appropriate block. Sellotape stuck to the surface of the block

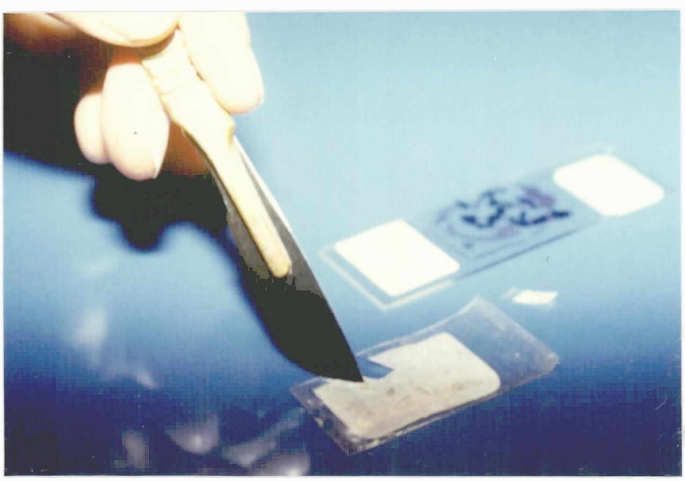
Photograph
Preparation of specimens for DNA FCM



2 blocks with the most poorly differentiated tumour chosen. Tumour bearing chips mapped out by the histopathologist (MCR)



50μm sections cut from adjacent tissue Note Sellotape on the face of the paraffin block



Tumour bearing chips cut out using the mapped H&E section as a guide

prevents the tissue section from curling up allowing easier handling. During the subsequent dewaxing procedure the Sellotape's adhesive is dissolved and it may be removed easily. Using the H&E slide as a reference the marked chips, ie those containing tumour were dissected from the 50 um section using a scalpel. These fragments were placed in a test-tube for dewaxing and digestion to make a cell suspension. This procedure is summarised in table 22.

Table 22
Preparation of cell suspension from paraffin embedded tissue

50 um paraffin sections

Dissect out the cancer bearing chips

Dewax with **Inhibisol** (Penetone Chemicals)
2 changes-glass tube

Rehydration	Absolute alcohol 2 changes
	95% Industrial Methylated Spirit
	70% " " "
	50% " " "
	Distilled water - remove supernatant

0.5% Pepsin pH 1.5 3ml 37^{0}C 30 mins

Intermittent vortex mixing

Remove supernatant Phosphate buffered saline 2 changes

Syringe via 19 then 21 gauge needle

Filter through 38 um nylon mesh

Incubate with 0.05% RNAase for 30 mins at 20^{o}C mins

Stain with 50 ug/ml propidium iodide

Dewaxing was achieved by the use of Inhibisol (Penetone

Chemicals) a proprietary dewaxing agent. Rehydration was performed using alcohol with increasingly higher water content and finally in distilled water. The enzyme digestion process described by Hedley 1983 was used. This has been used by a number of other workers and has become a standard technique (Fordham et al 1986, Watson et al 1985). The tissue is incubated for 30 minutes at 37^{o}C in a 0.5% solution of pepsin (P7012, Sigma Chemical Co Ltd, Fancy Road, Poole, Dorset) at a pH of 1.5. The sample is then centrifuged and resuspended in phosphate buffered saline twice over. Typically this produces a cell suspension with up to 10 million cells although occasionally it is much less than this. The "cells" when examined under the microscope are in fact predominately nuclei sometimes with a rim of adherent cytoplasm, a more accurate description would be a nuclear suspension. For each patient two samples were prepared for flow cytometry one from each of the two blocks selected by the histopathologist. From these two the most abnormal flow cytometry was used for subsequent analysis. The samples were treated with 0.1% RNAase (R4875 Sigma Chemical Co Ltd) at room temperature and stained with propidium iodide (Sigma Chemicals) 50 ug/ml 10 mins prior to flow cytometry. This is an intercalating dye binding between the strands of double nucleic acid. In cell nuclei this is predominantly DNA although there may be a little double stranded RNA. The latter is removed by treatment with RNAase. Samples prepared in the afternoon were kept unstained overnight at 4^{o}C and flow cytometry performed the following morning after adding propidium iodide and

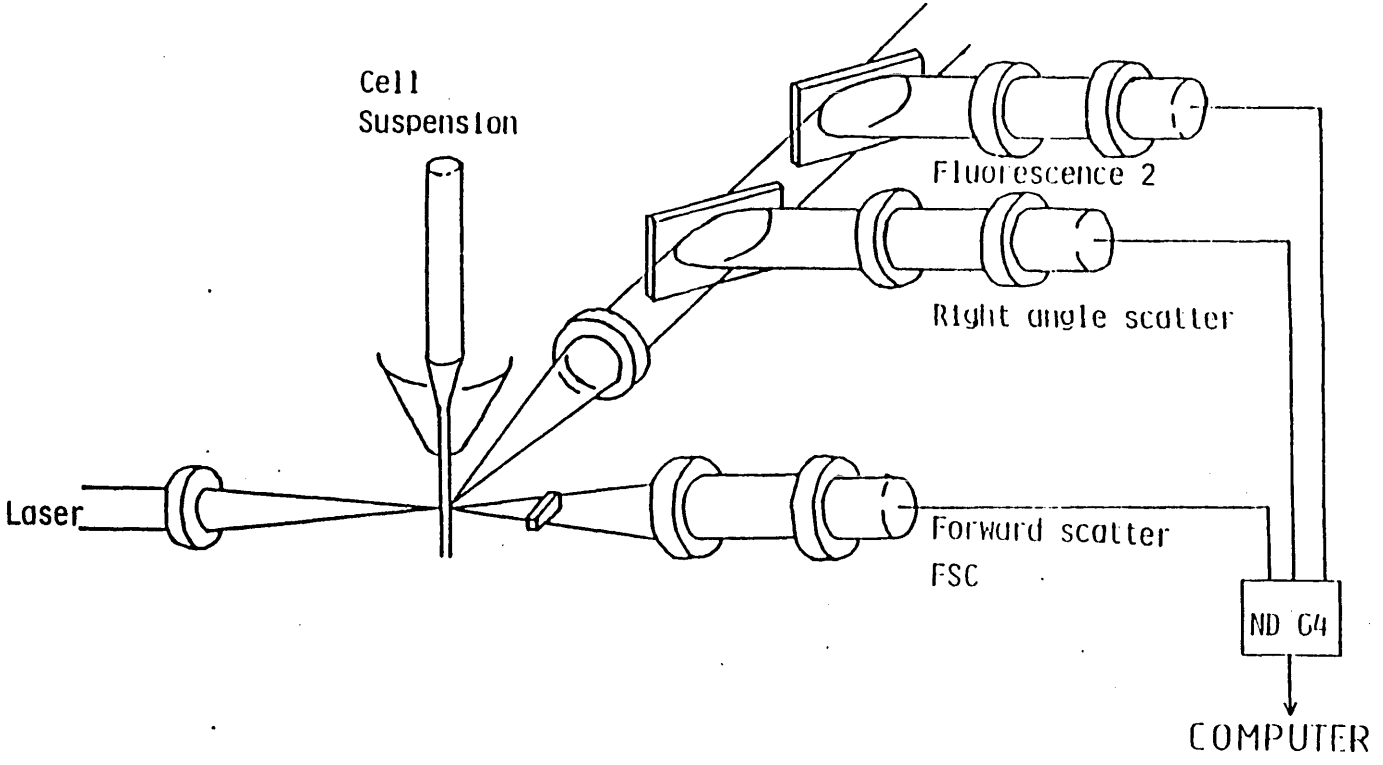
RNAase, those prepared in the morning were processed the same day.

Principles of flow cytometry

A Becton Dickinson FACS 420 with 3 right angle photomultiplier tubes and one forward scatter photodiode was used (Becton Dickinson Immunocytometry Systems, Mountain View, California). 400 mW of light from a 2W argon laser with a wavelength of 488 nm (blue-green) is shone through a stream of fluid containing the cell suspension (figure 18). The output from the photomultipliers and the photodiode is fed into a ND64 unit which records the relative light intensities on a scale from 0-255. Thus for each cell passing through the laser beam 4 separate light intensity measurements are made each on an arbitrary 256 point scale numbered 0-255. Each point on this scale is termed a channel. Interposed between the ND64 and the photomultipliers and photodiode are amplifiers, one for each. These have variable gain settings and can be switched to either log or linear mode. For the purposes of DNA FCM they were set on linear mode. The data is captured by a Hewlett Packard 9920 computer (Hewlett Packard, Palo Alto, California) and stored on tape with hard disk for analysis. This was performed using Consort 30 software (Becton Dickinson, Sunnyvale, Ca). The fluorescence due to the excitation of propidium iodide bound to DNA is measured by a photomultiplier at right angles to the stream (figure 18 fluorescence 2). A 625 nm band pass filter is used to ensure that only light of the appropriate wavelength is measured. As a linear amplifier

Figure 18

The flow cytometer



is used the channel number is proportional to the amount of fluorescence and therefore to the amount of double stranded DNA in the cell. The forward scatter of 488 nm light is thought to relate approximately to the volume of the particles passing through the laser beam. Note that the bar shown in the diagram excludes light that is transmitted directly through the stream with no deflection, only light that has been scattered or refracted is measured by the photodiode (FSC). A second photomultiplier mounted at right angles to the stream measures the right angle scatter of 488 nm light. The significance of this measurement is that it is related to the granularity of the cells under study. The use of the forward scatter and the right angle scatter corresponding to "size" and "granularity" has been used to discriminate between populations of mononuclear cells, principally between lymphocytes and monocytes. In prostate cancer others have reported that these two factors may relate to nuclear size and shape, known from light microscope studies to be prognostic indicators (Benson et al 1984). The third photomultiplier can be used to measure fluorescence at a second wavelength. Thus a fluorescein tagged antibody can be used in addition to propidium iodide.

Calibration and setting up

The FACS 420 was turned on and allowed to warm up at least 1/2 hour before samples were run. Fresh human lymphocytes and ethanol fixed chicken red cells were used for calibration purposes. The lymphocytes were stained with propidium iodide 50 ug/ml alone and Triton X100 0.9% to

lyse the cell membranes, the red cells with propidium iodide 50 ug/ml alone. The amplifiers of each photomultiplier can be adjusted in terms of gain, hence the need for calibration and setting up. In practice the system was remarkably stable and after calibration the gain settings were roughly the same from run to run. The forward light scatter gain was adjusted to get all the lymphocytes on scale. One or two of the samples were run as a trial and if necessary the gain was adjusted a little more to ensure that the vast bulk of the cells were on scale (figure 19). The right angle scatter was not formally calibrated. The fluorescence 2 was calibrated using fresh lymphocytes. The main peak (G0/1) was aligned with channel 100 on the ND 64 unit. Checking after the completion of each run revealed no significant drift with time. Chicken red cells were also used, these are nucleated cells and were found to have a main peak in channel 35. However the real value of the chicken red cells was in the determination of the electronic offset (see below).

