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Comparison of Results from Two Commercially Available In-House Tissue-Based Comprehensive Genomic Profiling Solutions

Research Use Only AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit and TruSight Oncology 500 Assay

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Increased adoption of personalized medicine has brought comprehensive genomic profiling (CGP) to the forefront. However, differences in assay, bioinformatics, and reporting systems and lack of understanding of their complex interplay are a challenge for implementation and achieving uniformity in CGP testing. Two commercially available, tissue-based, in-house CGP assays were compared, in combination with a tertiary analysis solution in a research use only (RUO) context: the AVENIO Tumor Tissue CGP RUO Kit paired with navify Mutation Profiler (RUO) software and the TruSight Oncology 500 RUO assay paired with PierianDx Clinical Genomics Workspace software. Agreements and differences between the assays were assessed for short variants, copy number alterations, rearrangements, tumor mutational burden, and microsatellite instability, including variant categorization and clinical trial-matching (CTM) recommendations. Results showed good overall agreement for short variant, known gene fusion, and microsatellite instability detection. Important differences were obtained in tumor mutational burden scoring, copy number alteration detection, and CTM. Differences in variant and biomarker detection could be explained by bioinformatic approaches to variant calling, filtering, tiering, and normalization; differences in CTM, by underlying reported variants and conceptual differences in system parameters. Thus, distinctions between different approaches may lead to inconsistent results. Complexities in calling, filtering, and interpreting variants illustrate key considerations for implementation of any high-quality CGP in the laboratory and bringing uniformity to genomic insight results. *(J Mol Diagn 2024, 26: 1018—1033; https://doi.org/10.1016/j.jmoldx.2024.08.001)*

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The sponsor, F. Hoffmann-La Roche Ltd./Roche Diagnostics Solutions, Inc., contributed to the design of this analysis. Data collected by the authors were analyzed by employees at Foundation Medicine, Inc. and GmbH, and F. Hoffmann-La Roche Ltd. Authors employed by the study sponsor collected and analyzed the data and wrote the manuscript.

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High-throughput parallel sequencing approaches, or next-generation sequencing (NGS), have been recommended for a number of tumor types by the National Comprehensive Cancer Network [NCCN; *https://www.nccn.org/home* (registration required), referenced with permission from the National Comprehensive Cancer Network, Inc. National Comprehensive Cancer Network, Inc. 2024. All rights reserved. Last accessed January 24, 2024. To view the most recent and complete version of the recommendations, go online to NCCN.org. NCCN makes no warranties of any kind whatsoever regarding their content, use or application and disclaims any responsibility for their application or use in any way] and European Society for Medical Oncology guidelines.[1] Comprehensive genomic profiling (CGP) is an NGS approach which allows for the detection of novel and known variants of all the main classes of genomic alterations in known cancer-related genes, including genomic signatures such as tumor mutational burden (TMB), microsatellite instability (MSI), and genome-wide loss of heterozygosity (gLOH).[2] Increased adoption of personalized medicine and an increase in the number and complexity of detected variants in advanced cancers have brought CGP solutions to the forefront, both for clinical practice and for research purposes.

Ideally, a CGP assay for formalin-fixed, paraffin-embedded (FFPE) tissue testing provides information both on established and emerging predictive molecular biomarkers; it maximizes all relevant information obtained from (limited) biopsy samples, and is straightforward to implement and operate in the laboratory. The latter is especially important in the context of an increasing effort to bring CGP in-house, locally. Effective implementation and standardization of CGP testing in-house can, however, be hampered by the abundance of available tests with different features, variability in bioinformatics systems (eg, secondary or tertiary analysis platforms) and data interpretation, and lack of standardized quality measures linked to published validation and utility data.[3,4] For example, having a robust method for predicting tumor purity improves the identification of copy number alterations (CNAs), and genomic signatures (TMB, MSI, and gLOH).[5] In addition, calculation of computational tumor purity enables quality metrics to be set, providing confidence in the results and allowing determination of assay sensitivity.[5–8] Although the secondary analysis platform version 1.0 (often integrated as part of the CGP assay) processes the CGP sequencing data and produces files with filtered variant calls and quality control metrics based on specialized algorithms, the tertiary analysis solution (to be paired with a CGP assay) provides variant reporting and visualization to enable the interpretation of genetic variants and reports clinical trial options. In essence, the challenge does not lie only in generating vast amounts of sequencing data, but also in establishing its relevance and significance in the context of the disease. Lack of uniformity in implementation of high-quality in-house CGP testing and integrated or paired bioinformatic systems may lead to inconsistent genomic findings.[5]

This study compared two commercially available, tissue-based, in-house CGP assays in combination with a tertiary analysis solution in a research use only (RUO) context: the AVENIO Tumor Tissue CGP RUO Kit (AVENIO CGP; Roche Molecular Systems, Inc., Branchburg, NJ) was paired with navify Mutation Profiler 2.3.1 [navify MP (RUO); Roche Sequencing Solutions, Inc., Pleasanton, CA] software and the TruSight Oncology 500 RUO assay (TSO-500; Illumina, San Diego, CA) was paired with PierianDx Clinical Genomics Workspace (CGW) version 6.21.0 software (PierianDx, Creve Coeur, MO). The aim of this study was to assess the agreement and differences in data for short variants (SVs), CNAs, rearrangements, TMB, and MSI, including variant categorization and clinical trial-matching (CTM) between the two assays used. For the observed differences, the origin and contributing factors related to both the assay and the bioinformatics analysis systems were explored, adding significantly to the understanding of key factors to consider when implementing in-house CGP testing.

Materials and Methods

Specimens and Samples

Samples used for this project were collected in the framework of the study "Somatische Mutationssignaturen in Soliden Tumoren" ("Somatic Mutation Signatures in Solid Tumors"; file number: Eth-oA 05/19 of the ethics committee of the State of Berlin Physicians Association, which waived the informed consent requirement for the anonymized sample collection with minimal clinical data; the ethics committees of the Brandenburg State Physicians Association, the Thuringian State Physicians Association, and the Saxony-Anhalt State Physicians Association concurred with this opinion). Nucleic acids were extracted from 180 macro-dissected FFPE tumor tissue samples. DNA and RNA used for TSO-500 assays (RUO; Illumina, Inc.; catalog number 20028216) were extracted using the Qiagen AllPrep DNA/RNA FFPE Kit (RUO; Qiagen GmbH, Hilden, Germany; catalog number 80234). DNA used for the AVENIO CGP Kit and the FoundationOne CDx assay [F1CDx; Foundation Medicine, Inc. (FMI), Cambridge, MA] was extracted using KAPA Express Extract Kit (RUO; Roche Diagnostics Cape Town, Cape Town, South Africa; catalog number KK7101). Pathologic review and all extractions were performed at Signature Diagnostics GmbH (Potsdam, Germany).

Comprehensive Genomic Profiling Assays

The TSO-500 assay is an RUO panel-based NGS assay that uses a hybridization-capture–based target-enrichment strategy and assesses 523 cancer-related genes.[9] In a single assay, it enables the detection of single nucleotide variants, multinucleotide variants, insertions or deletions (indels), and CNAs of 59 genes, measures genomic

signatures (including MSI and TMB), and supports RNA-based calling of fusions of 55 genes and alternative splicing events in FFPE tumor tissue samples.[9]

The AVENIO CGP Kit is an RUO panel-based NGS assay for genomic profiling of FFPE tumor tissue samples that assesses 324 cancer-related genes and detects all four mutation classes (single nucleotide variants, indels, CNAs, and rearrangements) across the whole panel (F. Hoffmann-La Roche Ltd., Basel, Switzerland; per manufacturer instructions). It also identifies three genomic signatures (TMB, MSI, and gLOH) in a single, DNA-only, hybrid capture workflow. The panel of genes in the AVENIO CGP Kit is designed to align with the gene content of the F1CDx panel, is powered by the FoundationOne Analysis Platform (developed by FMI, Cambridge, MA) for secondary analysis, and is based on the same algorithms that are also used in F1CDx.

F1CDx is a centralized NGS testing solution that has received approval as an *in vitro* diagnostic device. F1CDx has been approved by the US Food and Drug Administration for use as a companion diagnostic for the detection of selected genomic alterations in specific tumor types, as well as to assess *NTRK1/2/3* fusions, MSI, and TMB in all solid tumors (US Food and Drug Administration, *https://www.accessdata.fda.gov/cdrh_docs/pdf17/P170019S014C.pdf*, last accessed January 24, 2024). A high degree of alignment between the AVENIO CGP Kit and the F1CDx assay has been shown in a previous report, and F1CDx was used as a comparator assay on the same samples (Supplemental Table S1).[10]

Sequencing Analysis

Samples were sequenced on NextSeq500 instruments (Illumina) with eight (the AVENIO CGP Kit) or up to eight pairs of DNA plus RNA (the TSO-500 assay) libraries per sequencing run. Additional sequencing with a third assay was performed using F1CDx (Foundation Medicine GmbH, Penzberg, Germany). Assay details for the AVENIO CGP Kit and the TSO-500 assay are shown in Supplemental Table S2.

