Tumor Genomic Testing for >4000 Men with Metastatic Castration-resistant Prostate Cancer in the Phase III Trial PROfound (Olaparib)

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Statement of translational relevance

Precision medicine with poly(ADP-ribose) polymerase inhibitors is now available for men with metastatic castration-resistant prostate cancers (mCRPC) harboring homologous recombination repair gene alterations. Genomic sequencing to identify actionable mutations face different challenges in different cancers. The PROfound phase III trial (NCT02987543) molecularly prescreened patients with mCRPC for eligibility and to date constitutes the largest dataset of prostate cancer samples sequenced in real time for the purposes of determining eligibility for participating in a prospective clinical trial. In PROfound, 69% of patients had a next-generation sequencing (NGS) result. Evaluation of sample characteristics highlighted that the availability of a high-quality tumor tissue sample is key to obtaining an NGS result. The observations and learnings from PROfound will educate and provide guidance and encourage uptake of genomic testing in this disease area.

Abstract

Purpose: Successful implementation of genomic testing in clinical practice is critical for identification of men with metastatic castration-resistant prostate cancer (mCRPC) eligible for olaparib and future molecularly targeted therapies.

Patients and Methods: An investigational clinical trial assay, based on the FoundationOne®CDx tissue test, was used to prospectively identify patients with qualifying homologous recombination repair (HRR) gene alterations in the phase III PROfound study. Evaluation of next-generation sequencing (NGS) tissue test outcome against pre-analytical parameters was performed to identify key factors influencing NGS result generation.

Results: 4858 tissue samples from 4047 patients were tested and reported centrally. NGS results were obtained in 58% (2792/4858) of samples, equating to 69% of patients. Of samples submitted, 83% were primary tumor samples (96% were archival and 4% newly obtained). Almost 17% were metastatic tumor samples (60% were archival and 33% newly obtained). NGS results were generated more frequently from newly obtained compared with archival samples (63.9% v. 56.9%), and metastatic compared with primary samples (63.9% v. 56.2%). Although generation of an NGS result declined with increasing sample age, approximately 50% of samples aged >10 years generated results. While higher tumor content and DNA yield resulted in greater success in obtaining NGS results, other factors, including selection and preservation of samples, may also have had an impact.

Conclusions: The PROfound study demonstrates that tissue testing to identify HRR alterations is feasible and that high-quality tumor tissue samples are key to obtaining NGS results and identifying patients with mCRPC who may benefit from olaparib treatment.

Introduction

Metastatic castration-resistant prostate cancer (mCRPC) is a molecularly heterogeneous disease, with 20–30% of patients harboring deleterious alterations in DNA damage repair genes, including those with direct or indirect roles in homologous recombination repair (HRR) (1-4). The PROfound study (NCT02987543) is the first phase III, randomized, multicenter trial to show that a poly(ADP-ribose) polymerase (PARP) inhibitor, olaparib, improves radiographic progression-free survival (rPFS) and overall survival compared with control (physician's choice of next-generation hormonal agent [NHA] enzalutamide or abiraterone) in patients with mCRPC with alterations in genes involved directly or indirectly in HRR and disease progression on prior NHA (1,5). It is the largest PARP inhibitor study to date (6) to conduct central, prospective tissue next-generation sequencing (NGS) to screen patients to determine eligibility for enrollment. Patients were required to have a qualifying alteration in one or more of the 15 pre-specified genes for enrollment into one of two trial cohorts (Cohort A: *BRCA1*, *BRCA2*, *ATM*; Cohort B: *BRIP1*, *BARD1*, *CDK12*, *CHEK1*, *CHEK2*, *FANCL*, *PALB2*, *PPP2R2A*, *RAD51B*, *RAD51C*, *RAD51D*, *RAD54L*). Based on the findings of the PROfound study, the US Food and Drug Administration (FDA) approved olaparib for men with deleterious or suspected deleterious germline or somatic HRR gene-altered mCRPC (*PPP2R2A* not included) and disease progression following prior NHA. In addition, other agencies, including the European Medicines Agency (EMA), the Japanese Pharmaceutical and Medical Device Agency and the Australian Therapeutic Goods Agency, have approved olaparib for patients with *BRCA1* or *BRCA2* alterations and Health Canada has approved olaparib for patients with *BRCA1*, *BRCA2* or *ATM* alterations (7,8).

Identifying patients with mCRPC who may benefit from olaparib through molecular diagnostics can improve outcomes for patients with a poor prognosis. Guidelines from the National Comprehensive Cancer Network (NCCN), European Society for Medical Oncology (ESMO) and American Urological Association/American Society for Radiation Oncology/Society of Urologic Oncology (AUA/ASTRO/SUO), as well as other societies, have recently been updated to include recommendations of genomic tumor testing for HRR gene alterations in patients with prostate cancer (9-11). However, while tumor testing is the gold standard for molecular testing, ensuring it is routinely used remains a challenge. Identifying factors which may increase the likelihood of an NGS result being obtained is critical in encouraging routine uptake of tumor tissue testing in clinical practice.

Here, we report the results of molecular diagnostic testing of tumor tissue samples provided by over 4000 patients with mCRPC who were screened for the PROfound study. We discuss the real-world challenges to testing, and aim to provide insights into how to maximize generation of NGS results based on our findings.

Methods

Study design

The PROfound study (NCT02987543) is a phase III, prospective, randomized, open-label, multicenter, global trial. The study comprises two cohorts: Cohort A includes patients with alterations in *BRCA1*, *BRCA2* or *ATM* (assigned regardless of any co-occurring alteration in other genes), and Cohort B includes patients with alterations in 12 other HRR genes. Additional details of the study design can be found in the primary publication (1).

This trial was performed in accordance with the principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and the AstraZeneca policies on bioethics and was

approved by an institutional review board or ethics committee in the investigational sites. All patients provided written informed consent.

Sample collection

Clinical study sites were requested to submit tumor tissue to Foundation Medicine, Inc. (FMI) that was formalin fixed and paraffin embedded (FFPE) as a block or multiple 4–5 µm (*n* = 20) unstained, unbaked slides. An accompanying original hematoxylin and eosin (H&E) stained tissue section, where available, was also requested. If unavailable, H&E staining was performed at FMI. Archival tumor specimens were defined as those previously collected during routine diagnosis and care, while newly collected (referred to as recently obtained) samples were defined as tumor specimens collected solely for the purpose of the study. Furthermore, primary samples were those taken from prostate tissue, while all other non-prostate samples were considered as metastatic. For recently obtained samples, sites were instructed to use standard FFPE fixation (10% neutral-buffered formalin for 6–72 hours) and processing to preserve nucleic acid integrity. Strong acid decalcification was to be avoided for recently obtained bone biopsy and bone marrow trephine samples; instead, calcium extraction with ethylenediaminetetraacetic acid after formalin fixation was recommended. Details of the tumor sample (e.g., organ, collection method, and collection date) were recorded on the specimen collection module in the PROfound study electronic case report form as completed by participating sites.

