UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Research Report

EVALUATING THE CLINICAL UTILITY OF A SNP-BASED MICROARRAY PLATFORM – A COMPARATIVE STUDY

By

Minenhle Peniel Mayisela

1880919

Master of Science in Medicine in Genomic Medicine

Supervised by:

Ms Nkateko Mayevu

Dr Fiona Baine–Savanhu

NATIONAL HEALTH LABORATORY SERVICE

A research report (in the format of a “submissible” paper) submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Science in Medicine (Genomic Medicine)

Johannesburg, 2023

Declaration

I, Minenhle Peniel Mayisela, declare this research report (in the format of a “submissible” paper) is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Signature of candidate)

4th day in June 2023, in Johannesburg

Contribution of the candidate to the paper

Declaration: Student's contribution to article(s) and agreement of co-author

I, Minenhle Peniel Mayisela, student number 1880919, declare that this Research Report is my own work and that I contributed significantly towards research findings presented in the paper intended for publication below.

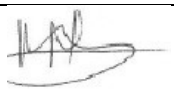
Signature of Student
Date: 4/06/2023
Name of Primary Supervisor: Ms Nkateko Mayevu

Signature of Primary Supervisor
Date: 4/06/2023

Signature of Co-Supervisor
Date: 4/06/2023

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of her Research Project.

Article Title: Evaluating the clinical utility of a SNP-based microarray platform – a comparative study

Authors	Name	Signature	Date
First Author	Minenhle Mayisela		4/06/2023
Second Author	Nkateko Mayevu		4/06/2023
Third Author	Fiona Baine - Savanhu		4/06/2023

Dedication

This research report is dedicated to my parents, Friction and Qondile Mayisela.

Abstract

Developmental disorders make up a majority of cases seen in genetic clinics at the National Health Laboratory Service (NHLS). Chromosomal microarray analysis (CMA) is the first line testing for individuals with developmental disorders and congenital anomalies. This study compared the clinical utility of single nucleotide polymorphism (SNP) - based array (CytoScan® Optima array) and a comparative genomic hybridization (CGH) array (SurePrint G3 Unrestricted CGH ISCA v2, 8x60 kb microarray) in diagnosis of developmental disorders. This was done by looking at the differences in copy number variant calls, segment size differences and gene content between the two microarrays. The cost to run each type of microarray test for diagnosis was also compared. The copy number variant calls made by the two platforms were comparable. The SNP-based array did provide additional information that would be useful in molecular diagnosis. The final recommendation was for the CGH array and the SNP-based array to be used interchangeably based on clinical requests at the Division of Human Genetics.

Acknowledgements

I would like to thank my supervisors for all their support and guidance this year. Thank you for your guidance in all my research assessments this year. To Nkateko Mayevu, thank you for all the time you put in towards my learning about microarrays. Thank you for your continued patience as I navigated my way in writing and reporting. Thank you for looking at my work even after work hours. Thank you for your support and help in my presentations for my coursework. Your reassurances throughout the year were one of the reasons I did not give up. To Dr Fiona Baine – Savanhu, thank you for all your guidance with my research this year and thank you for showing me and directing me towards good writing that I hope to achieve as I continue on my research journey - slowly but surely. It has been as honour to receive guidance and support from you, and I pursue my research journey.

Thank you to Ms Mirriam Hlongwane, Mr Daniel Nkwane and Mr Naseem Cassim for all their help in developing a costing tool for SNP-array testing. I appreciated the invaluable time you put in.

Thank you to Dr Robyn Kerr who helped with putting together the research ethics application.

Thank you to Prof Zane Lombard and Dr Khuthala Mnika for their support as course coordinators for this programme.

Thank you to my parents, Qondile and Friction Mayisela who funded my studies, and for their support and love throughout the journey. Thank you to my friends who always encouraged me to keep pressing on. Most importantly, thank you to God, for the strength to keep going.

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List of Abbreviations

CGH - Comparative genomic hybridization

ChAS - Chromosomal analysis suite

ClinGen – The Clinical Genome Resource

CMA - Chromosomal microarray analysis

CNV - Copy number variant

DECIPHER – Database of chromosomal imbalance and Phenotype in Humans Using Ensembl Resources

FISH - Fluorescence in-situ hybridization

HMM – Hidden Markov Model

ISCA - International Standards for Cytogenomic Arrays

LOH - Loss of heterozygosity

MLPA - Multiplex ligation-dependent probe amplification

NHLS - National Health Laboratory Service

OMIM – Online Mendelian Inheritance of Man

SV – Structural variants

UPD - Uniparental disomy

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Research report in the format of a “submissible paper”

This work has been written up as a paper for submission to The Journal of Molecular Diagnostics. The author guidelines such as abstract, referencing and word count have been adhered to and can be found in Appendix D. Tables and Figures have been included in the text for ease of reference.

Evaluating the clinical utility of SNP-based microarray platform – a comparative study

Minenhle Mayisela[1] *Nkateko Mayevu*[2] *Fiona Baine-Savanhu*[1, 2]

[1] Division of Human Genetics, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

[2] Division of Human Genetics, National Health Laboratory Service, Corner Hospital and De Korte Streets, Johannesburg, South Africa

Minenhle Mayisela
Email: 1880919@students.ac.za
Contact number: +27 78 788 8219

Nkateko Mayevu
Email: nkateko.mayevu@nhls.ac.za
Contact number: +27 11 489 9237

Fiona Baine-Savanhu
Email: fiona.baine-savanhu@nhls.ac.za
Contact number: +27 11 489 9158

Abstract for paper submission

Chromosomal microarray analysis (CMA) is the first line testing for individuals with developmental disorders and congenital anomalies. The evolution of cytogenetic techniques has brought about improvements in resolution and the turnaround time for genetic testing and disease diagnosis. The National Health Laboratory Service in Johannesburg, South Africa offers a testing service for South African patients with developmental disorders and congenital abnormalities and currently uses a Comparative Genomic Hybridization (CGH) array (SurePrint G3 Unrestricted CGH ISCA v2, 8x60 kb microarray). This study aimed to compare the clinical utility of a Single Nucleotide Polymorphism (SNP) array (CytoScan® Optima array) to a CGH array (SurePrint ISCA microarray) in the diagnosis of patients referred for microarray testing at the Division of Human Genetics, National Health Laboratory Service in Johannesburg, South Africa. This was done by looking at the differences in copy number variant calls, segment size differences and gene content between the two microarrays. The cost of running each type of microarray was also compared. The results of the study show that copy number variant calls made by the two platforms were comparable. The different microarrays were able to detect genomic losses and gains, including aneuploidies, in 17 samples. The SNP-based array provided additional SNP genotype information that would be useful in molecular diagnosis. The SNP-based microarray and the CGH array currently in use in the division may be used interchangeably as this comparison yielded similar results.

Introduction

Copy number variants (CNVs) are DNA segments greater than 50 bp in size that show a variable copy number compared to the reference genome[1]. Large CNVs that encompass dosage sensitive genes are likely to be disease causing[2]. Copy number variants play a role in the aetiology of developmental disorders, intellectual disability and congenital anomalies[3]. Cytogenetic techniques have long been used to identify and analyse structural and numerical rearrangements of chromosomes that cause losses and gains in genetic material[4]. The evolution of cytogenetic techniques has brought about improvements in resolution and the turnaround time for genetic testing and disease diagnosis.

Karyotyping allows the viewing of all chromosomes under a microscope and enables the detection of changes in chromosome number (aneuploidy), and large structural chromosome abnormalities by analysis of banding patterns[5]. It is still considered the gold standard for confirming and informing the mechanisms that underlie the gains and losses of genomic material in many chromosomal aberrations. Karyotyping is able to detect balanced and unbalanced chromosomal rearrangements because it uses banding patterns to visualise different regions of the chromosomes[5]. The technique is however limited to detecting CNVs that are greater than 5 Mb making it unsuitable for submicroscopic aberrations[5].

Fluorescence in-situ hybridization (FISH) has a higher resolution of between 100-200 kb compared to karyotyping[6]. This method uses a fluorescently labelled DNA probe to hybridize to its complementary segment on chromosomes and is visualised with a fluorescent microscope. Its limitation, however, is that the FISH probes are designed for specific locus on the chromosome.

This means that a disorder must be suspected for FISH to be used since one probe is used for one chromosome abnormality. Compared to FISH, multiplex ligation-dependent probe amplification (MLPA) has the advantage of simultaneously targeting multiple regions across the genome by quantifying dosage of DNA regions based on directed ligation[7]. Although MLPA probes have multiple targets, they are designed for known submicroscopic abnormalities related to specific diseases, meaning only conditions of known aetiology can be tested using this technique[8].

Chromosomal microarray analysis (CMA) has become a useful approach in the molecular diagnosis of developmental delay and congenital anomalies of unknown aetiology. This has led to a consensus by the International Standards for Cytogenomic Arrays (ISCA) Consortium recommending CMA as a first line test for the diagnosis of patients with developmental disorders and/or congenital anomalies[9]. Alternative methods of detecting chromosomal aberrations such as karyotyping, FISH and MLPA are limited by low resolution, low diagnostic yields and longer turnaround time for testing. Microarrays may have a resolution as high as 16 kb[10] depending on the probe number, spacing and distribution of its design[11]. A study undertaken to diagnose structural aberrations using CMA, gave a diagnostic yield of 15-20% compared to the 3% yield from karyotyping[9].