Secondary Analysis

The AVENIO CGP Kit sequencing data were analyzed using a proprietary software system version 1.0 (FoundationOne Analysis Platform) that was designed to detect all classes of genomic alterations, including base substitutions, indels, CNAs (amplifications and homozygous gene deletions), and select genomic rearrangements (eg, gene fusions), based on algorithms ('AVENIO CGP algorithms') previously described by Milbury et al.[5]

The TSO-500 assay sequencing data were analyzed using a software system developed by Illumina (TSO-500 version 2.2 Local App), run locally and as part of the PierianDx CGW software.

Both software systems used hg19 as a reference genome for reporting of variants.

Single Nucleotide Variants and Indels

SV calling for the AVENIO CGP Kit was performed using a *de novo* assembly-based algorithm,[5] which incorporated different quality control metrics (eg, number of reads mapping to an assembled variant, or read and mapping quality) followed by noise model-based filtering. Due to the performed *de novo* assembly, detected variants were not limited to those mapped by the upstream alignment algorithm (Burrows–Wheeler Aligner).[11] Thus, there was no theoretical upper limit for indel length.

The TSO-500 version 2.2 Local App used an algorithm called Pisces for SV calling.[12] It was based on scanning the original Binary Alignment Map file produced by the upstream alignment algorithm (Burrows–Wheeler Aligner, see previous paragraph) for discordances, and applying multiple quality and frequency filters to call putative variants. Pisces also has no restrictions on indel length but depends on the variant being present in the Binary Alignment Map file.

Copy Number Alterations

The AVENIO CGP Kit's CNA algorithm is an advanced version of the algorithm previously described by Milbury et al,[5] using preprocessed data for normalization instead of a process matched control sample. Using normalized sequence coverage levels and allele frequencies of known germline single nucleotide polymorphisms, a sample profile was produced. This profile was segmented, modeled, and then used to predict the copy number (CN) for each segment. Using the modeled profile and CNA levels, events were identified and called. The modeling also produced an estimate of tumor purity that could be used by downstream algorithms and for assessing the quality of the sample. In addition to the targeted genes, the AVENIO CGP Kit bait set included baits for single nucleotide polymorphisms and other regions to improve coverage across the genome for CNA calling. Thus, CNAs and homozygous losses could be called for all 324 targeted genes of the AVENIO CGP Kit.

CNA calling for the TSO-500 assay was performed using the CRAFT algorithm per manufacturer instructions, which identified amplification, reference, and deletion calling for target CNA genes within the assay (Illumina, Inc.). Coverage was counted for each target interval on the panel, and data were normalized, with the CNA status for each target gene and the fold change calculated. Biases, such as sequencing depth, target size, PCR duplicates, probe efficiency, guanine/cytosine bias, and DNA type, were corrected during normalization using normal FFPE and genomic DNA samples. A gene-specific threshold for amplification and deletion was determined for each target CNA gene based on *in silico* data. These thresholds were set to low levels per default; more stringent thresholds had to be defined when filtering the data downstream as tumor purity was not estimated by the CRAFT algorithm. CNAs were reported in 59 genes for the TSO-500 assay. Deletions were all marked as "LowValidation" in the variant call file but were still included in the downstream analysis.

Rearrangements/Structural Variants

The AVENIO CGP Kit called fusions and other relevant structural variants (eg, truncating rearrangements in tumor suppressor genes) based on a proprietary algorithm using clusters of discordant-read pairs that were further refined using the same *de novo* assembly approach that was used for SV calling. As the AVENIO CGP Kit does not include RNA sequencing data, rearrangement calling was based on DNA only. To identify structural variants, at least one of the breakpoints must be targeted. As breakpoints of most functional relevant fusions are located in intronic regions, selected introns of 34 genes were also targeted by the AVENIO CGP Kit, in addition to the exonic regions of 324 genes.

The TSO-500 assay calls fusions based on extracted RNA in 55 genes using the Manta fusion caller that facilitates discordant-read pair and split-read alignments, followed by RNA-specific filtering.[13] RNA sequencing data were also used to identify splice site variants in three genes (*AR*, *EGFR*, and *MET*).[14]

Fusion data sets were compared by automatically matching the involved genes and manual investigation of the resulting pairs, including removal of duplicate calls in both assays (eg, due to slightly different breakpoints or reciprocal events).

Tumor Mutation Burden

The AVENIO CGP TMB algorithm calculated TMB by counting the number of SVs with a variant allele frequency (VAF) of $\geq$5%, filtering out potential germline variants present in publicly available databases, or assessed by an algorithm utilizing the variants' VAF, as well as local CN, and estimated tumor purity from the CN algorithm. Additionally, potential driver mutations were removed to exclude bias. The count was then divided by the size of the targeted coding region (approximately 0.8 megabases).

The TSO-500 version 2.2 Local App used a similar approach, applying the same 5% VAF cutoff and removing potential germline variants using frequencies in public databases and an algorithm called 'Germline by Proxy.' 'Germline by Proxy' used the VAF of known germline variants in the same region if the variant in question was not present in a public database. The algorithm in the TSO-500 assays removed potential driver mutations by filtering variants with a count of $\geq$50 in the Catalogue of Somatic Mutations in Cancer (COSMIC) database.[15]

Microsatellite Instability

The AVENIO CGP Kit algorithm was used to determine MSI status by assessing repeat length at repetitive loci and comparing them to lengths previously assessed in training samples. The MSI score was then reported as a fraction of loci that were determined unstable and a predetermined threshold was used for calling instability.

The TSO-500 assay used a similar approach to calculate the fraction of unstable MSI loci, calibrated against previously processed samples.

Genome-Wide Loss of Heterozygosity

To assess potential homologous recombination deficiency (HRD) in ovarian tumor samples, the algorithm for the AVENIO CGP Kit calculated the percentage of gLOH, in addition to detecting variants in known HRD genes, such as *BRCA1/2*. Calculation of gLOH was based on the CN profile described in *Copy Number Alterations* and has been previously described by Milbury et al.[5] A threshold of 16% was applied to identify gLOH-positive samples.

While the TSO-500 assay does not provide a similar score, it does support calling variants in genes associated with HRD. Of note, there is a new version of the kit including a computational HRD score; however, this version was unavailable at the initiation of this analysis.

Variant Filtering

The AVENIO CGP Kit secondary results were filtered by the FoundationOne Analysis Platform for both quality metrics and variant frequencies observed in publicly available, population-genomic databases as well as the FMI data set. Quality filters are mostly built into the statistical models of the variant-calling algorithms, with addition of hard thresholds for the SV types: SVs: $\geq$5% for unknown variants (none for known variants); CNAs: CN $\geq$median ploidy +4 (+2 for *ERBB2*); rearrangements: $\geq$3 reads for known variants, $\geq$5 reads for unknown variants. For TMB, MSI, and gLOH, the status of the signature might be set to fail if certain quality metrics fail [eg, low tumor purity (<20%), low coverage (median, <500×), or contamination (>1%)]. To allow for a meaningful comparison of "raw variant calling" capabilities, intermediate output files without population genomic filtering were provided for the AVENIO CGP data set obtained by the AVENIO CGP Kit.

The TSO-500 Local App provides quality filters as part of the output but relies on downstream reporting solutions for filtering the variants, potentially including additional information, such as population-allele frequencies. DNA/RNA analysis outputs included TMB, variant call files for small and complex variants, MSI, gene amplifications, fusions, and splice variant call files.[16,17]

Tertiary Analysis

Two NGS-reporting solutions were used: navify MP 2.3.1 [Conformité Européene—In Vitro Diagnostic (CE-IVD); RUO in the United States] for the AVENIO CGP Kit, and a customized analysis pipeline within the CGW version 6.21.0 software platform from PierianDx/Velsera (RUO) for the TSO-500 assay.

For the AVENIO CGP Kit, navify MP imported data in different genomic builds and provided reports in hg38. A variant call file was uploaded with SV calls and a JSON file for all other mutations, including TMB and MSI status. To match the TSO-500 assay data, the same 1% VAF and population filters were used. However, as the AVENIO CGP Kit data were already filtered in secondary analyses,

the effect was minimal. The navify MP used the following databases for functional annotations: National Center for Biotechnology Information Reference (NCBI) Sequence Database (RefSeq) version 59, ROCHE 2.72.2, Clinical Interpretation of Variants in Cancer database (CIViC) 01-aug-2022, COSMIC version 96, NCBI database aggregating information about genomic variation and its relationship to human health (ClinVar) 20220804, Genome Aggregation Database (gnomAD) 2.1.1, Mitelman 27-jul-2022, The Cancer Genome Atlas 31.0, Database of Functional Predictions and Annotations for Human Non-synonymous and Splice Site SNVs (dbNSFP) 4.3a, and NCBI's database of human genomic Structural Variation (dbVar) 03-aug-2022.

