Prospective Study of Quantitation of Plasma DNA Levels in the Diagnosis of Malignant versus Benign Prostate Disease

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ABSTRACT

Purpose: **The aim of this study was to determine the potential of cell-free DNA levels as a diagnostic marker for prostate cancer, having first established the effect that blood sample processing has on this measurement.**

Experimental Design: **A total of 152 blood samples were collected prospectively from patients before their prostate biopsy and 25 from men in two distinct control groups. Blood was processed to yield three components: one-spin plasma (n = 68), two-spin plasma (n = 152), and serum (n = 56) samples.**

Results: **Having established the effect of sample preparation on the measured DNA level, the more reliable two-spin plasma sample was used to determine the relationship between the DNA level and the presence of prostate cancer. Those patients with cancer (n = 78) had a significantly higher level of DNA compared with the control groups ($P < 0.0001$ and $P < 0.0001$). However, DNA levels in patients with a benign biopsy (n = 74) were significantly higher than the 78 patients confirmed to have cancer ($P = 0.02$).**

Conclusions: **We conclude that the sample type used in the quantitation of cell-free DNA has an effect on the level reported. Elevated levels are present in the two-spin plasma samples of patients with prostate cancer compared with healthy controls but are not of diagnostic value during the management of prostate cancer.**

INTRODUCTION

Prostate cancer has become the most commonly diagnosed malignant disease in the Western world. Dramatic advances have been made in the diagnosis of this disease. However, these diagnostic tools lack sufficient specificity and sensitivity with which to diagnose all cases of prostate cancer. In particular, an elevated prostate-specific antigen (PSA) level is not specific to prostate cancer and can occur in a variety of pathologic conditions known to effect the prostate gland, including benign prostatic hypertrophy and prostatitis (1, 2). In addition, it has been shown that ~20% of men with organ-confined prostate cancer have a PSA <4.0 ng/mL, which is the cutoff normally used to indicate an elevated PSA (3). PSA levels are also subject to significant degrees of variability with recent studies quoting levels of coefficient of variation between 9% and 15% (4, 5). In addition to the problems with PSA measurements, standard transrectal ultrasound-guided biopsies have been reported to miss up to 23% to 30% of prostate cancers (6, 7). Even when attempts are used to decrease this figure by using extended biopsy regimens, up to 17% of cases may still go undiagnosed (8). It is clear, therefore, that the requirements for an additional diagnostic marker, in addition to PSA, in the management of this disease remain. The study of cell-free nucleic acids is a rapidly developing area of research, which offers the potential for providing such a marker.

Mendel and Metais (9) first reported the existence of cell-free nucleic acids within the human plasma of both normal subjects and those with various diseases in 1948. However, the relationship between an elevated cell-free DNA level and the presence of cancer was not investigated until 1975 (10). Since then, a number of studies have gone on to confirm this relationship in a variety of malignant processes, as summarized in Table 1 (11–18). From this table, it can be seen that a group of normal controls can expect to have a mean DNA level of between 0 and 100 ng/mL in their plasma/serum but that a similar group of cancer patients might expect to have a mean between 20 and 500 ng/mL. Sozzi et al. (18) confirmed the presence of increased levels of circulating DNA in patients with lung cancer compared with disease-free heavy smokers, with a real-time PCR technique. They have suggested that such measurements could be used as a new noninvasive approach for the early detection of lung cancer. The presence of elevated cell-free DNA levels have also been confirmed in prostate cancer patients (16). In this study, Wu et al. (16) reported a mean DNA level of 57 ± 30 ng/mL (mean ± SD) in a group of normal individuals compared with levels of 458 ± 790 ng/mL (n = 51) in a group of men with prostate cancer and a PSA level between 4 and 20 ng/mL.