Technical Aspects

Amplifiers and electronic offset

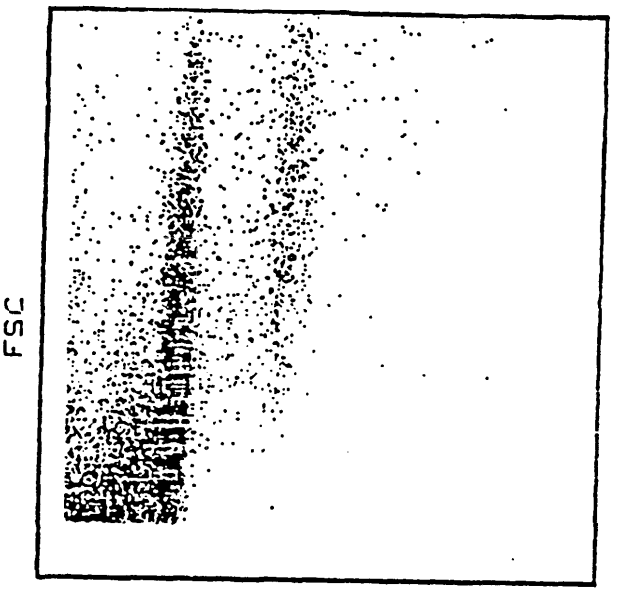
The linearity of our amplifiers was determined using fluorescent beads (Flow Cytometry Standards Corporation, USA). These were labelled with fluorescein and supplied with 4 different amounts bound to each of the beads. The amount of fluorescein per bead and the measured fluorescence were correlated in a linear manner. Furthermore we could confirm that the gain settings were

Figure 19

DNA Flow Cytometry
Example - paraffin embedded BPH specimen

Forward scatter

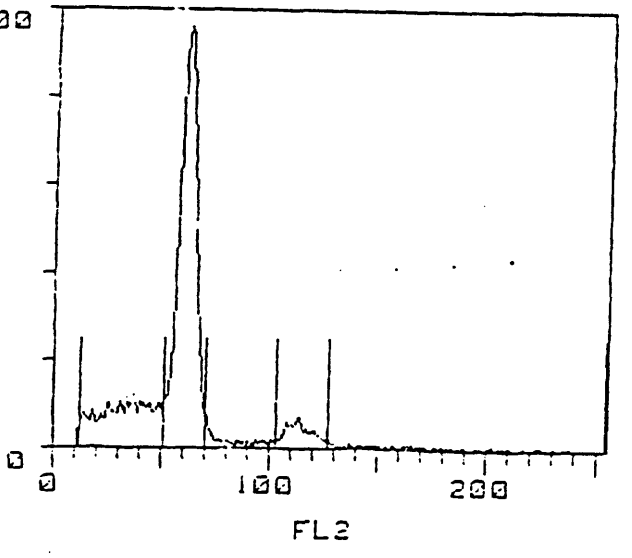
Each dot represents a cell/nucleus
n=10,000



Fluorescence 2 - DNA

A histogram of cell DNA content can be drawn

No of cells or particles



DNA content

Fluorescein calibration beads: fluorescein per bead vs fluorescence intensity (channel number) at different gain settings

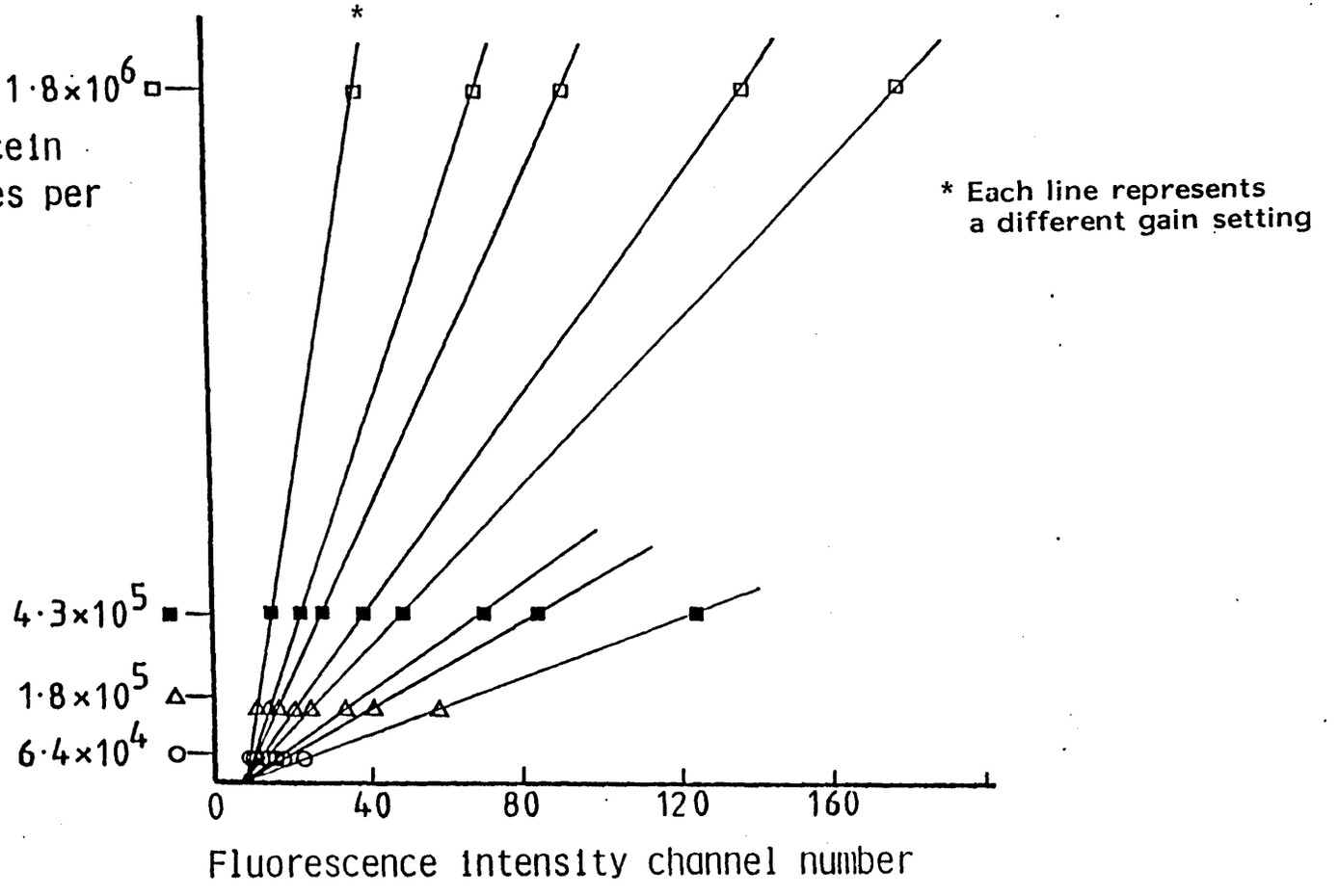


Figure 20

linear in that doubling the gain produced a corresponding increase in fluorescence reading. It was found however that whatever the gain setting the line connecting the florescence intensities for each type of bead did not pass through zero (figure 20).

The output from the photomultipliers passes via the amplifiers to the ND64 unit. This is in effect an analog to digital converter as it takes a continuously variable input, light intensity and gives it a digital value, from 0 to 255 ie a channel number. The greater the light intensity the higher the channel number. If zero light intensity is converted not into channel 0 but to channel 5 for instance then this will produce an offset which will affect the calculation of the ratio of one fluorescence intensity with another. It is this offset that explains why the lines described above do not pass through zero. Corroborative evidence was obtained using chicken red cells (Givan et al 1988). These cells tend to clump together in doublets or triplets. Since we know that the doublet peak should have twice the fluorescence of the singlet peak we can plot a graph of cell number, singlets or doublets against channel number (figure 21) to see at what point a line connecting the peaks crosses the X axis. This is the offset. If the offset is subtracted from the channel number of the singlet and doublet peaks then the channel number of the former will be exactly half that of the latter. Further confirmation is obtained by analysis of the triplet peak in the same way. We also found that debris in the specimen had its own peak and this appeared in the channel corresponding to the offset value, ie zero

DNA Flow Cytometry using chicken red cells:
The effect of different gain settings

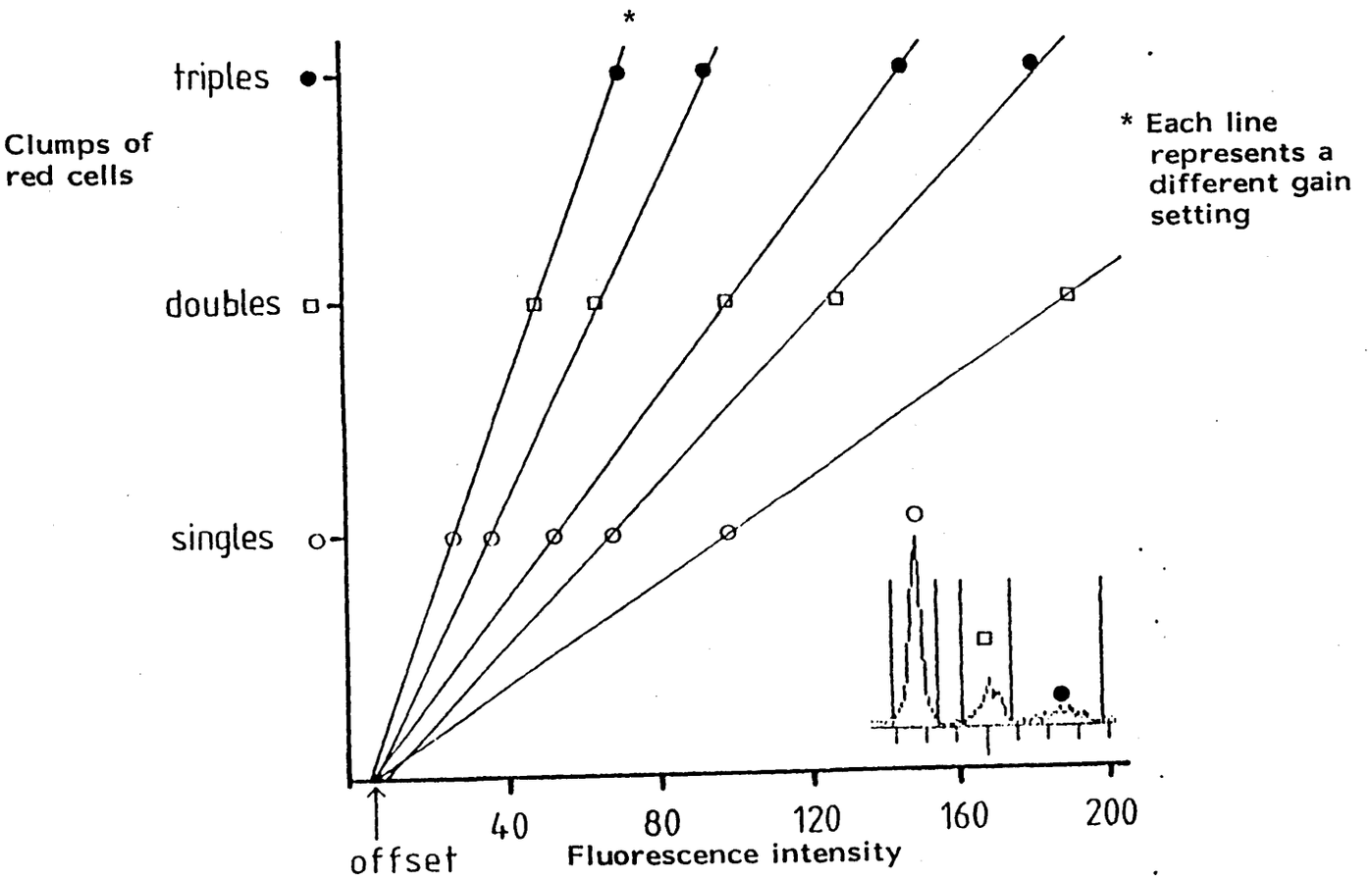


Figure 21

fluorescence. In setting up each run the offset was determined using chicken red cells and the appropriate corrections were made. It is possible to adjust the position of the offset but to reduce it to channel 0 exactly is not advised by the manufacturers service personnel as it may mean that with very slight drift the debris would appear not in channel 0 but 255. This artefact is due to the way the electronics are set up. A large amount of debris in channel 255 would upset some of the interpretation and therefore we preferred to correct for offset rather than adjust it to zero.