For the TSO-500 assay, the CGW version 6.21.0 software platform used hg19 as the reference genome for report creation. FASTQ files were uploaded via the CGW RunUploader and the CGW run with the TSO-500 version 2.2 pipeline. The following filter criteria were applied for reporting: for SVs, only those variants with a PASS filter were used; population germline allele frequency $\geq$1% was excluded (gnomAD total population frequency), and VAF $\geq$1% was retained. All called fusions and copy number variants with fold change $<$0.5 (default filter setting for losses) and $\geq$3.2 (as reported by Conroy et al[14]) for amplifications were included in the analysis. Data [eg, date of birth, sex, disease, tumor purity, and country for CTM (Germany)] were uploaded. CGW used the following databases for annotation: RefSeq version 105, COSMIC version 96, ClinVar 20220702, gnomAD r2.1, Exome Aggregation Consortium (ExAC) version 1.0, dbNSFP 4.3c, National Heart, Lung, and Blood Institute Exome Sequencing Project (NHLBI ESP) version 0.0.30, NCBI Single Nucleotide Polymorphism Database (dbSNP) 149, and Database of all potential human SNVs within splicing consensus regions and their functional annotations (dbscSNV) version 1.1.

Statistical Analysis

Wherever possible, three measures of agreement were used: average positive agreement (APA), average negative agreement (ANA), and overall percentage agreement (OPA), together with their 95% CIs. APA was defined as both matched pairs yielding the same biomarkers divided by the sum of both matched pairs yielding the same biomarkers plus one of the pairs yielding the same biomarkers over all samples. ANA was defined as both matched pairs did not yield the same biomarkers divided by the sum of both matched pairs not yielding the same biomarkers plus one of the pairs not yielding the same biomarkers over all samples. OPA was defined as the sum of all matched pairs yielding the same biomarker plus the sum of all matched pairs not yielding the same biomarker, divided by product of biomarkers and number of all samples. APA, ANA, and OPA were expressed as percentages and based on identical overlapping regions. Overlapping bases (AVENIO CGP Kit − TSO-500 assay) = 748,308.

TSO-500 assay blacklisted on overlapping bases = 17,746.

Variant Reporting Analysis

Key variant annotation data outputs were variant tier classification,[18,19] and NCCN and European Society for Medical Oncology Scale for Clinical Actionability of molecular Targets (ESCAT) guideline inclusion per tumor type.[20] The navify MP for the AVENIO CGP Kit and PierianDx CGW software for the TSO-500 assay both utilized the Association for Molecular Pathology/American Society of Clinical Oncology/College of American Pathologists guidelines for variant tiering.[18] Inclusion in the NCCN and ESCAT guidelines was determined manually.

Clinical Trial-Matching

For CTM, default settings were used: navify MP used phase 2 to 4 trials within Germany, genomic profile, age, sex, and disease; CGW used the participant's genomic profile, tumor type, and phase 1 to 4 trials within Germany. In CGW, it is not possible to choose the trial phases, whereas navify MP was only adjustable for each sample on its own. Note that navify MP matches participant age while CGW does not, and that CGW extends the trial location beyond the preferred region, which navify MP does not. CTMs were extracted from the exported report files from navify MP and PierianDx CGW. All trial identifiers were mapped to *https://clinicaltrials.gov* registry numbers [National Clinical Trial (NCT) identifier (ID)], and trial status and inclusion criteria were retrieved using the *https://clinicaltrials.gov* application programming interface. Both systems occasionally mapped the same trial under different identifiers [eg, once with European Union Drug Regulating Authorities Clinical Trials Database (EUDRACT ID), once with NCT ID], and these duplicates were aggregated. As both systems reported a variant/biomarker based on the trial that was matched, each trial match was categorized as "based on concordant variant" if the variant/biomarker was among the reported variants in both systems.

Results

Samples

Of 180 samples, aliquots of DNA (and separately RNA for the TSO-500 assay) from 144 FFPE solid tumor tissue specimens of various origins (prostate: n = 27; breast: n = 27; colon: n = 25; lung: n = 25; bladder: n = 7; head/neck: n = 7; ovarian: n = 6; melanoma: n = 6; liver: n = 5; pancreas: n = 5; and stomach: n = 4) (Supplemental Table S3) from trial participants passed quality measures and were analyzed successfully by both the AVENIO CGP Kit and the TSO-500 assay, with additional analysis using F1CDx as a reference method.

A scatter plot of matched pairs as represented by each circle showing median coverage for AVENIO CGP and

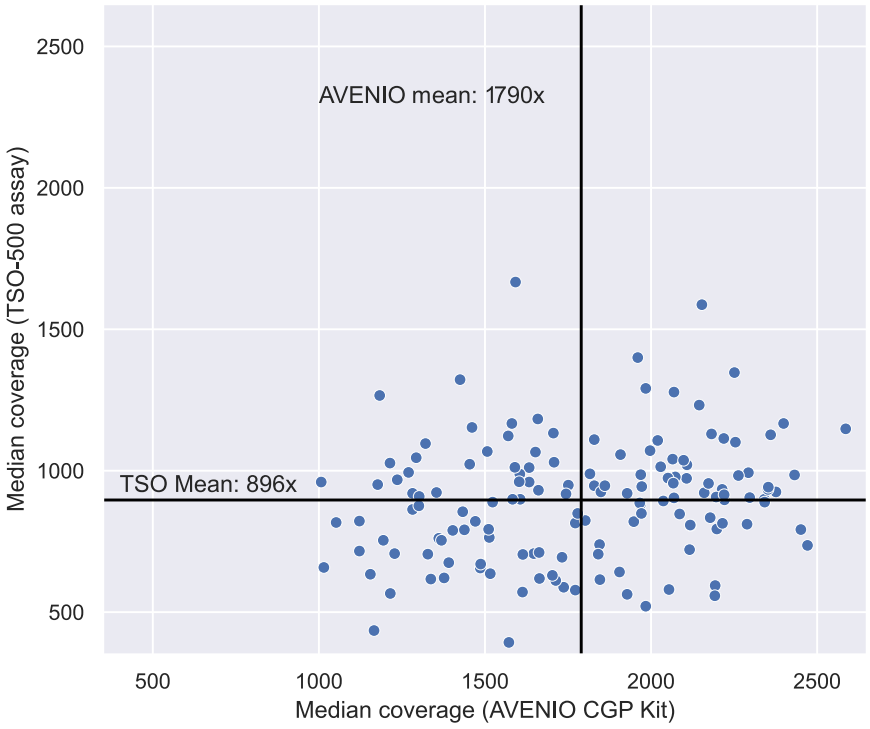

Figure 1 Scatter plot of matched pairs as represented by each circle showing median coverage for the AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit (AVENIO CGP Kit) and TruSight Oncology 500 assay (TSO-500 assay). Overall means of the median sequencing coverages are represented by the **vertical** and **horizontal lines**, respectively. While the eight DNA samples are sequenced on a NextSeq 500 using the AVENIO CGP Kit, eight DNA and eight RNA samples are sequenced on a NextSeq 500 using TSO-500. Therefore, the median coverage of the DNA samples has to be lower using TSO-500. However, the sequencing costs are identical for both kits.

TSO-500, with overall mean sequencing coverage, is represented by the reference lines and is shown in Figure 1.

Sample characteristics are shown in Supplemental Table S3.

Differences in variant calling and filtering between platforms is shown in Supplemental Figure S1.

Short Variant Agreement

Although both assay and bioinformatics solutions agreed on 39,625 SVs, they disagreed on 3412, leading to an APA of 92.1% (95% CI, 91.81%–92.33%) (Table 1). The APA for the 1161 indels and polynucleotide exchanges was 49.4% (95% CI, 46.44%–52.27%). The AVENIO CGP Kit detected 526 indels and polynucleotide exchanges that were not detected using the TSO-500 assay. In contrast, 61 indels and polynucleotide exchanges were detected by the TSO-500 assay that were not detected by the AVENIO CGP Kit. Given the large number of SVs, ANA tended to be high, driving OPA to 99.7%. The additional sequencing performed using the F1CDx showed similar high OPA/ANA/APA with the AVENIO CGP Kit (Supplemental Table S1).