Although these studies have contributed to our understanding of cell-free DNA levels, there remains considerable difficulty when attempting to compare such studies, particularly with regards to the DNA levels reported. Most of this difficulty arises from differences in the sample types measured (plasma versus serum) and the techniques used to perform these measurements. Thijssen et al. (17) and Lee et al. (19) have recently reported the importance of sample type on the DNA level measured, finding that serum samples often yield higher DNA levels than those obtained from plasma samples. Although the development of new techniques for measuring such parameters is a fundamental

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Note: A. Harris and J. Wainscoat contributed equally to the project.

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part of the research process and, therefore, difficult to avoid, attempts can be made to standardize the type of samples used and the process by which they are obtained. Furthermore, their application to clinically relevant decisions is not usually addressed (e.g., in the study on prostate cancer, medical students were compared with cancer patients; ref. 16).

This study was designed to determine the effect that sample collection has on the cell-free DNA level and then with the most reliable preparation, the diagnostic potential of cell-free DNA levels in the differentiation of prostate cancer from benign disease in the same symptomatic population.

MATERIALS AND METHODS

Patients and Samples. Men referred to the Royal Berkshire and Battle Hospitals in Reading, United Kingdom, for a prostate biopsy, after the finding of either an elevated PSA or abnormal digital rectal examination, were recruited into this prospective study, which had approval from the West Berkshire Local Research Ethics Committee.

Two control groups were obtained for the purpose of this study. The first, a low-risk benign group was obtained from a group of patients who had undergone a prostate biopsy 2 years previously. During the following 2 years, this group had been followed-up with serial PSA measurements, such that over a 2-year period they had received >12 PSA checks. At the end of the 2-year follow-up period, patients had their PSA velocity calculated with a linear regression line. Carter et al. (20) has previously shown that <5% of men without prostate cancer will have a PSA velocity of ≥0.75 ng/mL/y and ~70% of men with prostate cancer will have a PSA velocity of ≥0.75 ng/mL/y. Therefore, those men who had a PSA velocity <0.75 ng/mL/y were considered to be at low risk of having had a false-negative biopsy and therefore of having prostate cancer and were invited to donate a single blood sample for the purpose of this study. The second control group, called the healthy control group, were obtained from male volunteers under the age of 40 years with no previous history of prostatic disease.

All blood samples were collected from patients before any procedure in a 7 mL EDTA tube, which had either a single or double centrifugation step at 1,700 rpm (1,000 × *g*) for 10 minutes at 4°C (BR401 Refrigerated Centrifuge, Denley, Billingshursts, West Sussex, United Kingdom). All samples were spun within 1 hour of collection and stored immediately at −70°C (Model 925, Forma Scientific, Inc., Marietta, OH). Serum samples were collected and allowed to clot at room temperature for 30 minutes before undergoing a single centrifugation step at the same setting.

Sample Preparation. DNA was extracted from 600 μL serum or plasma sample with the QIAamp DNA Blood Mini kit (Qiagen, West Sussex, United Kingdom) according to the blood and body fluid protocol. Extracted DNA was stored at −20°C.

Real-Time PCR. DNA quantitation was conducted with a real-time PCR technique on an Applied Biosystems 5700 sequence detection system and a 5′-nuclease assay (Applied Biosystems, Warrington, United Kingdom).

Standard Curve. A standard curve was constructed in all experiments to allow for the absolute concentration of target DNA to be quantified. This was constructed with 5-fold serial dilutions of a known concentration of commercial male human DNA (Novagen, Madison, WI) at concentrations of 1,000, 200, 40, 8, 1.28, and 0.64 genome equivalents per microliter.

Identification of a Taqman Probe in Prostate Cancer Cases. An initial experiment was conducted on 23 two-spin plasma samples (12 cancer cases, 11 benign) with two gene probes, one targeting *Albumin* and the other the *APP* gene. Neither gene is located on a chromosome, which has been identified as developing genetic mutations during the development of prostate cancer. This provisional study revealed that both probes gave similar linear relationships of DNA levels across the samples used and that these levels were highly correlated ($r = 0.88$, $P < 0.001$; Fig. 1). The *APP* probe, however, gave levels 2.5-fold higher than those obtained with *Albumin*; thus, *APP* was chosen as the probe of choice. The observed difference in the DNA levels between the *Albumin* and *APP* experiments is most likely the result of the different binding efficiencies of both the primers and probe sets used.