Centralized prospective tumor sample testing

Prospective, central sample analysis was performed using an investigational clinical trial assay (CTA), based on the FoundationOne®CDx NGS test, developed by FMI. An H&E-

stained slide for each specimen underwent a specimen adequacy review by a trained pathologist to assess tumor nuclei percentage and available tissue volume. A minimum tumor content (ratio of tumor nuclei to all nuclei) of 20% and a minimum tissue volume of 0.2 mm^3 was required to proceed to DNA extraction for the CTA used on the PROfound trial. The cut-offs used on the PROfound trial were based on validated cut-offs for the clinical trial assay as defined by FMI. The majority of samples (~96%) assessed were, however, > 0.6 mm^3 (note: 0.6 mm^3 is the minimum tissue volume required for FoundationOne®CDx NGS, which was FDA-approved after the PROfound trial started) (12). For specimens with <20% overall tumor nuclei percentage, macrodissection to enrich tumor nuclei percentage above the required threshold was attempted if feasible based on tissue size and tumor distribution. Overall, a minimum of 50 ng of DNA post-extraction was required for samples to proceed to library construction (rare exceptions of samples yielding <50 ng of DNA that proceeded to testing were included). For each tumor specimen that passed tissue input adequacy requirements, subsequent library construction, hybrid capture and sequencing quality controls, a CTA report was generated specifying the presence or absence of qualifying gene alterations. In the PROfound study, a reported failed result referred to samples that did not meet pre-analytic tissue input adequacy requirements and/or did not pass analytic quality control metrics during processing at FMI, with reasons ranging from pathology review to computational analysis of sequencing data. For the purpose of analysis performed in this paper, generation of an NGS result was defined as samples that completed the FMI testing process according to FMI standards/test requirements and yielded an NGS result (NGS results included samples for which all sequencing quality control metrics were met irrespective of biomarker mutation status [‘pass’] or samples where one or more post-sequencing quality metrics (e.g., coverage,

computational tumor purity) did not meet pass criteria; however, NGS biomarker result was still deemed reportable but with potential for reduced sensitivity (‘qualified’ report).

Education for sample collection to improve generation of NGS results

Owing to the higher-than-anticipated rates of not obtaining an NGS result observed in the early stages of the PROfound study, a global WebEx session for study sites participating in the PROfound study was held in September 2017 to furnish insights into the current status of the samples with a view to providing advice on how to improve testing results. During the WebEx, the study sites were given advice on sample collection and processing prior to submission to FMI; they also had the opportunity to ask questions. Afterwards, they were provided with updated educational material in the form of an updated education manual and leaflets on best practices and learnings from PROfound up to that point. Education focused on advice for pathologists selecting the samples for testing, recommendations on sample collection and preparation for recently obtained samples, and best practices for slide sectioning to avoid cross-contamination. This included advice on collecting multiple core needle biopsies, creating blocks for cytology specimens using enrichment methods to maximize yield, minimizing tissue depletion of the blocks for initial diagnosis, guidance on using an 18-gauge needle and computed tomography for lymph node and soft tissue, guidance on using an 11- to 14-gauge bone-cutting needle (with or without a co-axial trocar) for bone samples, optimal sample preservation methods and preventing cross-contamination between samples. To evaluate whether this focus on education led to an improvement in generation of NGS results, data were analyzed for samples received for testing at FMI prior to and after October 1, 2017, at both sample and patient level.

Statistical analysis and modeling

Statistical analyses were performed using SAS version 9.4. For data on rates of NGS result generation, 95% confidence intervals (CIs) were obtained using the Clopper–Pearson method. For individual factors for which *P* values are reported, Chi-square and Wilcoxon and Kruskal–Wallis rank sum tests were applied as appropriate.

To evaluate the predictive power of the sample characteristics on obtaining an NGS result, a multivariate logistic regression model was generated. For the modeling analysis, the characteristics considered were sample type, organ site, collection method, sample age, total tissue volume, tumor content (nuclei) and DNA yield. The model was built, using data from the PROfound study, through selection of any significant two-way interactions of sample characteristics, as well as purposeful selection of single characteristics using available information from the tissue samples. As the model relied on complete information, any missing values were either excluded or imputed (to demonstrate the impact of exclusion on the overall model), and continuous variables such as DNA yield, tumor content, total tissue volume and sample age were log-transformed to remove skew. Any samples with variables that were recorded as zero were set to near non-zero values to enable log transformation (130 sample records had percentage tumor nuclei imputed from 0 to 1, and 21 records had tissue volume imputed from 0 to 0.01; 548 records with missing tumor DNA were excluded). The model was evaluated on the fit, taking into account a number of factors, including the Akaike information criterion for the whole (overall) model, the significance of the model factors (parameter estimates of effect likelihood ratio test/Wald tests), and the predictive power using receiver operating characteristic area under the curve (ROC AUC) assessment. A lack-of-fit test (Chi-square test) was also evaluated. Leave-one-out cross-validation was performed on the model and outputs were checked against the

study descriptive results for two-way interactions (tumor DNA, total tissue volume and tumor nuclei), such that the model can be used to predict outcomes when no real information is available.

Data availability

Data underlying the findings described in this manuscript may be obtained in accordance with AstraZeneca's data sharing policy described at https://astrazenecagrouptrials.pharmacm.com/ST/Submission/Disclosure. To protect patient privacy the raw data generated in this study are not publicly available. Individual requests will be considered, and data made available once the risk to patient identification has been assessed.

Results

Patients and sample testing

In total, 4425 patients were screened for the PROfound study at 206 sites in 20 countries. Samples from 4069 (92%) patients were received at FMI; samples were not available for 356 (8%) screened patients. Of the 4069 patients with available samples, 4047 (99.5%) were eligible for testing (i.e., proceeded to the first step in the test process, pathology assessment); the other 22 (0.5%) patients were ineligible for testing (e.g., incorrect sample submitted, such as fresh tissue or blood, or sample was received after the testing period was closed). From the 4047 patients with samples eligible for assessment, some patients submitted more than one sample (archival and more recently collected or multiple archival or multiple recently collected) resulting in a total of 4858 unique tumor samples evaluated at FMI with results (an NGS sequencing result or failed result) reported

to the FMI web portal. Generation of an NGS result was defined as samples that completed the FMI testing process according to FMI standards/test requirements and yielded an NGS readout (irrespective of biomarker mutation status). An NGS result was reported for 57.5% (2792/4858) of samples, equating to 69% (2792/4047) of patients overall. Of these, 316/4047 (7.8%) patients obtained an NGS result with a subsequent sample that was submitted after a failure of an initial sample test submitted. Additional analyses of the proportion of patients generating an NGS result by country are shown in **Supplementary Table 1;** however, due to small sample sizes from some countries, the data should be interpreted with extreme caution.

Tumor sample characteristics, collection methods, and challenges observed with providing sufficient samples

The majority of tested samples were obtained from archival primary prostate tissue (**Figs 1A–D**) and were collected by a variety of methods, with core needle biopsy (CNB) being the most common (**Fig. 1E**). Prostate CNB samples generally consisted of one to several residual cores of material remaining after primary diagnostic assessment, measuring < 1 mm in diameter and with lengths varying from 1 to 20 mm. There were a few anomalies with the reported collection methods, such that a small number of non-prostate-derived samples were recorded as having been collected during transurethral resection of the prostate (TURP), and non-bone samples were recorded as trephine biopsies; these were assumed to be incidental sampling during the procedure in question. The majority of prostate samples were archival CNBs collected as part of the initial patient diagnosis (see **Supplementary Figs 1A and B** for the distribution of collection methods used for each organ type and the organ type for each collection method, respectively). The age of samples ranged from < 1 to > 10 years (**Fig. 1F**); additional analyses of sample age by site

characteristics are shown in **Supplementary Table 2**. For example, of the non-prostate samples, bone samples had the lowest median age, whereas lung samples had the highest median age ($P < 0.0001$). Of the collection methods, the highest median age was seen for radical prostatectomy samples, while trephine biopsies had the lowest ($P < 0.0001$). **Fig. 2** shows a selection of histological images of samples collected in the study that did and did not generate NGS results.