Variant Categorization

The NCCN tier I/II variants ($n = 55$) and ESCAT tier I/II variants ($n = 33$) identified in this study with the AVENIO CGP Kit are listed in Supplemental Table S4. These variants were located in 14 genes following NCCN guidelines (*BRAF*, *BRCA1*, *BRCA2*, *CHEK2*, *EGFR*, *ERBB2*, *FGFR3*, *KRAS*, *MET*, *NRAS*, *PALB2*, *PIK3CA*, *RAD51C*, and *RAD51D*) and nine genes following ESCAT guidelines (*BRAF*, *BRCA2*, *CDKN2A*, *EGFR*, *ERBB2*, *KRAS*, *MET*, *PALB2*, and *PIK3CA*), and they occurred in a range of common solid tumor types (melanoma along with carcinomas of lung, prostate, breast, colon, head and neck, bladder, and ovary). All NCCN and ESCAT tier I/II variants that were detected by either the AVENIO CGP Kit or by the TSO-500 assay were identified by the AVENIO CGP Kit, whereas the TSO-500 assay did not detect 5 of 10 CNAs [4 *ERBB2* amplifications; sample IDs 63211, 62547, 62511, and 67815 using the stringent settings (Figure 2 and Supplemental Table S5) and a *BRCA2* deletion; sample ID: 62516 (Supplemental Table S5)]. Figure 2 shows the overlap of ESCAT and NCCN variants between the AVENIO CGP Kit and the TSO-500 assay. Of note, no gene fusions with ESCAT or NCCN tier I/II were identified with this analysis, reflecting the low prevalence of these variants in the tumor types included in this study.

For tier I/II variants using Association for Molecular Pathology/American Society of Clinical Oncology/College of American Pathologists guidelines, results for each assay are included in Supplemental Tables S4–S7. Tier I/II variants detected by the two assays overall were 719 SVs (single nucleotide and indel variants) (Supplemental Table S6), 251 CNAs (using the stringent threshold settings) (Supplemental Table S5), and 54 gene fusions (Supplemental Table S8). Fewer variants were identified by the AVENIO CGP Kit, mostly due to filtering of putative

Table 1 Agreement of SV, TMB, and MSI between the AVENIO CGP Kit and TSO-500 Assay

Biomarker	Statistic	Estimate, %	LCL	UCL
SV	OPA	99.5	99.48	99.52
	APA	92.1	91.81	92.33
	ANA	99.5	99.45	99.49
TMB	OPA	86.8	80.16	91.87
	APA	57.8	42.15	72.34
	ANA	83.9	76.00	90.02
MSI	OPA	99.3	96.19	99.98
	APA	80.0	28.36	99.49
	ANA	99.3	96.08	99.98

ANA, average negative agreement; APA, average positive agreement; AVENIO CGP Kit, AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit; LCL, lower confidence limit; MSI, microsatellite instability; OPA, overall positive agreement; SV, short variant; TMB, tumor mutational burden; TSO-500 assay, TruSight Oncology 500 assay; UCL, upper confidence limit.

benign variants (Supplemental Table S7) in addition to the expected variation based on panel design (eg, panel size). Analyses were performed by examining the agreement of tier I/II variants in a similar way to a recent publication.[19] In this study, a tiered variant list was presented to allow the comparison of each assay assessed against the other. In total, 281 tier I/II SVs were called as either tier I or tier II by both assays, whereas 112 and 326 were called as tier I or II only by the AVENIO CGP/navify MP solution and TSO-500/CGW solution, respectively. Examination of the 112 variants that were only called by the AVENIO CGP/navify MP solution showed that most ($n = 77$) were also called by the TSO-500 assay but categorized as tier III by CGW (Supplemental Table S6). Nearly all variants ($n = 76/77$) in this group were called tier II by the AVENIO CGP Kit, and several genes clustered in specific tumor types where alterations in those genes have been reported as common driver alterations. The remaining 35 variants were not called in the TSO-500 assay data set and were predominantly indels affecting larger than 10 bps or at homopolymer regions, suggesting that the TSO-500 assay subjects this kind of variant to more stringent filtering or exclusion. As an example, the *BRCA2* loss-of-function variant p.I605fs (NM_000059.4:c.1813dupA; *https://www.ncbi.nlm.nih.gov/nuccore/NM_000059.4*, last accessed August 15, 2024) within one of the lung adenocarcinoma samples (66835) occurred as a duplication of an 'A' nucleotide, within an 8-A homopolymer tract. The variant allele fraction of 48% for this mutation suggests that this may be an incidental germline variant. However, as neither assay requires a paired normal sample, a somatic origin cannot be entirely excluded. This variant was not called by the TSO-500 assay.

When looking at the 326 variants that were exclusively called and annotated as tier I/II by the TSO-500/CGW solution, nearly half ($n = 154$) were already filtered out "as benign" for the AVENIO CGP Kit by the FoundationOne Analysis Platform at secondary analysis (Supplemental Table S7). Closer investigation of the filtered variants for the AVENIO CGP Kit showed that most of these were filtered because of more stringent population frequency thresholds and/or custom filter rules based on the FMI database of genomic profiles. For example, the missense variant homo sapiens MET proto-oncogene, receptor tyrosine kinase (MET) NM_001127500.1p.T1010I (*https://www.ncbi.nlm.nih.gov/nuccore/NM_001127500.1*, last accessed August 15, 2024) was called in 9 of 144 samples (based on the VAF) eight times as heterozygous and once as homozygous germline variant, thus corresponding to an allele frequency of 3.45% in the sequenced population with no significant bias toward a specific type of cancer. However, the overall population frequency in Genome Aggregation Database, which was used for filtering in CGW, was slightly below the 1% threshold used for filtering (0.82%). One single variant (*KRAS* G12F mutation), characterized as tier I by the TSO-500/CGW solution, was identified by the AVENIO CGP Kit, but slightly below the 5% VAF cutoff that was chosen for reporting in navify MP (sample ID: 66850). This variant occurred in a sample with an assigned tumor type of lung adenocarcinoma and appeared to be a clonal mutation based on the assigned histologic tumor purity of 10% and

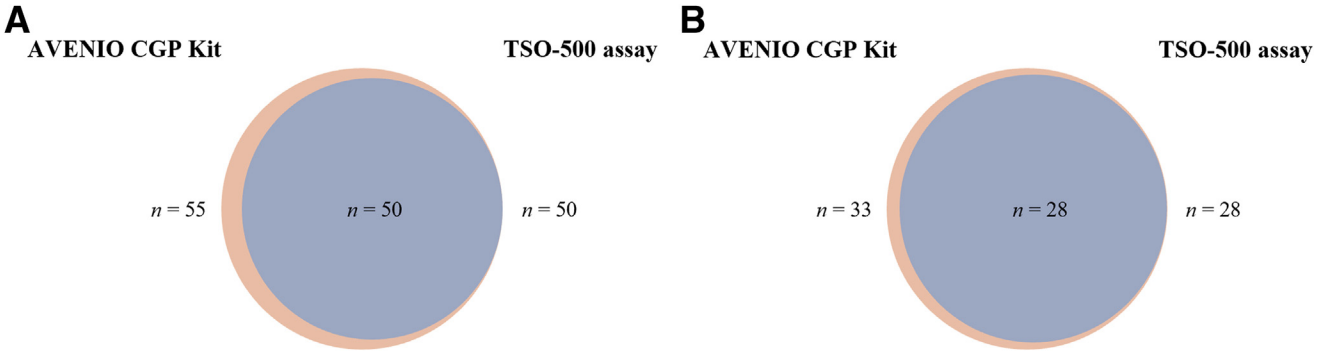

Figure 2 Venn diagram showing the proportion of National Comprehensive Cancer Network (**A**) and European Society for Medical Oncology Scale for Clinical Actionability of molecular Targets (**B**) variants between the AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit (AVENIO CGP Kit) and the TruSight Oncology 500 assay (TSO-500 assay).

variant allele fraction of another putative somatic driver mutation (STK11 E256*) present at a variant allele fraction of <10% with both testing platforms. Of note, unlike *KRAS* G12C, *KRAS* G12F is not currently cited in the ESCAT[21] or NCCN guidelines for non–small-cell lung cancer (NCCN; referenced with permission from the National Comprehensive Cancer Network, Inc. National Comprehensive Cancer Network, Inc. 2024. All rights reserved. Last accessed January 25, 2024. To view the most recent and complete version of the recommendations, go online to NCCN.org. NCCN makes no warranties of any kind whatsoever regarding their content, use or application and disclaims any responsibility for their application or use in any way). Additionally, 80 variants were in genes not targeted by the AVENIO CGP Kit panel, and 78 variants were categorized as lower tiers by navify MP. Of the remaining 14 variants, eight were slightly below the 5% VAF cutoff chosen for reporting in navify MP, and the remaining six variants were not called or were filtered as potential technical artifacts.