Fluorescence of material other than propidium iodide

Unstained cells were run through the machine on several occasions and fluorescence at 625 nm was measured. No fluorescence occurred. We can therefore be confident that the fluorescence we are describing does arise from the propidium iodide.

Concentration of propidium iodide

The concentration of propidium iodide used in this study is in line with that used by others (Fordham et al 1986, Taylor and Milthorpe 1980, Tribukait et al 1983). It has been shown that such a concentration provides saturation and that minor variations in propidium iodide concentrations will not affect the fluorescence measured. This was confirmed by our own experiment (see page 204).

Effects of formalin fixation and paraffin embedding

In the comparison of fresh tissue versus fixed material our investigations have been hampered by the lack of a good method to prepare cell suspensions from fresh tissue.

Others have reported good results using needle aspiration of the prostate in vivo and in vitro using mechanical disaggregation with or without chelating agents or enzyme digestion (table 23). We have tried finely mincing tissue with scalpel blades, ultrasound, EDTA, collagenase, trypsin and pepsin. None gave reliable and repeatable good quality disaggregation. Preparation of cell suspensions from paraffin embedded material is considerably easier. The results of the comparison of fresh versus fixed tissue are summarised in the results section.

Table 23
Methods of disaggregation for fresh prostatic tissue

Workers	**Methods**
Tribukait 1980 + 1983 Ronstrom 1981 Bichel 1982	Fine needle aspiration samples taken
de Vere White 1984	Mechanical disruption, aspiration through fine needle
Schultz 1985	Mincing, syringing thru fine needle + filtering
Roters 1980	Mincing + pepsin
Frankfurt 1984	Mincing + collagenase
Engelholm 1985	Mincing + Trypsin
Frankfurt 1985	Mincing + EDTA

Operator factors

Although DNA FCM produces impressive and seemingly objective histograms and other plots, it is in fact surprisingly operator dependent. Small problems with the flow through the nozzle or the laser alignment can dramatically affect the results. We are fortunate in the

Department of Surgery Newcastle University to have a scientist dedicated entirely to operating our FACS machine (Dr A Givan - AG), her experience with the FACS machine is now more than 3 years. Studies using DNA FCM have been carried out in breast cancer, hyperparathyroidism, and in numerous cell cycle experiments. As a further verification of our technique we have processed samples from other institutions in Birmingham, London and Sheffield.

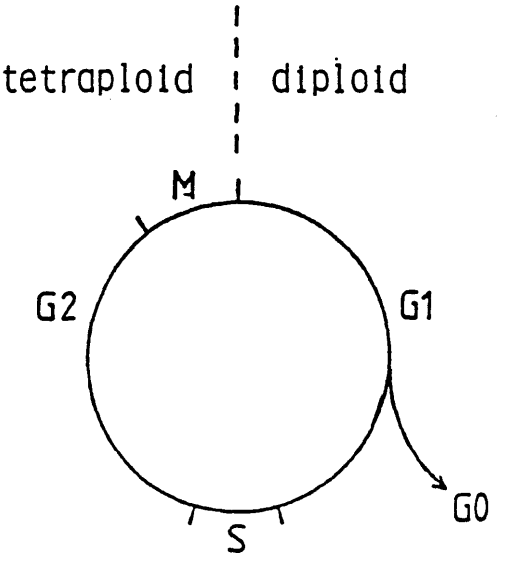
Interpretation of flow cytometry

Historically DNA FCM has been viewed as a means of determining the proportion of cells in different phases of the cell cycle. As a consequence much of the terminology of DNA FCM relates to the cell cycle and to ploidy (figure 22). Taking the histogram obtained from DNA FCM there are various indices that can be derived with the aid of a computer (figure 23). Whilst it is widely accepted that abnormal amounts of DNA are often found in cancer cells the precise boundary between normal and abnormal has not been defined in a universal way. Furthermore the best indices to be derived from the raw data have not been established. The indices used and the normal ranges for them as quoted by various authors is tabulated in table 24. In this study 3 indices were used, DNA index, percentage of cells in G2/M peak and the percentage of cells to the right of the G0/1 peak. The latter index was not adopted for the complete analysis (the reasons are discussed in "DNA FCM results"). Using reference ranges from the benign hyperplasias studied, DNA FCM results were classified into normal and abnormal groups or normal,

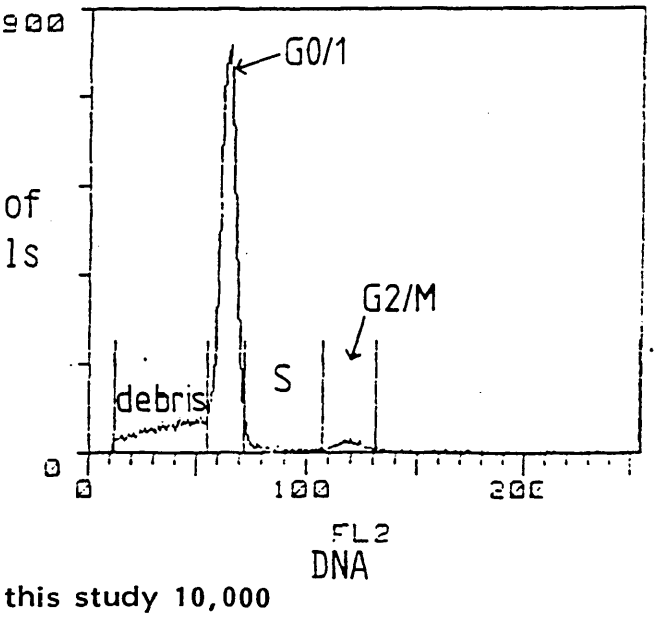
Figure 22

DNA Flow Cytometry Terminology

The cell cycle



DNA histogram

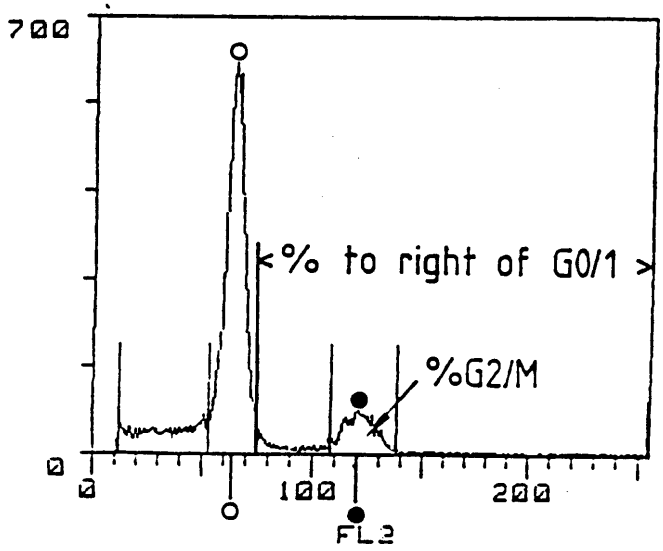


* In this study 10,000 cells were counted for each DNA FCM trace

Figure 23

DNA Flow Cytometry

Indices



DNA Index - % after correcting for offset
a tetraploid peak has a DNA index of 2

%G2/M - the % of cells in the G2/M peak
expressed as a % of all the cells counted
after subtracting the debris

% to right of G0/1 peak - the % of cells to the right
of the main diploid peak
This was later abandoned

abnormal and very abnormal groups.

Table 24

The interpretation of DNA flow cytometry

A variety of workers have used DNA flow cytometry to derive groups or sub-populations of patients on the basis of different indices. In many cases the normal range for indices such as the DNA index is not given.

Workers	Groups	Indices
Bichel et al 1979	Diploid, tetraploid and octoploid	Position of any discrete peak - no ranges quoted
Fordham et al 1986	Diploid, aneuploid	2nd peak >15% cells of DNA index shifted from tetraploid - range 1.875-2.125
Frankfurt et al 1984	Diploid,aneuploid	DNA index - no range given
Frankfurt et al 1985	Diploid, aneuploid tetraploid	DNA index diploid=single peak tetraploid= a G0/1 peak visible aneuploid=a 2nd peak with a DNA index other than 2 - no range
Ronstrom et al 1981 Tribukait et al 1983	Diploid, tetraploid aneuploid	Diploid=G2/M <7% of cells Tetraploid=G2/M>7% Aneuploid= a non-tetraploid peak - range not given
Roters et al 1981	Diploid, tetraploid one aneuploid	Diploid=G2/M <5% Tetraploid=G2/M >5% with tetraploid DNA index

Quality score

It soon emerged that some FCM traces were much better in terms of quality than others. This did not seem to vary. If a sample derived from the same paraffin block was processed on a separate occasion, then the quality of the trace remained consistent. It must relate therefore to some characteristic of the specimen either to the tissue itself or to the way in which it has been fixed and embedded. The possibility that the finding of a poor quality trace may of itself carry some prognostic significance could not be excluded. Therefore a means of scoring quality was developed to allow analysis of the data as a whole and also in subsets depending on the quality of the traces. A 4 point system was used, this is illustrated later (see DNA FCM results, page 198).

Benign standards

In the absence of universally agreed criteria for the interpretation of DNA FCM, especially in discriminating normal from abnormal it was decided that benign prostatic tissue must be studied to delineate the upper limits of normality for the various indices used. It seems likely that these limits may vary depending on the nature of the tissue under scrutiny and the way in which it is processed. 48 specimens from patients who were diagnosed to have benign prostatic hyperplasia in 1984 were processed. The results are detailed in the appropriate section (page 204).

Statistical methods

Data was processed on a PC computer using Oxstat II and

FAKAD (K I Macdonald, Nuffield College, Oxford OX1 1NF) statistical programs. A range of standard statistical tests have been used including student T, X^2 tests and regression coefficients. Life table survival curves were constructed as described by Peto et al 1977. Cox's proportional hazards model analyses were performed by Dr J Matthews, Reader in Medical Statistics at the Medical School, University of Newcastle using BMDP software (BMDP Statistical Software Inc, Los Angeles, California) on the University mainframe computer.

RESULTS AND DISCUSSION - 3

DNA Flow Cytometry

Technical aspects

- Indices measured
- Coefficients of variation (CVs)
- Debris
- Quality
- Fresh vs formalin fixed
- Concentration of propidium iodide

Results

- Benign disease
  - Setting of reference limits
- Prostate cancer
  - Correlation with progression and death
  - Correlation with histological grade and nuclear morphology

Discussion

- Technical aspects
  - Quality
  - Stability
  - Paraffin embedded tissue
- Interpretation
- Conclusions

DNA Flow Cytometry

Indices

From each DNA histogram a number of indices were derived. These are tabulated below and illustrated in figure 23 page 189.

Table 25
DNA Flow Cytometry - indices

* DNA index - corrected for electronic offset - see methods
* % cells in the G2/M peak after subtracting debris
* % cells to the right of the G0/1 peak after subtracting debris
* CV of main peak
* Quality score

Consort 30 software (Becton Dickinson, Sunnyvale, California) was used to count cells in each portion of the DNA histogram and to find the precise peak intensity of each peak. A region of interest, usually a peak is defined by the operator using a cursor. Once set the software calculates the number of cells in that region, gives the mode ie the highest point in the peak and also the coefficient of variation. The determination of quality was largely subjective using the guidelines given below. Examples are given later (figure 25) and the characteristics of each quality group are also given (table 27).

Coefficient of variation

The mean CV of the main G0/1 peak was 7.00 (+/- 1.55 SD), median 7.1, and the range was 3.2 - 12.8. The distribution of CV values appeared Gaussian (figure 24).