Generation of an NGS result by sample type

Of the 4858 FMI tested samples that had an outcome reported to the FMI web portal in the PROfound study, 2792 (57.5%) generated an NGS result. At sample level, the proportion of samples to give an NGS result was marginally lower for archival biopsy samples (56.9%) than for recently obtained samples (63.9%; **Fig. 3A**), and for primary samples (56.2%) than for metastatic (63.9%; **Fig. 3B**). There was no difference in obtaining an NGS result based on submitted sample format: 57.1% (95% CI, 55.2–59.0) for blocks ($n = 2679$) and 57.7% (95% CI, 55.6–59.8) for slides ($n = 2167$). Of the specimen tissue sites, samples collected from lymph nodes had the highest rates of NGS results obtained (74.7%), while bone samples had the lowest (42.6%; **Fig. 3B**). The sample collection method with the lowest rates of NGS results obtained was prostate CNB (52.4%), while a higher rate of obtaining an NGS result were reported for samples derived from radical prostatectomy (74.0%) and TURP specimens (69.8%; **Fig. 3C**). While trephine biopsies had the highest rate of generation of an NGS result of all collection methods (86.7%), these findings are based on only 15 samples, five of which were recorded as being derived from non-bone sites. Furthermore, many bone samples were recorded in the CNB category; therefore, this finding is not representative of all bone biopsies collected in the study. As expected, and in agreement with findings from the recently obtained versus archival

samples, a higher proportion of recently collected samples generated an NGS result, with a gradual decline with increasing sample age (**Fig. 3D**). The association of older sample age with greater chance of no NGS result being generated was demonstrated via Chi-square test for association ($P < 0.001$); for example, 68.1% (95% CI, 65.0–71.1) of samples that were < 1 year old generated an NGS result compared with samples that were > 10 years old at 47.3% (95% CI, 43.0–51.5). There was a similar age-related decline for CNBs when assessed separately that amplified the low tissue volume effect and further reduced the rate of generation of an NGS result in the CNB cohort from 65.5% (95% CI, 61.7–69.1) for samples aged < 1 year to 38.9% (95% CI, 33.2–44.9) for samples aged > 10 years (**Supplementary Fig. 2A**). In comparison, the rate of obtaining an NGS result was 74.7% (95% CI, 69.0–79.9) for samples < 1 year old and 56.6% (95% CI, 50.4–62.7) for samples > 10 years old for other collection methods (**Supplementary Fig. 2B**). Additional country level analyses of the proportion of samples generating an NGS result by sample type, collection method, and organ type are shown in **Supplementary Tables 3–5;** however, due to small sample sizes from some countries, the data should be interpreted with extreme caution.

Generation of an NGS result by percentage tumor content (nuclei), total tissue volume and DNA yield

Analysis of NGS result generation by tumor content (nuclei), total tissue volume and DNA yield indicated an increase in the proportion of NGS results obtained with increased tumor content and DNA yield (**Figs. 3E & G**). Despite one tissue volume grouping (1–5 mm^3) showing a slight decrease of NGS result obtained compared to the 0.6–1.0 mm^3 group there was a visible overall trend toward increased NGS result generation with increased total tissue volume as an individual factor. (**Fig. 3F**). Further evaluation of NGS

result generation by tumor content showed a corresponding increase in DNA yield with an increase in tumor content (**Supplementary Table 6**). It is worth noting that for DNA yield, there was a maximum input that would be analyzed by NGS (i.e., 50–1000 ng of DNA was required for library construction, with 1000 ng being the maximum input), so increased DNA yield would not necessarily always reflect increased input for NGS (for samples with DNA yield > 1000 ng) but may reflect a sample yielding higher-quality DNA. Mean and median percentage tumor content, total tissue volumes and DNA yields were evaluated for different sample categories (**Supplementary Table 7**). Higher mean and median DNA yields were observed for more recently obtained and non-prostate (metastatic) samples than with archival and primary (prostate) samples. Of the metastatic samples, those taken from lymph node samples had the highest mean/median tumor content and DNA yield, whereas bone samples had the lowest tumor content and DNA yield. Specifics of the decalcification methods for archival bone specimens were not well documented; however, histological appearance suggested use of strong acids in many cases, a likely contributor to the low DNA yields observed for bone samples. Notably, samples with total tissue volume > 0.2 mm^3 were eligible for evaluation in this study, whereas the recommended minimum total tissue volume for the FoundationOne®CDx NGS test is 0.6 mm^3. The majority (96.1%; 4669/4858) of samples collected in our study had a cut-off total tissue volume of > 0.6 mm^3. The overall proportion of samples generating an NGS for the cohort of samples with tissue volume ≥ 0.6 mm^3 was higher (58.9%; 95% CI, 57.4–60.3) than for the 0.2–< 0.6 mm^3 cohort (32.1%; 95% CI, 24.4–40.6), as was DNA yield (median 357.8 v. 88.3 ng, respectively; **Supplementary Table 8**).

Reasons for not obtaining NGS results

Of the 4858 unique samples that had a result reported in the PROfound study, testing failed to deliver a result in 2066 (42.5%) cases. **Fig. 4A** shows a flow chart of the processing stage at which samples failed and the proportion of samples failing at each stage. Overall, the main reasons for samples failing to meet FMI's standard requirements for the test process and consequently not obtaining an NGS test result were 'not meeting specimen adequacy requirements' (pathology review – a minimum tumor content of 20% and a minimum tissue volume of 0.2 mm^3) and failed DNA extraction (**Fig. 4B**). Archival samples were most likely to fail at the DNA extraction stage, whereas recently obtained samples were more likely to fail at the pathology adequacy review stage. The reasons for failure at pathology review stage were primarily insufficient tumor content/purity and insufficient tissue size; however, low cellularity or the sample being presumably exposed to high temperature during slide preparation (baked) were also factors. Reasons for failing to meet FMI's standard requirements for the test process varied across sample collection methods (**Fig. 4C**). For example, larger samples, such as from radical prostatectomy, were more likely to meet FMI's requirements at pathology review because of abundant tissue and typically larger foci of tumor, that could be enriched by macrodissection, than other collection methods; however, they were more likely to fail computational biology review because they were more likely to be archival and have lower DNA quality. Further analysis found that samples that generated an NGS result had a lower mean and median age than those that did not, and samples that failed to meet FMI requirements later in the testing process were older than those that failed at the beginning of the test process (**Supplementary Table 9**). For example, samples that failed at pathology review were typically younger in age (mean ± standard deviation [SD] 4.4 ± 4.6 years) than those that

failed to meet quality control metrics required during computational review of sequencing data (6.7 ± 4.7 years; **Supplementary Table 9**). Additional analyses of the reasons for failure by country are shown in **Supplementary Table 10;** however, due to small sample sizes from some countries, the data should be interpreted with extreme caution.

Modeling of factors affecting generation of an NGS result

To determine the factors impacting the generation of an NGS result of samples that passed pathology adequacy review, a logistic regression model (with log transformation of continuous variables) found that the combination of all sample variables had a substantial contribution to whether an NGS result was obtained (area under the curve [AUC] = 0.9172; **Fig. 5**). For this model, DNA yield was the main variable affecting if an NGS result could be obtained (AUC = 0.9061), with other variables such as sample age (AUC = 0.6292) and percentage tumor content (AUC = 0.5965) also having a potential effect but to a lesser extent (**Supplementary Fig. 3**). A model without log transformation of continuous variables was not as predictive of whether an NGS result was obtained (AUC = 0.8378); however, the ranking of individual factors did not change relative to the main model (**Supplementary Fig. 3**). The main model generated was based on complete records, such that any samples from which DNA was not extracted (e.g., failed pathology review) were not included. The impact of removal of incomplete sample data, imputing missing data and using DNA yield as a surrogate for NGS result generation is further described in the Supplementary Appendix (**Supplementary Figs. 4 & 5**). Despite the observations of changes in predictive power, the main model with no imputation of missing DNA was retained and any missing records excluded to ensure that the model was built on known records.