Copy Number Alterations

The AVENIO CGP Kit models tumor purity and ploidy to call CNAs without the need for the user to specify additional thresholds, whereas the TSO-500 assay CNA calls, when providing a single fold-change threshold, were dependent on purity (Spearman correlation coefficient: 0.53; $P < 0.0001$) (Figure 3). In 35 overlapping genes where both assays call losses or amplifications, the AVENIO CGP Kit called 131 amplifications compared with 37 amplifications called by the TSO-500 solution; all amplifications called by the TSO-500 assay were also called by the AVENIO CGP Kit. However, the TSO-500 assay called 148 losses compared with the 10 losses called by the AVENIO CGP Kit; there was an overlap of three losses. On 4756 of the total 5040 combinations of genes multiplied by samples, both assays agreed on a "no change" call. The weighted κ coefficient was 0.2395 (95% CI, 0.1784–0.3007), indicating a poor agreement on amplifications and losses. In total, both assays disagreed on 244 calls and agreed on 4796 calls.

When filtering for tier I/II variants in either navify MP or CGW and applying the default filters for the CGW software without a strict fold-change cutoff (Figure 4), 103 CNAs were called by both assays (Figure 4A). Of the remaining 150 calls that were unique to the AVENIO CGP/navify MP solution, most ($n = 118$) were in genes where the TSO-500/CGW solution does not call CNAs. In contrast, most of the 155 unique calls in TSO-500/CGW ($n = 139$) were in genes where the AVENIO CGP Kit was able to call CNAs but were not observed in the final data output.

When using the default filter settings from the CGW software combined with a fold-change threshold of 3.2, the overlap of tier I/II CNAs was much lower ($n = 39$) (Figure 4B). With these settings, 92 of the calls unique to the AVENIO CGP Kit were now below the fold-change cutoff of the TSO-500 assay. Only two CNAs were now unique to the TSO-500 assay, one of which was in a gene not included in the AVENIO CGP Kit. The other variant was a heterozygous *PTEN* loss. Investigation of the data showed that this was likely a heterozygous loss of all of chromosome 10, and such nonfocal events were not called by the AVENIO CGP Kit. It is important to note that losses were for information only in this version of the CGW software.

The different fold-change thresholds chosen in the preceding paragraphs and Figure 4 illustrate that, because of the dependency of the fold change on tumor purity and ploidy, setting a single threshold for all samples when using the TSO-500 assay might not be appropriate. For example, with the strict threshold in an invasive bladder transitional cell carcinoma sample, an *ERBB2* amplification (chromosome

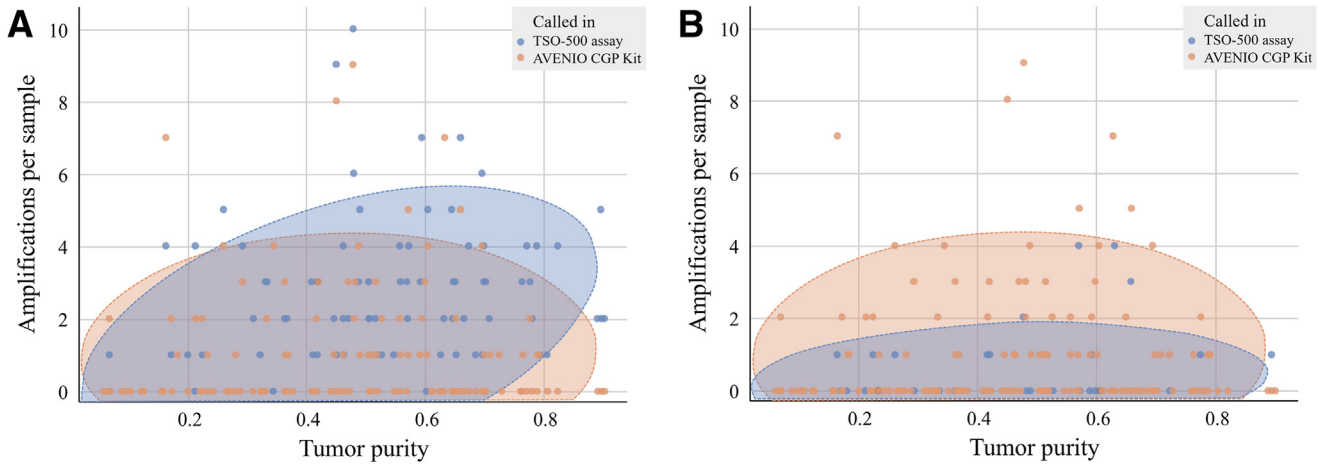

Figure 3 Copy number alterations per sample (*y* axis) versus tumor purity [from the AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit (AVENIO CGP Kit) results; *x* axis] for the TruSight Oncology 500 assay (TSO-500 assay; blue) and AVENIO CGP Kit (orange) assays. Variants were filtered for tier I/II and only genes where both assays called amplifications. Shaded areas depict covariance confidence ellipses, radius = 2 SDs. TSO-500 assay fold-change thresholds were set at 1.5 (**A**) and 3.2 (**B**).[14] No thresholds were required for the AVENIO CGP Kit.

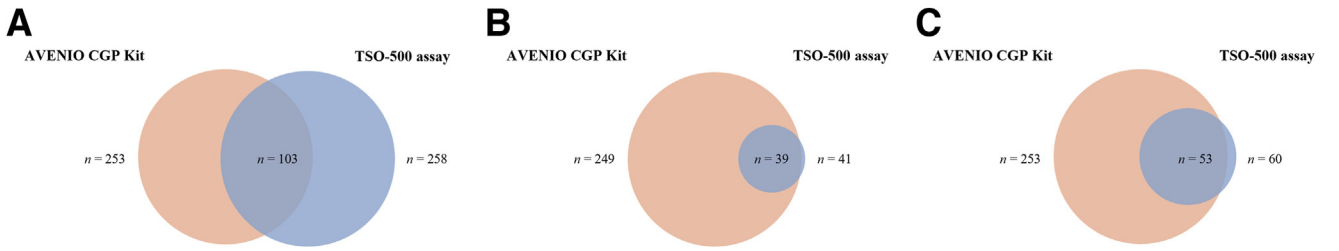

Figure 4 Copy number alterations when filtering for tier I/II variants in either navify Mutation Profiler or PierianDx Clinical Genomics Workspace (CGW) using default filters for the CGW software (1.5-fold change; **A**), default filter settings for the CGW software combined with a fold-change threshold of 3.2 (**B**),[14] and default filter settings for the CGW software combined with a fold-change threshold of 2.2 (**C**).[14] No thresholds were required for the AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit (AVENIO CGP Kit). TSO-500 assay, TruSight Oncology 500 assay.

17), which was called at CN = 17 by the AVENIO CGP Kit, was filtered out by the TSO-500/CGW solution because the fold change was 2.43. The sample had a comparably low tumor purity (estimated at 24.31% by the AVENIO CGP Kit) (Supplemental Figure S2). This variant would be included when using a third fold-change threshold of 2.2 that has also been previously used by Conroy et al[14] to call "indeterminate" amplifications. Using this threshold increases the number of overlapping CNAs to $n = 53$, while only increasing the number of calls unique to TSO-500/CGW to $n = 7$ (Figure 4C).

Rearrangements and Fusions

Overall, 12 fusions were called by both assays, whereas 57 rearrangements and 25 fusions were called only by the AVENIO CGP Kit and the TSO-500 assay, respectively (Supplemental Table S8). The difference can be explained by the different number of genes used for rearrangement calling (AVENIO CGP Kit: $n = 324$; TSO-500 assay: $n = 55$; this explains the $n = 44$ and $n = 1$ discordant calls, respectively) and the different supported variant types in the Kit (AVENIO CGP Kit: gene fusions and all other types of structural variants affecting the targeted genes; TSO-500 assay: fusions and alternative splicing events; this explains the $n = 8$ and $n = 15$ discordant calls, respectively). Oncogenic driver fusions in solid tumors are rare, especially those with potential relevance to the disease, such as fusions targetable with tyrosine kinase inhibitors. Given the size of the data set and that it was not specifically selected for harboring fusions, the ability for assessing the fusion-calling abilities of both assays is limited. The only relevant fusions previously described in the context of the investigated diseases were *TMPRSS2* fusions in prostate cancer samples, where there was 100% agreement between the two assays ($n = 9/12$ of the concordant variants; $n = 1/9$ *TMPRSS2-ETV1*; and $n = 8/9$ *TMPRSS2-ERG* fusions). However, two of eight (one from each assay) concordant *TMPRSS2* fusions were only added as concordant after manual investigation showed that they were concordant but annotated differently, highlighting the difficulties of interpreting complex variants from different sources even if these are well described in literature.

