The BARCODE 1 pilot study

Title: The Use of Genetic Profiling to Guide Prostate Cancer Targeted Screening

Short Title: BARCODE 1 Pilot

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1. Background

Introduction – the genetics of prostate cancer

Prostate cancer (PrCa) is now the commonest cancer in men in the Western world, with over 40,000 new cases per annum and a lifetime risk of 1 in 11 in the United Kingdom (UK) (Cancer Research UK CancerStats, 2012), as well as in the European Union with 397,000 new cases per annum, and 94 000 deaths (Globocan). However, its aetiology remains very poorly understood. The substantial worldwide variation in incidence rates suggests that lifestyle risk factors are important. To date, however, no definite lifestyle risk factors have been identified.

Aside from demographic factors, the only established risk factor for PrCa is family history. Genetic studies, in particular genome-wide association analyses have identified 77 genetic variants associated with PrCa risk (reviewed in Goh et al, 2012; Eeles et al 2013). The risk of the disease in first degree relatives of cases is approximately twice that in the general population (Carter et al., 1992; Goldgar et al., 1994; Eeles et al., 1999; Hemminki et al., 2002; Gronberg 2003; Edwards and Eeles, 2004). This familial risk is greater amongst young cases, being more than fourfold for cases below age 60. Higher risks have been shown for men with two or more affected relatives. There is a higher risk in Afro-Caribbeans who have a 2.87-3.19-fold increased risk compared with whites in the UK (Ben-Shlomo et al, 2008). Analyses based on the Nordic twin registries have found higher risks in monozygotic than dizygotic twins, supporting the hypothesis that much of this familial aggregation is due to genetic rather than shared lifestyle factors (Lichtenstein et al., 2000).

Genetic predisposition arises from rare highly-penetrant mutations, and/or from common variants conferring more moderate risks. We, and others, have found the former using direct candidate gene mutation analysis (e.g. Dong et al., 2003; Edwards et al., 2003, 2012; Guisti et al 2003; Cybulski et al., 2004; Kote-Jarai et al., 2011; Leongamornlert et al., 2012). Sequencing of a linkage region on 17q has revealed a high risk PrCa predisposition gene, *HOXB13* which has a relative risk of 4-20 in families and is present in about 3.4% of European populations (Ewing et al, 2012; Zu et al, 2012; Witte et al, 2013). Genome-wide association studies (GWAS) identify common variants, present in >5% of the population. In GWAS, susceptibility variants (usually single nucleotide polymorphisms (SNPs)) are identified by finding a difference in genotype frequency between cases and controls.

SNP Profiling

SNP genotyping is the measurement of genetic variations of single nucleotide polymorphisms (SNPs). It is a form of genotyping, which is the measurement of more general genetic variation. SNPs are one of the most common types of genetic variation. A SNP is a single base pair mutation at a specific locus, usually consisting of two alleles (where the rare allele frequency is >1%). SNPs are found to be involved in the etiology

of many human diseases. Because SNPs are conserved during evolution, they have been proposed as markers for use in association studies (genome wide association studies-GWAS).

Routine genotyping is expensive and requires more DNA and resources and time for analysis. New technologies such as highly multiplexed ligation-dependent PCR (LD-PCR) combined with high throughput next generation sequencing (NGS) technology offer an attractive alternative. This technology is called mass genotyping by sequencing technology (MGST) and can check numerous SNP positions in a number of samples combined in a single assay. MGST has the capacity to accommodate at least 100 SNPs and up to 10000 samples per assay of the Illumina NGS device.

Based on the estimated relative risks of currently known SNPs, approximately 30% of the familial risk of PrCa can now be explained and the top 1% of the risk profile has a 4.7-fold risk compared with the average of the population. It is estimated that nearly 2000 SNPs may be associated with PrCa risk (Eeles et al., Nature Genetics 2013) and the proposed Oncoarray initiative which will run 600 000 SNPs in 80 000 PrCa blood DNA samples and controls (cases:controls in a 3:1 ratio) is likely to find further hits. We have shown using theoretical modeling that genetic profiling of 27 SNPs in a population rather than the use of an age cut-off of 55 years for PrCa PSA screening would predict that 16% of men could avoid screening at the expense of missing 3% of cases (Pashayan, Duffy et al. 2011).

These results may have clinical implications for targeted screening and there are also potential implications for risk counselling. Individually each SNP confers a modest effect on relative risk, however, the combined effects of these SNPs are thought to be multiplicative and therefore may be substantial, and as other SNPs are identified it may be possible to define genotypes that are sufficiently predictive of risk to be useful clinically. MacInnis et al have described a model – the P model, which incorporates SNP data and family history (Macinnis, Antoniou et al. 2011).

Mathematical modeling has shown that if a population is genetically profiled for such variants, men who fall in the top 10% of the population genetic risk distribution have a 2.7-fold risk compared with the average of the total population. This risk is at a level where mammography is offered to women at higher breast cancer risk in populations. Taking this analogy, genetic profiling could therefore be considered to offer targeted prostate cancer screening in populations, hence this application which is at the cutting edge of translation of these findings.

Prostate Cancer Screening-present

PSA screening studies of the general population to date have reported conflicting effects on mortality from the disease.

To date there are several population based screening studies which have used a threshold of PSA to determine whether to undertake prostate biopsy (Andriole et al,

2009; Schroder et al, 2009; Hugosson et al, 2010; Schroder et al, 2012). The problem with PSA is that it has false positive and negative outcomes.

The European Randomised Study of Screening for Prostate Cancer (ERSPC) identified 182.000 patients through cancer registries from 7 European countries. In the screening group 82% of men received at least 1 screening PSA. The hazard ratio for death from prostate cancer was 0.8, which means that, in order to prevent 1 death from prostate cancer 1410 men need to be screened and 48 patients should be treated additionally for prostate cancer.(Schroder, Hugosson et al. 2009) The Goteborg trial, a subset of the ERSPC, with 20000 men recruited, reported a 56% difference in favour of the PSA-screened arm.(Hugosson, Carlsson et al. 2010)

The PLCO study (prostate, lung, colorectal and ovarian study), recruited 76,685 patients in the US and randomised them to PSA screening and digital rectal examination or no screening. After 7 years of follow up the incidence of prostate cancer was 116 per 10000 person-years in the surveillance group and 95 per 10000 person-years in the control group. The rate of death from prostate cancer per 10000person-years was 2 for the screening group and 1.7 in the control group with no statistically significant difference, despite the higher rate of cancer diagnosis.(Andriole, Crawford et al. 2009) Andriole et al published the latest update with 13 years follow up and there was no evidence of mortality benefit for annual screening compared to opportunistic screening. (Andriole, Crawford et al. 2012). On the contrary, Crawford et al, performing a subgroup analysis of the PLCO study data, showed that elective screening of individuals with no major comorbidities led to a significant decrease in the prostate cancer specific mortality. (Crawford, Grubb et al. 2011)

ASCO issued recommendations in 2012 advising against screening older men with a life expectancy of less than 10 years. The American Urological Society published guidelines in 2012, offering an informed decision making on PSA screening only to the age group of 55 to 69 year old men.(Basch, Oliver et al. 2012) The US Preventive Services Taskforce (USPSTF) took a step further discouraging routine PSA screening in all men.(Force 2012)

Targeted Screening

It is important to consider not just the number of cancers that are detected but the ability of a screening modality to distinguish between clinically significant disease, i.e. disease causing a significant risk to the patient's life or wellbeing, versus disease that would pose no threat if left untreated. The definition of clinically significant localized PrCa is defined using the NICE criteria for intermediate / high risk disease, which comprises a Gleason score of $\geq$7, and /or $\geq$T2b, N1, M1 (http://guidance.nice.org.uk/CG58).

The Targeted PSA Screening (TAPS) study looked at the feasibility of targeting screening at high risk groups (Melia et al, 2006) and identified a number of key issues. The aims of this study were to investigate the uptake rate of screening using prostate specific antigen (PSA) testing, and the referral rate in male relatives of men already

diagnosed with PrCa below the age of 65 years. This study recruited relatives of men with PrCa aged between 45-69 years and contacted eligible men via their affected relatives. The results of the study found that discussing the study in person with PrCa patients yielded a higher recruitment rate compared with postal invites. They also found that there was a high level of previous PSA screening within this cohort. Interestingly they found that men were far more likely to opt for screening within the study if they were married / co-habiting versus men who were single. The results of this study have important implications for the design of targeted screening programmes in higher-risk groups and highlights that further research is needed into the management of higher risk groups.

A study of men with at least one first or second degree relative with PrCa who underwent prostate biopsy showed that 25.3% had PrCa (Canby-Hagino et al., 2007). Nam et al (2009) studied the effect of 25 SNPs in men who had biopsy and PSA screening. In 3,004 patients, 1,389 (46.2%) were found to have PrCa. Fifteen of the 25 SNPs studied were significantly associated with PrCa on biopsy ($P=0.02\text{-}7x10^{-8}$). He selected a combination of 4 SNPs with the best predictive value for further study. After adjusting for other predictive factors, the odds ratio for patients with all four of the variant genotypes compared with men with no variant genotype was 5.1 (95% confidence interval, 1.6-16.5; P=0.006). When incorporated into a nomogram, genotype status contributed more significantly than PSA, family history, ethnicity, urinary symptoms, and digital rectal examination (area under the curve=0.74). The positive predictive value of the PSA test ranged from 42% to 94% depending on the number of variant genotypes carried ($P=1x10^{-15}$).

Biomarkers

PCA3 levels have been shown to correlate with positive prostate biopsies. Unlike serum prostate specific antigen the PCA3 score did not increase with prostate volume. PCA3 was independent of PSA reading s and previous biopsies. (Deras, Aubin et al. 2008) The PCA3 score was significantly correlated with total tumor volume in prostatectomy specimens (r = 0.269, p = 0.008), and was also associated with prostatectomy Gleason score (6 vs 7 or greater, p = 0.005).(Nakanishi, Groskopf et al. 2008) In a prospective European Study of 463 men the positive repeat prostate biopsy following an initial negative biopsy was 28%. It was found that the higher the PCA3 score, the greater the probability of a positive repeat biopsy. The PCA3 score (cut-off of 35) had a greater diagnostic accuracy than free:total PSA ratio.(Haese, de la Taille et al. 2008).