PCR Amplification. The DNA in the sample under investigation was measured with a real-time quantitative assay for the *APP* gene. The assay (21) included two primers: APP-F (APP-137F, 5′-TTTGTGTGCTCTCCCAGGTCT-3′) and APP-R (APP-210R, 5′-TGGTCACTGGTTGGTTGGC-3′) and a dual-labeled fluorescent Taqman probe, APP-P (APP-161T, 5′-FAM-CCCTGAACTGCAGATCACCAATGTGG-TAGTAMARA-3′; MWG, Ebersberg, Germany). PCR was done in a final volume of 25 μL and contained 12.5 μL Taqman 2× Universal PCR Master Mix (AmpliTaq Gold DNA polymerase, AmpErase UNG, deoxynucleoside triphosphates with dUNTP, passive reference 1, and optimized buffer components; Applied Biosystems), 100 nmol/L Taqman probe, 300 nmol/L of each primer, and 4 μL extracted DNA. Each sample was analyzed in triplicate. Triplicates of the standards were included in each run.

Data Analysis. The mean quantity of the triplicates was calculated by the 5700 sequence detection software. The DNA concentration, expressed in copies per milliliter, was calculated with the following equation (22):

$$C = Q \times \frac{V_{DNA}}{V_{PCR}} \times \frac{1}{V_{ext}}$$

where C is the target concentration in plasma (copies per milliliter), Q is the target quantity (copies) calculated by the 5700 sequence detection system, V_{DNA} is the total volume of extraction (25 μL), V_{PCR} is the volume of DNA used per PCR reaction (4 μL), and V_{ext} is the volume of plasma extracted (600 μL).

A conversion factor of 6.6 pg was then used to enable the cell-free DNA level to be expressed in terms of ng/mL. This value is based on the fact that one human genome is composed of 3×10^9 bp. Therefore, one cell with two sets of chromosomes has 6×10^9 bp. The average molecular weight of a single bp is 680 g/mol. The molecular weight of DNA in one cell is, therefore, 4.1×10^{12} g/mol ($6 \times 10^9 \times 680$). Finally, the mass of DNA from one cell can be calculated by dividing 4.1×10^{12} by Avogadro's number (6.23×10^{-23} g), giving rise to the conversion value of 6.6×10^{11} g (6.6 pg; ref. 23).

Table 1 Summary of studies quantifying cell-free DNA levels in patients with and without cancer

Author (reference no.)	Method of quantitation	Sample processing described	Sample type
Leon et al. (11)	Radioimmunoassay	Yes	Serum
Shapiro et al. (12)	Radioimmunoassay	No	Serum
Stroun et al. (13)	UV absorbance	No	Plasma
Jahr et al. (14)	Competitive PCR	Yes	Plasma
Sozzi et al. (15)	DNA Dipstick*	Yes	Plasma
Wu et al. (16)	Picogreen‡	No	Serum/plasma
Thijssen et al. (17)	Real-time PCR and Picogreen	Yes	Plasma and serum
Sozzi et al. (18)	Real-time PCR	Referenced	Plasma

*Invitrogen, Paisley, United Kingdom.
†Area under receiver operating curve.
‡Berthold Technologies, Bad Wild bad, Germany.

Statistical Analysis. Data were analyzed with Statview 5.0.1 (SAS Institute, Inc., Cary, NC) and Stata 7.0 (Stata Corporation, College Station, TX).

RESULTS

Effect of Sample Type on DNA Levels

Comparison of DNA Levels in Corresponding One-Spin and Two-Spin Plasma Samples. In 68 one-spin and two-spin samples, the DNA levels were highly correlated (Spearman, $r = 0.78$, $P < 0.001$; Fig. 2). The median difference between these values was 0.7 ng/mL (−6.2-84.8 ng/mL). In eight cases, a large discrepancy was present, with the one-spin value being 3-fold higher than the two-spin value. In no sample was the two-spin value 3-fold greater than the one-spin level.