Debris

Particles with less than diploid amounts of DNA appear to the left of the main or G1/0 peak on a DNA FCM histogram. Generally these consist of nuclear fragments or debris but abnormal hypodiploid nuclei can occur. However, in the material studied there was no discrete hypodiploid peak found. The number of particles to the left of the main peak may therefore be taken as an index of the amount of debris in the sample. In all cases a total of 10 000 particles were counted. The mean number of debris particles was 2719 (standard deviation 1157) with a range of 839 - 5958. The distribution of the amount of debris was broadly Gaussian with a possible slight skew to the left (lower amounts of debris). There was no correlation with the CV of the G1/0 peak. However analysis of the benign control material showed a correlation with the % of cells to the right of the G0/1 peak ($r=0.726$ $t=6.67$ $p<0.001$) (see figure 28).

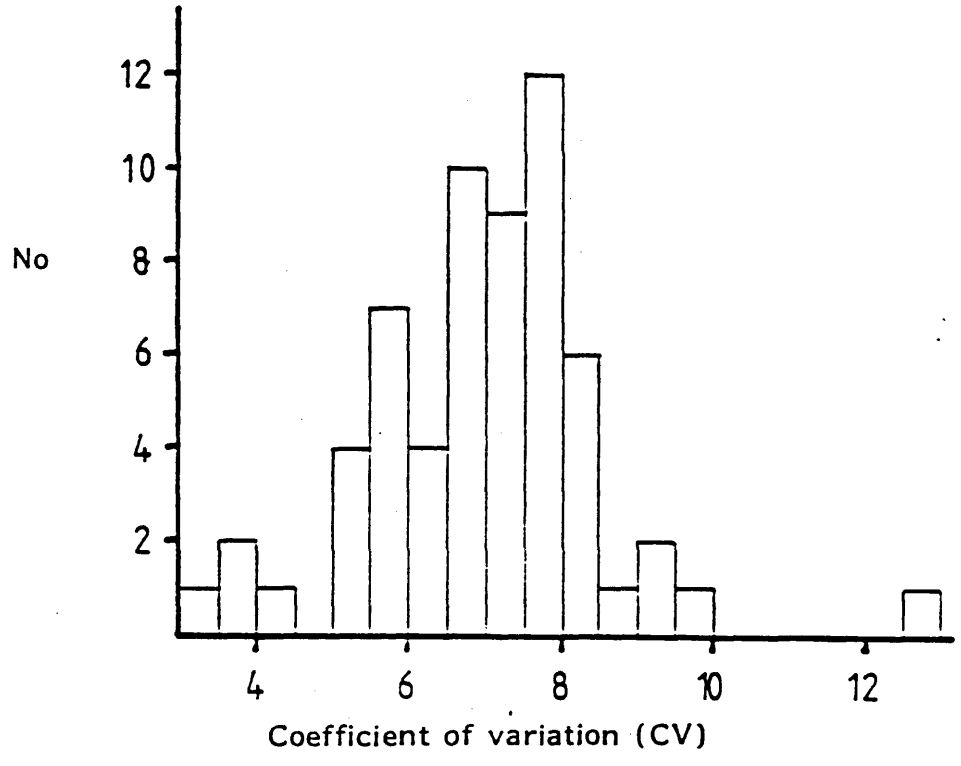
Quality

The quality of DNA histograms was divided into 4 groups.

1 High quality, clear narrow peaks. Very easy to delineate the peaks for analysis

2 Moderate quality but still possible to see with confidence the extent of each peak

Figure 24
DNA Flow Cytometry
CVs of the G0/1 peaks



3 Possible to see the pattern of histogram but the setting of markers at the beginning and ends of peaks subjective

4 Totally uninterpretable

See figure 25 for examples.

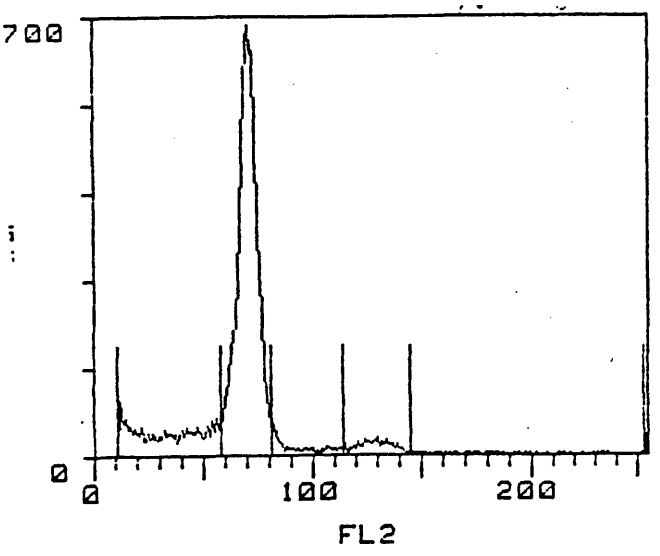
In most cancer cases 2 blocks of tissue were used for DNA flow cytometry. The block with the most abnormal flow cytometry was taken for further analysis providing it fell into categories 1 - 3. Single blocks were used for the benign control material. The quality scores obtained are shown in table 27.

Fresh vs formalin fixed tissue

This was hampered by the difficulty in disaggregating fresh specimens. It was difficult both to obtain a cellular suspension and to minimise the amount of debris. After experimentation with a variety of methods including mechanical disaggregation - mincing, straining and ultrasound, EDTA, collagenase and pepsin, 2 techniques were thought the best although neither was satisfactory. These were ultrasound and collagenase. Prostatic tissue from 5 patients was analysed both fresh and after formalin fixation and paraffin embedding. The latter were processed using the standard Hedley method as for the other paraffin embedded specimens in this study. Samples were left in formalin for 24 hrs, 1, 2 and 4 weeks. The resolution of the DNA FCM histograms was far superior after fixation and embedding (figure 26). Calibration was with fresh human lymphocytes set in channel 100. In

Figure 25
DNA Flow Cytometry
Quality Score

Quality Score 1



Quality Score 2

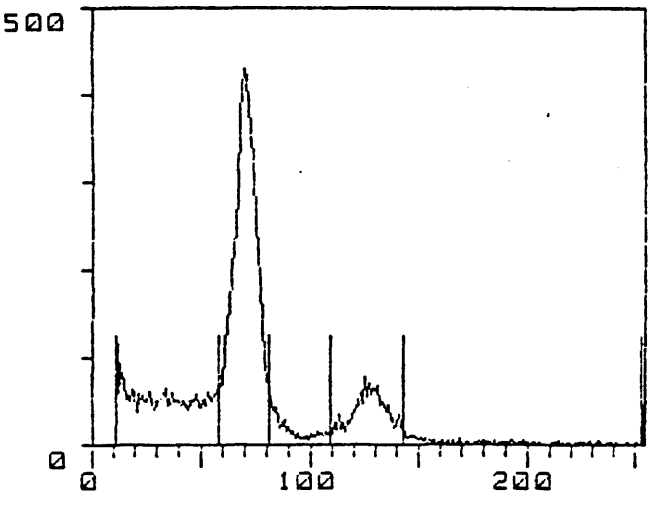
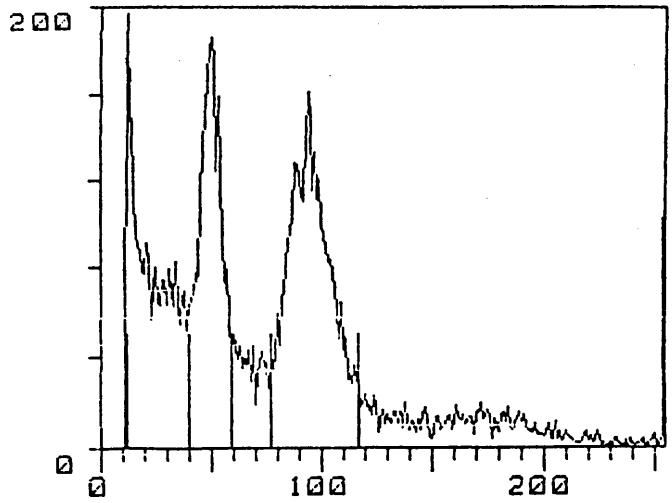


Figure 25
DNA Flow Cytometry
Quality Score

Quality Score 3



Quality Score 4

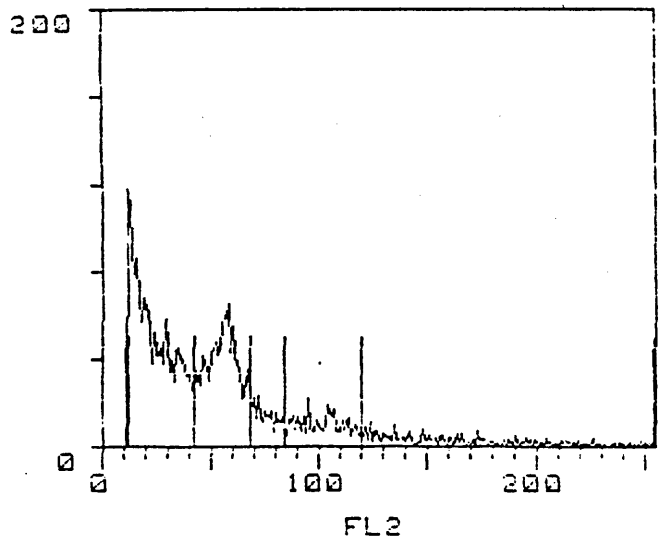
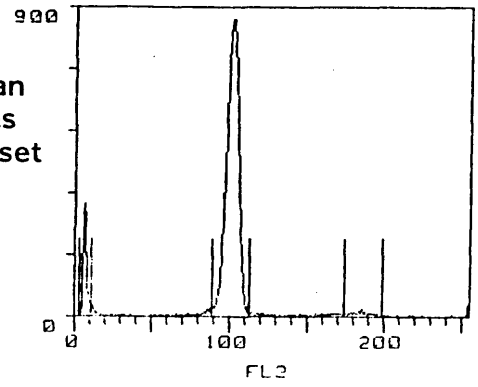
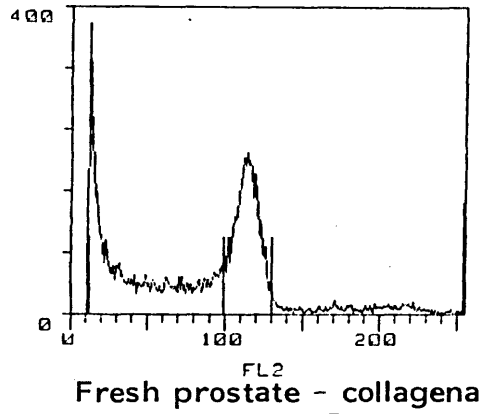


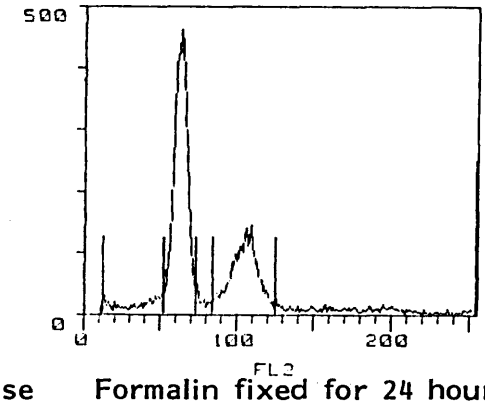
Figure 26
Fresh vs formalin fixed tissue
DNA FCM