The presence of the genetic rearrangement between transmembrane-serine protease gene (TMPRSS2) and the erythroblast transformation-specific (ETS) member ERG (v-ets erythroblastosis virus E26 oncogene homolog avian) has been demonstrated in almost half of PCa cases. This gene fusion is considered to be an early event in PCa development. (Perner, Schmidt et al. 2007) The prognostic value of this translocation is unclear, as studies report conflicting results. Prostatectomy specimens from 294 PrCa patients were evaluated using FISH and rearrangement was observed in 56.6% of

cases, and no association with biochemical progression or relapse free survival was found. (Fernandez-Serra, Rubio et al. 2013) In another study of 208 radical prostatectomy specimens ERG expression was assessed with immunohistochemistry and was identified in 23.7% of the samples. ERG expression was twice more likely to be present in higher tumour stage and patients with ERG expression were twice more likely to die of prostate cancer.(Weinmann, Van Den Eeden et al. 2013)

Prostate Cancer Screening-Future

The IMPACT study

A different approach to prostate cancer screening aiming to reduce mortality and morbidity is needed. A case control study rising to this challenge is IMPACT (The Identification of Men with a genetic Predisposition to Prostate Cancer: Targeted screening in *BRCA1/2* mutation carriers and controls). This is an innovative targeted screening study, which will help us formulate a novel approach to improving prostate cancer related outcomes (Mitra 2006).

IMPACT is an international collaboration amongst 52 worldwide centres which has recruited 350 men with *BRCA2* and 500 men with *BRCA1* and 850 controls. The aim of this study is to investigate the role of PSA screening in *BRCA* mutation carriers aged 40 to 69. PSA is checked annually and all men with PSA>3ng/ml are offered diagnostic 10 core trans rectal biopsy. This study is now extended to include patients with Lynch syndrome, 190 men with *MSH2*, *MSH6* and *MLH1* (MMR genes) and 190 controls (Impact).

The IMPACT trial group published data from the first 300 patients with 89 *BRCA1*, 116 *BRCA2* and 95 controls with median follow up of 33 months. The prevalence of prostate cancer was 3.3% with the positive predictive value of PSA screening of 47.6%. This preliminary report re-enforces the value of PSA screening in *BRCA* mutation carriers (Mitra, Bancroft et al. 2011).

The PROFILE Pilot study

The aim of the PROFILE study is to correlate germline genotypes in men with an increased risk of PrCa due to a genetic predisposition with biopsy outcome and also to assess the additional contribution of DW-MRI and new biomarkers to PrCa screening in this group. An initial pilot has been completed. The aim of the pilot PROFILE study was to conduct a feasibility study in 100 men with a positive family history of PrCa (at least one first degree relative affected at <70 years, with diagnosis verified) to determine the interest in the study, biopsy uptake and complication data. The rationale behind the study design of this protocol was identifying at risk groups based on family history and retrospectively profiling rather than taking a specific SNP profile as a criterion for screening and biopsy. The pilot PROFILE study recruited eligible men aged 40-69 years with a family history of PrCa over a two year period. After informed consent, patients provided blood samples to measure PSA level and for DNA extraction. All participants were asked to undergo a 12 core prostate biopsy regardless of baseline PSA result.

Participants without previous prostate biopsy or who underwent biopsy >1 year ago were also offered a T2-weighted with DW-MRI prior to biopsy in 50 of the participants.

In total 116 men were recruited and 102 biopsies completed. All patients were asymptomatic. Based on SNP analysis of 39 PrCa risk SNPs, a total of 53 men had a predicted relative risk <1 (median age 55 yrs; median PSA 1.20). In this subgroup, 8 men (15.1%) were diagnosed with PrCa (median age 62.0 yrs, median PSA 2.50). Amongst the 48 men with a relative risk >1 (median age 51.0 yrs; median PSA 1.4) 13 PrCas (27.1%) have been identified (median age 56.0 yrs, median PSA 2.7). T2 weighted in conjunction with DW-MRI had 33% false positives and 10% false negatives. The AUC of T2 weighted in conjunction with DW-MRI was 0.83. Twelve men with PrCa had a PSA <3 (52%). No adverse psychosocial variables were noted.

The main conclusion from this pilot study was that prostate biopsy as a means of PrCa screening is feasible and acceptable in men with a family history of PrCa. The findings support a larger study investigating the use of SNPs in PrCa risk stratification for targeted screening. The PROFILE study is currently being rolled out to include 2 cohorts, the first one would address the issue of family history and the second cohort the issue of African-Caribbean ancestry.

2. Study overview and rationale

The BARCODE 1 study has been developed to investigate the role of genetic profiling for targeting population screening. This study forms a pilot of 300 men, with the view to continue to a future study of 5000 men.

The primary endpoint is the association of biopsy result with genetic risk score in men having targeted prostate screening based on SNP risk profiling.

Secondary endpoint would be the comparison of results with population based PSA screening will be analysed to determine if there is a higher proportion of clinically significant disease than is identified in population based studies. This study will also explore the acceptability and logistical issues around using genetic profiling on a population level to target a cancer screening programme.

Initially we aim to recruit 300 men with the assistance from participating GPs. Men aged 55-69 years who are likely to be eligible for the study will be identified by their GP from medical records. They will be contacted via a letter from their GP and if interested in the study will be asked to fill in a questionnaire to confirm they are eligible to participate. This questionnaire can be completed in hard copy and men will also be given the option to fill in an online version. If eligible, men will then be sent a DNA collection kit. Saliva kits will be analysed with SNP profiling for the known 99 clinically relevant SNPs. Men with a genetic risk equivalent to the top 10% of the population distribution (approximately 30 men in total) will be invited for a TRUS prostate biopsy, plus further biological samples. Biopsy results will be correlated with the genetic score. PSA and other

biomarkers will be integrated into results to assess combined effects of genetic score and markers.

It is well known that the response rate to questionnaires sent in the post is low, but with the support of primary care practitioners and also with the option of an online eligibility questionnaire, we aim to improve the uptake rate. This will be a pilot study and will help us to identify problems with recruitment and SNP profiling. Saliva kits have been shown to yield enough extracted DNA to perform SNP profiling for all the candidate alleles. Provided that the initial cohort is recruited smoothly and the top 10% successfully undergoes biopsies then we aim to expand the study.

The expanded study will recruit a total of 5000 men and men with a genetic risk equivalent to the top 10% of the population distribution (approximately 500 men in total) will have been invited for a TRUS prostate biopsy, plus further biological samples.

Additional blood, urine, saliva and tissue samples will be taken for research purposes in order to investigate new biomarkers in this population using biochemistry, proteomic, metabolomic and microarray approaches. Samples will be collected from urine for further studies, for example biomarker studies PCA3 and the TMPRSS2 ERG translocation to correlate these with SNP profile, but biopsy decisions will not be made on these results.

All participants will also be invited to participate in a sub-study that aims to provide valuable information about the psychosocial and behavioural impact of genetic risk-profiling in the general population, deduce information needs of men undergoing testing and develop decision support tools accordingly. The results of this sub-study will be used alongside the results of the pilot BARCODE 1 study to inform the design of the main BARCODE 1 study in which the decision support tools will be trialled and refined. The background, rationale, methodology are outlined in detail in the BARCODE 1 psychosocial sub-study protocol. A separate consent form is also provided for this sub-study.

3. Aims

Primary aim:

- To determine the association of biopsy result with genetic risk score in men having targeted prostate screening based on SNP risk profiling

Secondary aims:

- To determine the incidence and aggressiveness of PrCa in men within the top 10% of the genetic score
- To determine the association of biological sample biomarker profile with prostate biopsy result in men at genetically higher PrCa risk undergoing targeted PrCa screening

4. Study Design

This screening study is designed to look at the role of genetic profiling for targeting population screening. The aim is to evaluate genetic profiling using the known 99 SNPs as a means for offering targeted screening for PrCa in men at a genetically higher risk. Additionally the study aims to integrate serum and/or urine markers to genetic profiling for those identified in the top 10% risk category. Biomarkers with established prognostic value will be checked including PSA, PCA3, hK2 and free: total PSA ratio.

5. End Points

Primary Endpoint:

- To determine the association of SNP genetic risk score with prostate biopsy results

Secondary Endpoints:

- To assess the incidence and the aggressiveness of prostate cancer amongst men within the highest 10% of the genetic score of new markers including quantitative imaging biomarkers e.g. apparent diffusion coefficient metrics and their association with the results of prostate biopsy
- To determine the association of the biomarker profile with genetic score and biopsy results
- To explore the use of genetic profiling to target prostate cancer screening in a clinical environment

6. Inclusion/ Exclusion Criteria

Number of subjects:

- 300 men willing to undergo genetic SNP profiling

Inclusion Criteria:

- Men aged 55-69 years
- Caucasian ethnicity
- WHO performance status 0-2 (see Appendix A)
- Absence of any psychological, familial, sociological or geographical situation potentially hampering compliance with the study protocol and follow-up schedule

Exclusion criteria

- Non-Caucasian ethnicity (including mixed race or Jewish)

- Previous diagnosis of cancer with a life-expectancy of less than five years
- Prostate biopsy in the past year
- Previous diagnosis of prostate cancer
- Co-morbidities making prostate biopsy risk unacceptable (anticoagulants or antiplatelet medication like Warfarin or Clopidogrel, poorly controlled diabetes or cardiovascular disease)

Subject Withdrawal

- Subjects may withdraw from the study at any time if they so wish without giving a reason. No further data will be collected about that individual, and any unused samples will be destroyed. Data collected up to that point will be retained for audit purposes.

7. Methodology

Please refer to Figure 1: Pilot study algorithm on page 14 for a summary of the study methodology. For the purpose of the pilot study we aim to recruit 300 men aged 55-69 years. Eligible men will be identified through their General Practitioners. They will receive information in lay terms about the study through the post. Those who consent and are eligible will be sent a saliva collection kit to provide a DNA sample.

Genetic profiling of known prostate cancer predisposition SNPs will be performed. Those within the top 10% of the risk profile will be offered a clinic appointment to discuss prostate cancer screening and will be offered a prostate biopsy. All men, regardless of their genetic profile score, will be followed for five years via cancer registries to track development of cancer in the future.

All men will also be invited to participate in the psychosocial sub-study, regardless of their genetic profile result. The methodology is outlined in detail in the sub-study protocol. Men will be invited to complete four questionnaires over the course of the study, the first upon enrolment in the main study, the second following receipt of the genetic risk profile results, the third at 6 months following these results and the fourth at 12 months following these results.