This model was used to evaluate various sample characteristic combinations to predict outcome in specific scenarios, even where data were not available (**Table 1**). The selection

of cut-offs was based on selecting a range of minimum requirements required (50 ng DNA input; 0.2 mm^3 and 20% tumor content) and expected optimal conditions (200 ng input; 1 mm^3 tissue volume), as well as a range of tumor content spanning various potential observations in a real-world setting. As a supplement to the observed outcomes in PROfound, the model enabled us to evaluate various scenarios of sample characteristics that may be observed in a real-world setting. The two-way interaction model comprises purposefully selected key sample characteristics such as DNA yield, percent tumor nuclei, tissue volume, and age, irrespective of their significance in the model. Within the characteristics evaluated in Table 1, age < 5 years was shown to predict increased likelihood of acquiring an NGS result for the categories outlined in **Table 1**, including those with the lowest total tissue volume and percentage tumor content. Our model demonstrated that selecting an increased tissue volume when combined with the other characteristics shown in Table 1 is not predicted to impact the likelihood of obtaining an NGS result if considered in combination with the factors: collection method, DNA yield category, age category, and log-transformed percent tumor nuclei. The influence of tissue volume as an individual factor is, however, visible in the analysis of the actual data (**Fig. 3F**). Furthermore, even for samples aged < 5 years, an increase in the proportion of NGS results obtained was predicted as other categories, such as tumor content and DNA yield, were increased. Interestingly, however, for samples > 5 years, the model predicted minimal impact of different percentage tumor content when the DNA input was between 50 and 200 ng but a predicted increase in likelihood of obtaining an NGS result when the DNA was > 200 ng and increased tumor content. Additional modeling that included factors such as tumor grade and Gleason score was also performed (see details below and Supplementary Appendix).

Model testing

Imputation of missing DNA or removal of DNA

An additional model assessed imputation of missing DNA data at results less than the required minimum of 50 ng. By imputing the DNA yield to 0.01 or 49 ng, while the general trend of individual factors influencing NGS result generation was similar to the main model, there was a shift in ranking of sample age and percentage tumor nuclei specifically compared with the main model (**Supplementary Figs. 4A and 4B**, respectively). With no imputation for DNA (i.e., the main model), the predictive power for percentage tumor nuclei was AUC = 0.5965, which increased to AUC = 0.6763 with imputation of tumor DNA to 0.01 ng. This increase is in line with that observed for the overall main model prediction (from AUC = 0.9172 to AUC = 0.9400). By removing DNA yield completely from the model, the predictive power of the model decreased (AUC = 0.7604), but there was a shift in ranking of sample age and percentage tumor nuclei compared with the main model (**Supplementary Fig. 4C**).

Assessment of different DNA yields

Given that DNA yield had the greatest predictive power of NGS result generation, an additional model was evaluated using DNA yield as a surrogate for NGS result generation. Using a DNA yield of >50 ng as the cut-off value, the predictive power of the overall model decreased from AUC = 0.9172 to AUC = 0.7749, suggesting that it is not just sufficient DNA that is required for an NGS result to be obtained and that other factors should be considered (**Supplementary Fig. 5A**). In this model, the predictive power of tumor nuclei increased (AUC = 0.6785) relative to the main model (AUC = 0.5965). Conversely, evaluation of higher DNA yield cut-off values (>100 and >200 ng) found that the predictive

power of the overall model decreased further, as did the predictive power of tumor nuclei (**Supplementary Figs. 5B and 5C**, respectively).

Reliability of the model

To check the reliability of the model, we evaluated the predicted model outcomes against data observed in the study, and high concordance was observed (**Supplementary Table 11**).

Assessment of other clinical factors

While DNA yield and percentage tumor nuclei were higher for samples that had an NGS result, when evaluated at organ level, it was noted that some metastatic samples (e.g., liver and lung) had less overlap in the range of percentage tumor nuclei and DNA yields for samples yielding an NGS result over samples where an NGS result was not obtained (data not shown), suggesting that other clinical factors (e.g., tumor grade and Gleason score) may potentially influence generation of a test result. To investigate further, another model evaluated available data including primary tumor grade information and Gleason score as additional factors (**Supplementary Fig. 6**). These data were primarily available for patients randomized in the PROfound study; therefore, the dataset was biased to a population of patients with samples yielding NGS results (with a select few who did not have an initial result) and should thus be interpreted with caution.

Generation of NGS results following investigator educational session

Evaluation of rates of NGS result generation for all samples found an 8% improvement from 51.9% (95% confidence interval [CI], 49.2–54.6) for the 1380 samples received at FMI on/before 1 October 2017 to 59.7% (95% CI, 58.0–61.3) for the 3478 samples analyzed after October 1, 2017, following the educational sessions in September 2017

(**Supplementary Table 12**). A similar improvement was observed at patient level, whereby the proportion of samples where an NGS result was obtained increased from 61.1% (95% CI, 58.2–63.9) for the 1172 patients whose samples were received before October 1, 2017, to 69.9% (95% CI, 68.3–71.6) for the 2968 patients whose samples were received on/after October 1, 2017. Evaluation of the change in the percentage of samples not obtaining an NGS result over time showed a gradual decline from 48.1% at September 30, 2017 (1380 samples) to 42.5% by December 31, 2018 (4858 samples) at sample level (**Supplementary Fig. 7A**), and from 38.9% at September 30, 2017 (1172 patients) to 31.1% by December 31, 2018 (4047 patients) at patient level (**Supplementary Fig. 7B**). These results suggest that educating pathologists and laboratory staff on the preparation of samples is a key aspect of improving generation of an NGS result.

Discussion

The PROfound study is the largest study to date to screen patients prospectively for a qualifying alteration in a panel of genes with a direct or indirect role in HRR by central tumor tissue NGS testing. The PROfound study has demonstrated the feasibility of molecularly targeted therapy in men with mCRPC (when tumor tissue is available), with an NGS result generation rate of 69% when calculated at patient level (1), comparable to that observed in several other prostate cancer studies utilizing similar standardized operating procedures (13-15). We sought to evaluate the observations from the PROfound study as successful implementation of genomic testing in clinical practice is critical to identify men with mCRPC for whom there is a significant unmet clinical need and who might be eligible for precision medicine with PARP inhibitors, immunotherapy, and targeted agents.

Most tissue samples analyzed in the PROfound study were from archival tissue, and although the rate of NGS results generated was marginally higher in more newly obtained samples, generating an NGS result was possible with both. Furthermore, while most samples were taken from primary prostate tumor specimens, the rate of generation of an NGS result was higher for non-prostate metastatic samples. This may be a result of metastatic samples having higher tumor content and cellularity and incidentally higher DNA yield, as well as being more recently obtained and assessed. Despite lower rates of NGS result generation with primary and archival tissue, evaluation of HRR alterations in these samples is achievable for some patients and appropriate even after mCRPC progression as the majority of HRR alterations in prostate cancer appear to occur early in the disease and prior to metastasis (16-18). We observed a decline in the rate of NGS results obtained with increased sample age, which may be a result of DNA degradation over time. The main reason for not obtaining an NGS result for archival samples was failure to extract sufficient DNA, possibly because of DNA degradation, with exacerbation of the effect by tissue collection techniques that yield smaller available tissue input volumes, such as CNB. Despite these findings, the generation of an NGS result was reported in almost half of samples aged >10 years, especially in samples of larger volume or high tumor content, suggesting that optimizing collection methods may help with obtaining NGS results from archival samples. The generation of an NGS result is also influenced by collection methods. For example, CNB is increasingly being used to obtain prostate tissue samples as it is less invasive than many other collection methods, and it was the most common method in the PROfound study; however, rates of obtaining an NGS result were lower than for other collection methods. Several features specific to prostate CNB are likely to contribute to not obtaining an NGS result for samples, such as not meeting the requirements for adequate

specimen volume and percentage tumor nuclei. The benign prostate gland comprises abundant fibromuscular stroma and interspersed secretory glands with large lumens, leading to an overall low density of nuclei compared with other anatomical sites. In contrast, areas with prostatitis and prostate adenocarcinoma, particularly those with higher Gleason grades, have higher density of nuclei. When a prostate CNB is obtained, depending on the path it traverses, there can be a large variance in the amount of tissue with low versus high nuclei density and, therefore less of a predictable relationship between tissue volume and DNA extraction yield. Thus, in relatively large cores with small foci of tumor, volume of tissue and percentage tumor nuclei can be sufficient but total nuclei insufficient to yield the minimum DNA required. The observations from this study suggest that generation of an NGS results is achievable in CNB as long as this effect is accounted for and other factors are adhered to (e.g., selecting the most recently collected samples with highest tumor content where possible). Other considerations, such as embedding multiple CNBs into a single FFPE block, may increase the likelihood of generating an NGS result through increasing DNA yield (19). Macrodissection of the tumor area is also possible and is recommended to increase the tumor content of the sample if the overall volume and cellularity of sample is sufficient (20). Furthermore, with the increasing use of magnetic resonance imaging (MRI)-guided biopsies, higher tumor cellularity with likely higher tumor DNA yield will be found in CNBs, which will inevitably improve NGS result acquisition for this sample type (21).