There were three more concordant potential fusions of *ERBB2-STARD3*, *TP53-DHX33*, and *TP53-CLUH,* respectively. Interestingly, *TP53*, *DHX33*, and *CLUH* are not part of the RNA target list.

The majority of discordant variants were rearrangements other than fusions and, because of the different technologies used for rearrangement calling, they may not have been called by the other assay or they may have been reported differently. The TSO-500 assay calls alternative transcripts/splice variants in a select list of three genes (*AR*, *EGFR*, and *MET*). In the 144 samples investigated, 15 such variants were called (*AR*: $n = 6$; *MET*: $n = 5$; and *EGFR*: $n = 4$).

As the AVENIO CGP assay does not use RNA, it cannot directly detect differential splicing events; however, it might be able to identify the underlying genetic variants causing the alternative splicing event, such as larger deletions or SVs affecting canonical splice sites. In total, five *MET* ×14 skipping events were detected by the TSO-500 assay, based on RNA analysis. For two of these, both the AVENIO CGP Kit and TSO-500 assay called a known *MET* ×14 splice site SV. Notably, these two samples were lung adenocarcinoma cases. The other three *MET* ×14 skipping events were called with a low read count close to the calling threshold in samples from other cancer types for which this kind of event has not been described, suggesting that they could be false positives or low-level subclones.

Concordance of other structural variants detected by the AVENIO CGP Kit without a qualitative effect on the RNA could not be assessed. However, there are some instances where such variants are likely the driver mutation for tumorigenesis. The AVENIO CGP Kit identified a putative truncating deletion segment with one breakpoint in *PTEN* exon 1 and the other breakpoint in an intergenic region in a prostate cancer sample without other known driver mutations. *PTEN* loss is a well-known driver in prostate cancer.[22] Note that this variant was not supported by the version of the navify MP used in this analysis.

Tumor Mutational Burden Differences

TMB with the TSO-500 assay was significantly higher versus the AVENIO CGP Kit (average pairwise difference, 3.4 mutations/megabase; $P < 0.001$) (Table 1). The TSO-

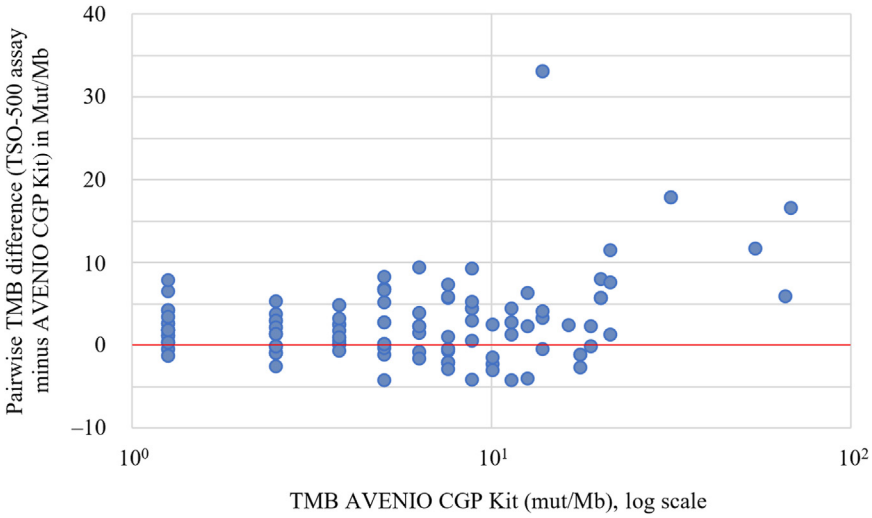

Figure 5 Comparison of tumor mutational burden (TMB) scores between the TruSight Oncology 500 assay (TSO-500 assay) and the AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit (AVENIO CGP Kit). *x* Axis, AVENIO CGP Kit TMB (log scale), and *y* axis: pairwise difference. The **red line** indicates no difference (0). Note that a single outlier sample with a very high TMB (>>100) was excluded. Mb, megabase; mut, mutations.

500 assay called 41 of 144 samples TMB >10 mutations/megabase versus 31 of 144 samples for the AVENIO CGP Kit (Figure 5). While the Pearson correlation of TMB values yields a coefficient of 0.99169, the Spearman correlation coefficient was much lower with a 0.85333 coefficient. This suggests that only 73% of the variance of TMB values can be explained by a linear regression. The reasons for differences between the TSO-500 assay and the AVENIO CGP Kit are shown in Table 2, with the most important contributors being differential filtering of potential germline variants and, especially, known/likely driver variants. The AVENIO CGP Kit showed a high degree of agreement [OPA, 90.91% (95% CI, 80.94%−98.54%), APA, 75% (95% CI, 42.81%−94.51%), and ANA, 91.18% (95% CI, 76.32%−98.14%)] with the F1CDx assay for these samples (Supplemental Table S1 and Supplemental Figure S3).

Other Biomarkers and Variants

The OPA for MSI was 99.3% (95% CI, 96%−100%). TSO-500 detected four of five MSI-high samples detected with the AVENIO CGP Kit (Table 1). The disagreement on one sample stems from the cutoff of 20% unstable loci for the TSO-500 assay, where the sample has 20 unstable loci of 122 usable loci (16.39%).

A comparison of gLOH could not be performed because the TSO-500 assay did not offer a comparable biomarker to measure HRD; however, four samples were available with an ovarian cancer disease ontology for which the gLOH signature has been validated in F1CDx (Supplemental Table S1). One of these samples did not yield a valid gLOH result because of low tumor purity. Of the remaining three samples, two of the gLOH scores were below the threshold

Table 2 Reasons for Differences between the TSO-500 Assay and AVENIO CGP Kit for TMB Testing

Assay	Reason	*n*
Variants included in TMB calculation by AVENIO CGP Kit	AF below threshold in the TSO-500 assay	54
	Close to 5% AF threshold	31
	Gene not targeted by the TSO-500 assay	80
	In TMB file, but excluded from score	3
	MNVs are excluded by the TSO-500 assay	11
	Other	49
	TSO-500 assay filter: GermlineFilterProxi	64
	TSO-500 assay filter: no CodingVariant	6
Variants included in TMB calculation by TSO-500 assay	AF below threshold in AVENIO CGP Kit	100
	ExAC allele count above threshold (>2)	52
	Gene not targeted by the AVENIO CGP Kit	967
	Known/likely/ambiguous variant in the AVENIO CGP Kit	266
	No potential germline call in the AVENIO CGP Kit	42
	Other	43
	Potential germline variant in the AVENIO CGP Kit	89

AF, allele frequency; AVENIO CGP Kit; AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit; ExAC, Exome Aggregation Consortium; MNV, multinucleotide variant; TMB, tumor mutational burden; TSO-500 assay, TruSight Oncology 500 assay.

established for F1CDx of 16% (0% and 14.1%, respectively), whereas the other one had a score above the threshold (26.8%), which could indicate the applicability of poly (ADP-ribose) polymerase inhibitors. Of note, a *BRCA1* frameshift variant was also identified in this sample by both the TSO-500 assay and the AVENIO CGP Kit, which is also indicative of HRD.

Clinical Trial-Matching

In total, 1056 trials were matched by the AVENIO CGP/navify MP solution (average seven trials per participant), and 543 by the TSO-500/CGW solution (average four trials per participant); however, these included trials that were not currently recruiting [$n = 169$ (16%) in navify MP and $n = 163$ (30%) in CGW], trials that were not taking place in Germany [$n = 286$ (53%) in CGW], and trials where the participant was not eligible because they did not meet the age inclusion criteria [$n = 65$ (6.2%) in navify MP and $n = 136$ (25%) in CGW]. After discarding nonappropriate trials, 825 valid trials remained for the AVENIO CGP/navify MP solution and 218 for the TSO-500/CGW solution (Supplemental Table S9); of these, 85 were identical (ie, present in both systems). The rest were either matched only by navify MP ($n = 740$) or only by CGW ($n = 133$). An overview of trial concordance and discordance between the two assays is presented in Table 3. The differences in results are mainly driven by disagreements in the underlying reported variants (discordant variants) and the use of extended search terms such as HRD. The rest of the discordances are explained by subtle differences in the design of the reporting solutions for the CTM approach (eg, use of databases, selected trial phases, or type of intervention, etc.).

Data Availability

For up-to-date details on Roche's Global Policy on the Sharing of Clinical Information and how to request access to related clinical study documents, see here (*https://www.roche.com/innovation/process/clinical-trials/data-sharing*, last accessed September 4, 2024).