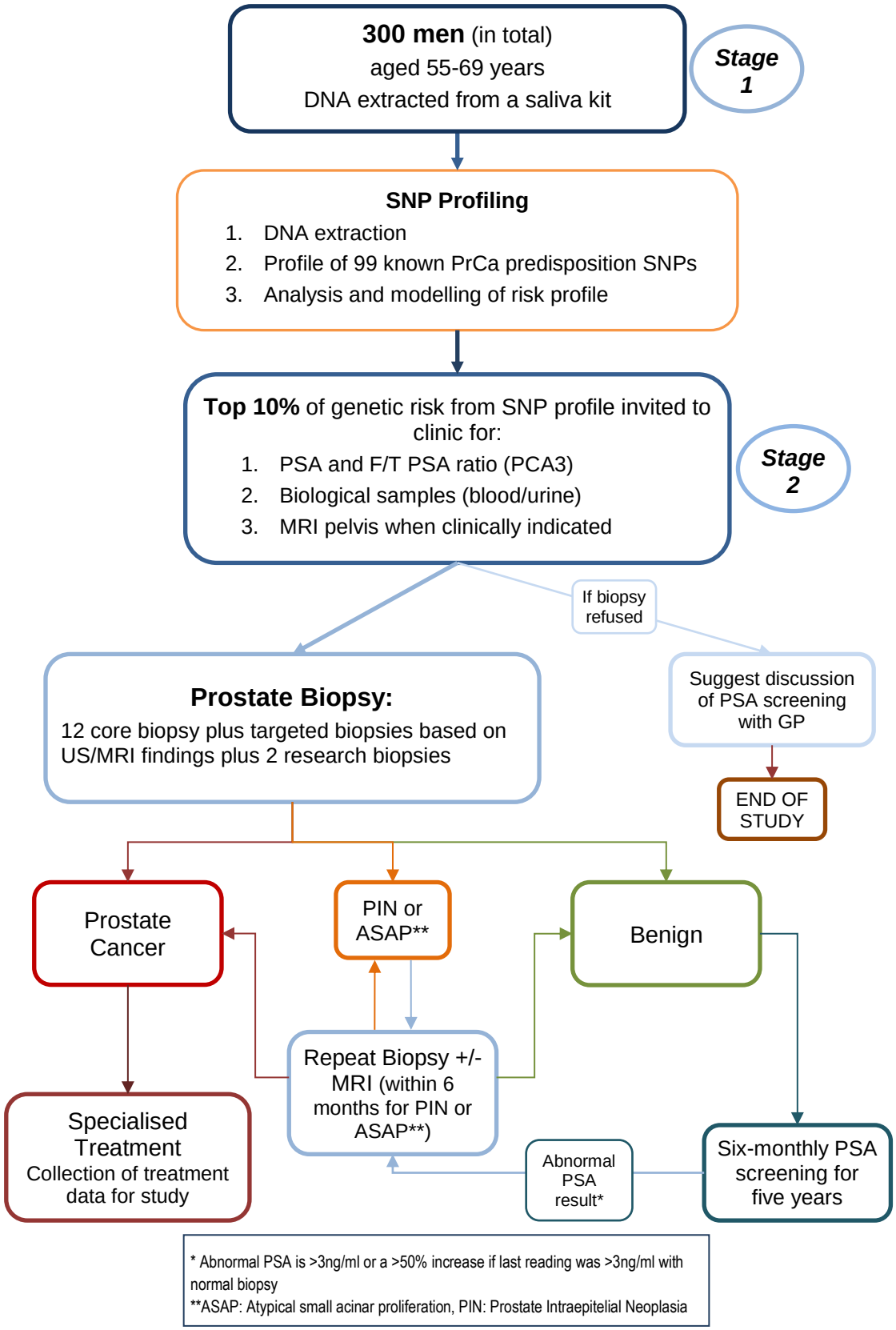
300 men (in total)
aged 55-69 years
DNA extracted from a saliva kit
Stage 1
SNP Profiling
1. DNA extraction
2. Profile of 99 known PrCa predisposition SNPs
3. Analysis and modelling of risk profile
Top 10% of genetic risk from SNP profile invited to clinic for:
1. PSA and F/T PSA ratio (PCA3)
2. Biological samples (blood/urine)
3. MRI pelvis when clinically indicated
Stage 2
If biopsy refused
Prostate Biopsy:
12 core biopsy plus targeted biopsies based on US/MRI findings plus 2 research biopsies
Suggest discussion of PSA screening with GP
END OF STUDY
Prostate Cancer
PIN or ASAP**
Benign
Repeat Biopsy +/- MRI (within 6 months for PIN or ASAP**)
Specialised Treatment
Collection of treatment data for study
Abnormal PSA result*
Six-monthly PSA screening for five years
* Abnormal PSA is >3ng/ml or a >50% increase if last reading was >3ng/ml with normal biopsy
**ASAP: Atypical small acinar proliferation, PIN: Prostate Intraepitelial Neoplasia

Figure 1: Pilot study algorithm

Enrolment

Stage 1

Initially we plan to run a pilot study with 300 men. For further details on the recruitment process, pages 15 & 16 (Figure 2: Recruitment Algorithm and Figure 3: Eligibility Letter algorithm). We aim to recruit patients via GPs. An invitation letter with a participant information sheet, an eligibility questionnaire and consent form will be sent in the post through the GP surgery.

The option to fill in the questionnaire and give provisional consent to the study online will also be provided. Participants who complete the questionnaire online will also be asked two additional questions about this experience. This information will be used when considering the use of online tools for the main BARCODE 1 study.

Once the questionnaire is received, the study team will determine if the patient is eligible and reply via letter. If necessary, a member of the study team will contact the patient to clarify information from the questionnaire and determine if he would be suitable for a prostate biopsy.

If the patient is eligible for the study, a saliva kit will be sent to provide a DNA sample. DNA extraction and SNP profiling will follow. The participant will be informed by letter of whether they fall into the top 10% risk category or not. Those in the top 10% risk category will then receive a follow-up telephone call before attending the hospital to discuss screening options and be offered a prostate biopsy.