In our study, surgical specimens, radical prostatectomies and TURP were found to contain adequate tissue volume, thus often resulting in relatively high levels of NGS result acquisition; however, the percentage tumor content of these large specimens can be low, so macrodissection to enrich samples can sometimes be insufficient for obtaining an NGS

result. Differences in rates of obtaining an NGS result across organ types were noted, with lymph nodes having the highest rates of NGS result acquisition and bone samples the lowest, similar to previous reports (13). The improved NGS result acquisition reported for lymph node samples may be the result of a combination of higher nuclear content per volume of tissue and metastatic prostate cancer deposits being localized in subcapsular, high-purity nodules, making them more easily visualized and accessible for biopsy (i.e., tumor sites are better localized with imaging for extraction with needle biopsies) and thereby having the highest tumor content and DNA yields compared with other organ sites. While the low rates of obtaining an NGS result observed for bone samples may be associated with difficulties in collection of sufficient tissue (22-24), leading to lower tumor content and tissue volume, the low DNA yields observed may be a result of other pre-analytical factors unique to the collection of bone samples. Avoidance of strong acid decalcification in bone biopsies of tumor samples collected for genomic testing is critical for molecular testing (25). As a consequence, there was a recommendation for this study that decalcification should not be performed on recently obtained bone samples where possible; however, for archival bone samples, it was not possible to control for decalcification as information regarding processing of these samples was not routinely available. Country-level analyses of samples provided at screening found that those countries whose samples had an overall lower rate of NGS result generation had more failures after pathology review, suggesting that while the samples may have met the tissue sample requirements as assessed by the pathologist, other factors such as sample age and/or poor sample preservation may have resulted in failures later in the process.

Our modeling analysis confirmed the findings that multiple factors impact the generation of an NGS result, with some having a greater prediction value than others; for example,

DNA yield and percentage tumor nuclei had a strong influence on generating an NGS result in the model. Furthermore, while the model predicted that generation of an NGS result would be higher with younger samples (< 5 years), regardless of tumor content, total tissue volume or DNA yield, predicted rates of obtaining NGS results are still encouraging for older samples (≥ 5 years), particularly when other characteristics are considered. However, we did not evaluate other factors, such as biopsy procedure (i.e., MRI- or transrectal-ultrasound-guided biopsy), imaging procedure (i.e., prostate-specific membrane antigen or computed tomography guided) and experience of the radiologist.

To improve on the rates of NGS result generation observed in the PROfound trial in the initial few months, a live education session and updated instruction materials were provided to participating sites, based on input from both FMI and medical experts (see Supplementary Appendix for more information). Results suggested that educating pathologists and laboratory staff on the preparation of samples is a key aspect of improving generation of an NGS result. A recent review detailing practical considerations for molecular diagnostic testing in prostate cancer highlights other aspects of sample collection and processing that should be considered for improving the rates at which NGS results are obtained in mCRPC, as well as guidance on DNA extraction and processing to optimize DNA yield and quality for molecular diagnosis (20). In addition, the importance of communication between clinicians and pathologists, as well as continued training and feedback between oncology, interventional radiology and pathology teams, has also been emphasized (26).

Many institutions are establishing tumor gene panel testing and molecular characterization of prostate samples for HRR alteration status, and this is anticipated to increase given that guidelines, such as those from the NCCN, ESMO and

AUA/ASTRO/SUO, are now including recommendations for tumor testing for HRR gene alterations in patients with prostate cancer (10,11,27). Additionally, recent advances in cell-free DNA based tumor profiling based on blood samples (liquid biopsy) to identify alterations in circulating tumor DNA may also provide an additional test option for patients where an NGS result cannot be obtained from tumor samples or for whom no tumor tissue is available, although plasma DNA frequently has a tumor content below 30%, with most liquid biopsy NGS assays not being cleared for copy number analyses (28-31). It is important to note that should cell-free DNA be negative it does not rule out tissue positivity.

The majority of samples that progressed through testing and obtained an NGS result in the PROfound study met the validated quality control criteria of the molecular test used in the trial with very few exceptions. While it is unclear how representative the testing experience and NGS result outcome metrics reported here will be for other clinical laboratories, which may have alternative pipelines, different thresholds for sample metrics and/or may choose to test samples in exception of quality control requirements (32), this study does demonstrate some key learnings and factors that may predict the generation of an NGS result and should be considered for collection of samples for testing, irrespective of the test used. Sample age, tumor content and DNA input were the three factors that had the biggest impact on obtaining an NGS result, both in our actual data and our modeled data. These three factors in particular should be considered when selecting samples for NGS testing. If multiple samples are available, consider selecting the younger samples if the tumor content is similar. For older samples >5 years; consider using samples that have the highest tumor content and from locations likely to produce high tumor content and subsequently high DNA yield (e.g., from lymph nodes). For newly collected samples, use of standard formalin fixation and avoidance of decalcification (for bone samples) may help to

preserve nucleic acid integrity. Men should be counseled that this test pipeline only delivers an interpretable result approximately two-thirds of the time; that is, screening archival samples or submitting to new biopsies may not enable access to personalized targeted treatment. With FDA and EMA approval of olaparib for treatment of patients with HRR gene (7) and BRCA alterations (8), respectively, genomic testing is a key element in treatment planning for patients with mCRPC, and so should be taken into consideration at sample collection.

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Table 1. Two-way interaction model of NGS result acquisition by age (<5 and ≥ 5 years) and DNA category (50–200 and >200 ng)

DNA	Total tissue volume, mm^3	Tumor content, %	Estimated least-squares mean difference ± SD, logit	*P* value	Proportion of samples generating an NGS result, %	
					Age < 5 years	Age ≥ 5 years
50–200 ng						
	0.2	20	0.4328 ± 0.2191	0.0482	69.87	60.06
	0.2	40	0.8394 ± 0.1575	<0.0001	75.87	57.60
	0.2	80	1.2460 ± 0.2396	<0.0001	81.01	55.09
	1	20	0.4328 ± 0.2191	0.0482	67.53	57.43
	1	40	0.8394 ± 0.1575	<0.0001	73.83	54.93
	1	80	1.2460 ± 0.2396	<0.0001	79.28	52.39
> 200 ng						
	0.2	20	0.7283 ± 0.1920	0.0001	85.28	73.67
	0.2	40	1.1349 ± 0.1617	<0.0001	95.46	87.11
	0.2	80	1.5414± 0.2669	<0.0001	98.71	94.23
	1	20	0.7283 ± 0.1920	0.0001	83.87	71.51
	1	40	1.1349 ± 0.1617	<0.0001	94.96	85.84
	1	80	1.5414 ± 0.2669	<0.0001	98.56	93.61

Abbreviation: SD, standard deviation

Figure legends

Figure 1.