Microarrays use deoxyribonucleic acid-based methods to detect CNVs as well as the size of the aberrations present caused by unbalanced chromosomal rearrangements (submicroscopic) and aneuploidies[9]. There are two types that are commonly used: comparative genomic hybridization (CGH) array and single nucleotide polymorphism (SNP) microarrays. The CGH array, referred to as a true comparative hybridization array, directly compares the DNA from a test sample to a normal reference sample[11]. The microarrays emit a fluorescent light that is converted to a log2 ratio value. The copy number changes of this array are represented graphically as a log2 ratio which informs the type of copy number change i.e., a gain or a loss.

The SNP microarray on the other hand, only requires the test sample and no reference[12]. This array functions by directly hybridizing the test DNA sample to a microarray slide that contains probes designed to be complementary to target DNA segments flanking SNPs[13]. The results of the array show the gain or loss of DNA segments as well as the different SNP genotypes for each SNP locus[13]. Since SNP arrays provide genotyping information, they can be used to detect loss of heterozygosity (LOH) in genomic regions and can also detect low levels of mosaicism[14].

The CGH array test was implemented in 2020 as the first line testing strategy for patients presenting with developmental disorders and congenital abnormalities at the Division of Human Genetics at the National Health Laboratory Service (NHLS), a state diagnostic laboratory in the Republic of South Africa. The CGH array used is the SurePrint G3 Unrestricted CGH ISCA v2, 8x60K microarray (Agilent technologies, Santa Clara, CA). For the initial testing and validation of the system, the CytoScan® Optima array (Thermo Fisher Scientific, Waltham, MA), a low-resolution microarray designed specifically for prenatal testing was included.

This project aimed to compare the detection capacity, ease of use and cost of the CytoScan® Optima array to the SurePrint CGH ISCA, 8x60K array currently used for diagnostic testing of postnatal samples in the Division. This was done by testing 17 samples with known CNVs detected by the CGH array. The CytoScan® Optima contains both copy number and SNP probes and thus provides SNP genotype which may be used to detect LOH. SNP arrays have also been shown to perform better in the detection of low-level mosaicism.

Materials and methods

Sample/Patient selection

This comparative study used residual DNA samples from patients referred to the Division of Human Genetics diagnostic laboratory for CMA testing. Microarray testing records were reviewed and samples with a range of chromosomal abnormalities previously detected using the CGH array were selected. The patients had clinical features such as intellectual disability, dysmorphic features and a variety of congenital anomalies. The total number of samples investigated was 17 and all samples were tested using the Cytoscan® Optima array. The kit used has the capacity to test 24 samples. The 17 samples analysed here were selected from the 24, to represent a range of chromosomal abnormalities (including structural variants and copy number variants). The data previously generated from the SurePrint G3 Unrestricted CGH ISCA v2, 8x60 kb microarray were compared with data from the Cytoscan® Optima array. The SurePrint ISCA array has a probe-spacing of approximately 60 kb and analyses eight samples on a single slide. The study was granted ethics approval by the Human Research Ethics Committee (Medical) of the University of Witwatersrand, certificate number: M220295.

CytoScan® Optima Array

The Cytoscan® Optima Array (Thermo Fisher Scientific, Waltham, MA) was used to generate copy number data and SNP genotype data. This is an Affymetrix SNP-based array that consists of 18 018 copy number probes to detect any gains or losses as well as 148 450 SNP probes which target SNP sites at different polymorphic locations[15]. For comparison, the SurePrint ISCA array (Agilent technologies, Santa Clara, CA) has 60 000 copy number probes concentrated at genomic regions of clinical relevance as determined by the ISCA consortium. The laboratory workflow for the CytoScan Optima array (including hybridization, washing and scanning) was performed by staff members at the Division according to the manufacturer's protocol for the 17 selected samples. The GeneChip® Scanner 3000 7G (Thermo Fisher Scientific, Waltham, MA) generates CEL files. CEL files are files that summarise probe intensity signals from the scanner. These raw data CEL files were provided to the candidate and analysed using Chromosome Analysis Suite (ChAS) software v4.3 (Thermo Fisher Scientific, Waltham, MA).

Chromosome Analysis Suite (ChAS) v4.3

The 17 CEL files were analysed using ChAS v4.3 software (Thermo Fisher Scientific, Waltham, MA). This a cytogenetics analysis software that analyses chromosomal changes across the genome. It automatically converts CEL files into cychp files which contain information on copy number, LOH, mosaicism and genotype calls. Before further analysis, the cychp files must meet all the quality control metrics: median absolute pairwise >/= 0.25, waviness </= 0.12 and SNP quality control >/= 15. The software uses computational statistical models to determine copy number state and SNP genotype for each CNV call. The algorithms used for copy number state and SNP genotyping are the Hidden Markov Model (HMM) and the Bayesian Robust model respectively. Normalised log2 ratio (weighted log2 ratio) for each probe is plotted and the copy number determined from the graph using the Bayesian HMM algorithm.

Copy number variant calling

The information provided by the copy number probes and the SNP probes is displayed on different tracks in the ChAS browser (Thermo Fisher Scientific, Waltham, MA). The log2 ratio and the copy number state tracks show information obtained from the copy number probes. The B allele frequency, allele difference and smooth signal tracks show the information provided by the SNP probes.

The CNVs are shown as gains or losses based on the log2ratio displayed by the tracks on the graph in the ChAS browser. A normal diploid copy number has a log2 ratio of 0. The ratio used to calculate a gain is 3/2 therefore a single copy gain has a log2 ratio of 0.58 and the ratio used to calculate a loss is 1/2 therefore a single copy loss has a log2 ratio of -1.

SNP genotype information is derived from the allele difference and B allele frequency given by the ChAS software. The alleles are designated A and B, and each assigned a value of 0.5. The allele difference is the difference between the number of two alleles using the formula A – B. The B allele frequency is the number of B alleles divided by the total number of allele combinations of a SNP (AA, BB or AB), and is calculated using the formula B/(A+B) is used to calculate it. The smooth signal is a smoothened calibrated copy number estimate. Genomic imbalances were annotated

against the primary genome assembly, Genome Reference Consortium Human Build 37 (GRCh 37). In this study, the software was set to call CNVs that are greater than 200 kb and LOH regions greater than 8 Mb in size. It is recommended that larger size thresholds provide more sensitivity and reduce false positive rates[16].

Genotype-phenotype correlation of CytoScan Optima results

The patients' clinical features were made available to ensure the molecular finding could be associated with the phenotype i.e., genotype-phenotype correlation. The public databases embedded in the ChAS software provide information on the associated CNV syndrome if any, pathogenicity, deleterious genes contained, dosage sensitivity of the genes, and existing data on patients with the same/similar CNV. The databases used are Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER)[17], Clinical Genome Resource (ClinGen)[18], ClinVar[19,20] and Online Mendelian Inheritance of Man (OMIM)[21].

DECIPHER is a clinical research tool that helps in interpreting data from CNV analysis. DECIPHER facilitates the sharing of information between clinical researchers across the world to enable progress in defining new syndromes and gene function[17] and is accessible here: https://www.deciphergenomics.org/browser). ClinVar is a public archive of human variants and the interpretation of their relationship to disease and other conditions. Many different organisations, including laboratories and patient registries, submit variants to ClinVar for public access. Submissions include a description of CNV syndrome for which variant was interpreted as well as the clinical significance of the variant[19,20]. ClinVar is accessible here: https://www.ncbi.nlm.nih.gov/clinvar/). Clinical Genome Resource (ClinGen) is a curated collection of data on copy number variants and sequence variants derived from publications of case studies looking at CNV syndromes. ClinGen provides an interconnected community for resources to improve understanding of genomic variants and improve its use in clinical care[18]. ClinGen is accessible here: https://clinicalgenome.org/).

Online Mendelian Inheritance of Man (OMIM) is a primary repository of information on genes and genetic phenotypes derived from clinical studies. OMIM synthesizes

and summarises new and important information based on expert review of biomedical literature. OMIM also plays a role in naming and classifying genetic phenotypes[22] (https://www.omim.org/). Altogether, these databases contain genetic and phenotypic information of variation in the genome. They allow for storage, sharing and comparison of genetic data across institutions and between researchers.

Costing

The cost of performing the SNP array test was determined using the NHLS costing tool. This tool is designed to standardise the costing of diagnostic tests within the organisation. The calculation considered the cost of the array reagents, consumables, labour and fixed equipment. This price was then compared to that of the CGH array already in use in the Division.

Results

Copy number variants detected by the CytoScan Optima SNP array

In the sample of 17 patients, 24 genomic copy number variants were observed by the CytoScan Optima SNP array alone. This included 12 losses and 12 gains. The genomic coordinates of the copy number variants for each sample tested can be found in Table 1. Some of the CNVs detected are associated with known syndromes and most are suspected to have occurred de novo.