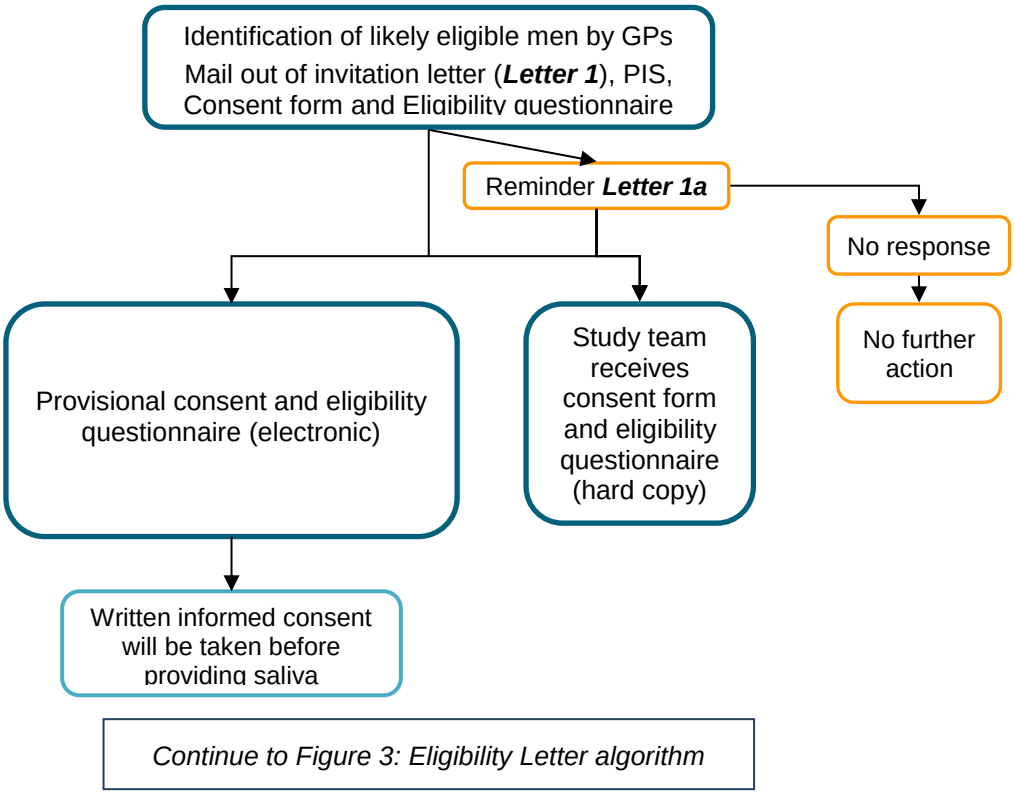

Figure 2: Recruitment Algorithm

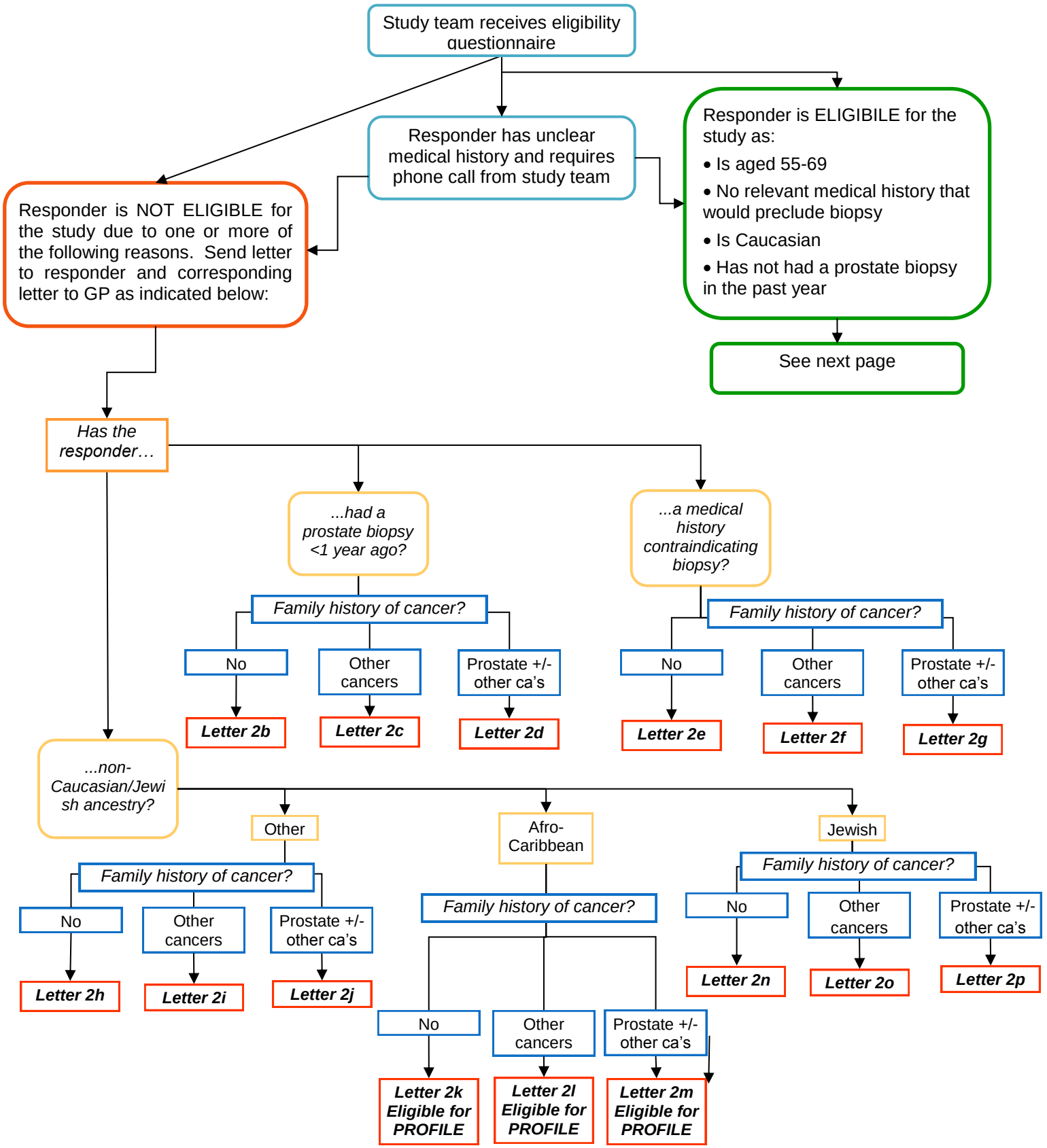

Figure 3: Eligibility Letter algorithm

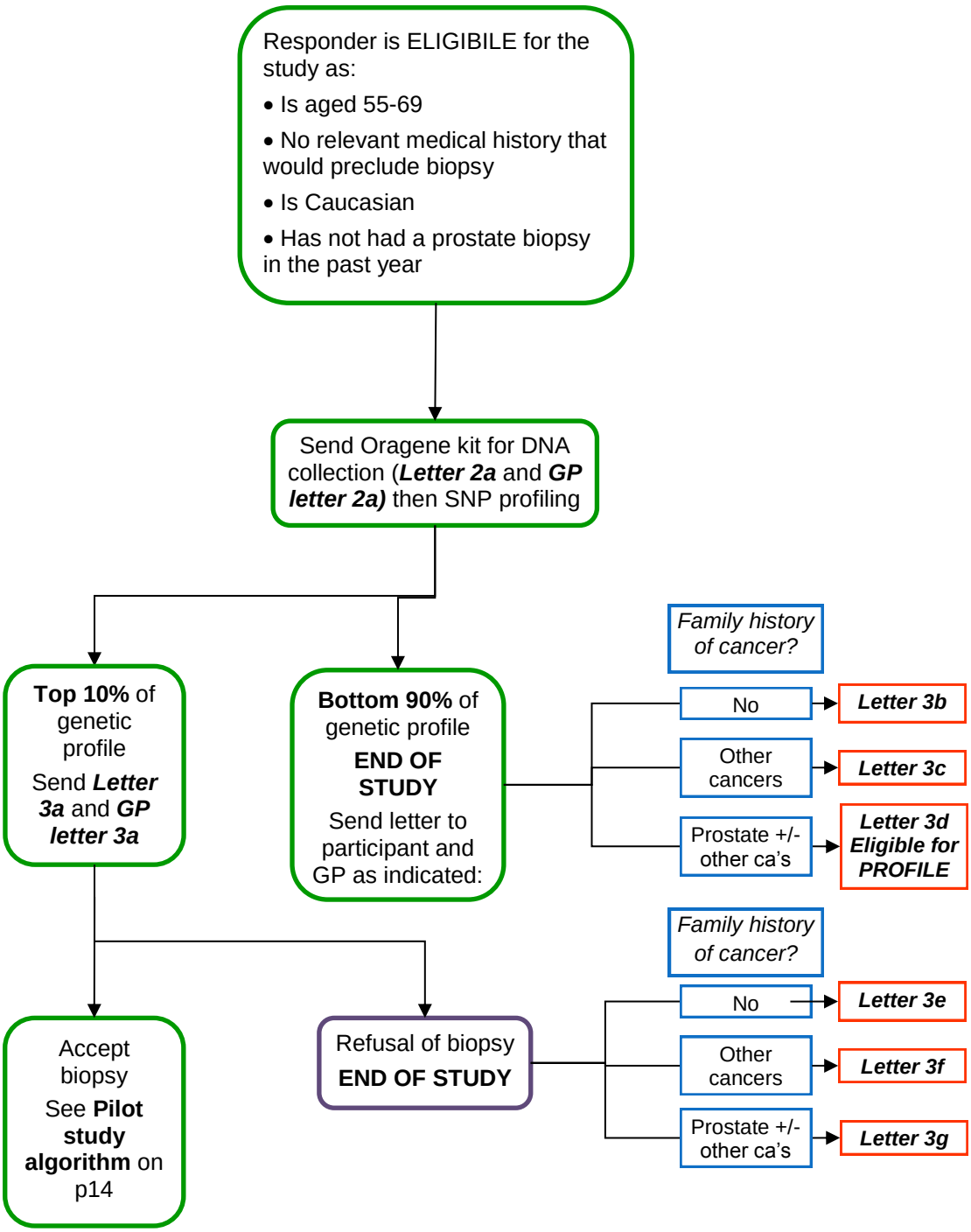

Figure 4: Participant letter algorithm

Stage 2

In the second stage, the 30 men in the top 10% of the genetic risk score will be offered a hospital clinic appointment (see page 14, Figure 1: Pilot study algorithm). Those in the bottom 90% of the risk score would have no further follow up as part of the study. If they have a family history of prostate cancer, they will be referred via letter back to their GP, and will also be offered entry into the PROFILE study (CCR4045, REC reference 13/LO/1787).

Those in the top 10% will be offered four or five hospital appointments at the Royal Marsden Hospital, London. During their first appointment, which will last approximately 45-60 minutes, the participant will be offered a prostate biopsy and also a discussion about the pros and cons of other types of prostate cancer screening, for example PSA testing. They will be counselled about all the study procedures including the potential side-effects of biopsy. Men can decide whether they would like proceed with the study and the biopsy during this first appointment or they can have the opportunity to go away and consider their options. For the latter, if the team has not heard from them within two weeks they will be telephoned to answer any further questions and to either schedule another appointment or to confirm that they do not wish to take part in the study further.

Biological Samples

Biological samples, including blood and urine, will be taken in order to measure PSA levels and the free:total PSA ratio at an appointment prior to biopsy. Research blood and urine samples will also be taken and stored for the study. These samples are taken at the Royal Marsden Hospital, London by trained clinical research fellows and research nurses.

MRI prostate

MRI prostate will be offered to participants when that is considered clinically relevant and to those who are medically suitable.

Digital Rectal Examination

A digital rectal examination ideally should be performed in all participants who are being considered for a prostate biopsy, provided that they consent to it. A prostatic massage is also necessary, in order to acquire first pass urine for PCA3 and TMPRSS-ERG assays.

Prostate Biopsy

A twelve core trans-rectal ultrasound guided biopsy (see Appendix C) will be taken for diagnostic purposes (with additional targeted biopsies where appropriate) and a further 2 samples obtained for research. Consent to take the 2 extra samples for research will be sought before the biopsy procedure commences (optional for patient) and will be immediately snap frozen in dry ice for future DNA and RNA analyses.

In the case of a visible anterior prostate suspicious lesion on MRI, then a template biopsy upfront would be preferable, in view of the risk of a false negative TRUS biopsy in this setting.

All biopsies will be reviewed by one pathologist at the Royal Marsden Hospital using an agreed standardised procedure for our unit's research studies (see Appendix D). If any of the 12 cores identify the presence of PrCa, the subject will receive treatment as advised by their local hospital if they do not wish to have treatment at the Royal Marsden Hospital. All cases will be scanned into a virtual central review database for review by a panel of expert urological pathologists.

Those cases where the first biopsy detects Atypical Small Acinar Proliferation (ASAP) or High Grade Prostatic Intra-epithelial Neoplasia (HG-PIN) will be re-biopsied within 6 months, or sooner according to local (Royal Marsden) guidelines. A repeat DW-MRI could be performed, adding in extra cores depending on the MRI appearance. The repeat biopsy will either be a template biopsy or TRUS biopsy depending on the MRI findings.

Outcome of template biopsy

1. Prostate cancer – treatment as advised by local centre (likely the Royal Marsden Hospital, unless patient wishes to be treated local to their home)
2. ASAP / HG PIN detected – repeat DW-MRI and biopsy in 6 months to 1 year.
3. No abnormalities identified - PSA follow up 6 monthly for 5 years

Refusal of prostate biopsy

For those who do not wish to proceed with prostate biopsy, we will write to them and their GP to recommend a discussion about the option of PSA screening. Their care will be discharged to their GP.

Follow-up after normal biopsy

In light of their increased genetic risk of prostate cancer, men who have a normal biopsy will be offered six-monthly PSA for five years as part of the study. The algorithm for further biopsy has been piloted in our PROFILE pilot study. If men have a PSA >3ng/ml or a >50% increase after a previous PSA >3ng/ml with a normal biopsy, they will be offered a repeat biopsy.

Study follow-up

For men in the top the 10% of risk profile, we will request an update on their medical history from their GP for five years after study completion. For all men, we will monitor any development of cancer through cancer registries.

Review of genetic profiling results

As new information becomes available about the genetic basis of prostate cancer it is possible that we may revisit samples from study participants, for example if a new gene causing prostate cancer is discovered. Where this new information is felt to be clinically relevant we will re-contact the men involved in the study to inform them of the results.