Fresh human lymphocytes G0/1 peak set at 100

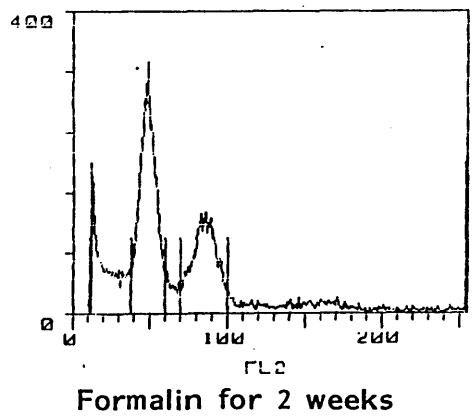




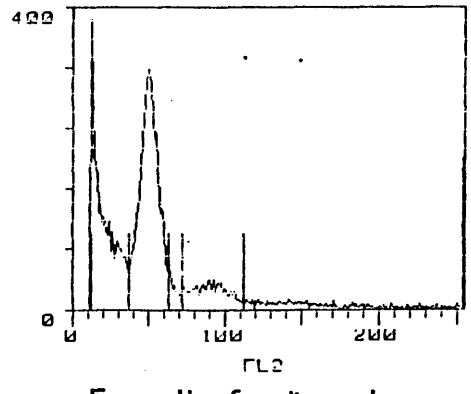
Fresh prostate - collagenase disaggregation Poor quality with low yield of cells



Formalin fixed for 24 hours Paraffin embedded + pepsin Good quality trace



Formalin for 2 weeks Much poorer quality



Formalin for 4 weeks Poor quality with some loss of the second peak

general the channel number of the fresh G1/0 peak was also close to 100. After formalin fixation this fell to about 70. Prolonged fixation in formalin seemed to result in poor quality flow cytometry traces. Detailed comparison was not really possible, given the poor quality of traces obtained from fresh tissue.

Concentration of propidium iodide

A DNA FCM histogram with 2 clear peaks was used to evaluate the effect of varying the concentration of propidium iodide. Over a wide range the character of the trace and the DNA index of the second peak remained unchanged (table 26 + figure 27).

Table 26
Effect of propidium iodide concentration

Concentration of PI ug/l	DNA index
6.25	1.95
12.5	1.93
25	1.98
50	1.92
100	1.88
200	1.88
400	1.92

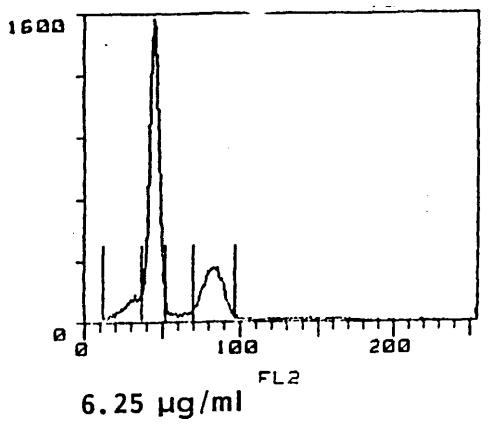
Mean DNA index 1.92 sd 0.0359

Results

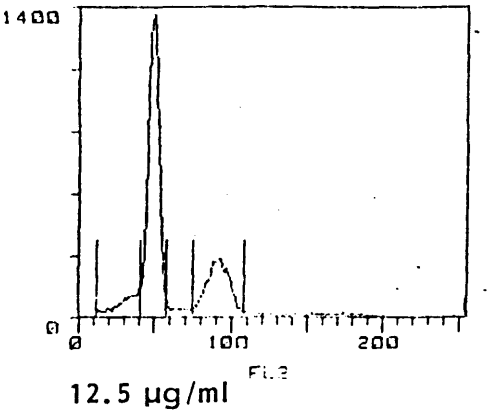
Benign disease

Paraffin embedded prostate chips from 48 patients with benign prostatic hyperplasia were processed. All were unselected consecutive patients from 1984 (patients page 85). 7 patients had quality grade 4 DNA FCM traces and were therefore excluded from subsequent analysis. Of the remaining 41, 12 had no second or G2/M peak and therefore

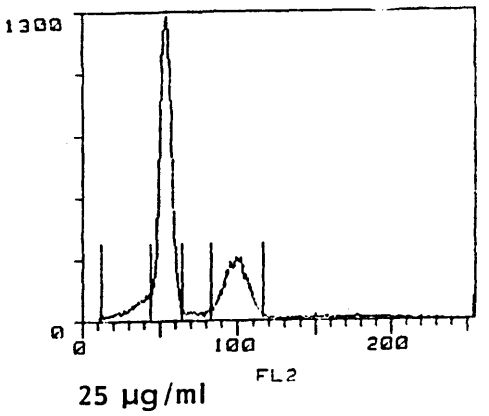
Figure 27
DNA FCM Prostate cancer
Propidium iodide concentration



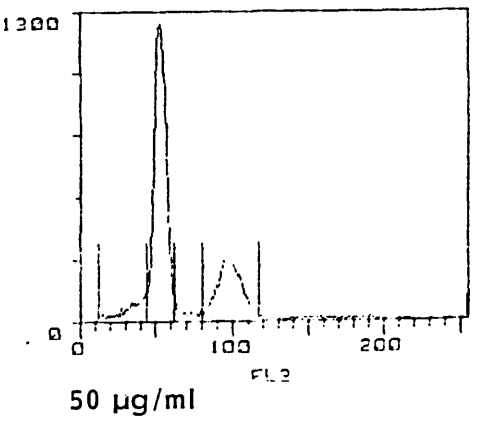
6.25 µg/ml



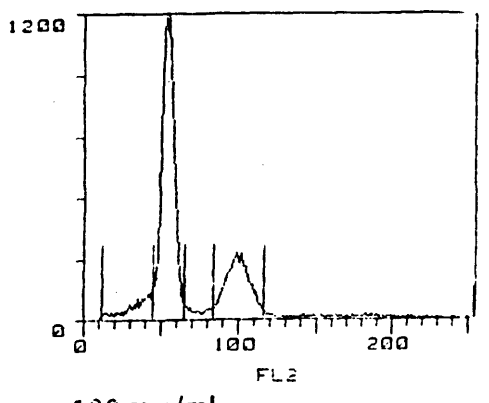
12.5 µg/ml



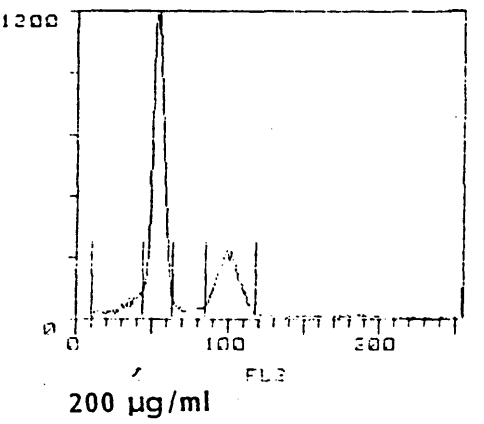
25 µg/ml



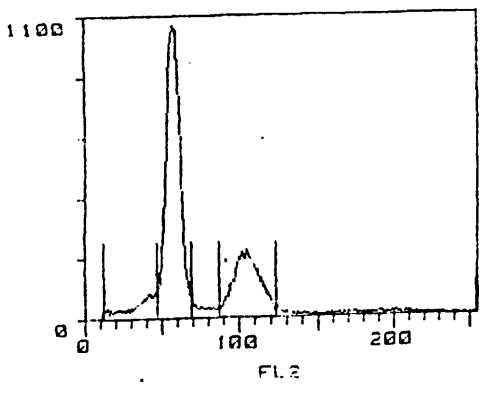
50 µg/ml



100 µg/ml



200 µg/ml



400 µg/ml

the calculation of DNA index or % of G2/M cells was not possible.

The mean CV for the main peak was 6.88, range 5.1 - 10, SD 1.068. The quality score, debris, and CV are shown in table 27.

Table 27
Benign prostatic hyperplasia

Quality score	mean CV (SD)	mean debris (SD)	No
1	6.26 (0.31)	1066(538)	7
2	6.54 (0.72)	2130 (780)	23
3	8.01 (1.19)	3210 (699)	11
4	-	-	7

It was found that the % of cells to the right of the main peak correlated well with the amount of debris. For this reason this parameter was not used in later analyses (figure 28).

Setting reference limits

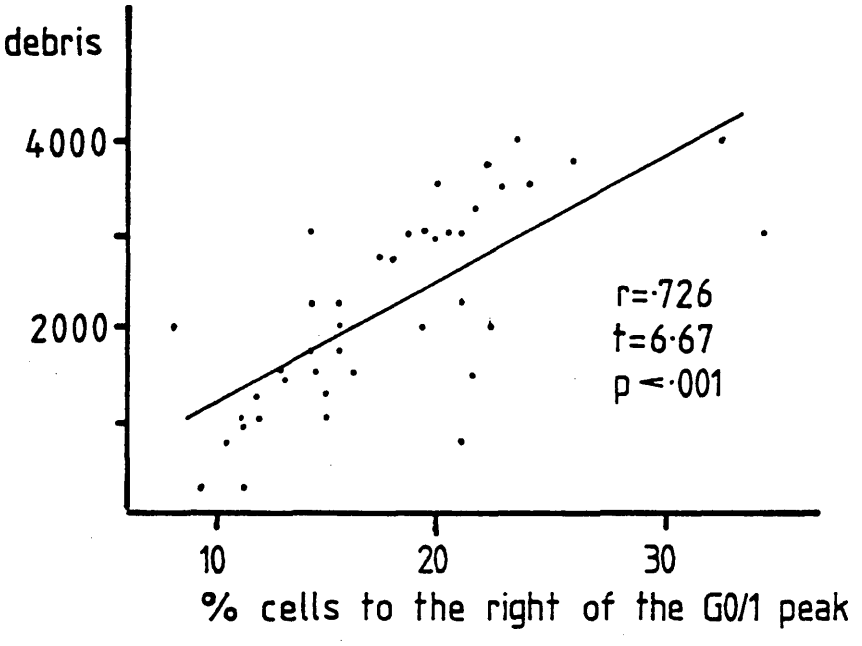
DNA index

The mean corrected DNA index for the 29 cases in which calculation was possible was 1.97 (SD .043). The distribution appeared Gaussian and therefore the limits were set at +/- 2 SDs ie 1.88 - 2.06.

% of cells in G2/M peak

The mean % cells in the G2/M peak was 5.80 (range 3.51 - 12.0). The distribution was slightly skewed and therefore logs were taken before adding 2 SDs to the mean to determine an upper limit. This was set at 9.12% rounded up to 10% for convenience.

Figure 28
DNA Flow Cytometry
Debris and % cells to right of G01 peak



Prostate Cancer

Histological material from the retrospective study group of patients was analysed (see patients page 84). Of the 61 patients 1 had a quality grade 4, uninterpretable DNA histogram and another had no remaining histological material available. Thus 59 patients were studied - see table 28.