Comparison of DNA Levels in Corresponding Plasma and Serum Samples. Forty of the above cases had plasma and serum taken. The DNA levels in the 40 samples with corresponding one-spin and two-spin plasma components correlated well (Spearman, $r = 0.91$, $P = 0.001$), but a relationship could also be seen between the one-spin plasma and serum samples (Spearman, $r = 0.68$, $P < 0.001$) and the two-spin plasma and serum samples (Spearman, $r = 0.56$, $P < 0.001$). However, in all samples, the serum component yielded a much higher level of DNA, which was highly variable in its extent, as shown in Fig. 3. The median serum DNA level in the 40 samples was 27.1 ng/mL (2.9-242.7 ng/mL) compared with 2.9 ng/mL (0.0-9.9 ng/mL; Spearman, $P = 0.084$) in the 40 one-spin plasma and 2.6 ng/mL (0.0-11.8 ng/mL; Spearman, $P = 0.083$) in the 40 two-spin plasma samples.

Relationship between DNA Levels and the Presence of Prostate Cancer

One-Spin Plasma and Serum Samples. No relationship was seen between the DNA level and the presence of cancer in the prostate biopsies in either the 68 one-spin plasma or the 59 serum samples available for statistical analysis (Mann-Whitney, $P = 0.4$ and $P = 0.6$, respectively; Fig. 4A).

Two-Spin Plasma Samples. In the 152 two-spin plasma samples, the 78 patients diagnosed with prostate cancer had a significantly higher level of DNA than the two control groups, low-risk benign ($n = 15$; Mann Whitney, $P < 0.0001$) and the healthy controls ($n = 10$; Mann-Whitney, $P < 0.0001$). However, the DNA levels in the patients with a benign biopsy ($n = 74$) were significantly higher than the 78 patients confirmed to have prostate cancer (Mann-Whitney, $P = 0.02$; Fig. 4B). Both the age and referral PSA level in these 152 patients correlated with the presence of prostate cancer, as would be expected. Median age of the cancer group was 69 years (range, 56-86 years), whereas the median age of the benign group was 66 years (range, 45-84

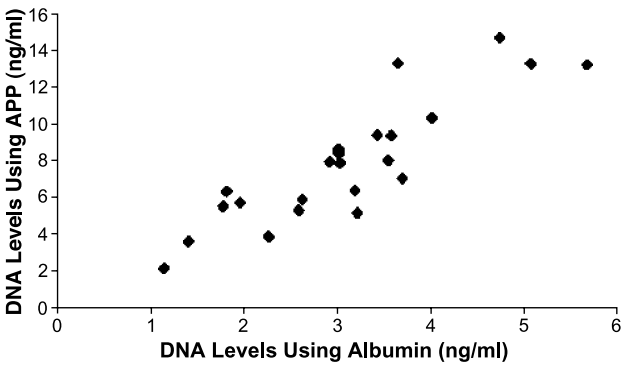

Fig. 1 Correlation plot revealing the degree of correlation in DNA levels measured with the *Albumin* and *APP* probe ($n = 23$; $r = 0.88$, $P < 0.001$).

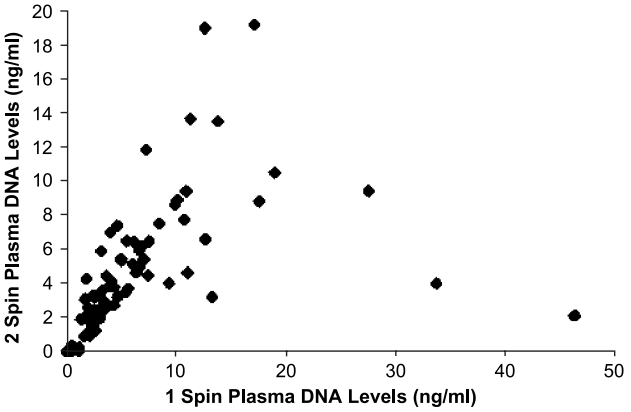

Fig. 2 Correlation plot revealing the degree of correlation in DNA levels measured with one-spin and two-spin plasma samples ($r = 0.78$, $P < 0.001$).