8. Data Acquisition

Stage 1a: Eligibility and Enrolment

- Sign study consent form (hard copy) OR
- Provide provisional consent for the study (electronically)
- Complete eligibility questionnaire that includes information about family history and medical history (electronic or hard copy)

Stage 1b: SNP Profiling

- Sign study consent form (hard copy for those not already provided)
- Saliva sample given
- SNP profiling performed on DNA extracted from saliva
- The genetic profiling results will be disclosed to the participant via letter then via telephone for those in the top 10% of the profile.

Stage 2a: Enrolment (for those in top 10% of genetic profile)

- Offer prostate biopsy and imaging
- Provide blood samples for PSA testing and other biomarkers and 30ml urine sample (first pass) pre and post prostatic massage for PCA3 and other studies (Appendix B – Guidelines for Sample Collection)

Stage 2b: At Biopsy

Each subject will complete the following:

- Sign the local hospital biopsy consent form
- Sign the study-specific biopsy consent form
- Sign MRI consent form (for those offered)

Stage 2c: Biopsy Results

- The biopsy results will be discussed with the participant either in person or by telephone (for negative results only and at the clinician's discretion)

If PrCa is diagnosed

The staging and further investigation of the disease is as directed by the collaborating uro-oncology unit. Management is based on the immediately available pathology report, not on the later central review.

Minimum information required by the study centre will be:

- Clinical T stage

- Gleason score of biopsy and extent of involvement (in percentage of tissue involved an absolute length of core in millimeter)
- Treatment and management plan
- Radiological TNM stage
- Histopathology report for men undergoing radical surgery
- Slides should be scanned into PathXL for central review after the local clinical report has been issued
- Following a diagnosis of PrCa, a treatment questionnaire will be required annually

Stage 3: Study follow up

We will follow all men in the study for development of cancer either through their GP or cancer registries.

Potential adverse events

Side-effects of biopsy:

TRUS biopsy should be carried out in accordance with the study protocol (Appendix C) and antibiotic prophylaxis should be given as per local (Royal Marsden) hospital protocol.

The procedure is uncomfortable and associated with the following risks

- Painful or difficult voiding 13%
- Haematuria 11%
- Fever/sweats 6%
- Septicaemia 3%
- Acute urinary retention 1%

(Taken from Crundwell et al, 1999)

For this reason subjects will be followed carefully and be able to contact the urology department in case of problems.

Venepuncture

Venepuncture a risk of

- Feeling faint,
- Bruising at venepuncture site,
- Excessive bleeding,
- Hitting a nerve,

- Hitting an artery

The procedure should be carried out by those with adequate training and in accordance with local (Royal Marsden) hospital protocol.

9. Data Analysis

- All biopsy interventions and results will be reported to the data centre as they occur. Biopsy results will be reviewed by a central team of pathologists.
- PrCa diagnosis will be reported immediately. The diagnosis and treatment will be based on histological confirmation. A later research central review will be undertaken by a central team of pathologists. If there is disagreement the local diagnosis will be the overriding one for treatment.
- Data completeness (Questionnaires and CRFs) will be evaluated
- Initial translational studies will use the stored serum/urine samples.
- An Independent Data Monitoring Committee will review the study data 6 monthly

10. Study Organisation/ Trial Monitoring and Management Strategy

Administrative Responsibilities

The CI, Clinical Fellow and Study Coordinator (in cooperation with the Data Centre) will be responsible for writing the protocol, submitting to the Committee for Clinical Research and for local management R&D approval, reviewing all case report forms and documenting evaluation forms, discussing the contents of the reports with the Statistician, and for writing the draft of the study results. The CI will also generally be responsible for answering all clinical questions concerning eligibility, treatment, and the evaluation of the subjects.

Steering Committee

It will be the responsibility of the CI to report changes to the protocol and data updates to the study Steering Committee. The Steering Committee will consist of the co-investigators as described in the first page.

Independent Data Monitoring Committee (IDMC)

An Independent Data Monitoring Committee will be set up to regularly review and scrutinise available data and advise on appropriate action.

11. Adverse Events

Definitions

Adverse Events (AE) are any untoward medical occurrence or experience in a patient or clinical investigation subject which occurs following participation in the trial regardless of the causal relationship. This can include any unfavourable and unintended signs or symptoms, an abnormal laboratory finding (including blood tests, x-rays or scans) or a disease temporarily associated with the use of the study, for example:

- death
- a life-threatening event (i.e. the subject was at immediate risk of death at the time the reaction was observed)
- hospitalisation or prolongation of hospitalisation
- persistent or significant disability/incapacity
- any other medically important condition (i.e. important adverse reactions that are not immediately life threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed above)

12. Reporting procedure

Non-serious adverse events

All Adverse Events (AE), occurring during the study until the end of the period of follow-up must be recorded on an adverse event form. All adverse events will be reported to the data centre and logged in accordance with to the local sites Standard Operating Procedures for Adverse Events.

The Chief Investigator will decide if those events are related to the study intervention (i.e. unrelated, unlikely, possible, probable, definitely and not assessable) and the decision will be recorded on the adverse event forms. AEs definitely not study related (i.e. reported as unrelated) will not be considered as adverse events in study analyses, but reported separately. The assessment of causality is made by the investigator using the following definitions:

Relationship	**Description**
Unrelated	There is no evidence of any causal relationship
Unlikely	There is little evidence to suggest there is a causal relationship (e.g. the event did not occur within a reasonable time after administration of the trial medication). There is another reasonable

	explanation for the event (e.g. the subject's clinical condition, other concomitant treatments).
Possible	There is some evidence to suggest a causal relationship (e.g. because the event occurs within a reasonable time after administration of the trial medication). However, the influence of other factors may have contributed to the event (e.g. the subject's clinical condition, other concomitant treatments).
Probable	There is evidence to suggest a causal relationship and the influence of other factors is unlikely.
Definitely	There is clear evidence to suggest a causal relationship and other possible contributing factors can be ruled out.
Not Assessable	There is insufficient or incomplete evidence to make a clinical judgement of the causal relationship.

Serious adverse events

All Serious Adverse Events (SAE), related or not to the study, occurring during the study period and within 30 days after the last study intervention (eg. biopsy) will be reported and logged in accordance with to the local sites Standard Operating Procedures for Adverse Events.

Original SAE reports will be filed in the BARCODE 1 trial masterfile.

13. Statistical Considerations

Sample size

This is a pilot study of 300 men, with approximately 30 in the top 10% genetic risk category. This sample size has been chosen pragmatically to allow us to recruit 100 men from each of the 3 collaborating GP practices.

For the main study (to be a submitted for approval on completion of the pilot study), a sample size of 5000 men will be required to identify the approximately 500 men within the top 10% of the genetic risk score, who will undergo a prostate biopsy. Power calculations show that 99 SNPs have a polygenic variance of 0.44 and therefore those in the top 10% risk category will include 27% of PrCa cases. We estimate that based on our PROFILE pilot study data that we will identify between 38 to 49 new PrCa in the 500 men biopsied, 3 times that of population screening. Our clinically significant cancers would be at least double that expected by PSA screening.

Recruitment timeframes

It is anticipated that the study will complete recruitment within 24 months. The study team will meet monthly to discuss recruitment and will report to the Steering Committee and Data Monitoring Committee six monthly. If there are problems with meeting the target recruitment this will be discussed at the Steering Committee meetings.

Descriptive Statistics

In this pilot study we will be using a variety of descriptive statistical tools to analyse our data. We will first calculate the uptake of the genetic test and also the proportion of men finally accepting a prostate biopsy. We will calculate the Positive Predictive Value (PPV) of prostatic biopsy based on genetic profiling and the cancer detection rate. Both these variables will be treated as binomial probabilities with exact confidence intervals. We will compare our findings to published data from other screening studies. Furthermore, we will assess the correlation of the calculated risk score to our findings.

End of study

The end of study is defined as the date of the last appointment of the last participant.

14. Regulatory & Ethics Committee Approval

Subject protection

The responsible investigator will ensure that this study is conducted in accordance with the Good Clinical Practice (GCP) guidelines, the Data Protection Act 1998 (DPA) and the Human Tissue Act 2004 (HTA) and Codes of Practice for consent issued by the Human Tissue Authority.

All staff at each Trust are required to abide by the Data Protection Act 1998 and also in accordance with the Confidentiality Code of Practice and Data Protection Policy and Procedure. The protocol will be approved by the Committee for Clinical Research at the Royal Marsden NHS Foundation Trust and Institute of Cancer Research and the Research and Ethics Committee.

Subject identification

Once men are found to be eligible for the study and consent to participant, a sequential identification number will be automatically attributed to each subject registered in the trial. This number will identify the subject and must be included on all case report forms. In order to avoid identification errors, subjects' initials (maximum of 4 letters), date of birth and hospital number (if available) will also be reported on the case report forms.

Informed consent

All subjects will be informed of the aims of the study, the possible adverse events, the procedures and possible hazards to which he will be exposed. Each participant will be

informed about the strict procedures used to protect the confidentiality of his patient data, and that his medical records may be reviewed for trial purposes by authorised individuals other than their treating physician.

It will be emphasised that participation is voluntary and that the subject is allowed to refuse further participation in the protocol whenever he wants. This will not prejudice the subject's subsequent care. Documented informed consent must be obtained, according to the principals of GCP, for all subjects included in the study before they are registered at the Data Centre.

The informed consent procedure must conform to the ICH guidelines on Good Clinical Practice. This implies that "the written informed consent form should be signed and personally dated by the subject or by the subject's legally acceptable representative".

Assessment of family history provided to the study

A brief family history will be collected for the purposes of the study, however the study team will **not** provide a detailed, clinical assessment of this family history as part of the study as this is a clinical service beyond the remit of a research team. However, given the nature of the study and that it is being conducted by a genetic research group, it would be irresponsible not to highlight when a referral to a clinical genetics service may be warranted based on reported family history. All family histories reported by participants will be checked by a genetic counsellor/nurse and if a referral to a clinical genetics department is indicated, it will be suggested to the participant to discuss with their GP.

SNP profiling in the study and its clinical utility

It is highlighted throughout the participant materials that the genetic testing (i.e. SNP profiling) provided in the study is a **research** test and as yet the results are not fully understood and are subject to change. Assessment of genetic risk based on SNP profiling is **not** currently a technique used within clinical genetic practice in the UK. The research team will only suggest a referral to a clinical genetics service based upon strength of family history, regardless of SNP profile result.