Table 28
Prostate cancer patients n=61

Quality score	mean CV (SD)	mean debris (SD)	No
1	5.72 (1.50)	2143 (1294)	12
2	7.24 (1.50)	2613 (1083)	36
3	7.66 (1.09)	3620 (742)	11
4	-	-	1
		Total	60*

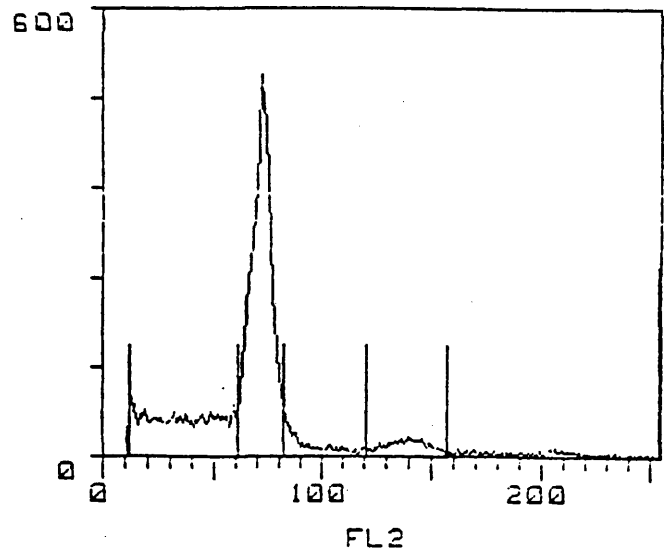
* No histological material available for 1 patient

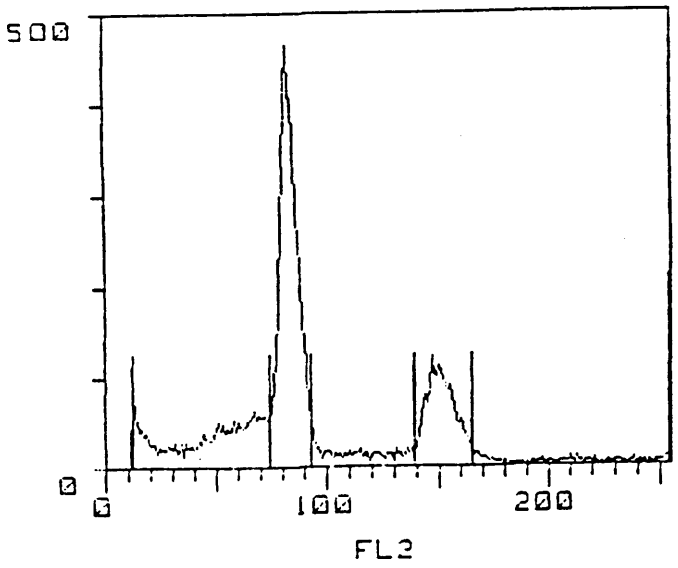
DNA FCM from the patient with quality score 4 could not be analysed so he was excluded

Overall there were 25 DNA FCM histograms judged to be normal and 34 abnormal on the basis of the reference limits given earlier. Of the 34 abnormal histograms all had raised % of cells in the G2/M peak (or in an aneuploid second peak) and 12 showed aneuploidy ie a corrected DNA index outside the defined range. It can be seen that the predominant abnormality was tetraploidy. Florid aneuploidy, that is large numbers of nuclei containing bizarre amounts DNA was uncommon. (Figure 29 shows examples). Most cases of aneuploidy had second peaks

Figure 29
DNA Flow Cytometry
Examples

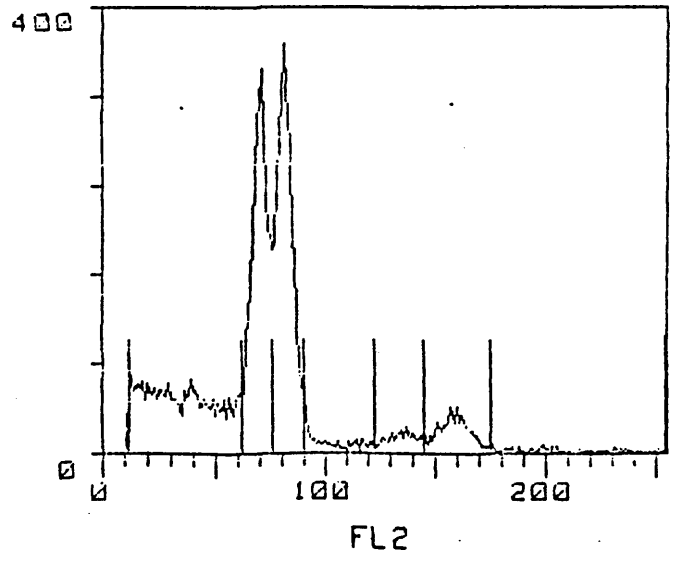
Diploid





Abnormal
(Tetraploid)

Abnormal
(Aneuploid)



just marginally outside the limits for DNA index.

Correlation with progression and death

The results have been tabulated against the patients outcome (table 29).

Table 29
DNA FCM - Outcome

	Cancer deaths *	Other deaths +	Symptomatic progression	No progression
Normal	3	5	5	12
Abnormal	10	4	15	5

X^2 10.054 p=0.01 - excluding deaths from other causes but including both prostate cancer deaths and those treated for symptomatic progression who did not die.

* As noted previously (page 159, footnote to table 19) one patient with T4 M0 disease died at home under the care of his General Practitioner. The precise cause of death was unclear, therefore he is included in the cancer deaths group. He did not receive hormone manipulation before his death.

+ One patient received treatment for symptomatic progression but died from histologically proven adenocarcinoma of the large bowel (see page 159).

In total 33 patients received treatment for progression, 20 remained alive, 12 died from prostate cancer and 1 died from other causes.

Symptomatic progression requiring treatment occurred in 33 patients. The relationship between DNA FCM and the time to progression can be plotted as a life table curve (figure 30). Progression was significantly more likely in those patients with abnormal flow cytometry (p=0.01).

Death from prostate cancer can be used as the end point for a similar analysis (figure 31). Although there was a clear trend with increased probability of death from

Figure 30
Progression requiring treatment
DNA FCM life table analysis

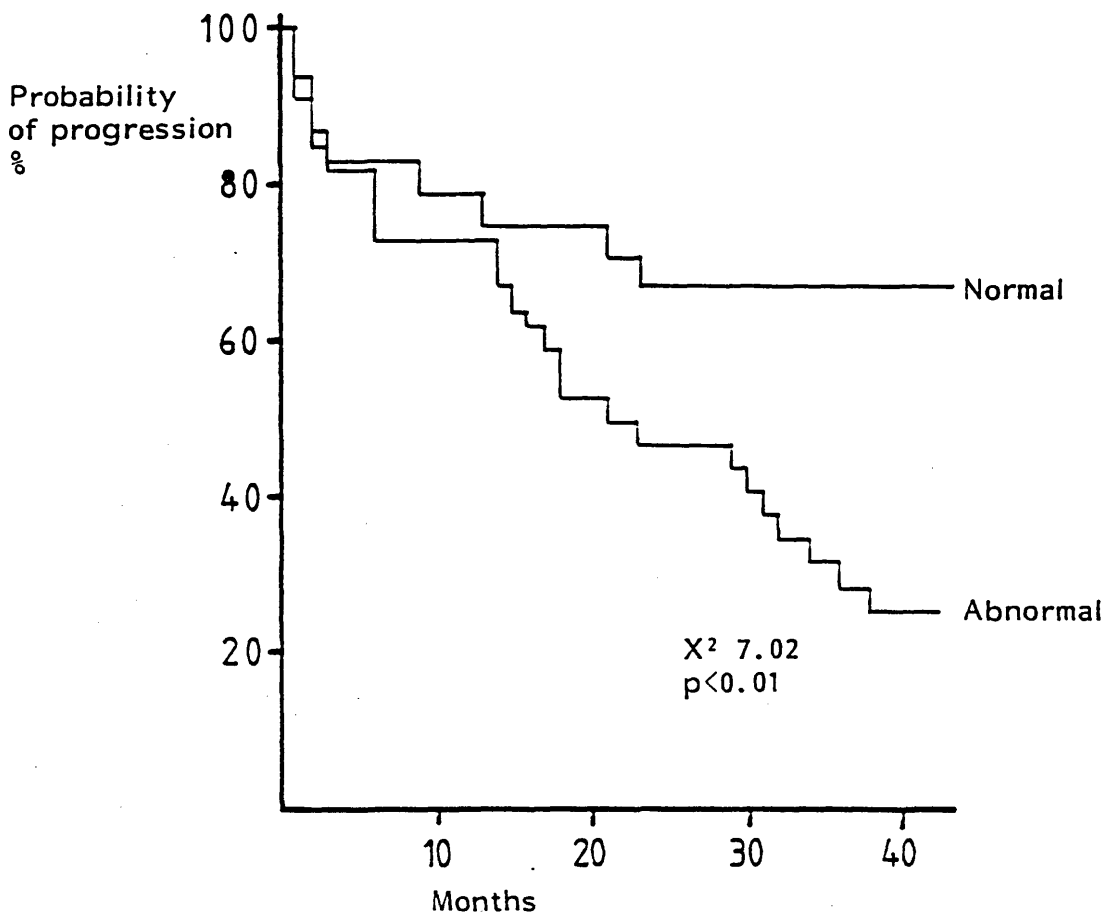
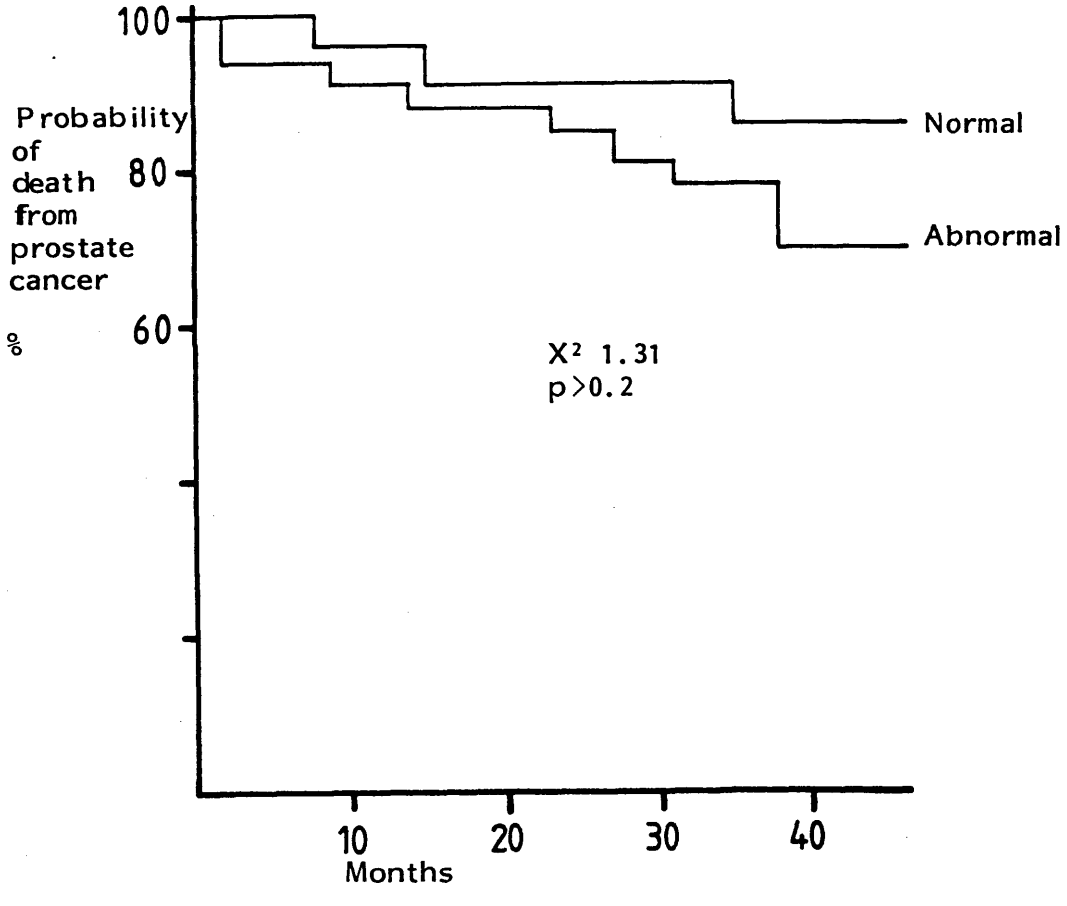


Figure 31
Death from prostate cancer
DNA FCM life table analysis



prostate cancer in the abnormal DNA FCM group this did not reach statistical significance.