Proportional comparisons by sample characteristics from 4858 tumor samples (taken from 4047 patients with samples eligible for assessment): (**A**) specimen tissue type; (**B**) time of sample collection (archival/recently obtained); (**C**) stage of disease (primary/metastatic); (**D**) collection time and stage of disease (archival/recently obtained/primary/metastatic); (**E**) specimen collection method; and (**F**) sample age*.

*Age was calculated as time from biopsy date to test report date; †Trephine biopsy is bone marrow biopsy by trained individuals following a standard operating procedure. TURP, transurethral resection of the prostate

Figure 2.

Example histological images from samples received for genomic testing in the PROfound study.

(**A**) and (**B**) are from prostatectomy samples of a patient with prostatic acinar adenocarcinoma. The tumor area is indicated by the circled area, and benign glands by the arrows. (**C**) and (**D**) are from a newly collected TURP sample. The boxed area in (**C**) is magnified in (**D**) to show the high tumor purity and edge cautery effect typical of TURP. (**E**) and (**F**) are from a good-quality CNB sample. (**E**) shows two cores of 0.8 mm diameter and total length of ~30 mm with more than 60% of tissue containing tumor, and the boxed area in (**E**) is magnified in (**F**), showing less cellular fibromuscular stroma adjacent to the tumor, which demonstrates how nuclear density varies between the uninvolved and tumor-containing areas of the core. Histological images in A–F were from samples that obtained an NGS result. (**G**) and (**H**) are from poorer-quality CNB samples from prostate that produced failed results. (**G**) and (**H**) show two examples of samples that failed during pathology review due to low cellularity, these samples did not proceed to downstream analysis. (**I**) and (**J**) are from CNBs taken from the lymph node with abundant

metastatic prostate carcinoma. The boxed area in (**I**) is magnified in (**J**), showing that the lymph node architecture is completely effaced by metastatic disease. (This lymph node sample obtained an NGS result.) (**K**) and (**L**) are taken from newly collected bone samples that contain abundant tumor. The boxed area in (**K**) is magnified in (**L**), showing residual calcification of the edge of the bone specular (purple) and osteoblasts in lacunae with well-defined nuclear features, which suggest that strong acid decalcification was not used on this sample. This bone sample yielded an NGS result. In contrast, (**M**) and (**N**) are taken from newly collected bone excision, showing extensive decalcification. In (**M**), the arrows indicate decalcified bone spicules, which stain uniformly with eosin (pink), as does the rest of the sample, including areas heavily infiltrated with metastatic high-grade tumor. The boxed area in (**M**) is magnified in (**N**), showing the decalcified spicules eroded by lytic nests of metastatic prostate adenocarcinoma staining minimally with hematoxylin (purple) and with almost no nuclear detail. This sample failed DNA extraction despite high tumor content, yielding < 10 ng of DNA. CNB, core needle biopsy; TURP, transurethral resection of the prostate

Figure 3.

Proportion of samples generating an NGS result by: (**A**) time of sample collection (archival/recently obtained); (**B**) stage of disease (primary prostate and metastatic overall and split by specimen tissue type); (**C**) specimen collection method; (**D**) sample age; (**E**) tumor content; (**F**) total tissue volume; and (**G**) DNA yield.

Data are presented as % (95% CI). $P < 0.0001$ for Chi-square test with hypothesis of general association for NGS result generation for time of sample collection, stage of disease, collection method, sample age, tumor content, total tissue volume and DNA yield. The Chi-square test demonstrates a significant difference in the distribution of the NGS result among the categories above. TURP, transurethral resection of the prostate.

Figure 4.

(**A**) Flow chart of the processing stage at which samples failed and the proportion failing at each stage, (**B**) NGS test outcome/reasons for all samples, archival samples and recently obtained samples, and (**C**) failure rates/reasons for each sample collection method.

Compbio, computational biology; LC/HC, library construction/hybrid capture; NGS, next-generation sequencing; QC, quality control; TURP, transurethral resection of the prostate

Figure 5.

Logistic regression model of factors contributing to generation of an NGS result: main model with log transformation of continuous variables.

Figure 1

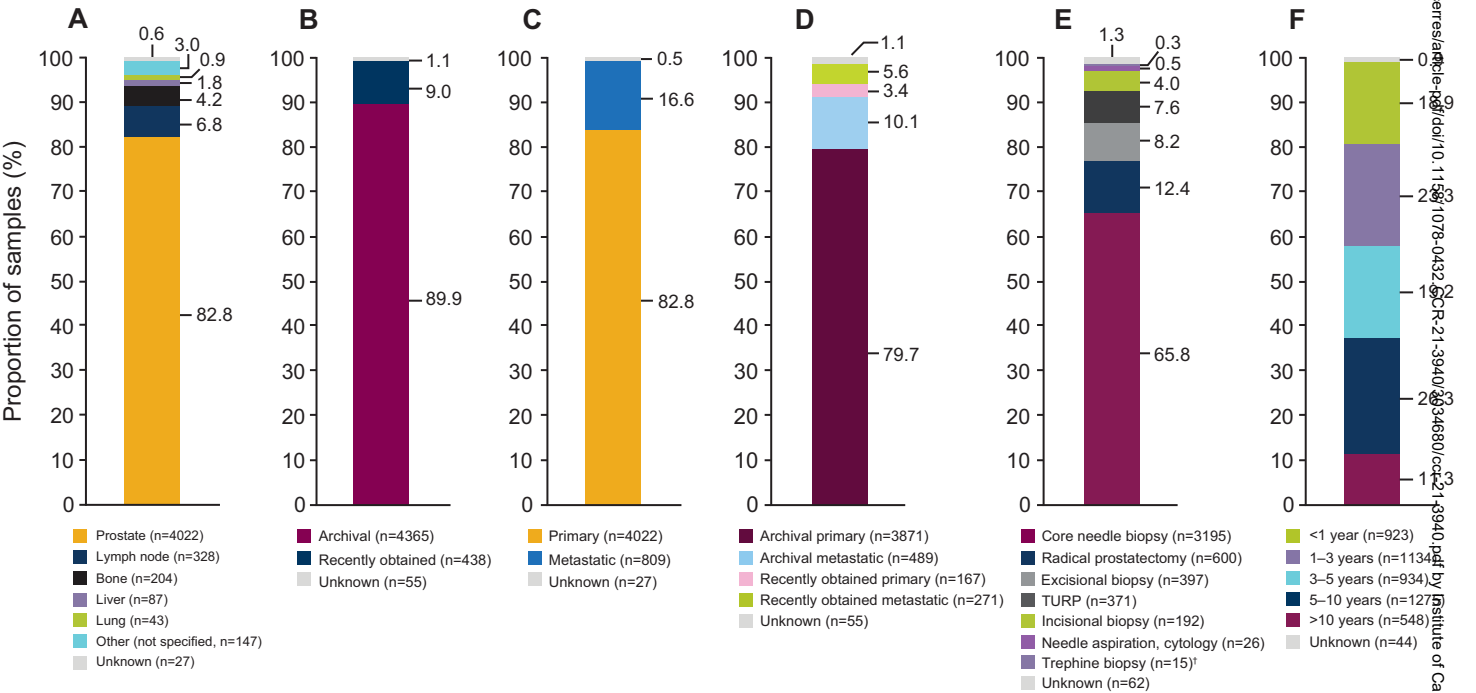
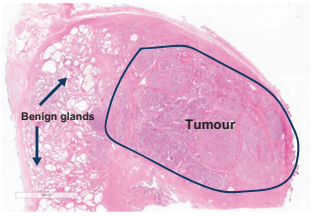