Queries and concerns of participants and their family members based on SNP profiling result

It is possible that participants and/or their family members will have queries or concerns if the participant falls within the top 10% risk category of the SNP profile. These concerns are very valid given the genetic nature of the risk that is being tested and reported. By its nature, the risk described by SNP profiling is inherited is a very different manner to a monogenic mutation that confers a large increase in risk by itself. The full clinical implications for a family member of someone who has a high risk based on a SNP profile are not fully understood. Given this, clinical assessment of risk is currently based upon family history in the absence of a known high penetrance mutation in a

family and a referral to a clinical genetics service for assessment of such a risk would not be appropriate.

The study team comprises of genetic specialists, including geneticists, genetic counsellors and genetic nurses who have extensive experience in clinical genetics and translational genetics research studies and so are well versed in conversing with patients about these issues. Any concerns raised by the participant or their family about the SNP profiling results can ably be discussed by the research team.

Over diagnosis of prostate cancer

One limitation of prostate screening is the detection of PrCas that would not otherwise have been detected and that may not be of clinical significance. However, these are cohorts of men at genetically higher risk of PrCa. Based on our pilot data we expect up to 50 new PrCa diagnoses in the 500 men biopsied. We estimate that the number of PrCa diagnosed will be 3 times that detected with population screening and the number of clinically significant cancers 2 times the number with population screening. Therefore while this risk of over diagnosis is recognised it is felt to be justified in this particular cohort. This will be discussed with every participant during the consent process as well as all potential treatment options.

We are currently not in a position to predict which of the low grade PrCa diagnosed will develop into a more aggressive tumour. Active surveillance follow up strategies aim to address this issue. Patients are followed up regularly and undergo repeat imaging and prostatic biopsies at regular intervals. Active surveillance is a safe and less invasive approach compared to radical treatment. Biomarkers predicting for an aggressive phenotype are currently in development and will make surveillance much easier. Increasingly, there is an argument that early diagnosis can have a positive impact on outcomes.

15. Data Handling, Record Keeping and Study Samples

Control of data consistency

Data forms will be entered in the database at the Data Centre. Computerised and manual consistency checks will be performed on newly entered forms; queries will be issued in case of inconsistencies. Consistent forms will be validated by the Data Manager to be entered on the master database. Inconsistent forms will be kept "on-hold" until resolution of the inconsistencies.

Use of online data collection

Those who choose to fill in the eligibility questionnaire online will do so via an online interface. The online system utilised will comply with EU data protection requirements (encompassed by the Data Protection Act in the UK), will be approved by the sponsor's

(The Institute of Cancer Research) IT Security team and will undergo a Privacy Impact Assessment.

External review of histology

Histological assessment of prostate biopsies is subject to inter observer variation, particularly with reference to assessing Gleason grade. For this reason biopsies will routinely be reviewed and representative samples will be re-examined by the study pathologists. Clinical decisions will be based on local assessment and a routine review to confirm diagnosis will not be required. If the review in retrospect reports a cancer which was not reported locally then this case will be subject to expert pathological review by the study panel pathologists in conjunction with the local reporting pathologist and an MDT decision taken as to the outcome.

Transfer and storage of data

The Data Centre is the Oncogenetics Team at the Institute of Cancer Research, Surrey. Electronic data will be stored on the ICR network which is routinely backed up. Hard copy data with identifiers will be stored in locked, fireproof cabinets within the ICR, with access limited to staff working on the study who are trained in Data Protection policies and legislation. Transfer of data between the Data Centre and recruitment sites (i.e. GP practices) will take place using password protected files via encrypted Iron Key or encrypted email. Passwords will be communicated by a separate method as per ICR data protection policy.

Retention and destruction of data

Raw data will be retained for 30 years. The Institute of Cancer Research and Royal Marsden Hospital guidelines for archiving of data resulting from non-clinical trials refer to the Research Governance Framework for Health and Social Care in the UK (2005). Clause 2.3.5 of this policy states:

"Data collected in the course of research must be retained for an appropriate period, to allow further analysis by the original or other research teams subject to consent, and to support monitoring by regulatory and other authorities."

Given the study is examining genetic factors associated with cancer and cancer risk, data will be kept for a period of 30 years. Our rapidly changing and growing knowledge of cancer genetics indicates that the genetic results from the study will also evolve and further incidental findings may result. Further review of the data with updated information may be necessary in the future.

Following this period, data will be destroyed according to The Institute of Cancer Research policy, with all hard copy data shredded and electronic data deleted to MoD standards.

Collection, Transfer and Storage of Samples

Blood, urine and biopsy samples will be collected at the Royal Marsden Hospital by trained clinical research fellows and research nurses. Saliva samples are provided by the participant using well validated collection kit in their own home and sent to the study team at The Institute of Cancer Research, complying with biological sample transfer guidelines of the European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR).

Biological samples will be stored at The Institute of Cancer Research, Surrey in accordance with the joint Institute and Royal Marsden Hospital policy for removal, storage, use and disposal of human tissue for research, Blood, urine and biopsy samples will be stored in -80 freezers and saliva samples at room temperature in storage facilities on site. No samples will be transferred en masse from other research sites. Retention and destruction of samples

Participants are given the option of consenting to the use of their biological samples in this research study and an additional option of 'generic' consent for use in future studies, subject to ethical approval. This is in line with guidance from the Health Research Authority, which encompasses requirements of the ICH Good Clinical Practice, the European Clinical Trial Directive 2001/20/EC, the UK Medicines for Human Use (Clinical Trials) regulation 2004. Participants can request their samples be withdrawn from future study use and destroyed at any time.

If requested, samples will be destroyed in a manner appropriate for biological waste according to ICR guidelines and a record kept of this destruction.

16. Financing, Indemnity & Insurance

This study has received funding from the European Union within the ERC Advanced Grant 2013. This is funding dedicated to support innovative studies.

The standard NHS indemnity procedures will apply at each collaborating hospital. Each participating site is responsible for ensuring insurance and indemnity arrangements are in place to cover the liability of the Principal Investigator.

Liability rests with the study sponsor – the Institute of Cancer Research and a Research Agreement will be in place with each collaborating centre specifying the liability arrangements.

The study sponsor, the Institute of Cancer Research has no special compensation arrangements for this study. The NHS Litigation Authority covers standard clinical negligence of NHS employees, staff and health professionals under its Clinical Negligence Scheme for Trusts.

17. Publication Policy

The Chief Investigator together with the team at the data centre will write the final publication of the study results. A draft manuscript will be submitted to all co-authors (the study team, two named individuals from each collaborating centre and all members of the steering committee) for comments. After revision by all co-authors the manuscript will be sent to a major scientific journal.

The CI, the Study Coordinator and the Data Centre must approve all publications, abstracts and presentations based on subjects included in this study. This is applicable to any individual subject registered in the trial, or any subgroup of the trial subjects.

18. Appendices

APPENDIX A

WHO scale for performance status

Grade	Performance scale
0	Able to carry out all normal activity without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out light work.
2	Ambulatory and capable of all self-care but unable to carry out any work; up and about more than 50% of waking hours.
3	Capable of only limited self-care; confined to bed or chair more than 50% of waking hours
4	Completely disabled; cannot carry on any self-care; totally confined to bed or chair.

APPENDIX B

GUIDELINES FOR SAMPLE COLLECTION

For all samples blood should be drawn:-

- Prior to any manipulation of the prostate
- At least 24h following ejaculation (if within 24h the time should be noted)
- 6 weeks after resolution of prostatitis

Each centre must record for each sample:-

- The tube used to collect the sample (should include full details of tube type and manufacturer)
- Time of blood draw
- Time and temperature of centrifugation (where appropriate)
- Time and temperature of storage

Samples to be collected:

Please note that ideally all samples should be processed and frozen as soon as possible on the day that they were taken. If samples cannot be processed on the day then samples should be processed in the lab chronologically.

All blood tubes should be gently inverted (10-15 times) before being placed in the centrifuge.

1. Serum for routine quality control

Collection tubes: Plain –BD Vacutainer SST II Advance 8.5ml (sterile, gel, plain to promote clotting, plastic) is recommended.

Centrifuge: Leave the sample to clot for approximately 30 minutes and then centrifuge at ~2200rcf for 15 minutes.

Aliquots: Remove serum with a sterile pipette and aliquot into 4 equal volumes (approximately 0.5mL) in 1.8mL Nunc Cryotubes

Storage: The aliquots should be transferred to a -80^{o}C freezer as soon as possible. (The samples may be stored at 4^{o}C for up to 24 hours).

2. Plasma Heparin

Collection tubes: Plasma Heparin – BD Vacutainer LH PST II 8.0ml (sterile, gel, heparin to prevent clotting, plastic) is recommended

Centrifuge: Leave the sample to clot for approximately 30 minutes and then centrifuge at ~2200rcf for 20 minutes.

Aliquots: Remove plasma with a sterile pipette and aliquot into 4 equal volumes (approximately 0.5mL) in 1.8mL Nunc Cryotubes

Storage: The aliquots should be transferred to a -80^{o}C freezer as soon as possible. (The samples may be stored at 4^{o}C for up to 24 hours).

3. Serum

Collection tubes: Plain –BD Vacutainer SST II Advance 8.5ml (sterile, gel, plain to promote clotting, plastic) is recommended.

Centrifuge: Leave the sample to clot for approximately 30 minutes and then centrifuge at ~2200rcf for 10-20 minutes.

Aliquots: Remove serum with a sterile pipette and aliquot into 4 equal volumes (approximately 0.5mL) in 1.8mL Nunc Cryotubes

Storage: The aliquots should be transferred to a -80^{o}C freezer as soon as possible. (The samples may be stored at 4^{o}C for up to 24 hours).

4. Plasma EDTA

Collection tubes: EDTA –BD PPT, K2E 15.8mg, 8.5ml (sterile, gel, EDTA to prevent clotting, plastic) is recommended.

Centrifuge: Centrifuge at ~2200rcf for 20 minutes as soon as possible.

Aliquots: Remove serum with a sterile pipette and aliquot into 4 equal volumes (approximately 0.5mL) in 1.8mL Nunc Cryotubes

Storage: The aliquots should be transferred to a -80^{o}C freezer as soon as possible. (The samples may be stored at 4^{o}C for up to 24 hours).

5. Sodium Citrate

Collection tubes: Vacutainer Light Blue top 2.7ml tubes with 0.109m Sodium Citrate (pH 5.7) #363083) is recommended.