Table 1 Summary of studies quantifying cell-free DNA levels in patients with and without cancer (Cont'd)

Controls (mean DNA level, ng/mL)	Type of cancer	Cancer (mean DNA level, ng/mL)	Statistical significance
13 ± 3 (n = 55)	Mix	180 ± 38 (n = 173)	$P < 0.005$
Normal: 14 ± 3 (n = 88)	Gastrointestinal	412 ± 63 (n = 199)	Not calculated
Benign: 118 ± 14 (n = 187)			$P < 0.001$
None detected (n = 50)	Mix	1.2-437 μg/20 mL (n = 56)	Not calculated
Most < 2.0 (n = 20)	Mix	219 (n = 30)	Not calculated
18 (n = 43)	Non–small cell lung cancer	318 (n = 84)	AUC-ROC = 0.844†
57 ± 30 (n = 74)	Mix	PSA 4-20: 458 ± 790 (n = 51)	Not calculated
	(Figures for prostate cancer)	PSA > 20: 490 ± 471 (n = 34)	
Serum: 12.9 ± 10.7 (n = 28)	Mix	Serum: 47.6 ± 46.1 (n = 26)	$P < 0.001$
Plasma: 4.8 ± 3.6 (n = 28)	(All with liver metastases)	Plasma: 10.6 ± 14.2 (n = 26)	Not significant
Median: 3.1 (n = 100)	Non–small cell lung cancer	Median: 24.3 (n = 100)	AUC-ROC = 0.94†

years; Mann-Whitney, $P = 0.0002$). The median referral PSA in the cancer group was 8.4 ng/mL (range, 2.4-1,400 ng/mL) and that in the benign group was 7.2 ng/mL (0.3-55.6 ng/mL; Mann-Whitney, $P = 0.0275$).

The low-risk benign group (n = 15) had a significantly higher DNA level than the healthy control group (n = 10; Mann-Whitney, $P < 0.0001$), although significantly lower level than the benign group (n = 74; Mann-Whitney, $P < 0.0001$).

DISCUSSION

Despite reports on the potential that cell-free DNA levels have as a new diagnostic marker in cancer, few studies have been conducted to explore its role in the management of prostate cancer. The limited number of studies that have been done have concentrated on the search for tumor-specific genetic alterations in the cell-free DNA (24, 25).

The current study, which set out to determine the diagnostic value of cell-free DNA levels in the management of prostate cancer, recruited men from a group of patients who were considered at risk of having this disease. These patients, who had either an elevated PSA or abnormal digital rectal examination, were being referred for a prostate biopsy to investigate this further.

The first part of the study was aimed at investigating the effect of sample processing on the cell-free DNA level, with three distinct sample types, including one-spin plasma, two-spin plasma, and serum samples. The median DNA level found in the 40 corresponding one-spin plasma, two-spin plasma, and serum samples, regardless of diagnosis were 2.9 ng/mL (0.0-9.9 ng/mL), 2.6 ng/mL (0.0-11.8 ng/mL), and 27.1 ng/mL (2.9-242.7 ng/mL), respectively. Although these values seem lower than most (Table 1), they perhaps more importantly correlate well with those reported in the recent study by Sozzi et al. (18), which also used a real-time PCR technique on plasma samples, which had been subjected to a similar processing technique (2,500 rpm for 10 minutes at 4°C).