The copy number state and SNP genotype information for each software call were visualised using the ChAS v3 browser. Figure 1 shows the different tracks that can be seen on the ChAS browser and the information it provides. The copy number state represents the copy number variation in the target genome relative to the reference genome. It is displayed as a log2 ratio based on the intensity of the probe represented by that genomic region. The SNP genotype information is represented by calculation of the allele difference and B allele frequency, as well as smooth signal.

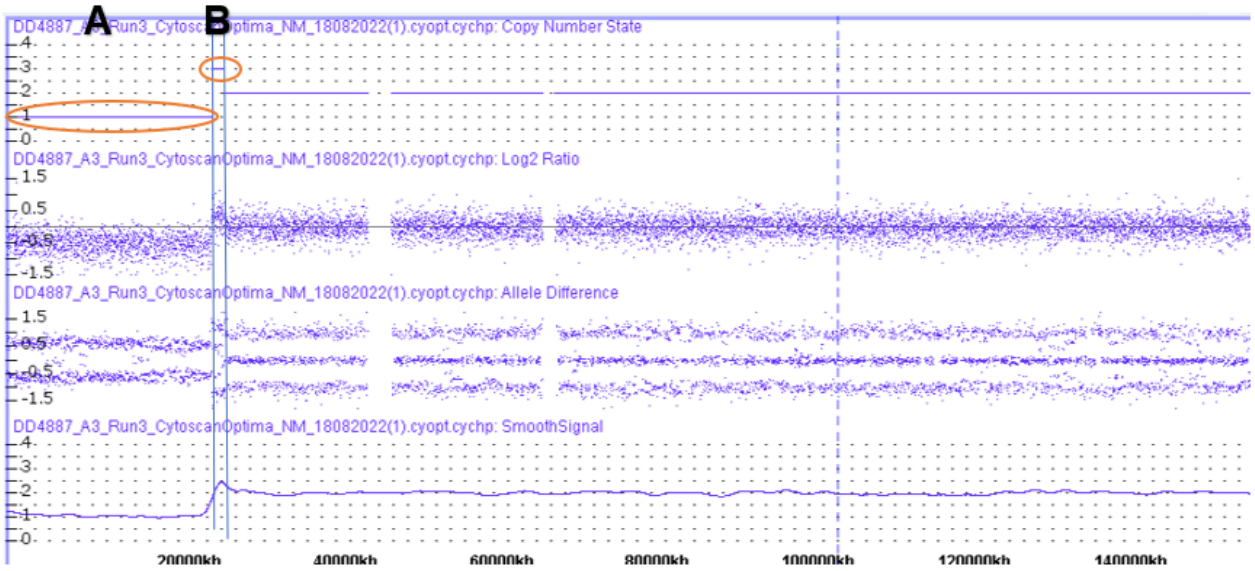

Figure 1. Screenshot from the chromosome analysis suite v4.3 showing the copy number graph data, the log2 and smooth signal of chromosome 5. The graph shows chromosome 5 with a 26 Mb terminal loss adjacent to a 1 kb gain on Patient 14. **A)** A copy number loss. Copy number state is 1.0 which indicates a loss of that genomic region. The loss is indicated by the red circle. The allele difference shows two tracks only indicating that there are two genotypes for that segment indicating a loss. **B)** A copy number gain on Patient 14. Copy number state shows 3.0 which indicates a gain of that genomic region. The gain is indicated by a red circle. The allele difference shows four tracks indicating that there are four genotypes for that segment indicating a gain.

Comparing CGH array and SNP-based array

The SNP array (CytoScan Optima) used in this study detected 24 CNVs. Of these, 23 were previously detected by the SurePrint ISCA CGH array used for diagnostic testing. Table 1 presents the genomic breakpoints for each CNV call. The genomic coordinates of the copy number changes were compared and found to be similar between the two arrays for a majority of the patients. The OMIM database, DECIPHER genome browser and ClinGen were used to look at the gene content of the CNVs detected. The coordinates of each CNV detected were entered into the DECIPHER genome browser and gene content assessed linked were provided directly to the OMIM database for genes known to be associated with mendelian disorders.

The SNP array detected a 981.958 kb gain of 15q26.2 in Patient 10 that the CGH array did not detect. Upon looking up this CNV on the public databases, it was found to not be clinically significant and therefore was not the molecular cause of the patient's phenotype.

Table 1. Genomic breakpoints. Size (kb) and associated CNV syndromes detected by CytoScan Optima array and SurePrint ISCA array.

Patient	CytoScan Optima array results			SurePrint ISCA array results			
	Breakpoint coordinates	Copy number state	Segment size (kbs	Breakpoint coordinates	Segment size	Size difference	Associated CNV syndrome
1	arr[GRCh37] 11p14.3p13(24385583_33146139)x1	1	8760.556	arr[GRCh37] 11p14.3p13(24381118_33162974)x1	8781.856	21.3	WAGR syndrome - 11p12del
2	arr[GRCh37] 3q13.2q22.3(111612191_136676525)x3	3	25064.334	arr[GRCh37] 3q13.2q22.3(111848681_136983572)x3	25134.891	70.557	
3	arr[GRCh37] 13q11q12.11(19436287_31787356)x1	1	12351.069	arr[GRCh37] 13q12.11(20407295_21861250)x1	1453.955	10897.114	
3	arr[GRCh37] 18p11.31(3412357_5191958)x1	1	1779.601	arr[GRCh37] 18p11.31(3415823_5195070)x1	1779.247	0.354	
3	negative			arr[GRCh37] 18p11.32p11.31(236820_3414693)x1	3177.873	3177.873	
4	arr[GRCh37] 2q26q27 (208150499_242783384)x1	1	34632.885	arr[GRCh37] 2q33.3q37.3(208174860_243068396)x3	34893.536	260.651	
4	arr[GRCh37] 6q26q27 (161069941_170919482)x1	1	9849.541	arr[GRCh37] 6q26q27(161176622_170921089)x1	9744.467	105.074	
5	arr[GRCh37] 8q21.11q22.1(75661636_94779028)x1	1	19117.392	arr[GRCh37] 8q21.11q22.1(75624070_94892477)x1	19268.407		8q21.11 microdeletion
6	arr[GRCh37] 22q11.21(18166423_21108369)x1	1	2941.946	arr[GRCh37] 22q11.21(18917031_21443283)x1		2941.946	22q.11.2 microdeletion
7	arr[GRCh37] 7p22.3p11.1(43361_58006205)x3	3	57962.844	arr[GRCh37] 7p22.3p11.2(54185_56174888)x3	56120.703	1842.141	7p trisomy
8	arr[GRCh37] 5p15.33p14.1(113577_27953367)x1	1	27839.79	arr[GRCh37] 5p15.33p14.1(22149_27908206)x1	27886.057	46.267	Cri Du Chat syndrome
9	arr[GRCh37] 13q11q34(19436287_115107733)x3	3	95671.446	arr[GRCh37] 13q12.11q34(20407295_115092648)x3	94685.353	986.093	13q trisomy
10	arr[GRCh37] 15q26.2(95721415_96703373)x3	3	981.958	negative		981.958	
10	arr[GRCh37] 16p12.2p11.2(21620626_29379768)x3	3	7759.142	arr[GRCh37] 16p12.2p11.2(21475060_29331350)x3	7856.29	97.148	16p11.2p12.2 duplication
11	arr[GRCh37] 4q12q35.2(58793349_190957473)x3	3	132164.124	arr[GRCh37] 4q12q35.2(58881742_190896674)x3	132014.932	149.192	
11	arr[GRCh37] Xq28(147973535_155233731)x1	1	7260.196	arr[GRCh37] Xq28 (147991745_155232907)x1	7241.162	19.034	
12	arr[GRCh37] 6q27(167711355_170610394)x1	1	2899.039	arr[GRCh37] 6q27(168364343_170906796)x1	2542.453	356.586	
12	arr[GRCh37] 17p13.3p13.2(150733_4116672)x3	3	3965.939	arr[GRCh37] 17p13.3p13.2(11807_4045078)x3	4033.271	67.332	
13	arr[GRCh37] 17q12(34831963_36350005)x3	3	1518.042	arr[GRCh37] 17q12(34611352_36248918)x3	1637.566	119.524	17q12 duplication syndrome
14	arr[GRCh37] 5p15.33p14.1(113577_26368797)x1	1	26255.22	arr[GRCh37] 5p15.33p14.1(22149_26287349)x1	26265.2	9.98	
14	arr[GRCh37] 5p14.1(26380734_27858577)x3	3	1477.843	arr[GRCh37] 5p14.1(26422464_28052827)x4	1630.363	152.52	Cri Du Chat syndrome
15	arr[GRCh37] Xq13.1q21.1(70902832_83032256)x2	2	12128.424	arr[GRCh37] Xq13.1q21.1 (71030284_83015968)x3	11985.684	142.74	
16	arr[GRCh37] 1q44(246267566_249224684)x3	3	2957.118	arr[GRCh37] 1q44(246081208_249212668)x3	3131.46	174.342	
16	arr[GRCh37] 10q25.1q26.3(109292731_132504534)x3	3	23211.803	arr[GRCh37] 10q25.1q26.3(109312155_132468422)x	22156.267	1055.536	
16	arr[GRCh37] 10q26.3(132516104_135427143)x1	1	2911.039	arr[GRCh37] 10q26.3(132580596_135434178)x1	2853.583	57.456	
17	negative			negative			

Genotype-phenotype correlation

The clinical features of the patients were provided to match the molecular diagnoses to the phenotype. Of the 17 patients, six were found to have CNVs were associated with known microduplication/deletion syndromes. Microdeletion syndromes were confirmed in four patients: Patient 1 – Wilms tumour, aniridia, genitourinary anomalies and mental retardation (WAGR) syndrome (OMIM: 194072)[23,24] from a 8.7 Mb interstitial deletion, Patient 5 - 8q21.11 microdeletion syndrome (OMIM: 614230)[25] from a 19.1 Mb interstitial deletion. Patient 6 - 22q11.2 microdeletion syndrome (OMIM: 611867)[26,27] from a 2.9 Mb interstitial deletion, Patient 8 – Cri Du Chat syndrome (OMIM: 123450)[28–30] from a 27.8 Mb terminal deletion and Patient 14 – Cri Du Chat syndrome (OMIM: 123450)[28–30] from a 1.5 Mb terminal deletion. Microduplication syndromes were confirmed in two patients: Patient 10 - 16p11.2 duplication syndrome (OMIM: 614671)[31] from a 7.8 Mb gain and Patient 12 – 17q12 duplication syndrome (OMIM: 614562)[32] from a 3.9 Mb kb gain. The clinical features for each of these patients matched the phenotype of the confirmed disorder, as shown in Table 2.