Centrifuge: Centrifuge at ~2200rcf for 20 minutes as soon as possible.

Aliquots: Remove serum with a sterile pipette and aliquot into 4 equal volumes (approximately 0.5mL) in 1.8mL Nunc Cryotubes

Storage: The aliquots should be transferred to a -80^{o}C freezer as soon as possible. (The samples may be stored at 4^{o}C for up to 24 hours).

6. Whole Blood for DNA extraction

Collection tubes: EDTA –BD Vacutainer KTE 10.8mg, 6ml (sterile, EDTA to prevent clotting, plastic, for DNA extraction) is recommended.

Storage: No processing required. Transfer to -80^{o}C freezer as soon as possible. (The samples may be stored at 4^{o}C for up to 24 hours).

7. Saliva for DNA extraction

Collection tubes: Oragene saliva collection tubes

Storage: No processing required. The samples should be stored at room temperature until DNA extracted.

RNA for expression studies

Collection tubes: Either PAXgene™ Blood RNA Tube, PreAnalytiX GmbH, Homobrechtikon, CH, 2.5ml, Vacutainer Brand plug.

Or Applied Biosystems

Storage: No processing required. The samples should be left overnight at room temperature before freezing at –80^{o}C.

8. Urine collection pre prostatic massage

Collection tubes: 30ml in a universal plastic container.

Procedure: The first pass urine should be collected

Storage: The samples should either be or kept in the original container (if freezable) or decanted directly into two approximately 10 ml aliquots in freezable falcon tubes and transferred to a –80^{o}C freezer as soon as possible. Do not overfill these containers as this could cause cracking on freezing due to volume expansion.

9. Urine collection post prostatic massage (for PCA3)

Massage prostate three times with finger via DRE

Collection tubes: 40mL in a 60mL universal plastic container.

Procedure: The first pass urine will be collected following a DRE. The physician will perform a DRE as follows. *Apply firm pressure on the prostate from the base to the apex and from the lateral to the median line of each lobe. Apply enough pressure to slightly depress the prostate surface. Perform exactly 3 strokes per lobe.*

Following DRE, the subject will collect the first 40 mL of urine in a labelled 60 mL urine collection cup. If the subject cannot stop his urine flow and provides more than the 40 mL, the entire volume will be kept. If the subject is unable to provide this quantity, collect at least 20 mL. Record the time and volume of urine collection on the Case Report form.

In order to test the urine sample with the PCA3 assay, the sample must be processed with the urine specimen collection kit per the PCA3 assay package insert instructions. Urine samples should be maintained at 2 to 8°C and refrigerated for no longer than 4 hours if not processed immediately.

1. Invert urine sample (in urine collection cup) 5 times to re-suspend cells.

2. Using the transfer pipette, transfer 2.5 mL of urine to an appropriately labelled PCA3 transport tube. The correct volume of urine has been added to the transport tube when the fluid level is within the black fill lines.

3. Screw cap on the PCA3 transfer tube tightly, then invert the transport tube 5x to mix.

4. Two additional aliquots of processed urine specimens will be made by following the same procedures in steps 1 through 3 above, volume permitting. There should be a total of 3 processed urine specimens; extra processed urine specimens will be used for repeat testing, if necessary, and research studies as described in this protocol.

5. Screw cap on the PCA3 transfer tube tightly, then invert the cup 5x to mix.

6. The remaining urine will be transferred to a 50 mL transfer tube with orange cap containing 4 mL of 0.5M EDTA.

7. Screw cap on the 50 ml transfer tube tightly, then invert the transport tube 5x to mix.

Storage: TBC.

10. Biopsy samples

Collection tubes: Each sample should be placed directly into a 1.8mL Nunc Cryotube with no preservative and placed immediately into dry ice.

Storage: The cryotubes should be transferred to a -80^{o}C freezer as soon as possible after the procedure.

APPENDIX C

The 12 biopsy cores should be taken from the following locations:

Right peripheral zone base

Right transitional zone base

Right peripheral zone mid

Right transitional zone mid

Right peripheral zone apex

Right transitional zone apex

Left peripheral zone base

Left transitional zone base

Left peripheral zone mid

Left transitional zone mid

Left peripheral zone apex

Left transitional zone apex

In addition to these 12 standard cores, cores should also be targeted according to MRI findings (where applicable).

Two research cores should be obtained in addition to the above, one from the right side and one from the left side of the prostate. For storage / processing, see Appendix B.

APPENDIX D

Processing and Reporting Prostatic Biopsies

By Professor Chris Foster

Number of Cores

Multiple reports form the U.S. and Europe have confirmed that "sextant" sampling methods "miss" a significant percentage of cancers in the first biopsy procedure and that an extended biopsy approach yields higher detection rates. The number of cores recommended in these studies is variable ranging from a minimum of 8 cores to extensive biopsy schema. Most reports have advocated 10-12 cores (Fink, Hutarew et al. 2001, Stewart, Leibovich et al. 2001, Bott, Young et al. 2002, Durkan, Sheikh et al. 2002, Haggarth, Ekman et al. 2002, Taylor, Gancarczyk et al. 2002, Matlaga, Eskew et al. 2003). It might be argued that the precise technique adopted in an individual patient depends upon whether radiographic abnormalities have been identified within the prostate or whether prostatic biopsy is being employed as a "blind" screening procedure following detection of an elevated PSA or digital rectal abnormality. However, if performed correctly, a standard protocol-based procedure should identify, locate and map all the essential information with respect to the majority of prostate cancers. At the initial biopsy, a minimum of 12 cores should be taken in standard positions with extra cores targeted to areas of MR abnormality (Damiano, Autorino et al. 2003). The use of 12 as opposed to 6 cores increases prostate cancer detection frequency by 23.5% and the greatest benefit is in those with a PSA of <4ng/ml which is the most likely scenario in PROFILE (Thiesler et al., 2007)

Location, Anatomic Source of the Cores

All the above-cited studies reported significantly improved cancer detection when the most lateral "subcapsular" peripheral zone of the prostate including the anterior "horns" and the apex were biopsied. Sampling these compartments according to different studies results in reducing the sextant false negative rates by 20-35%, with a recent report indicating that the extended biopsy schemes minimizes PSA and age related detection rates. The recommended scheme i.e. a modification of that introduced by Presti et al, comprising 10 biopsies, (6 sextant and 2 lateral and apical on each side) (Presti, Chang et al. 2000). This approach limits the biopsy scheme to 6 central cores with an emphasis on the lateral peripheral zones (de la Taille, Antiphon et al. 2003). This 10-core biopsy protocol that emphasises lateral and apical placement (Bauer, Zeng et al. 2000) enhances detection of peripheral zone cancers, as we demonstrated in a comparative study (Philip J et al, 2004). We further confirmed the positive effect of sampling the peripheral region of the prostate, even when using a 12-core technique (Philip J et al., 2006). Without this lateral direction, 12-core biopsies may be negative despite a very high index of suspicion of prostatic malignancy (Abd, Goodman et al. 2011, Serefoglu, Altinova et al. 2012). This is probably because many cancers originate peripherally (Presti, Chang et al. 2000). Any hypoechoic areas in the peripheral zone should be included in the biopsy strategy. In addition, it may be necessary to perform digitally guided biopsies of an indurated or suspicious area. Recommendations to maximise cancer detection have included strategies incorporating more regions such as transition and lateral peripheral zones (Epstein, Walsh et al. 1997, Levine, Ittman et al. 1998).

Considerations for Gland Volume

Detecting prostate cancers in larger prostates is often more difficult than in smaller glands. While more studies suggest that obtaining more cores from larger prostates can increase the rate of cancer detection, a recent report on 750 patients acknowledged the inverse relationship between

gland volume and ability to detect prostate cancer in larger glands, disputes the value of more core biopsies (Durkan, Sheikh et al. 2002). Thus, it may be beneficial to obtain more biopsy cores from large volume glands. However, there are no objective evidence-based data to support such a presumption.

Length and Diameter of Cores, Type of Needles Used

It is important to provide adequate diagnostic material with an effort to obtain intact cores. This is directly dependent on the type of needle biopsy gun employed and the training and dexterity of the operator. Assessment of training and efficiency should be monitored by audit.

Maintaining Source Identification of Individual Cores When Sent for Pathological Examination

To alleviate workload in the laboratory, it has been suggested that cores from the apex, mid and base from one side of the prostate can be submitted in one container and reported collectively. Adopting such a protocol is suboptimal and contravenes established WHO (Bostwick, Foster et al. 2000) and European (Boccon-Gibod, van der Kwast et al. 2004) guidelines. Whatever the employed protocol, it is important to maintain separation of biopsy samples according to side (right/left) throughout submission and pathology reporting. Samples obtained via modifications of the sampling protocol (such as few cores from a palpable abnormality), need to be oriented and kept separately for processing and reporting.

Assessment of a patient as a potential candidate for locus-specific treatment (i.e. radical prostatectomy or selective radiotherapy) requires the comprehensive accumulation of data from several distinct clinical, radiological and pathological sources. Key to this assessment is a detailed understanding of the precise location, and possible extent, of an identified prostate cancer. Therefore, individual prostatic tissue core biopsies, taken separately, should be retained and processed separately and not "lumped together" in single cassettes. Furthermore, the practice of attempting to arrange multiple needle-cores of tissue into single cassettes in some sort of sequence marked by the presence of some identifiable agent, or non-prostatic tissue (e.g. mouse liver has been suggested) should be discouraged as unnecessary:

- Introduction of unwarranted complexity.
- Increased likelihood of error with respect to identification of individual cases.
- Increased handling of tissues.
- Increased need to cut multiple sections to fully examine each of the tissue cores with consequent loss of tissue for additional studied (e.g. immuno-histochemistry).

While apparently pragmatic, it is probable that a cost-benefit assessment of "tissue aggregation" is likely to indicate the compromise of detailed information for the unlikely gain of speed in tissue processing, and hence should be discouraged.