When the levels of cell-free DNA were compared between sample types, those in the one-spin and two-spin plasma samples seemed to correlate well ($r = 0.78$, $P < 0.001$). The one-spin samples were, however, seen to give occasionally much higher levels of DNA than the corresponding two-spin sample. This observation is most likely the result of a technical error, occurring when the plasma is removed too close to the buffy layer, resulting in contamination with cellular DNA. In contrast to these findings, a recent study by Chui et al. (26), which also investigated the effect of different processing procedures on the cell-free DNA level, found that plasma samples subjected to only one centrifugation process (1,600 × g) had significantly higher levels of DNA than those subjected to two centrifugation steps (1,600 × g + 16,000 × g). This

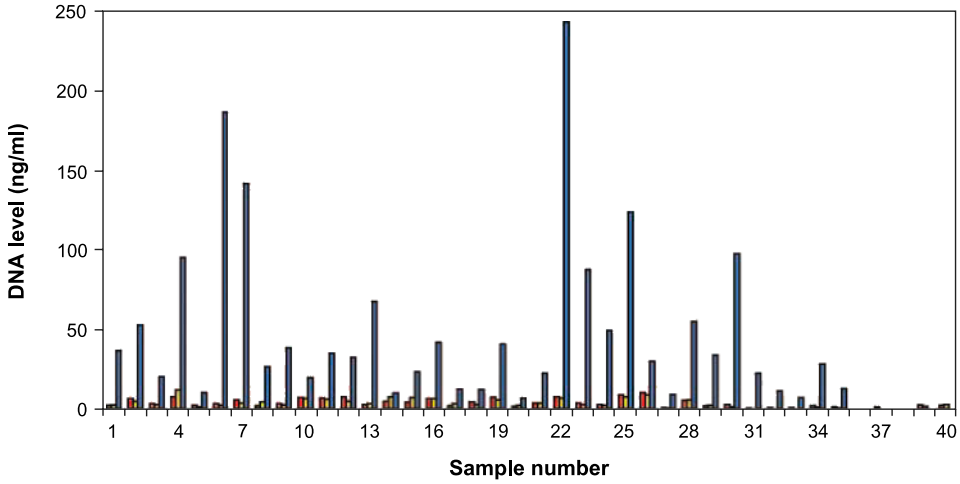

Fig. 3 Bar chart showing the different DNA levels (*columns*) measured in the three corresponding sample components: one-spin plasma (*red*), two-spin plasma (*yellow*), and serum samples (*blue*).

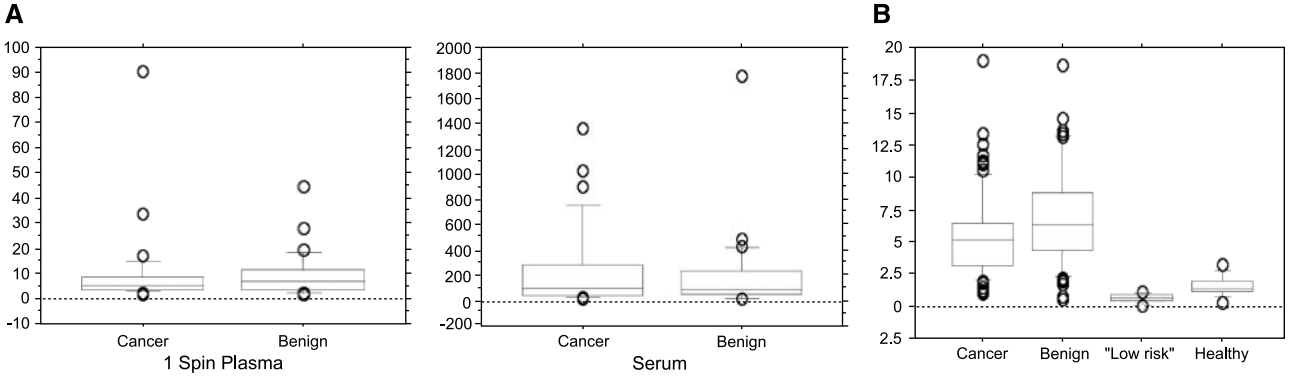

Fig. 4 *A*, box plots representing the relationship between the one-spin plasma (n = 68; P = 0.4) and serum (n = 59; P = 0.6) DNA levels (ng/mL) and the biopsy diagnosis. *B*, box plot representing the relationship between the two-spin plasma DNA level (ng/mL) in the four groups: cancer (n = 78), benign (n = 74), low-risk benign (n = 15), and healthy controls (n = 10).

discrepancy between the two studies may be explained by the different centrifugation settings used or perhaps may reflect the careful removal of the plasma from the one-spin plasma samples in the current study. Although the one-spin and two-spin plasma samples seemed to correlate well, the serum samples were seen to give consistently higher levels of DNA, which were highly variable between patients. It is likely that this extra DNA arises through the process of coagulation, which occurs during the preparation of the serum sample and it is unlikely, therefore, to be controllable.