For five of the patients: Patient 3, Patient 4, Patient 11, Patient 15 and Patient 16, there were no syndromes associated with the CNVs detected. The CNVs detected in these patients had no clinical significance.

Table 2. CNV syndromes confirmed in the sample of patients in this study.

Patient	CNV syndrome	Clinical features of the patient	Features of syndrome	Reference
1	WAGR 11p13p13 syndrome (OMIM: 194072)	overweight-for-age, developmental delay, dysmorphic features, hypotonia, cryptorchidism,aniridia, behavioural problems	Wilms tumour, Aniridia, genital anomalies, mental retardation, cryptorchidism	23,24
5	8q21.11 microdeletion syndrome (OMIM: 614230)	severe-profound developmental delay, self-injurious behaviour, bilateral eye abnormalities ((R) buphthalmos and (L) microphthalmia), dysmorphic features, craniosynostosis	Intellectual disability, dysmorphic features, camptodactyly, syndactyly, hypotonia, sensoneural hearing loss	25
6	22q11.21 microdeletion syndrome (OMIM: 611867)	premature baby with tracheoesophageal fistula, cyanotic heart with common atrium, pulmonary atresia, abnormally shaped low set ears, long fingers and toes, intermediate fontanelle	Cardiac anomalies, immunodeficiency, speech delay, hypocalcaemia, neurological and developmental behavioural issues	26,27
8	Cri Du Chat syndrome (OMIM: 123450)	global developmental delay, failure to thrive, microcephaly, dysmorphic features (including strabismus, microstomia and marked microretrognathia), chronic upper airway obstruction, high-pitched cry	Microcephaly, round face, hypotonia, psychomotor symptoms, macrostomia, mental retardation, high pitched cry	28–30
10	16p11.2 Microduplication (OMIM: 614671)	postnatal short stature, moderate-to-severe developmental delay, dysmorphic features, limb abnormalities	Speech delay, microcephaly, autism, epilepsy, diaphragmatic hernia, smooth philtrum, small ears, developmental delay	31
13	17p13.3 duplication syndrome (OMIM: 614562)	prenatal short stature, microcephaly, mild-moderate developmental delay, subtle dysmorphic features, CNS abnormalities, limb abnormalities	Hypotonia, dysmorphic features, failure to thrive, poor growth, brain abnormalities	32
14	Cri Du Chat syndrome (OMIM: 123450)	overweight-for-age, microcephaly (postnatal onset), dysmorphic features, cryptorchidism, structural brain abnormalities (hydrocephalus and Dandy-Walker variant), and mild developmental delay	Microcephaly, round face, hypotonia, psychomotor symptoms, macrostomia, mental retardation, high pitched cry	28–30

Patient 3

Several findings from the results from Patient 3 aided in the distinguishing of the SNP array and the CGH array. These were associated with CNV calls, genomic coordinates and size breakpoints, gene content from CNVs, and genotype-phenotype correlation.

The CGH array detected a loss of 18p11.32p11.31 in Patient 3 that the SNP array did not detect. This CNV was considered clinically significant as it included the OMIM morbid gene *SMCHD1* (OMIM :614982). Haploinsufficiency of *SMCHD1* is associated with Bosma arrhinia microphthalmia with coloboma, arhinia and endocrine findings [33]. This patient presented with the following overlapping clinical feature: choanal atresia – a narrowing of the nasal passageway, which is feature of Bosma arrhinia microphthalmia syndrome.

There was a notable difference for Patient 3 who was found to have a loss of 13q12.11 detected by both arrays. The genomic coordinates determined by the SNP array were 13:19436287-31787356; while CGH array coordinates were 13:20407295-21661250. This equated to a CNV of 12351 kb on SNP array compared to 1454 kb on CGH array; a difference of 10897 kb. Table 1 shows the genomic coordinates and the sizes of all CNVs detected in the sample patients by the CGH array and SNP array. The observed differences in genomic coordinates and CNV size are indication that the SNP array and the CGH array differ in probe design and placement across the genomic regions they target.

A difference in gene content can be observed in instances where both arrays detect the CNV but because of probe placement differences and distribution, one array includes a gene or part of a gene that is not shown by the other microarray.

Patient 3 had a CNV loss of 18p11.32p11.31 detected by the CGH array copy number probes that the SNP array copy number probes did not detect. This may be because the SNP arrays copy number probes may not have covered that region. However, the smooth signal and BAF tracks derived from SNP probes from the SNP

array showed evidence of this loss. The gene(s) contained in the CNV included the OMIM morbid gene *SMCHD1.*

Aneuploidies

Two of the 17 patients were confirmed to have trisomies: Patient 9 had a mosaic partial trisomy 13q, shown by a gain of the entire q arm (95 Mb) at approximately 63% mosaicism and Patient 7 had partial trisomy 7p, shown by a gain of the entire p arm (57 Mb). Partial trisomies can result from a gain in relevant large portion of a chromosome[34] due to chromosomal rearrangements. Both the SNP array and the CGH array detected these large chromosomal changes and estimation of mosaicism for Patient 9 was comparable between the two and is addressed below.

Mosaicism

Patient 9 was found to have mosaic trisomy 13 at approximately 63% based on the CytoScan Optima SNP array data on chromosome 13. The ChAS software is able to estimate the level of mosaicism by averaging the smooth signal values of the CNV detected. The SurePrint ISCA array detected the gain 13q; however, the level of mosaicism has to be manually estimated by working backwards from the log2 ratio. Patient 9 was diagnosed with trisomy 13, in approximately 60% of cells. This is comparable to the automated software estimate of 63% made by ChAS.

In order to investigate the ability of the SNP array to detect mosaicism, Patient 17 was included in this study because of the reported clinical phenotype (skin pigmentary abnormalities) which is strongly suggestive of mosaicism. SNP arrays are more reliable at detecting mosaicism because they give SNP genotype information unlike the CGH array. Like the CGH array, the CytoScan® Optima array used in this study was unable to pick up mosaicism in this patient. Patient 17 was therefore reported to have no clinically significant CNVs detected by the microarrays.

Patient 3 had been previously diagnosed with a loss of 18p11.32 using the SurePrint (Agilent Technologies, Santa Clara, CA). This deletion was no detected by the CytoScan® using copy number probes. The SNP probes however, showed this as a potentially mosaic loss. In Figure 2, there is no copy number variant call made by the software (see copy number state track at the top); however, the smooth signal track

is lower than 2 and the BAF track shows a split suggesting the presence of a mosaic loss. The 18p11.32 (1.7 Mb) deletion was estimated to be approximately 59% mosaic.