Guidelines for Adequate Prostatic Needle Biopsy Processing

Irrespective of any screening programme, heightened awareness of prostate cancer in the general population, together with increased digital rectal examination and use of PSA testing has increased the detection of early prostatic neoplasia. By definition, many of these lesions tend to be smaller in size and to approximate closer to the normal range of morphological appearances, thus making diagnosis more difficult (Epstein 2004). Some guidance is suggested that might assist in resolving this dilemma:

The number of biopsies embedded in one cassette

Urologists want to know at which site the prostate cancer is located. This information may help to decide whether a unilateral nerve sparing prostatectomy is possible. In cases of lesions suspect for adenocarcinoma, it is important to know their localization for site-specific repeat biopsy. It is considered preferable that each biopsy core is embedded in a manner that it may be identified uniquely. Originally, this was considered to be separately (Boccon-Gibod, van der Kwast et al. 2004). However, indelible colour-marking at the time of grossing and cassetting allows several cores to be aligned parallel to one another and processed simultaneously. This recommendation was not given explicitly in previous guidelines (Bostwick, Foster et al. 2000).

The procedure of embedding of needle biopsies into paraffin wax

The objective is to achieve a maximum amount of tissue for microscopic evaluation since this correlates with the cancer detection rate (Iczkowski, Casella et al. 2002, van der Kwast, Lopes et al. 2003). However, needle biopsies tend to become curved after fixation and flat embedding of the biopsy cores enhances the amount of tissue that is examined by the pathologist. Strengthening of biopsy cores can be achieved by stretching the needle biopsy tissue between two nylon meshes or by wrapping them in a piece of paper. This can be done even after initial formalin fixation. Such manipulations are not recommended because manual handling, however minimal, is associated with traumatisation to the tissue and impaired morphology.

The number of sections from each biopsy core (levels of sectioning)

Earlier reports (Bostwick, Foster et al. 2000, Iczkowski, Casella et al. 2002) have demonstrated that it is mandatory to cut several sections of each biopsy core at different levels in order not to miss small foci of adenocarcinoma. Cutting biopsy cores at different levels may allow a definite diagnosis of adenocarcinoma when a small focus is found at a single level. Practically, laboratories need to agree a single strategy for cutting and staining prostatic needle biopsy specimens. Reyes and Humphrey provide strong evidence that complete histologic sampling with serial sections entirely through the paraffin wax block is unnecessary (Reyes and Humphrey 1998). Their study of 200 consecutive cases showed that the initial three slides, each containing several sections, identified all of the contained cancers, thus making further work redundant. Furthermore, after an initial diagnosis of pure high-trade PIN, generation of additional sections is also unnecessary. Rather, the patient should undergo clinical follow-up and full rebiopsy. It is recommended that sections of a core at two different levels are sufficient. Ribbons between the two levels can be stored for cases where additional histologic slides or immunohistochemistry are required.

The length of each biopsy core should be recorded as an integrated part of the macroscopic description for comparison with the length on the glass slide.

Guidelines for Uniform Reporting of Prostate Lesions

Reporting of the histopathology of prostatic needle biopsies is performed in accordance with ISUP 2005 guidelines (Epstein et al 2005) and should be as unequivocal and concise as possible. This means that the nomenclature of prostatic lesions in pathology reports should be uniform. Terms like “atypical glands”, “glandular atypia”, “probably malignant”, but “benign not excluded” should be avoided, since it is not clear to the urologist, which further action should be taken. The adequacy of prostatic needle biopsies should be mentioned in the pathology report. An inadequate prostatic core biopsy core is defined as a core lacking glandular structures, is traumatized or is fragmented such that a diagnosis of prostate cancer cannot be reliable

confirmed or excluded. The underlying terms seem to have proven their value and consistency in the last several years:

Benign

This includes fibromuscular or glandular hyperplasia, various forms of atrophy as well as foci of chronic (lymphocytic) inflammation. Although multiple biopsies with post-atrophic hyperplasia may be reported as such, in itself this finding has no clinical consequence. Distinctions between the above entities are of limited clinical relevance and subject to considerable inter-observer variation (Oppenheimer, Kahane et al. 1997). Pathologists should make themselves aware of benign prostatic lesions that mimic carcinoma (Foster and Sakr 2001).

Acute inflammation

This lesion is characterized by damage to glandular structures. This finding might explain increased serum PSA levels.

Chronic granulomatous inflammation

Includes xanthogranulomatous inflammation. This condition can cause strongly elevated PSA levels and cause a false positive digital rectal examination.

Adenosis

Adenosis fortunately is a very rare finding in peripheral zone derived needle biopsies. Adenosis which is characterised by a condensation of small glands surrounded by sporadic basal cells is also known as atypical adenomatous hyperplasia (Bostwick, Srigley et al. 1993). The latter term is not recommended because the term “atypical” may suggest a relation with malignancy.

Prostatic intra-epithelial neoplasia (PIN)

Although initially low grade and high grade PIN were distinguished, only (high grade) PIN is reported. Cytological and nuclear abnormalities contributing to the various entities recognised as “low grade” PIN has no prognostic relevance. Only “high grade” PIN is associated with an adverse risk of developing prostate cancer. Therefore, HGPIN is now reported simply as ‘PIN’. The extent and architectural pattern of PIN may also be reported, since some of these variants (solid, comedo and cribriform) may be associated with unfavourable prostate cancer as they may represent intraductal spread of high-grade cancer [(Cohen, McNeal et al. 2000)]. Isolated diagnosis of HG PIN necessitates a repeat biopsy within six months. There is a strong association of previous PIN with cancer (Meng, Shinohara et al. 2003). Men with PIN have been reported to have up to 36% cancer detection rates in subsequent biopsies (Davidson, Bostwick et al. 1995, Goeman, Joniau et al. 2003).

Atypical small acinar proliferation (ASAP)

This entity is not *per se* malignant, but may be a harbinger, if not a precursor, of malignancy and therefore requires to be identified and reported. Prostate needle biopsies occasionally contain cytologically and architecturally atypical small acinar proliferations (ASAP) that are suspicious for, but not diagnostic of, adenocarcinoma. These histological appearances include the number of acini per focus of ASAP, number of foci, variation in acinar size, nuclear enlargement, presence of luminal mucin, crystalloids, adjacent focal chronic inflammation, adjacent atrophy, and adjacent

prostatic intraepithelial neoplasia (PIN). Stratification of suspicion in cases of ASAP without PIN results in "favor benign", "uncertain", and "favor carcinoma". In an otherwise benign biopsy, the high predictive value of ASAP for subsequent adenocarcinoma promotes a repeat biopsy. Nevertheless, no single clinical or pathologic feature has been identified that increases the likelihood of subsequent cancer.

Adenocarcinoma

The location(s) of the foci of adenocarcinoma should be recorded. In this way the number of positive biopsies is implicitly known to the clinician. If a small focus (< 3 mm) of adenocarcinoma is present in only one needle biopsy this may be recorded in the conclusion as "focal adenocarcinoma". It is also recommended to estimate the proportion of tumour involvement of the needle biopsies, particularly with the advent of quantitative prostate biopsy for prediction of organ confined disease (Haese, Chaudhari et al. 2003). The extent of cancer involvement may be given in percentage of the biopsy core lengths (e.g. > 5%, 10%, 20%, etc).

Appearance suspicious, but not diagnostic, of adenocarcinoma

If the lesion is too small and/or lacks sufficient criteria to be able to make a definite diagnosis of adenocarcinoma (Cheville, Reznicek et al. 1997, Epstein 1999).

The possibility of other malignancies, including carcinosarcoma, sarcoma and adenocarcinoma of the colon etc. masquerading as prostatic carcinoma should be considered. When adenocarcinoma, high grade PIN, or lesions suspicious for adenocarcinoma are present at separate sites, these should also be reported separately.

Reporting grades of differentiation

It is recommended to use the Gleason scoring system. Advantages of this grading system are its general use and the large amount of data in the literature on its prognostic impact and accuracy. As advocated by Epstein (Epstein 2000) Gleason scores of 2 to 4 to prostatic adenocarcinoma should not be attributed on peripheral zone needle biopsies. It is recommended that the lowest Gleason growth pattern that can be assessed in needle biopsies is growth pattern 3, implying that a Gleason score of 6 is the lowest possible on peripheral zone needle biopsies (Epstein, Allsbrook et al. 2005).

An important feature of the Gleason system is that it takes into account the heterogeneity of prostate cancer by including the two most prominent growth patterns. Thus, in sextant needle biopsies the Gleason score can range from 6 to 10. The location of a separate area of high grade (Gleason growth pattern 4 or 5) cancer should always be reported irrespective of its extent in the needle biopsy (Srigley, Amin et al. 2000). In radical prostatectomy specimens a second growth pattern that comprises less than 5% of the tumour area is not included in the Gleason score. This rule does not apply for high-grade cancer in prostatic needle biopsies: Irrespective of the amount of the second growth pattern it is included in the Gleason score. If, in addition to growth pattern 3, both pattern 4 and 5 are present in the needle biopsies the pattern 5 will be included in the Gleason score (i.e. 3 + 5 = 8).

Immunohistochemistry

Of all special investigations available to diagnostic surgical pathologists only immunohistochemistry has yet found a regular place in the compendium of techniques routinely-

accepted techniques. Antibodies to detect high-molecular weight cytokeratins (Brawer, Peehl et al. 1985, Purnell, Heatfield et al. 1987, Grignon, Ro et al. 1988, Hedrick and Epstein 1989, Devaraj and Bostwick 1993) and to αMeCo racemase (Xu, Stolk et al. 2000, Jiang, Woda et al. 2001, Luo, Zha et al. 2002, Rubin, Zhou et al. 2002) are principally employed. Antibody 34βE12 (previously known as "keratin 903" and generated by Gown and Vogel in 1982 (Gown and Vogel 1982) reveals absence of basal cells from glandular epithelial structures to be indicative (but not diagnostic) of malignant change. Conversely, enhanced expression of αMeCo racemase (identified as P504S and first reported by Xu et al. (Xu, Stolk et al. 2000) occurs in neoplastic prostatic epithelial cells of both luminal and basal types (Evans 2003). Both reagents should be used by experienced immunohistochemistry and interpreted with caution by experienced diagnostic pathologists to avoid erroneous interpretation of appearances. It cannot be emphasized strongly enough that underpinning such diagnostic adjuncts is the "Gold Standard" of good morphological assessment.

Quality control indicators

The standardization of processing and reporting on prostate needle biopsies, will be increasingly important in order to assure quality and to avoid medico-legal complications.

As a quality indicator the average length of needle biopsies and the percentage of inadequate biopsies can be used. The frequency of suspect lesions might give an indication as to the level of certainty reached by the pathologist. This is of course related to several factors, including the population under study, the quality of needle biopsies and their processing as well as the staining and the confidence of the pathologist. The percentage of suspect lesions should not rise above 5% since this will lead to a too frequent indication of repeat biopsies.

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