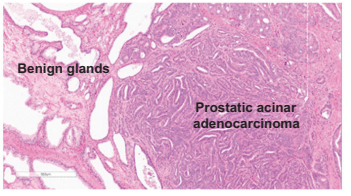
Figure 2

A Prostatectomy (NGS result obtained)



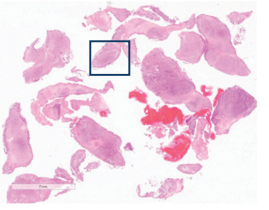
Total magnification 4x

B Prostatectomy (NGS result obtained)



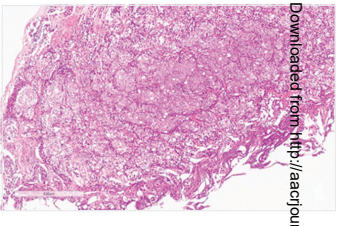
Total magnification 40x

C TURP (NGS result obtained)



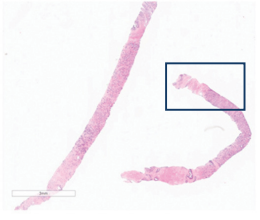
Total magnification 5x

D TURP (NGS result obtained)



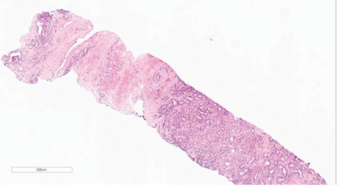
Total magnification 50x

E Prostate core needle biopsy (NGS result obtained)



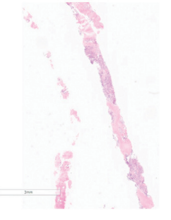
Total magnification 2x

F Prostate core needle biopsy (NGS result obtained)



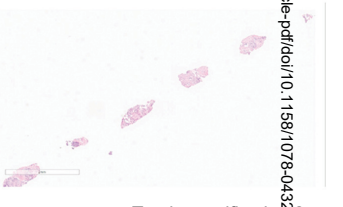
Total magnification 40x

G Prostate core needle biopsy (failure: no NGS result)



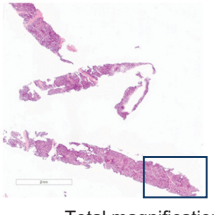
Total magnification 2x

H Prostate core needle biopsy (failure: no NGS result)



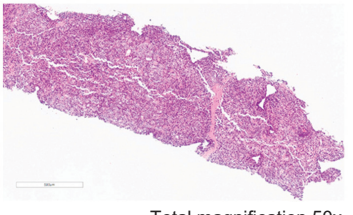
Total magnification 2x

I Lymph node core needle biopsy (NGS result obtained)



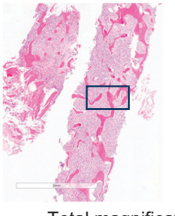
Total magnification 2x

J Lymph node core needle biopsy (NGS result obtained)



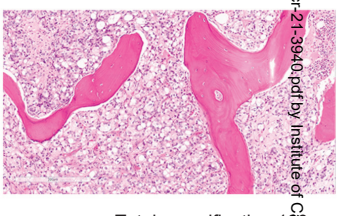
Total magnification 50x

K Bone biopsy (NGS result obtained)



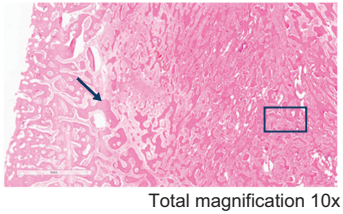
Total magnification 10x

L Bone biopsy (NGS result obtained)



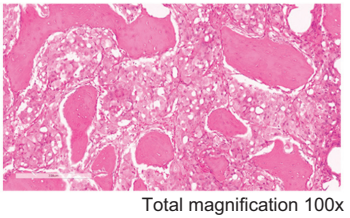
Total magnification 10x

M Bone biopsy (failure: no NGS result)