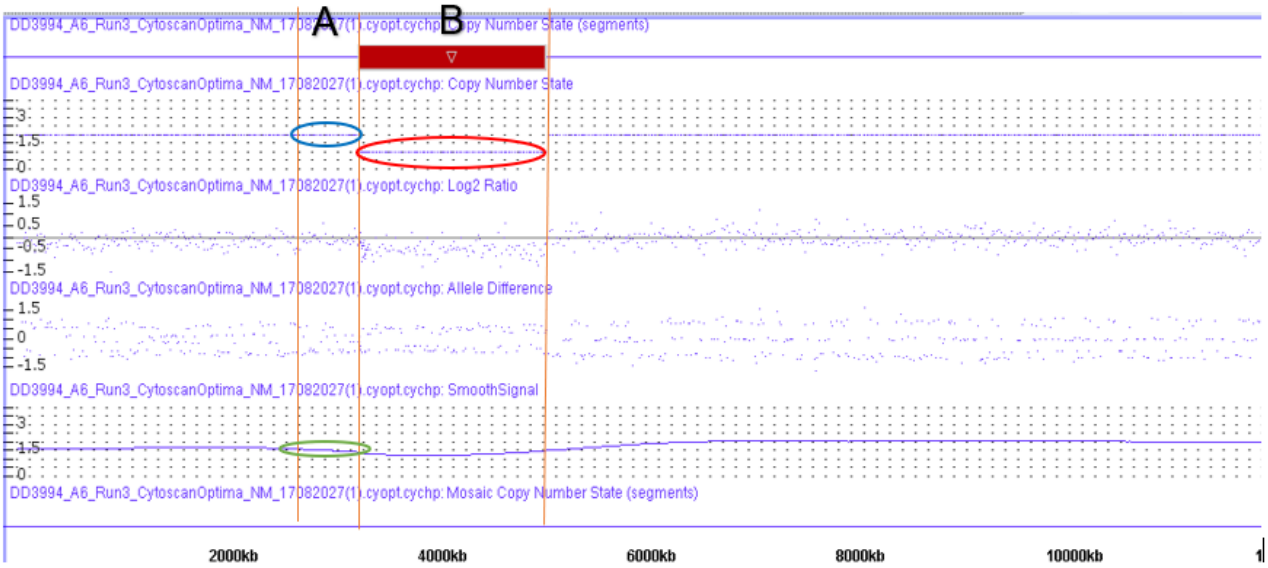

Figure 2. Screenshot of chromosome analysis suite v4.3 showing a CNV loss at chromosome 18p11.31. **A)** The copy number state indicates a normal number of copies for the region (blue circle) however the smooth signal is lower than 2 (green circle) indicating the possibility of a mosaic loss in that region **B)** The copy number state of 1.0 for that segment indicates a loss of that genomic region (red circle). The allele difference track has two tracks instead of three, indicating that there are two possible genotypes for that segment i.e., a loss of genomic material.

Mechanisms underlying CNVs

The mechanisms underlying the CNVs detected by both the SNP array and the CGH array can be inferred from visualising the CNVs in the respective software. This applies to patients with more than one CNV e.g., a terminal loss on one chromosome and a terminal gain on another chromosome is suggestive of an unbalanced translocation. Table 3 presents the list of patients with multiple CNVs and their inferred mechanism.

Table 3. List of rearrangements and the inferred mechanisms.

Patient	CNVs	Inferred mechanism
4	2q26q27 terminal loss 6q26q27 terminal loss	Unbalanced translocation
11	4q12q35.2 terminal gain Xq28 terminal loss	Unbalanced translocation
12	6q27 terminal loss 17p13.3p13.2 terminal gain	Unbalanced translocation
14	5p15.33p14.1 terminal loss 5p14.1 terminal gain	Inversion
16	1q44 terminal gain 10q25.1q26.3 interstitial gain 10q26.3 terminal loss	Complex rearrangement

Loss of Heterozygosity

The SNP array was able to detect loss of heterozygosity greater than 8 Mb based on filter settings. The LOH was visualised using the ChAS browser. The LOH regions are determined by the SNP genotype information: BAF and allele difference displayed as tracks in the ChAS browser which can be seen in Figures 5, 6 and 7. Nine of the 17 patients, had LOH regions larger than 8 Mb as presented on Table 4.

Table 4. List of LOH segments greater than 8 Mb identified in the patient sample.

Patient	Sex chromosomes	LOH segment	Corresponding deletion
1	Male	Xp22.33p11.1 X11.1q28	-
4	Female	6q25.3q27	6q26q27
8	Female	5p15.33p14.1	5p15.33p14.1
10	Male	Xp22.33p11.1 X11.1q28	-
12	Female	Xp11.23p11.1	-
13	Male	Xp22.33p11.1 X11.1q28	-
14	Male	5p15.33	5p14.1
		Xp22.33p11.1 X11.1q28	-
16		Xp22.33p11.1 X11.1q28	-
17	Female	22q11.23q12.3	-

For three of the patients, the LOH regions were associated with a deletion in the same region. The LOH tracks on the browser indicated LOH was present, however the allele difference track did not support LOH, but rather a deletion (Figure 3). The allele difference indicates only indicates the presence of A and B genotypes, which indicates a loss.

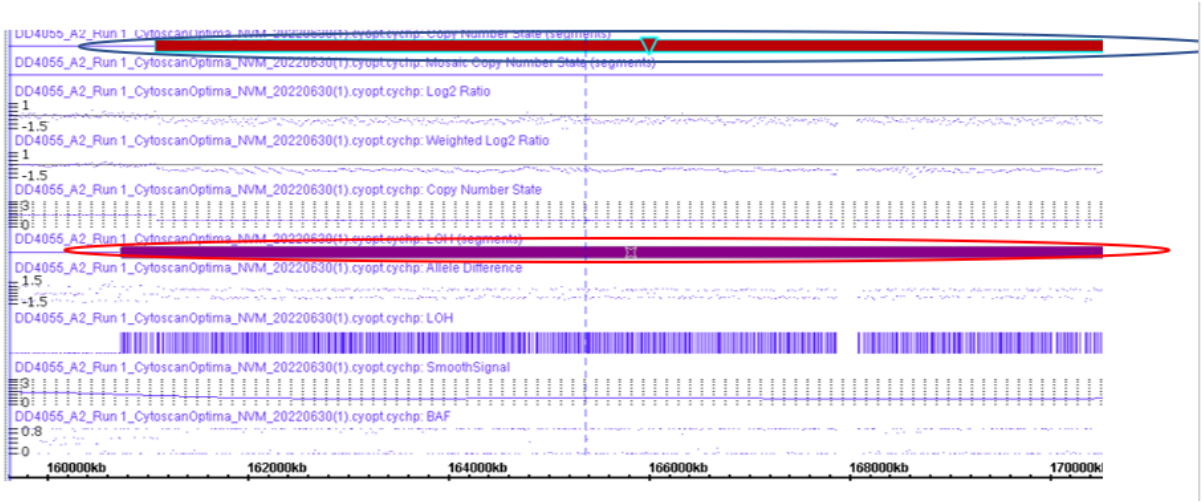

Figure 3. Screenshot of chromosomal analysis suite v4.3 showing the LOH (segment), allele difference, LOH and BAF tracks of chromosome 6q33.3q37.3. The LOH segment (red circle) track show that the LOH segments correspond to a loss (blue circle) in the same region at chromosome 6q33.3q37.3. The LOH tracks indicate a presence of LOH in the region however, the allele difference tracks do not support LOH.

Of the nine patients found to have LOH, only three patients had regions greater than 8 Mb that were associated with a deletion. Figure 4 is a visual representation of a segment of chromosome 22 of Patient 17. On the browser, both the LOH and allele difference track indicate a LOH on chromosome 22q11.23. The allele difference tracks show AA and BB genotypes only, indicating there is neither loss nor gain therefore there is loss of heterozygosity in that region. It is important to note that the CGH array is unable to detect LOH therefore the CGH test result was negative for any abnormalities for this patient. Unfortunately, there is no published information on the effect of LOH on the 22q11.23 region therefore LOH studies on 22q11.23 need to be done. Patient 12 also had an LOH region at chromosome Xp11.23p11.1 that was not associated with a deletion and had AA and BB genotypes.

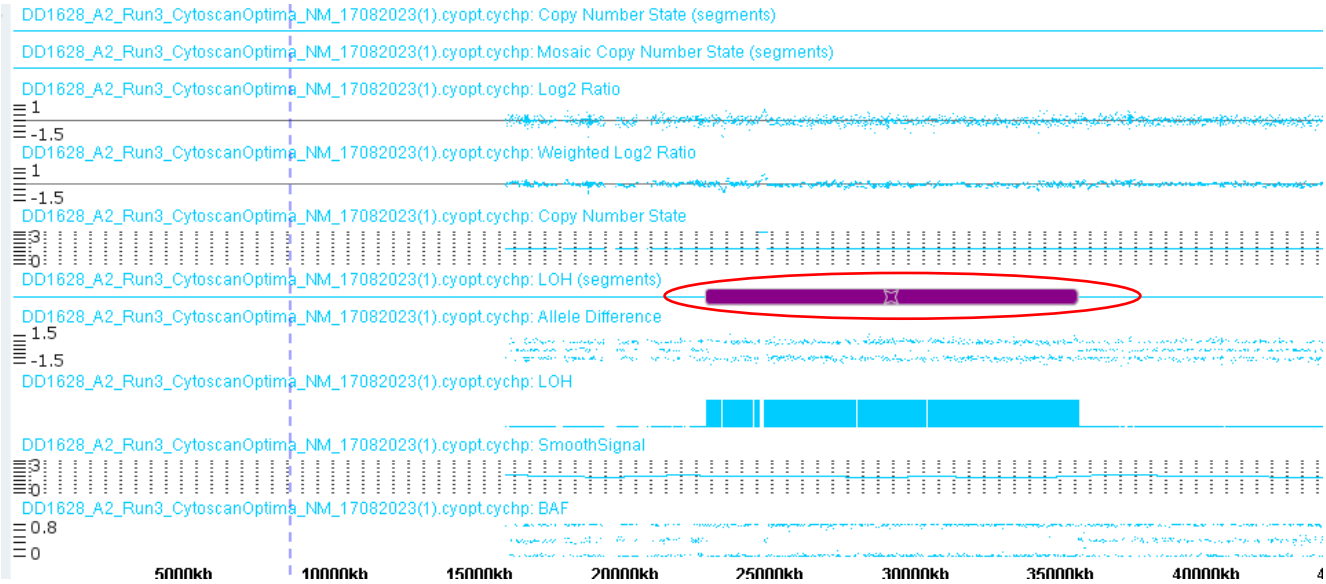

Figure 4. Screenshot of chromosome analysis suite v4.3 showing the LOH (segment), allele difference, LOH and BAF tracks of chromosome 22. The tracks show that the LOH segments (red circle) were associated with a loss in the same region, chromosome 22q11.23q12.3. Both the LOH and allele difference tracks indicate a presence of LOH in the region.