In light of the apparent consistency of DNA levels in the two-spin plasma samples compared with the serum samples and with the effect of cellular contamination being minimized in contrast to the one-spin plasma samples, two-spin plasma was chosen as the sample of choice for the second part of this study. This choice of sample type is also supported by the recent study by Chui et al. (26) who observed that plasma samples subjected to two centrifugation steps provide DNA levels comparable with those undergoing filtration, where cellular contamination is essentially eliminated. In that study, Chui et al. (26) also showed that two-spin plasma samples (1,600 × g + 16,000 × g) do not show any significant degrees of SD on a day-to-day basis, when compared with the filtered samples, in contrast to samples subjected to only a single centrifugation step (800 × g). Therefore, not only are two plasma samples less likely to suffer cellular contamination, but they would also seem to show less day-to-day variability.

The second part of the study showed that cell-free DNA levels are higher in patients with prostate cancer compared with either a group of healthy controls or men at low risk of having prostate cancer. However, surprisingly, those men with benign prostatic pathology had a significantly higher DNA level when compared with the prostate cancer group. One explanation for this may be that a number of the men found to have benign prostatic pathology on their biopsies, who were therefore included in the benign group, may have in fact had a false-negative biopsy and be harboring as yet undiagnosed prostate cancer. Inadvertently categorizing patients in the wrong group can be expected to affect the overall results of this study. However, this potentially confounding factor was overcome in this study by recruiting a large patient group. The findings of a clear correlation between the presence of prostate cancer and both the age of the patient and the referral PSA value in our study population also suggests that this was a representative group in which to compare DNA levels. When the DNA levels in the one-spin plasma and serum samples were compared with the presence of prostate cancer, no statistically significant difference was found between the two groups. Therefore, these findings clearly show that the quantitation of cell-free DNA levels is not a useful diagnostic tool for those patients suspected of having prostate cancer. Unlike other patient groups undergoing investigation for the presence of a malignant process, those found to have benign prostatic disease often have some other form of pathology within the prostate gland, such as prostatitis, which may be elevating the cell-free DNA level perhaps to a greater degree than that of prostate cancer.

Only one study has been published with the specific aim of quantifying the level of cell-free DNA in prostate cancer patients. In this study, Wu et al. (16) extracted cell-free DNA from 1 mL serum/plasma samples with the QIAamp 96 Spin Blood minikit (Qiagen), and quantitation was done with a PicoGreen DNA detection kit. The mean cell-free DNA level obtained from the control group, composed of normal individuals undergoing annual health checks, was 57.1 ± 30.6 ng/mL (mean ± SD). The 51 patients with prostate cancer who had a PSA level between 4 and 20 ng/mL had a mean DNA level of 458 ± 790 ng/mL and the 34 patients with a PSA >20 ng/mL had a mean DNA level of 490 ± 471 ng/mL. However, the method by which the serum/plasma samples were processed in this study was not described and, indeed, there is some confusion as to which sample type was used during quantitation.

In conclusion, our study shows the need for careful sample selection and processing in this developing field of research, as well as endorsing the need for adequate documentation of sample preparation in future studies. We also find that when applying this assay to a clinically relevant question of trying to detect cancer patients in a similar symptomatic population prospectively, the level of cell-free

DNA is lower in those men diagnosed with prostate cancer compared with those with benign prostatic disease. Previous studies have not evaluated DNA in this way and future studies will need to be appropriately designed.

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