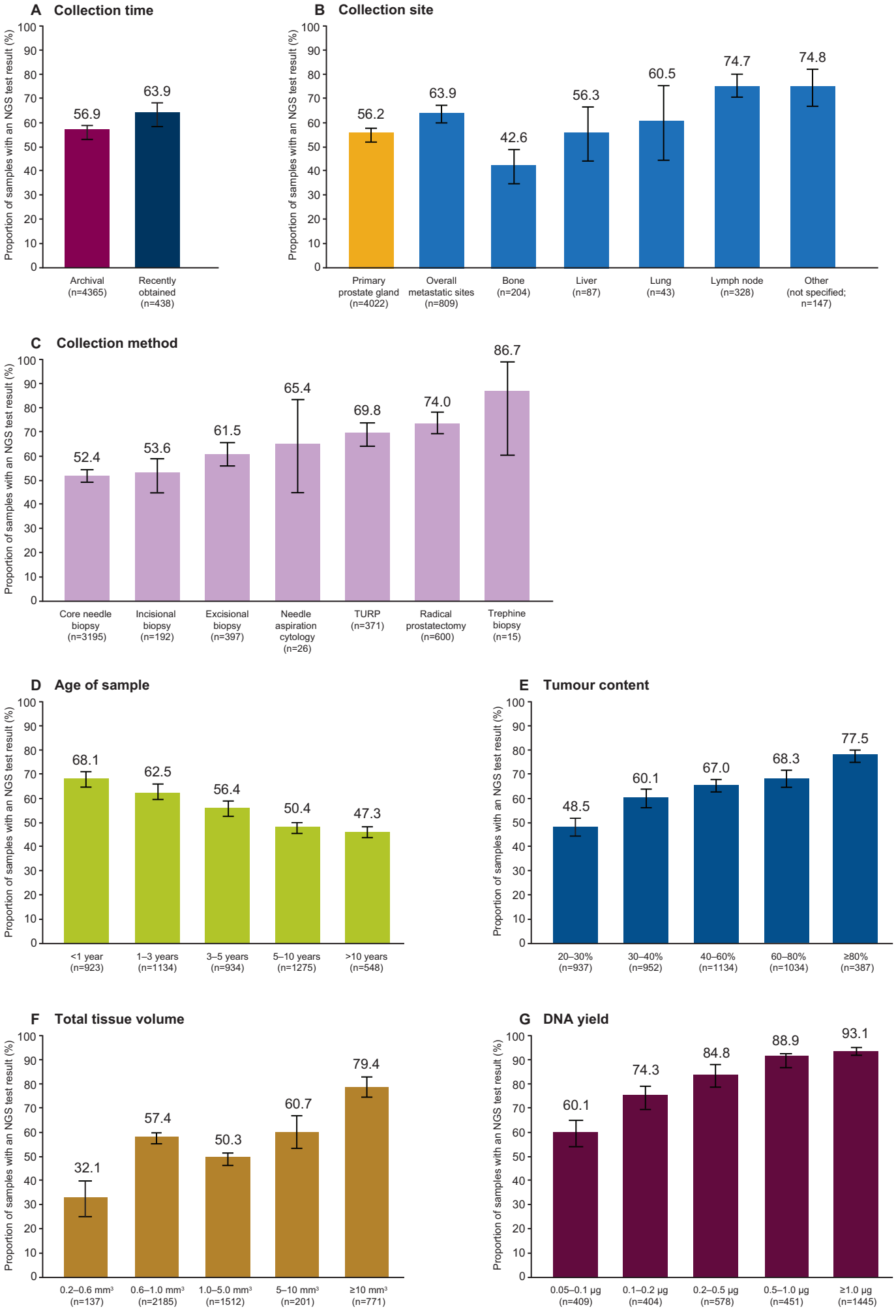
Total magnification 10x

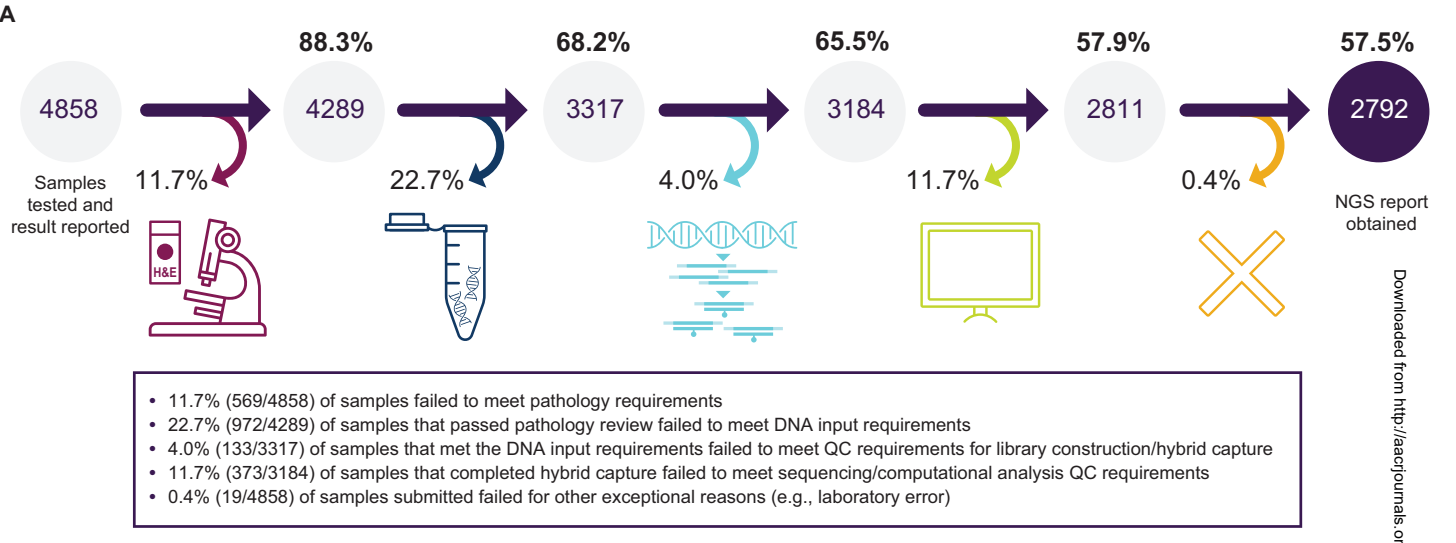
N Bone biopsy (failure: no NGS result)



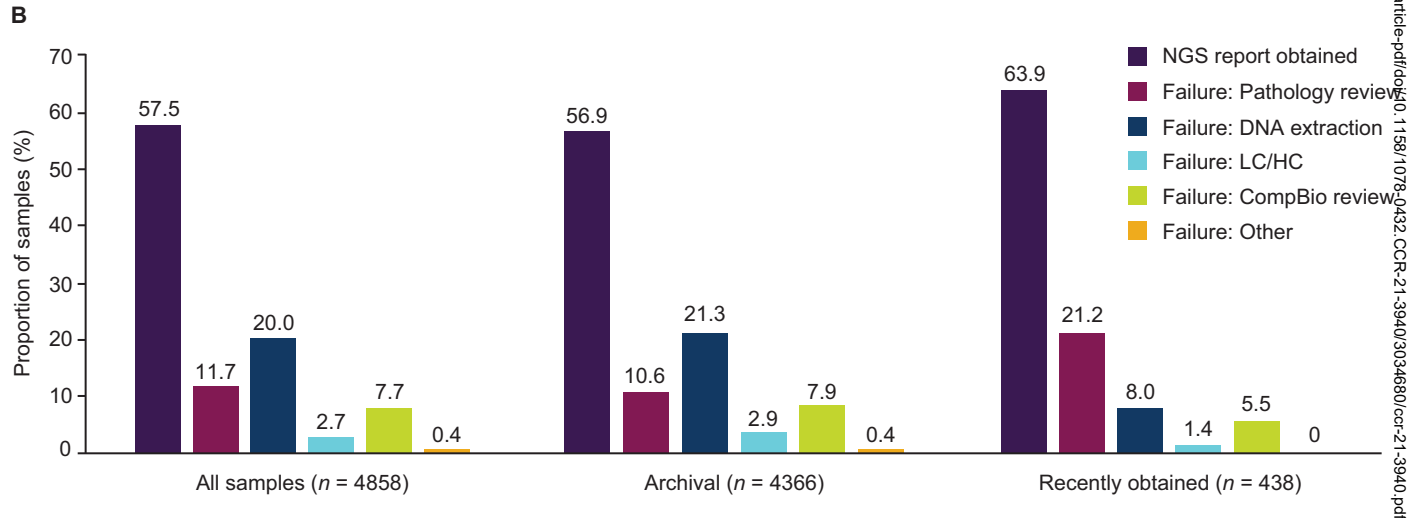
Total magnification 100x

Figure 3





- 11.7% (569/4858) of samples failed to meet pathology requirements
- 22.7% (972/4289) of samples that passed pathology review failed to meet DNA input requirements
- 4.0% (133/3317) of samples that met the DNA input requirements failed to meet QC requirements for library construction/hybrid capture
- 11.7% (373/3184) of samples that completed hybrid capture failed to meet sequencing/computational analysis QC requirements
- 0.4% (19/4858) of samples submitted failed for other exceptional reasons (e.g., laboratory error)



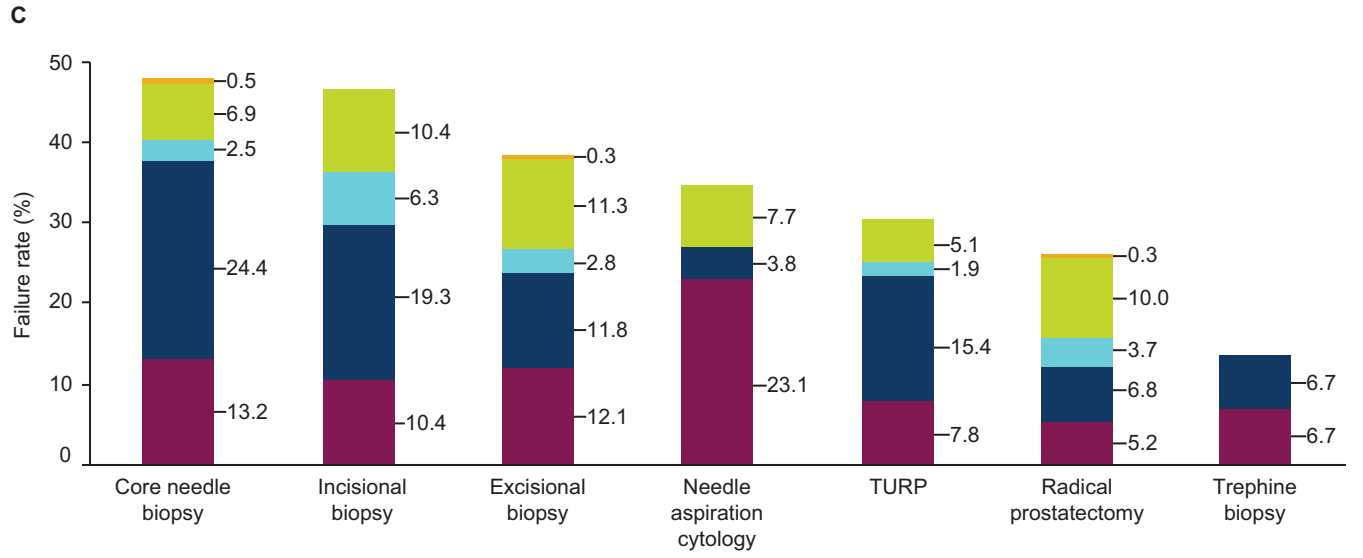


Figure 5