The ChAS browser indicated LOH for the entire X chromosome in all male patients in this sample as seen in Figure 5. This may have been due to the settings of the ChAS browser.

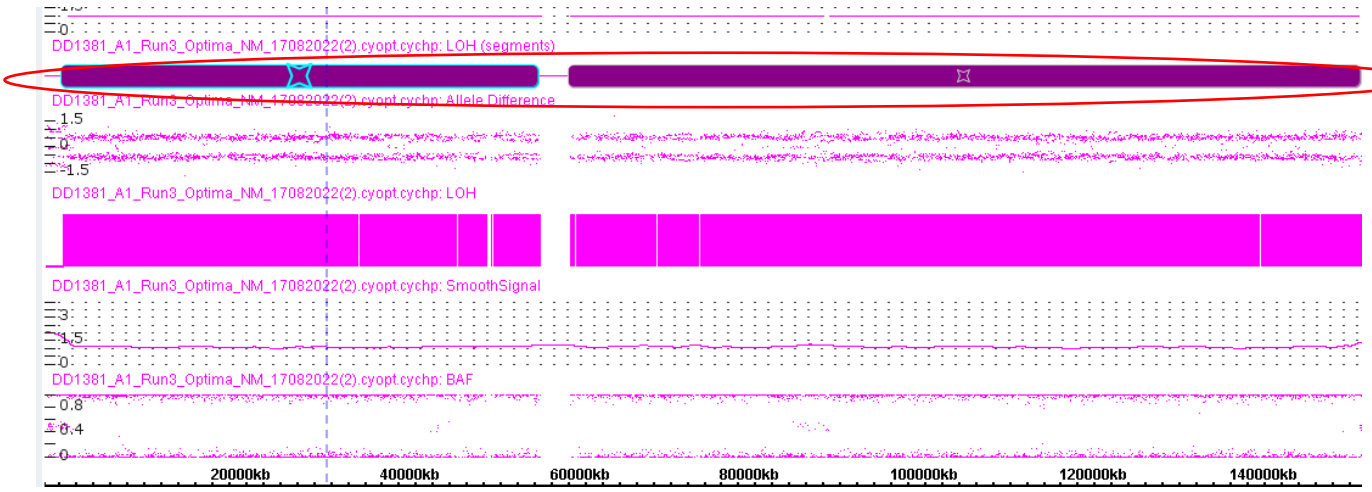

Figure 5. Screenshot of chromosome analysis suite v4.3 showing the LOH (segment), allele difference, LOH and BAF tracks of chromosome X in Patient 1. The tracks show that the LOH segments (red circle) runs on the entire X chromosome.

Costing

The price of a single sample SNP array test was determined to be $352.33 (R5601.57) at exchange rate $1 = R15.91 (9 January 2023) with DNA extraction included. This price was determined using the NHLS costing tool which considers the consumables and reagents, labour (medical scientists and genetic counsellor) and fixed equipment. Table 5 shows the breakdown of the amounts calculated for the different pricing components. The cost of the CGH array already implemented in the Division is $533.94 (8494.98) at exchange rate $1 = R15.91 (9 January 2023) with DNA extraction included. Table 6 shows the breakdown of the costs of the CGH array test. This makes the cost of SNP array $181.61 (R2889.41) at exchange rate $1 = R15.91 (9 January 2023) lower than the CGH array. The price breakdown for each test shows that the difference in total price between the two microarrays is due to a difference in the reagents and consumables used for each test.

Table 5. The cost of running a SNP-based array test

Component	**Cost for each test**
Reagents and Consumables	$ 268.93
Labour	$ 37.29
Fixed equipment	$ 13.59
DNA extraction	$ 32.52
TOTAL	$ 352.33

Table 6. The cost of running a CGH array test

Component	**Cost for each test**
Reagents and Consumables	$ 450.54
Labour	$ 37.29
Fixed equipment	$ 13.59
DNA extraction	$ 32.52
TOTAL	$ 533.94

Discussion

Chromosomal microarray analysis has proved to be useful in the detection of CNVs that cause developmental delay in individuals. In this study, the clinical utility and cost of a SNP-based array, the CytoScan® Optima microarray was compared to that of the SurePrint G3 Unrestricted CGH ISCA v2, 8x60K microarray, which is currently being used at the Division of Human Genetics, NHLS. The CNV calls made by each array type were compared for genomic coordinates, segment size and gene content. The genotype-phenotype correlations were only made for patients with associated syndromes.

The SNP array detected 24 CNVs in total and the CGH array detected 24 CNVs. Between the CGH array and the SNP array, there were 23 common CNVs detected. The SNP array was unable to detect the 18p11.32p11.31 loss that was detected by the SNP array and the CGH array was unable to detect the 15q26.2 gain that was detected by the SNP array. The ability of a microarray to detect genomic imbalances is determined by its design[35]. Although the designs of the two microarrays investigated are different, the CNVs detected were comparable.

The difference in design also suggests that there are likely to be differences in the genomic coordinates of the CNVs detected. This also explains the observed differences in the segment sizes of the CNVs detected by the two microarrays. An example of a difference in the starting breakpoint detected was seen in Patient 3 where the CytoScan® Optima detected a 13q11q12.11 deletion at coordinates 13:19436287-31787356 whereas the SurePrint ISCA array detected a 13q12.11 deletion at coordinates 13:20407295-21661250. The size difference in starting breakpoint was 9.71 Mb.

The sizes of the segments detected by both the CytoScan® Optima and the SurePrint ISCA array were comparable. In this study sample, the CytoScan® Optima array detected CNVs ranging in size from 0.98 Mb to 94.36 Mb. Other studies have reported the CytoScan® arrays detecting CNVs as small as 0.76 Mb[36].

Patient 2 was previously confirmed to have a gain at 3q21 by karyotyping, however karyotyping does not provide the breakpoint of the genomic imbalance. The two microarrays defined this CNV as a gain of 3q13.2q22.3. Microarrays have the advantage of being able define the exact genomic breakpoints of the CNV as shown in Table 1. This capacity to precisely characterize breakpoints is important in accurately determining the specific genes included in the CNV and therefore the genetic disorders that may be caused by that CNV[37].

The probe-spacing and design of the arrays compared here differs. For this study, a comparison between the CytoScan® 750K array (Thermo Fisher Scientific, Waltham, MA) and SurePrint ISCA may have been more suitable because similar to the SurePrint ISCA, the CytoScan® 750K array (Thermo Fisher Scientific, Waltham, MA) is designed for postnatal analysis.

The SNP array is designed with copy number probes alongside SNP probes therefore, unlike the CGH array, this array is able to genotype the SNP locus in that genomic region. This allows the detection of LOH in patients. Loss of heterozygosity is the loss of one of the pair of alleles at a constitutional heterozygous locus; it is assessed by detecting small DNA sequence changes at specific chromosomal loci[38]. LOH has been detected in events of UPD[39]. UPD is when the two homologous chromosomes are inherited from one parent with no contribution from the other parent[40]. LOH studies show that UPD has a prevalence of 1 in 176 in patients with developmental delay[41]. The American College of Medical Genetics (ACMG) recommends reporting LOH greater than 5 Mb[40]. In this study we analysed LOH regions greater than 8 Mb to decrease cases of false positives. This was necessary as false positives may lead to incorrect diagnoses.

Long regions of LOH greater than 8 Mb were detected in 9 patients. Seven of the LOH were false positives as the allele difference track that indicates genotype was not indicative of LOH region but a loss or the hemizygous X chromosome in males. Care must be taken in interpreting the results as shown deletions may be called as LOH due to the fact that in that region there is only one allele represented due to the loss of genomic material on the other chromosome.

Although the ChAS software was able to detect true LOH regions, there no evidence to support how this was the underlying cause of the patient phenotypes. The LOH region found in chromosome 22q11.23q12.3 in Patient 17 does not include the three known imprinting genes – *DGCR6L, DGCR6* and *FLJ204* found on chromosome 22[42]. Furthermore, the 36 genes in the LOH region on chromosome Xp11.23p11.1 in Patient 12 are not reported to be imprinted genes.

Being able to detect LOH is also important during diagnosis of patients because of the important information on consanguinity that may be derived Individuals born of a consanguineous union have segment of their genomes that are homozygous as a result of inheriting identical ancestral genomic segments through both parents[43]. Identifying LOH caused by consanguineous unions can help in diagnosing recessive conditions. This is why it is important to do trio testing whereby the maternal and paternal parent are also tested in order to determine whether a CNV was de novo or inherited. Information on family history and trio studies were not provided in this study and therefore the significance of LOH in the patients was not determined.