There was no correlation between the quality of DNA FCM and the outcome. This suggests poor quality is a largely random event probably related to the original fixation technique.

Correlation with histology

The prostate tissue was originally examined by various histopathologists in 1984. Each was given a grade from 1-3 but these were arbitrary. Accordingly the material was reviewed by a single pathologist and 2 grading systems used. In total therefore there are 3 ways in which the histological grade may be expressed. The correlation with DNA FCM is tabulated below.

Table 30
Prostate cancer
DNA FCM vs grade (G1-G3)

	Normal	Abnormal
G1	14	7
G2	6	12
G3	5	15

X^2 8.15 p=0.02

DNA FCM vs Gaeta
Maximum score

	Normal	Abnormal
1	2	2
2	10	10
3/4	13	22

X^2 2.22 p=NS

Table 30 continued

DNA FCM vs Gaeta
Nuclear morphology score

	Normal	Abnormal
1	3	3
2	12	13
3/4	10	18

X^2 2.65 p=NS

DNA FCM vs Gaeta
Glandular architecture score

	Normal	Abnormal
1	3	2
2	10	14
3/4	12	18

X^2 3.02 p=NS

DNA FCM vs Gleason
Total score

	Normal	Abnormal
2-4	9	4
5-6	4	9
7-8	8	15
9-10	4	6

X^2 6.28 p=0.01

The percentage of prostate tissue involved showed some correlation with the DNA FCM but this did not reach significance (table 31). It was not possible to allow for the fact that poorly differentiated tumours tend to be diffuse.

Table 31
DNA FCM vs % of prostate tissue replaced by cancer

	Normal	Abnormal
<50%	12	10
51-75%	6	9
76% +	7	15

X^2 4.67 p=0.1

DNA Flow Cytometry Discussion

Technical Aspects

Quality

Flow cytometry can produce varying results depending on the technical competence of the operator, the fluorochrome and the nature of the cellular material used. It is reassuring that when many centres in the USA were sent samples of lymphocytes they produced comparable results (Homberger et al 1989). However, solid tissue presents a problem in that disaggregation is required. It would seem paradoxical that in the case of a tough fibrous tissue such as the prostate, fixed cells disaggregate easier than fresh ones. I would suggest that fixation makes the nuclei more resistant to damage particularly from proteolytic enzymes such as pepsin. Cells that occur naturally as a single cell suspension such as lymphocytes produce excellent DNA FCM histograms with no debris and very narrow defined peaks, the mean CV being 3.31 in the US series cited above. In this study the prostate material typically produced debris and a rather wide peak with an mean CV of 7.0 (range 3.0-12.8). Comparison with other workers has shown that this does not represent a technical failure but is inherent in the tissue and the technique. For example using paraffin embedded material and the Hedley disaggregation technique McIntire et al (1988) obtained average CVs of 11.9 for aneuploid and 13.2 for diploid tumours in contrast to the CV for lymphocytes which was 2.0. The problem with the comparatively poor resolution is that an abnormal peak might occur so close to the G0/1 peak that they are not visible separately.

Vindelov et al (1983) found that in order to discern 2 distinct peaks there must be a percentage difference in DNA index of about twice the CV. In other words if the CV is 3 to see a bimodal not a wide peak there must be a 6% difference in the DNA index. There are 2 reasons why this is unlikely to be happening to any great extent. Firstly the proportion of abnormal DNA FCM traces that we have found is in line with other workers, suggesting that significant numbers of abnormalities have not been overlooked. The normal/abnormal DNA FCM ratio in this study was 42/58%, roughly comparable with the results of others 59/41% (McIntire et al 1988), 54/46% (Ritchie et al 1988), 47/53% (Fordham et al 1986). It remains possible however that some yet to be discovered technique improves resolution and reveals a systematic underappreciation of DNA abnormality by all workers. Secondly when one looks at the outcome of patients with wide peaks they do not form a group that fares especially badly. If we are missing subtle abnormalities they do not seem to have much clinical significance.

In this study I have scored quality to allow subsequent stratification of analysis. The exclusion of poor quality traces from analysis can only be justified if we can be confident that the quality reflects some random technical problem such as inadequate fixation rather than a biological phenomenon relating to the tumour. Should the latter be the case then a poor quality trace might predict outcome and should not be discarded. Within the constraints of the small numbers studied it did not appear that quality correlated with biological tumour behaviour.

If one looks at quality score, amount of debris or width of CV there is no correlation with outcome over the period studied.

Stability

It is possible that the flow cytometric characteristics of a tumour may change with the passage of time or vary within different parts of the tumour. This problem has not been specifically addressed in this work but evidence from elsewhere suggests that the flow cytometry of a given tumour remains constant. In ovarian cancer Friedlander et al (1984) found a consistency both spatial ie within the tumour and temporal. Further tumour samples were obtained from 9 of their patients, biopsied at relapse or taken at post mortem. In breast cancer Auer et al (1980) demonstrated that the primary tumour and its metastases demonstrated the same DNA FCM pattern. There is no published work that confirms this to be the case in prostate cancer although this would seem likely.

Paraffin embedded tissue

The poor results with fresh tissue did not allow a full scale comparison of fresh vs formalin fixed and paraffin embedded tissue. Within the limited study I performed there was no evidence of a large difference in the character of the histograms although the quality was much better using paraffin embedded material. De Vere White et al (1984) also found that fixed tissue gave the best results. Using the Dunning rat prostate tumour he found no difference between fresh and fixed tissue. It is well established that this also applies to many other tissues

such as lymphomas (Camplejohn and Macartney 1985) and a variety of human tumours including ovarian cancers (Hedley et al 1983). Studying breast cancer, Lawry et al (1987) did find some difference between fresh and fixed tissue. The percentage population of cells in each part of the cell cycle in non diploid traces did not correlate statistically n=14. However the ploidy index (similar to the DNA index) was not different in fresh or fixed tissue. It remains possible that some quantitative aspects of DNA FCM may differ when using fresh rather than fixed tissue but the overall character of the trace and whether it would be classified as normal or abnormal would probably be unaffected.

Interpretation

Throughout the literature there are widely differing methods of analysing data from DNA FCM. No single method seems universally accepted. Most authors ultimately describe traces as normal or abnormal with very little emphasis on the degree or extent of abnormality. This work therefore was undertaken with no fixed concept of how normality should be determined. The DNA index seemed a clear parameter to measure and providing allowance is made for the electronic offset of the analyser it should be reproducible and not subject to observer bias. Computer analysis has been described (Watson et al 1987) but was not available for this study. A simpler system was to use the Consort 30 software to determine the number of cells in each region of the histogram. Regrettably, this does involve observer bias in the setting of the lines dividing

the different regions to be evaluated. Normal or reference limits could only be determined by using comparable control material and the same techniques. The use of benign prostate tissue was therefore mandatory. The % of cells in the G2/M peak seemed the most reliable parameter. It was striking that in the BPH series the % of cells to the right of the main peak correlated with the amount of debris. We did not have access to software that may have helped in selecting out the debris from the trace (Beck 1980). It must be said that any manipulation of the histogram data involves assumptions that theoretically seem correct but are difficult to prove.

Results

Benign disease

Abnormalities amongst these patients as described by de Vere White and his associates (Deitch et al 1989) were not found. In particular no case with a peridiploid abnormal peak was detected although these were reported in a quarter of their patients with BPH.

In the interpretation of DNA flow cytometry from cancers it seems unlikely that there are "normal" values that can be applied universally and therefore the use of benign tissue as a reference is inescapable. It was disappointing that so many of the benign prostates had poor traces. When prostatic tissue that initially produced quality grade 4 DNA FCM (uninterpretable) was processed for a second time the same results were obtained in all cases. This implied that the problem lies in the original fixation. The cancer patients had generally 4 blocks of tissue from which to

chose. The 2 with the greatest amount and most poorly differentiated cancer were selected. Should one or both produce a grade 4 trace then a different block could be used. In only one case was all the histological material available unsuitable in that it produced only uninterpretable traces despite repeated attempts.

Prostate cancer

It has been shown in this study that DNA FCM predicts progression requiring treatment. Within the time scale available it did not however predict death from prostate cancer. This is because 33 patients progressed and were treated but only 13 died from prostate cancer. Presumably if the follow up were longer those who progressed would die and FCM would therefore predict death as well. It is interesting however that many other means of determining prognosis can predict death within the study timescale.

Ritchie et al (1988) found that in localised prostate cancer DNA FCM did not help to predict recurrence or death within the study period. Adolfsson describes a similar experience to my own, that is a correlation with progression but not with survival (Adolfsson J 1989, Karolinska Hospital, Box60500, 104 01 Stockholm - personal communication). However, using tissue from pelvic lymph node metastases, Winkler et al (1988) found a strong correlation between abnormal DNA ploidy and progression.

Fordham et al (1986) demonstrated a markedly diminished probability of survival amongst aneuploid tumours $p<0.001$. Looking at nodal metastases Stephenson et al

(1987) found a definite correlation between DNA FCM and survival. Using archival material from the primary tumour dating back to 1958 Lundberg et al (1987) also demonstrated a correlation between ploidy and survival. Thus there is general agreement that DNA FCM is a prognostic indicator but its clinical value is not clear. This is further evaluated later when the impact of other prognostic determinants are taken into account (see multifactorial analysis).

In my study DNA FCM correlated well with grade. In particular there was a significant correlation with the Gleason score, a finding in contrast to that of Fordham et al (1986). Although Ritchie et al (1988) did not conclusively demonstrate such a correlation, there was a trend approaching significance, $p<0.063$. Winkler et al (1988) demonstrated correlation between aneuploidy and grade using the Mayo system ($p<0.003$) but oddly not when using the Gleason system, the reason for this disparity is not clear. It seems likely, therefore, that there is an overlap between the light microscopy findings and those of DNA FCM. Helpap (1981) has shown that histological appearance of prostate cancer and the nuclear morphology correlate well with the cell cycle activity of the tumour as shown by H_3 thymidine uptake studies. Should it be that much of the prognostic value of DNA FCM evaporates when histological findings are taken into account then this clearly limits its value.

In conclusion DNA FCM does have some prognostic value.

Whether this is useful in the clinical setting can only be assessed by taking into account the other prognostic determinants that the clinician will have at hand when the patient is reviewed.

Conclusions

DNA Flow Cytometry

Paraffin embedded prostate tissue produces reasonable quality DNA flow cytometry traces

Abnormality is associated with progression and probably death

There seemed to be a correlation with histological grade

It remains to be seen whether this technique is relevant clinically

RESULTS AND DISCUSSION 4

Multivariate Analysis

Cox's proportional hazards model analysis

Discussion

- Prostate specific antigen
- DNA flow cytometry

Concluding discussion

- Study design
- Future prospects
- General conclusions

Cox's proportional hazards model analysis

This analysis was performed by Dr J Matthews, Division of Medical Statistics, University of Newcastle. The University mainframe computer was used in conjunction with BMDP2L software, BMDP Statistical Software Inc, 1984, 1440 Sepulveda Blvd, Suite 316, Los Angeles, California 90025, USA. Initially single variables were reviewed. Two different end points were taken; death and progression requiring treatment. The analysis produces 2 statistics, the coefficient b which measures the size of the effect and p which is the probability of observing an effect of such magnitude if in fact there was no real effect and the observation was due merely to chance (ie if the null hypothesis holds true). From b one can derive the relative risk given by e^b which is easier to interpret. If the relative risk is greater than 1 then high values of that factor carry increased risk. Conversely, a relative risk of less than 1 carries a decreased risk. The results obtained are tabulated below.