Discussion

Implementation of high-quality, in-house CGP testing solutions is essential to enable the consistent use of genomic insights to support a shift toward precision oncology. When using one assay across different laboratories, a high intralaboratory and interlaboratory reproducibility can be achieved, as shown by a recent report of the AVENIO CGP Kit used in five independent laboratories.[23] However, various CGP assays, bioinformatics solutions, and variant reporting analysis methods are available in the market today. Differences in these systems and a lack of understanding of the complex interplay between them are a significant challenge for the implementation of CGP testing in the laboratory and achieving uniformity across laboratories. In this study, the data outputs from two commercially available in-house RUO CGP pan-cancer solutions paired with a reporting analysis software were compared to determine the agreement in data and identify the origin of the differences observed. Good understanding of the different approaches and specific features of both the assay and a paired bioinformatics system are key for an accurate implementation of an in-house CGP workflow and obtaining high-quality results enabling a more complete comprehensive genomic understanding of the cancer.

The ESCAT tiers classification, as agreed by leading cancer specialists in Europe and North America, is widely used in guidelines, supports the discussion and decision processes by institutional molecular tumor boards, and may serve as a support tool for reimbursement. Of the total 33 ESCAT tier I/II variants identified with both assays, the AVENIO CGP Kit detected all, whereas the TSO-500 assay did not detect 5 of 10 CNAs [4/10 CNAs in *ERBB2* (*HER2*) and a deletion in *BRCA2*], as discussed below. Utilizing NCCN, rather than ESCAT, guidelines yielded similar results. When comparing Association for Molecular Pathology/American Society of Clinical Oncology/College of American Pathologists tiering of the two solutions, both assays called a substantial number of tier I/II variants that were tiered differently or not called by the other solution. While the majority of variants exclusively detected by the

Table 3 Overview of Trial Concordance between the AVENIO CGP Kit/navify MP and TSO-500 Assay/CGW

	navify MP	CGW	Overall
Total valid trials matched, *n*	825	218	1043
Discordant trials, *n* (%)	740 (89.7)	133 (61.0)	873 (83.7)
Based on discordant variant, *n* (%)	282 (34.2)	113 (51.8)	395 (37.9)
Based on extended search term, *n* (%)	153 (18.5)	0 (0)	153 (14.7)
Based on concordant variant, *n* (%)	458 (54.8)	20 (9.2)	478 (45.8)
Concordant trials, *n* (%)	85 (10.3)	85 (39.0)	170 (16.3)
Based on discordant variant, *n* (%)	5 (0.6)	5 (2.3)	10 (1.0)
Based on concordant variant, *n* (%)	80 (9.7)	80 (36.7)	160 (15.3)

Percentages in parentheses refer to the total valid trials matched.

AVENIO CGP Kit, AVENIO Tumor Tissue Comprehensive Genomic Profiling Kit; CGW, PierianDx Clinical Genomics Workspace; navify MP, navify Mutation Profiler; TSO-500 assay, TruSight Oncology 500 assay.

AVENIO CGP Kit were a result of the tiering process itself, the biggest factor for variants only detected by the TSO-500 assay was differences in filtering processes. The TSO-500 assay secondary pipeline filters use quality metrics only, whereas the AVENIO CGP Kit pipeline includes filters for function and frequency to exclude putative benign variants, and utlilizes internal FMI data. Depending on the use case, both approaches have their advantages and disadvantages. The unfiltered data from the TSO-500 assay allows a more flexible use of the data downstream, whereas the data from the AVENIO CGP Kit are ready to use for downstream tertiary reporting applications.

TMB reported with the TSO-500 assay was significantly higher than with the AVENIO CGP Kit. With the exception of different target areas, which are controlled for when calculating TMB, the biggest differences originated from filtering the underlying variants. Both assays removed potential driver mutations, as recommended in TMB harmonization efforts.[24] However, the AVENIO CGP Kit algorithm used a less stringent definition of driver mutations (eg, including loss-of-function variants in tumor suppressor genes). Another important difference is the removal of potential germline variants. The AVENIO CGP Kit used a statistical model based on tumor purity and CN modeling,[25] whereas the TSO-500 assay used information of known germline variants close to the variant in question ("Germline by Proxy"), which leads to the inability of calling a germline status if no such variant is nearby or if the tumor purity is $\geq$80%.[15] These differences lead to different numbers of TMB-high cases when applying the established threshold of 10 mutations/megabase[17]: thirty-one of 144 and 41 of 144 samples for the AVENIO CGP Kit and the TSO-500 assay, respectively. The AVENIO CGP Kit has been shown to have a high degree of agreement [weighted κ coefficient = 0.88 (95% CI, 0.78−0.98; asymptotic SE: 0.05)] with the F1CDx assay in a previous study,[10] for which the range of TMB values is well described.[10,26–28]

Calling of CNAs is complex, and having a robust method for predicting tumor purity is critical.[5,29] Tumor cells typically undergo a collection of large-scale (chromosome-wide or chromosome-arm-wide) and small-scale genomic amplifications and losses. Tumor samples also typically consist of a mixture of tumor and normal cells (range, approximately 20% to 80%), with normal cells tending to be diploid. The tumor component of a tumor sample tends to correspond mostly to a single dominant clone, and many tumors undergo a genome-wide amplification as part of their evolution, so a range of overall ploidies is possible. The AVENIO CGP Kit bioinformatics pipeline uses coverage ratios and allele frequencies from common germline single nucleotide polymorphisms to model the underlying purity and ploidy of the tumor and subsequently call CNAs. By controlling for purity and ploidy as part of the calling procedure, the CNA calls by the AVENIO CGP Kit are not dependent on these factors, and the user can use the estimated CN value directly. However, the current method applied by the TSO-500 assay requires the user to define a threshold for the coverage ratio (fold change) of each gene and each sample. Arguably, very distinct CNAs can also be called with this approach by applying the same strict cutoff for all samples and genes and thereby controlling for specificity. As shown in this study, this could lead to missing potentially relevant variants, such as *ERBB2* amplifications. However, it has been shown previously that biologically relevant amplifications tend to have higher CNs, thereby making any limitations in CNA calling potentially less significant in the overall context.[30,31]

Gene fusions and other rearrangements with biological relevance to the disease are rare in solid tumors. As this study aims to be representative of real-world use of CGP assays and includes only a limited number of samples, the number of actionable fusions identified here, such as targetable *ALK*, *ROS*, *RET*, *NTRK*, and *FGFR* fusions, is, as expected, limited. The only relevant fusions detected were *TMPRSS2* fusions in prostate cancer samples,[32] where concordance following manual interpretation was 100%. The majority of discordant variants were rearrangements other than fusions. When interpreting potential rearrangements not previously described in the literature, variability in data quality can lead to additional complexity, and hence careful interpretation is recommended. Both assays have subsets of rearrangements with low numbers of supporting reads, close to the limit of the detection. While these could be due to subclones, at least some of these variants are likely false positives. For example, it is not easy to distinguish a *TP53-CLUH* fusion that was detected by both assays and is therefore likely to be real but had only 14 supporting reads in the TSO-500 assay, which is close to the limit of detection (seven unique reads).

The TSO-500 assay uses RNA for fusion calling, whereas the AVENIO CGP Kit uses DNA, and both approaches have their advantages and disadvantages. DNA is more stable than RNA, requires no additional wet-laboratory work or sequencing, exact breakpoints can be identified, and other rearrangements that are not expressed in RNA, such as truncating deletions, can be called. While gene fusions are arguably the most important structural variants in cancer, truncating variants, such as the phosphatase and tensin homolog (PTEN) rearrangement identified in this study,[33] have also shown to be relevant to the disease. However, DNA is potentially less sensitive when using targeted panels (as at least one of the breakpoints must be targeted), and specific targets need to be added to a panel to capture fusions (ie, certain introns of interest in the AVENIO CGP Kit baitset increase the panel size and thus the cost of sequencing). This might not be viable in diseases, like hematologic cancers or sarcomas, that are mainly driven by a diverse set of fusions with a comparably long list of target genes. Additionally, it is difficult to capture highly repetitive regions of intronic DNA, where some breakpoints for common fusions may be located. RNA analysis, on the other hand, has a higher sensitivity as it is not necessary to capture or target the exact breakpoint. RNA analysis is a

quantitative method that can also reveal if a potential fusion transcript is expressed. However, RNA analysis may fail in 9% to 50% of FFPE samples, especially in poorly processed/preserved FFPE, as RNA degrades more quickly than DNA and the sensitivity to detect fusion gradually decreases with decreasing sample quality.[34–40] Use of RNA for sequencing also represents a second workflow with additional costs when compared with DNA sequencing alone, analysis is limited to fusion and splicing events (ie, other arrangements like truncations of tumor suppressor genes cannot be detected), and the exact breakpoints cannot be identified. Even though the power of this study to assess fusion concordance is limited, the variants that were identified in both RNA and DNA show that a carefully designed DNA-based assay, which includes important introns paired with high-quality bioinformatics methods, can yield comparable results to RNA-based assays in solid tumors.[41] The results are in line with a recent study assessing large structural variants in cancer genomes[42]; however, variable results have been reported in the literature.[43–46] It is important to note that in driver-negative tumors or in diseases driven by a broader set of potential fusions, such as sarcomas or hematologic cancers, RNA-based assays should always be considered.