The difference in design between the two microarrays is influenced by the purpose for which it is intended. The CytoScan® Optima array was designed as a low-resolution prenatal array. This means it is limited to large and known pathogenic CNVs. The SurePrint ISCA array was designed for postnatal CNV detection and therefore has a much higher resolution as CNVs may be analysed in association with clinical features. This difference informs the probes and markers that manufacturers include in the microarray. For the CytoScan® Optima array, probe coverage needs to be sufficient for the detection of aneuploidies, large chromosomal aberrations and clinically relevant regions underlying known syndromes associated with congenital anomalies and developmental disorders.

The samples selected for this project were predominantly from patients with known CNVs including confirmed microduplications and microdeletion syndromes. Of the 17 patients, four had microdeletion syndromes and two had microduplication syndromes

confirmed diagnoses, and two confirmed trisomy of chromosome 7p in Patient 7 and trisomy 13q of chromosome 13q in Patient 9.

Inferences on the mechanism leading to a loss or gain can be made from the data from both microarrays. A quarter of the CNVs were suggested to be a result of unbalanced translocation which are known to cause developmental disorders[44]. Translocations are structural variants (SV) caused by the incorrect joining of co-existing chromosome breaks[45]. The joining together of chromosome breaks is facilitated by DNA repair mechanisms. Translocations are a result of irregularities in the proteins involved in the cellular mechanisms used for repair of chromosome breaks. Mechanisms include non-homologous end joining and homologous recombination[44]. The analysis software used to display the microarray results allow one to visualise positions of these structural variants. Microarrays can only infer the mechanism underlying unbalanced structural aberrations. Confirming the mechanism may be helpful in determining recurrence rate of the CNV for risk assessment during genetic counselling.

The two arrays compared in this study are able to detect mosaicism. The main difference is that the SNP-based array used SNP genotype data to provide a more reliable estimate of mosaicism. Using ChAS mosaicism can be displayed as a segment on the browser or inferred from the smooth signal, BAF and the allele difference track. Mosaicism on the CGH array can only be inferred from the log2 ratio.

Another difference is how the level of mosaicism detected for a particular CNV is estimated. In the CytoGenomics software of the Agilent platform, the level of mosaicism is manually calculated from the log2 ratios whereas in ChAS, the level of mosaicism is estimated by the software from the average of the smooth signal values. In this study, two samples were found to present with mosaic chromosomal aberrations, Patient 9 with gain of 13q at 63% mosaicism and Patient 3 with loss of 18p11.32 at 59%. SNP-based arrays are reported to be able to detect mosaic levels of as low as 4%[46]. The detection of mosaicism is important in diagnosis, predicting

recurrence rate, as well as in genetic counselling. Failure to identify low levels of mosaicism during diagnostic testing may lead to inaccurate interpretations of genetic results[47]. Mosaicism in copy number change have been reported in some developmental conditions[48,49]. SNP arrays are known to detect lower levels of mosaicism and being able to do that could lead to more accurate reporting genetics results to patients.

The two microarrays did not have 100% concordance for the known CNVs in the sample of patients investigated in this study. For Patient 3, the SurePrint ISCA array detected a deletion that was linked to the patient's clinical features (choanal atresia and subtle dysmorphism). This region included the gene *SMCHD1*; loss of function mutations in this gene causes Bosma arrhinia micropthalmia syndrome (OMIM: 603457), a rare condition of the eyes and nose[50]. Choanal atresia is one of the reported features in affected patients. In addition, there is one case recorded on ClinVar with a loss of the *SMCHD1* gene causing the disease[51].

The ChAS software did not call this deletion as a copy number change. However, the smooth signal and BAF values on the browser were indicative of a change in copy number. This may be explained by the lower resolution of the CytoScan Optima array compared to the SurePrint ISCA array. Although the smooth signal track and BAF track are able to indicate a copy number change, this may be missed by an inexperienced analyst.

The differences in the CNV calls made following analysis by the CGH array and the SNP-based array are important to note because as seen in Patient 3, the CNV that the ChAS software did not call contained the gene that explained the phenotype of the patient. This suggests the difference in the genomic regions that each array has a higher density of copy number probes in. This shows that both microarrays are relevant in detection of CNVs associated with neurodevelopmental and congenital disorders as they have the capacity to detect regions that are relevant to these disorders.

Patients 3, 4, 11, 15 and 16 presented with clinical phenotypes related to congenital disorders and developmental delays, however, the CNVs associated with the phenotypes were not clinically significant therefore a molecular diagnosis was not reached. This may be due to public databases not having enough information on a CNV, patients with that CNV not having a clinical diagnosis or insufficient information on the dosage sensitivity of the genes in the CNV. Cytogeneticists who analyse CNVs in clinical settings use public databases to inform the classification of a CNV and reach a molecular diagnosis for the patient. These databases having insufficient information limits the correlation of certain CNVs with the patients clinical features and/or condition.

The CytoScan® Optima array has benefits in that it is much easier to use. The washing step of the workflow happens in a closed fluidics system as opposed to the SurePrint ISCA array workflow whereby the washing step is done manually. The wash buffer conditions need to be temperature controlled therefore this step in the SurePrint ISCA is prone to error. A limitation of the CytoScan® Optima array is that it is labour intensive and has sensitive enzymes.

The price of detecting CNVs using a SNP array is $181.61 less than using the CGH array. This is the cost that patients will have to pay in order to have the test conducted on them if they present with intellectual disabilities and developmental delays and congenital anomalies. A cheaper price for these patients would be more beneficial as the NHLS services the public sector; a cheaper price may mean improved affordability for lower income patients.

Other studies that have compared the utility of CGH array and SNP array have found that the CGH array is better suitable for detection of CNVs because its probe is more clinically targeted. This means its usage leads to better results and coverage for target sites[52]. Another study however, found that the SNP array was more suitable for detection of copy neutral abnormalities such as UPD, however, the fewer the SNP probes on the array, the more likely LOHs that are due to identity by descent will be called[53].

Limitations

1. The study analysed a small sample size (17) due to:
   a. The CytoScan Optima kit used has the capacity to test only 24 samples.
   b. The enzymes used for the CytoScan Optima test are very sensitive therefore some tests were unsuccessful.
2. The public databases used to interpret and classify CNVs are limited; in some cases, there is little or no information and a classification may not be made.

Conclusion

The utility of the CytoScan Optima array was studied by comparing it to the SurePrint ISCA array that is already in use for diagnostic service at the Division of Human Genetics, National Health Laboratory Service in Johannesburg. The two microarrays investigated here have comparable detection capacity. Compared to CGH arrays, SNP arrays provide information on LOH that could be relevant in the diagnosis of genetic disorders. The SNP array costs less than the CGH array which would make it more advantageous to the lower income group. It is suggested that the SNP-based array and the CGH array be used interchangeably based on clinical requests. Since the CytoScan® Optima was designed specifically for prenatal studies, it can be used for prenatal testing to limit the detection of CNVs you cannot interpret. The Division should consider incorporating SNP probes into the microarray testing strategy for a suspected chromosomal abnormality.

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Appendices

Appendix A. Approved research protocol

CANDIDATE'S SURNAME: MAYISELA	FIRST NAME/S: MINENHLE		STUDENT NUMBER: 1880919
CURRENT QUALIFICATIONS: BSC (HONS) GENETICS AND DEVELOPMENTAL BIOLOGY			
TEL:	CELL: 078 788 8219	E-MAIL: 1880919@STUDENTS.WITS.AC.ZA	FAX:
DEGREE FOR WHICH PROTOCOL IS BEING SUBMITTED: MSC (MED) GENOMIC MEDICINE			
PART-TIME OR FULL-TIME: FULL-TIME			
FIRST REGISTERED FOR THIS DEGREE: 2022	TERM: 1	YEAR: 2022	
DEPARTMENT: HUMAN GENETICS			
TITLE OF PROPOSED RESEARCH: EVALUATING THE CLINICAL UTILITY OF A SNP-BASED ARRAY PLATFORM – A COMPARATIVE STUDY			
CANDIDATE'S SIGNATURE:		DATE: 28 APRIL 2022	
SUPERVISOR 1 (NAME & SURNAME): NKATEKO MAYEVU		% SUPERVISION: 60%	
SUPERVISOR'S QUALIFICATIONS: MSC (MED) MEDICAL BIOCHEMISTRY			
SUPERVISOR'S DEPARTMENT: HUMAN GENETICS			

SUPERVISOR'S ADDRESS / TEL / E-MAIL: DIVISION OF HUMAN GENETICS, NHLS BRAAMFONTEIN / 011 489 9237 / NKATEKO.MAYEVU@NHLS.AC.ZA	
SUPERVISOR 2 (NAME & SURNAME): FIONA BAINE-SAVANHU	% SUPERVISION: 40%
SUPERVISOR'S QUALIFICATIONS: PHD	
SUPERVISOR'S ADDRESS / TEL / E-MAIL: FIONA.BAINE@WITS.AC.ZA	

SYNOPSIS OF RESEARCH:*(Brief summary of proposed research project; between 200-300 words only; with sub-headings: an introduction and justification for study, aim/s, proposed methodology and expected outcome/s)*

Introduction and justification of study:

The proposed study will compare the clinical utility of a SNP-based array (Cytoscan® Optima) to that of the comparative genomic hybridization array (aCGH) currently used by the Division of Human Genetics. The aCGH platform has been used by the Division of Human Genetics, National Health Laboratory Service as a diagnostic method for conditions caused by copy number variants and structural abnormalities. The long-term goal of this project is to make recommendations regarding whether the Division should implement a SNP-based array in diagnostic setting.