Table 32

Cox's proportional hazards analysis - single variables
Time to death

Variable	No	b (SE)	e^b	p
age	61	0.052(0.028)	1.05	0.056
PSA(log)	61	0.791(0.292)	2.20	0.006
PAP(log)	61	0.821(0.319)	2.27	0.007
Alkaline phosphatase	55	1.327(0.671)	3.77	0.044
Bone scan +/-	61	1.40(0.31)	4.06	0.001
Grade G_1vsG_2	61	0.546(0.585)	1.72	0.30
G_1vsG_3		0.946(0.543)	2.62	0.19
T stage				
T_{0-2} vs T_3	61	0.983(0.642)	2.67	0.12
T_{0-2} vs T_3		1.801(0.709)	6.05	0.021
Gaeta	61	0.474(0.304)	1.61	0.12
Gleason	61	0.202(0.109)	1.22	0.059
Mode of presentation		0.195(0.62)	1.22	0.919
Haemoglobin	61	-0.142(.105)	0.87	0.176
Creatinine	61	-2.14(2.41)	0.11	0.31
Length of history	60	-0.396(0.314)	0.674	0.20
Flow cytometry Normal/abnormal	59	0.176(0.450)	1.19	0.69
% cells in G2/M	59	0.12(0.019)	1.01	0.41

Taking a significance limit of 0.05 it can be seen that there are 5 factors that have been shown to predict death, age, PSA, PAP, alkaline phosphatase and a positive bone scan. Other factors that would be expected to predict prognosis have not reached significant levels given the numbers available.

DNA FCM does not appear to predict death in this study. It must be said that the large standard error of b raises the possibility that the failure to demonstrate an effect is due to inadequate numbers. It is possible that a larger study or one with longer follow up would demonstrate that DNA FCM has a significant predictive value. It should be noted however that other prognostic factors such as the presence of metastases and serum PSA have been shown to be prognostic over the same timescale.

If time to progression is used as an end point the results are somewhat different.

Table 33

Cox's proportional hazards analysis - single variables
Time to progression requiring treatment

Variable	No	b (SE)	e^b	p
age	61	-0.021(0.019)	0.97	0.27
PSA(log)	61	1.405(0.264)	4.07	<0.001
PAP(log)	61	1.455(0.263)	4.28	<0.001
Alkaline phosphatase	55	2.038(0.646)	7.67	0.0015
Grade G_1vsG_2	61	0.293(0.372	1.72	0.45
G_1vsG_3		0.210(0.356)	3.35	0.0009
T stage				
T_{0-2} vs T_3	61	0.899(0.379)	2.46	0.02
T_{0-2} vs T_3		1.394(0.489)	4.03	0.0083
Gaeta	61	0.615(0.190)	1.85	0.0011
Gleason	61	0.329(0.079)	1.39	<0.0001
Mode of presentation		-0.771(0.47)	0.46	0.23
Haemoglobin	61	0.0258(0.087)	1.026	0.767
Creatinine	61	-0.616(1.44)	0.54	0.668

Table 33 continued

Variable	No	b (SE)	e^b	p
Length of history	60	0.0699(0.195)	1.07	0.72
Flow cytometry Normal/abnormal	59	0.795(0.310)	2.26	0.0088
% cells in G2/M	59	0.042(0.0115)	1.04	0.0001

It can be seen that as expected DNA FCM has shown itself to be a prognostic indicator when taken in isolation and looking at time to progression. The advantage of this analysis is that it allows one to look at the prognostic value of a set of variables then add another. It can then be seen whether the addition gives further prognostic information.

As before 2 end points were studied.

Table 34
Multivariate model
Time to death

	Coeff b (SE)	e^b
age	0.058(0.03)	1.06
Hb	-0.168(0.106)	0.84
Bone scan	1.339(0.48)	3.81
G_1 vs G_2	0.474(0.59)	1.61
G_1 vs G_3	1.251(0.58)	3.49

Overall p=0.0018

Adding DNA FCM either normal vs abnormal or %G2/M does not add any information p=0.197 and p=0.46 respectively. Adding PSA, PAP or alkaline phosphatase does not give

further prognostic information. However both PSA and PAP can be substituted for the bone scan and not diminish the prognostic value of the combination.

The situation is similar if time to progression is used.

Table 35
Multivariate model
Time to progression

	Coeff b (SE)	e^b
age	-0.012(0.018)	0.989
Hb	-0.005(0.082)	0.995
Bone scan	2.163(0.463)	8.70
G_1 vs G_2	0.009(0.388)	1.009
G_1 vs G_3	1.084(0.37)	2.958

Overall $p<0.0001$

Again putting DNA FCM into the model does not add significantly p=0.205 for normal vs abnormal and p=0.18 for %G2/M.
As before PSA and PAP could replace the bone scan but they add little if performed in addition to the above combination.

Discussion

The danger of using a sophisticated analysis technique is that the data can be overinterpreted and conclusions drawn that are tenuous. The basis of the analysis is the quality of the data that is entered at the beginning. There is no doubt that this is accurate but the need to be rigorous has reduced the number of patients studied. The relatively

small numbers raise the possibility of a type 2 error. This will be borne in mind in the subsequent discussion.

Prostate specific antigen

This retrospective study can only be expected to answer one question of PSA. It is, what is the prognostic value of a serum PSA taken at the time of diagnosis? In this context one might regard PSA as an adjunct to the usual staging procedures. It has been shown that a raised PSA is associated with an increased risk of progression and death. Furthermore this multivariate analysis establishes that PSA appears to be of value in that it detects disseminated disease. Numbers do not permit meaningful analysis of subgroups but one might speculate that the PSA level in Mo patients provides additional prognostic information. Certainly review of the data in the PSA retrospective study indicates that this is likely.

Several authors have stated that the routine follow up bone scans are a burden on health resources and often of little or academic value only (Lund et al 1984, Corrie et al 1988). The use of PSA would provide a more acceptable alternative. It may be possible to go further. A patient who after TURP of an apparently localised prostate cancer has a very low PSA less than 21 ng/ml probably does not need a bone scan. It is very unlikely that he has disseminated disease.

DNA FCM

There is no doubt that DNA FCM predicts prognosis. There seems to be however some differences between various workers as to the degree to which it determines prognosis.

Fordham et al (1986) showed dramatic differences in prognosis dependent on the DNA FCM and these were also the findings of Tribukait et al (1983). Others have not demonstrated the same magnitude of prognostic value Ritchie et al (1988). The reasons for this difference are obscure. The technique of FCM used in this study was identical to that used by Fordham et al. The explanation may be that the patients had different characteristics or that they were treated differently. The characteristics of the patients in this study have been well defined and the management policy of deferred treatment was also uniform and unchanging.

As to the real power of DNA FCM in predicting prognosis contributions from other workers may be required to finally settle the question. A further dimension is whether this technique really provides extra information to the clinician that he does not already have. The multivariate analysis performed here would suggest that it does not provide such information but the numbers of patients studied leave open the possibility that it may be of some value although small and probably not clinically useful. However, using a similar multivariate analysis Lundberg et al (1987) demonstrated prognostic value for DNA flow cytometry after taking histological grade into account. Whether this finding at the limit of significance indicates that DNA flow cytometry will be useful in the future remains unanswered.

Concluding Discussion

Study design

The design of this clinically based study made full use of the facilities available at the Freeman Hospital, Newcastle. It set out to evaluate PSA and DNA FCM in the prediction of prognosis. This task was addressed against a background of a deferred treatment policy for prostate cancer in which a large proportion of patients progressed and needed treatment. A principal goal was to identify those at risk of progression. For this reason experimental work was confined to the technical aspects of the PSA assay and the performance of DNA FCM. An experimental approach may be appropriate however in enhancing these techniques in the future. Animal studies would be unlikely to be helpful.

The numbers of patients included in this study was limited by practical considerations. The original retrospective group of cancer patients comprised 93 patients. Rigorous reappraisal of the data relating to these reduced the number to 61. Only patients with prostate cancer managed by a deferred treatment policy were evaluated. The principal reason for exclusion was treatment at the time of diagnosis (n=20)(given to patients with symptoms), some were treated prior to being studied and therefore should never have been included (n=6). Two patients had benign histology on review. The remaining 5 cases excluded had insufficient data. Although the number of patients evaluated was 2/3 of the number planned the study retains validity, however its power is diminished.

Future prospects

Routine patient management

The findings of this study would support a rather parsimonious use of resources in the prediction of prognosis. In particular the routine bone scan seems unnecessary outside clinical trials. The use of simple parameters such as histological grade and serum PSA should enable the clinician to identify patients at high risk of progression.

Further information regarding prognosis

The use of larger numbers would allow a more powerful study of PSA and DNA FCM. The ideal opportunity would be in parallel with a multicentre trial. Many modern trials however include large numbers of tests thought to carry prognostic significance and the extra workload for the clinician is a disincentive to entering patients into the trial. However, once a similar analysis to that described here has been repeated with large numbers, multivariate analysis would enable a small selection of tests to be kept and the rest discarded. The need to obtain large numbers for prostate cancer trials means that the protocols must be simple (Kirk D, Glasgow - personal communication 1990). This approach would allow for sufficient prognostic data to check the comparability of the treatment arms and to perform stratification into subgroups for analysis whilst keeping the extra workload to a minimum.

Technical developments

PSA

It has been shown that patients with BPH can have moderately high levels of PSA. It may be possible to increase the specificity of the assay and distinguish PSA derived from benign tissue from that originating from prostate cancer. One suggested method is the use of lectin binding to remove PSA with certain sugar residues (PSA is a glycoprotein). It is hoped that PSA from malignant tissue may have an altered sugar composition (I Gibb, Principal Biochemist, Royal Victoria Infirmary, Newcastle - personal communication 1989).

DNA FCM

The advent of gene probes and other molecular biological breakthroughs tends to make the concept of DNA FCM, measuring total nuclear DNA content, seem rather crude. What is needed is a rapid means to detect the degree of genetic disorder within prostate cancer cells and determine its prognostic significance. The work presented here has shown DNA FCM to be of little value in predicting prognosis in its present form. Its value may be enhanced in the future by the use of antibodies directed at oncogene proteins. In general, however, it seems likely that sophisticated DNA analysis at the genetic level will supersede this technique.

General Conclusions

The work described here was carried out against a background of management by a deferred treatment policy. Prostate cancer is characterised by extreme variability in

its natural history, some patients progress rapidly, others are relatively untroubled by their disease. We can now to some extent identify those at high risk of progression and those who are relatively safe and can be seen infrequently.

Bone metastases especially with a markedly raised PSA were associated with rapid progression. Such patients should be treated at diagnosis. A patient with an equivocal bone scan in the presence of a low or moderately raised PSA could be managed expectantly, for instance a single hot spot in a rib and the PSA was less than 21 ng/ml.

For routine follow up PSA should replace bone scanning. As PSA levels correlate with the time to progression patients with very low levels may be seen less often.

PSA should not be used diagnostically. It should not be measured routinely in all patients with bladder outflow obstruction as a diagnostic screen.

DNA flow cytometry has yet to earn its place as a routine prognostic tool.

The advent of any new means of determining prognosis is accompanied by considerable enthusiasm and often over optimistic reports are written. Careful evaluation preferably with multifactorial analysis is required.

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ERRATA

Page 106	Line 8	(table 11) should read (table 8)
Page 227	Line 15	T_3 should read T_4
Page 228	Line 25	T_3 should read T_4