Numerous new treatments and evolving biomarkers are being evaluated in clinical trials. To increase the efficiency of the CTM exercise, a good understanding of the high value and impactful parameters of the bioinformatics system is required. Although both navify MP and CGW use similar input variables for CTM (disease and genetic variants of the participant), the two systems have low overall agreement in the obtained trial listing. The differences are partly driven by disagreements in the underlying reported variants (resulting from different assay designs, variant calling, and tiering approaches) and partly by conceptual differences in CTM, such as selection of intervention type and/or regions, filtering of trial phases, use of trial databases, and inclusion of participant factors for trial eligibility. Both systems have significant differences in these features: i) CGW reports trials with more targeted interventions, whereas navify MP includes trials with a wider range of interventions; ii) the navify MP uses multiple databases, whereas CGW only uses *https://clinicaltrials.gov*; iii) the CGW extends the geographic search radius stepwise and ultimately matches trials in any location if no trials are available in the preferred location (eg, 53% of trials reported by CGW did not have a study site in Germany), whereas the navify MP limits the results strictly to the indicated preferred region; iv) CGW does not consider participant age for trial eligibility, whereas the navify MP does; and v) the CGW includes phase 1 trials, whereas the navify MP does not. These conceptual differences explain why the navify MP showed a higher percentage of valid trials in the desired location and for which the participants were eligible. As CTM algorithms in combination with different assays can display huge variability, it is important that the managing physician or research staff understand the factors with the highest impact on the matching efficiency of the platforms. The outcome generated through any CTM software needs to be reviewed in context of the interpretation and actionability of the identified tumor mutations, ideally by a molecular tumor board to find the best study match for the participant in a rapidly evolving trial landscape.

This study compares two RUO CGP solutions using real-world FFPE samples. Although this allows for the realistic assessment of the capabilities of both solutions, it is hard to assess which solution is correct in cases of discordance. In cases such as differences in tiering of the same variant, assessment is highly dependent on the individual needs of the laboratory running the test. Secondary analysis discordances are equally difficult to assess, because a gold standard does not exist. For example, $n = 3/4$ of the discordant ESCAT/NCCN tier I/II *ERBB2* amplifications could be evaluated using digital PCR and only $n = 1/3$ could be confirmed. Closer investigation showed that the two discordant amplifications were modeled as equivocal whole chromosome arm events at copy number 5, which is right at the limit of detection and does not allow for an unambiguous decision on whether the events are true or false positives. Utlilizing F1CDx as a comparator (eg, for TMB) allows the investigator to lean on higher degrees of validation and published studies. However, it must be acknowledged that the similarity of the AVENIO CGP Kit to F1CDx is high by design. If possible, a correlation of results with known prevalences of specific variants in diseases of interest should be taken into account to assess potential systematic discordances. Although this does not allow judging in single cases, it does reveal systematic overcalling of certain variants that can be used to subsequently adjust filters, as illustrated using the MET NM_001127500.1p.T1010I variant.

In conclusion, the two commercially available RUO CGP solutions paired with a tertiary reporting analysis software showed a good overall agreement for detection of SVs, known gene fusions (limited by available variants in data set), and MSI, while important differences were obtained in TMB scoring, ESCAT variant detection (specifically for CNAs), and CTM. Differences in variant and biomarker detection could mainly be explained by different bioinformatic approaches to variant calling, filtering, tiering, and normalization. Differences in CTM results were driven by the different underlying reported variants and conceptual differences based on system parameters. For TMB, MSI, and gLOH, the computational tumor purity fraction was important for interpretation of the signature and was only available in the AVENIO CGP Kit, showing a high degree of alignment with F1CDx. The overall agreement between the two CGP kits needs to be further considered in context of the practical implications of a single DNA protocol versus separate parallel RNA/DNA workstreams. Different approaches may lead to inconsistent results across CGP tests if not properly understood and controlled. This is further complicated by the

constant and rapid evolution of assays and secondary and tertiary analysis solutions. For example, while this publication has been prepared, a new version of the TSO-500 assay, which includes an HRD score and potentially improved CNA calling, has been released.[29] Also, the FoundationOne Analysis Platform, based on the same algorithms used in F1CDx, is expected to have regular updates in the pipeline, potentially further enhancing the performance of the AVENIO CGP Kit in the future.

Ultimately, it is very important, disregarding the method used, to ensure the quality of the results obtained. Both assays compared in this study are for RUO. The complexities in calling, filtering, and interpreting variants as highlighted in this publication illustrate key considerations for the implementation of any high quality CGP in the laboratory and support the endeavor to bring uniformity in genomic insight results.

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Author Contributions

H.-P.A., M.C.H., J.O., and T.W. conceived the study; H.-P.A., M.C.H., and F.F. curated the data; H.-P.A., F.F., and T.W. formally analyzed the data; J.O. and H.S. acquired funding; H.-P.A. and K.H. performed investigations; H.-P.A. developed the methods; U.S. provided resources; H.-P.A., F.F., and H.S. provided software; H.-P.A. validated the data; H.-P.A., M.C.H., and T.W. visualized the data; H.-P.A., M.C.H., and M.T. analyzed data from the clinical trial-matching and prepared the respective sections of the manuscript; J.M.A. and M.C.H. interpreted the National Comprehensive Cancer Network and European Society for Medical Oncology Scale for Clinical Actionability of molecular Targets tiers for the genomic variants in their disease context; F.F., J.O., M.D.V., E.S., and T.W. wrote the original draft; and all authors critically revised subsequent drafts, interpreted the data, and take full responsibility for the accuracy and integrity of this work.

Disclosure Statement

H.-P.A., F.F., and U.S. are employees of Signature Diagnostics GmbH (H.-P.A. at the time of the analysis, but now retired). M.C.H., J.O., and J.M.A. are employees of Foundation Medicine, Inc. (J.O. at the time of the analysis, but is no longer an employee), and M.C., S.O., and T.W. are employees of Foundation Medicine GmbH; all hold stock in F. Hoffmann-La Roche Ltd. K.H. is an employee of Helios MVZ Pathologie Erfurt GmbH. M.T. is an employee of F. Hoffmann-La Roche AG and holds stock in F. Hoffmann-La Roche Ltd. D.G. holds stock in Univ8 Genomics Ltd.; has received honoraria (institution) from Roche, Illumina, Novartis, AstraZeneca, Eli Lilly, Amgen, and Janssen; has participated in a consulting or advisory role (institution) for AstraZeneca and Novartis; has participated in a speakers' bureau (institution) for AstraZeneca; and has received travel, accommodations, and expenses (institution) from Roche, AstraZeneca, and Illumina. E.P. received compensation for speaking engagements and advisory roles, with payments made directly to his affiliated university, from Roche, AstraZeneca, Novartis, and MSD. H.S. and M.D.V. are employees of Roche Diagnostics Solutions, Inc., and M.D.V. holds stock in F. Hoffmann-La Roche Ltd. E.S. reports lectures for Bio-Rad, Seracare, Novartis, Roche, Biocartis, Illumina, Eli Lilly, Janssen-Cilag, Pfizer, AstraZeneca, and Agena Bioscience; he is consultant in advisory boards for MSD/Merck, GSK, AstraZeneca, Astellas Pharma, Sysmex, Roche, Pfizer, Novartis, Bayer, Bristol Myers Squibb, Eli Lilly, Amgen, Biocartis, Illumina, Agena Bioscience, Janssen-Cilag (Johnson & Johnson), Sinnovisionlab, Diaceutics, and CC Diagnostics; and received research grants from Pfizer, Biocartis, Invitae-ArcherDX, AstraZeneca, Agena Bio-science, Bristol Myers Squibb, Bio-Rad, Roche, Boehringer Ingelheim, CC Diagnostics, and Abbott (all paid to University Medical Center Groningen account); and travel reimbursements from Bio-Rad, Abbott, Illumina, Agena Bioscience, and Roche. All authors received support in the form of third-party writing assistance for this manuscript, furnished by Nucleus Global, an Inizio company, and provided by F. Hoffmann-La Roche Ltd./Roche Diagnostics Solutions, Inc.

Supplemental Data

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