Aim:

The aim of the study is to evaluate the clinical utility of a SNP-based array for diagnostic CNV detection compared to an CGH array.

Proposed methodology:

The study will analyse SNP-based array data using the Chromosomal Analysis Suite (ChAS) v4.3, which is an Affymetrix copy number variant (CNV) calling tool. The CNV analysis and interpretation will consider the size of the CNV, the gene content of the CNV and the use of databases such as database of Genomic Variants (DGV). A comparison of the available CNV results obtained from the CGH array and the SNP-based array by analysing the data from 10 samples with known CNVs that have been identified on the CGH array platform. Lastly the cost of offering the SNP-based array test in the Division of Human Genetics will determined using the National Health Laboratory Services (NHLS) costing tool.

Expected outcome:

A direct comparison of a SNP-based array and CGH array for diagnostic CNV detection

This project will explore the use in using SNP microarrays for detecting copy number variants in our human DNA sequences. These copy number variants are cause of disease for many South Africans and most of the time they go undiagnosed because of lack of technology to detect the disease. This project will show why using SNP array will improve diagnostic rate of disease and this is important because diagnosis of disease informs prognosis and will be helpful in offering personalised therapy and even prompt scientist to start clinical trials to treat the genetic cause of disease.

WITS ETHICS NOT REQUIRED: Yes No WITS ETHICS PENDING: Yes No WITS ETHICS APPROVED: Yes No (circle appropriate symbol)* ***Please note the final human ethics clearance certificate or animal ethics certificate must be available prior to starting research**		IF Y SUPPLY ETHICS CLEARANCE CERTIFICATE AS ATTACHMENT AND INCLUDE ETHICS NUMBER HERE: **M220295**
As supervisor/s, I/we confirm that I have read the protocol which has been submitted for assessment.		
SIGNATURE OF SUPERVISOR/S:		
SIGNATURE PG OFFICE STAFF ……..	REGISTERED YES….. NO…..	STAMP

SYNOPSIS OF RESEARCH CONTINUED

Research Proposal

Evaluating the clinical utility of a SNP-based array platform – a comparative study

By

Minenhle Mayisela

1880919

Master of Science in Medicine in Genomic Medicine

Supervised by:

Ms Nkateko Mayevu

Dr Fiona Baine - Savanhu

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List of abbreviations

CGH – comparative genomic hybridization

CMA – Chromosomal microarray analysis

CNV – copy number variants

FISH – Fluorescence in situ hybridization

kb – kilobases

LOH – loss of heterozygosity

Mb – Megabases

MLPA – Multiplex ligation-dependent probe amplification

PCR – Polymerase chain reaction

SNP – single nucleotide polymorphism

UPD – Uniparental disomy

ISCA - International Standards for Cytogenomic Arrays

1. Introduction

1.1. Copy Number Variants

Copy number variants (CNVs) are DNA segments that are greater than 50 bp in length and show variable copy number compared to reference genome [1] This suggests that the genome of individuals not only varies in DNA sequence but also in the number of copies of a particular DNA segment [54] . A greater proportion of CNVs are part of normal variation and do not cause disease.

The CNVs that are disease-causing are usually large, encompassing many genes and/or are found in dosage sensitive genes [54]. They play a role in the aetiology of diseases such as developmental disorders [55]. The study of CNVs in relation to disease contributes to determining the copy loss or gains that cause disease [55]. It also involves discovery of rare de novo alterations that cause neurodevelopmental disorders and congenital anomalies [55]. These CNVs are detected using several laboratory techniques.

Cytogenetic methods have been used for a long time to detect CNVs that cause disease in humans. Over time, the methods have evolved from conventional cytogenetic methods such as karyotyping to molecular cytogenetics methods such chromosomal microarray platforms.

2. Evolution of Cytogenetics

Cytogenetic techniques are used to identify and analyse structural rearrangements of chromosomes caused by cellular processes such as incorrect recombination or DNA repair. They detect balanced and unbalanced structural changes to chromosomes such as CNVs, translocations and inversions [4]. The techniques have evolved over the years, with each successive technique improving resolution and turnaround time for genetic testing and disease diagnosis.

2.1.1. Karyotyping

Karyotyping allows scientists to view all chromosomes under a microscope and detect numerical and large structural chromosome abnormalities by analysing banding patterns [5]. Giemsa/G banding is a commonly used technique in karyotyping. The

giemsa stain and trypsin solution create dark and light banding patterns on the chromosomes [5]. This helps visualise the different regions of the chromosomes and to study any structural changes. More advanced molecular cytogenetic techniques have been introduced; however, karyotyping is still considered the gold standard for confirming and informing the mechanism underlying many chromosomal aberrations. The resolution of karyotyping is between 5-10 Mb, meaning it is unable to detect submicroscopic chromosomal abnormalities [5]. Improved cytogenetic techniques have helped detect these abnormalities over the years.

3. Fluorescence in situ hybridization (FISH)

The development of fluorescence in situ hybridization (FISH) made the detection of submicroscopic chromosomal abnormalities possible, addressing some of the limitations of the karyotype. It is a molecular cytogenetic technique that uses fluorescently labelled probes hybridized on chromosomes to detect known structural changes in chromosomes [56] The chromosomes are analysed under a fluorescent microscope. The resolution of FISH is between 100 – 200 kb, depending on the probes used [57]. The FISH probes are designed for specific regions of interest on the chromosome; thus, to employ FISH, there should be a suspected disorder [58]. Another drawback is that FISH can only test for one suspected chromosomal abnormality at a time making it less cost effective. T

4. Multiplex Ligation-Dependent Probe Amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) is a technique that quantifies the dosage of DNA regions based on directed ligation [7]. It has the advantage of targeting multiple regions on the genome, which FISH fails to do so. This makes MLPA more cost effective. The MLPA technique uses probes specific to the region of interest to assess the relative copy number of the DNA sequence [8]. The method uses two probes that are ligated if hybridization takes place [8]. Ligated probes are then amplified by a polymerase chain reaction (PCR) and the products visualised using specialised software called Coffalyser.

The results are generated as peaks on a graph, which are then compared to separate peaks from control probes [7]. The MLPA probes can be used with sequence targets of between 50 – 100 bases long. This means that this technique may be useful in fragmented DNA as well as in detecting single gene changes that cause disease and cannot be detected by FISH [7].

Although MLPA has multiple targets, the probes are designed for known submicroscopic abnormalities related to specific diseases, meaning only those conditions can be tested using this technique. With the field of genetics rapidly changing, testing strategies and techniques have also evolved to provide more information for clinical diagnosis. A broader technique which targets the whole genome - the microarray - makes it possible to detect de novo aberrations.

5. Chromosomal Microarray

In 2010, the International Standards for Cytogenomic Arrays (ISCA) Consortium recommended chromosomal microarray analysis (CMA) as the first line of testing for patients that present with developmental delays, congenital anomalies, intellectual disabilities and autism spectrum disorder [59]. Microarray addressed the resolution limits imposed by conventional cytogenetic techniques such as karyotyping and FISH [11]. The CMA technique is a deoxyribonucleic acid-based method that detects CNVs as well as the size of the aberrations present [60].

Microarrays detect CNVs, unbalanced chromosomal rearrangements and aneuploidies. The resolution depends on probe number, spacing and distribution when designing the microarray [11].

There are two microarray approaches used: array comparative genome hybridization (aCGH) or single nucleotide polymorphism (SNP) array [61]. SNP-based arrays specifically are capable of detecting loss heterozygosity (LOH) and uniparental disomies (UPD) [61]. A study undertaken to diagnose structural aberrations in patients with Down syndrome using CMA, gave a diagnostic yield that was 15-20% higher compared to the 3% yield from conventional methods, specifically karyotyping [59,62].

5.1.1.1. Comparative Genomic Hybridization array

The aCGH is referred to as true comparative hybridization because it demonstrates the amount of DNA at various areas of the genome by comparing a test sample to a normal reference sample [11]. This is done by differentially labelling the reference DNA and the patient's DNA with fluorophores and the two samples are then co-hybridized onto a microarray (a glass slide containing multiple probes from various regions of the genome) [63]. The results indicate the fluorescence ratio of the amount of reference sample to the test sample. A higher presence of reference sample indicates that there is a loss of DNA and a higher presence of the test sample indicates a gain of DNA [63] .

The results are represented graphically as a $\log_2$ ratio. Important to note is that aCGH requires both a test and normal reference in order to determine if there is a CNV at a region [11].

5.1.1.2. Single Nucleotide Polymorphism array (SNP array)

The SNP-based microarray requires only the test sample, in what is called a one – colour test [12]. The SNP array functions by directly hybridizing the test DNA sample to a microarray slide that contains probes designed to bind to a target DNA sequence flanking SNPs [13]. The fluorescent colour intensity is dependent on the amount of target DNA in the sample. The results are displayed as a graph with two tracks. One track shows the exact copy number of the gene region of the sample represented by a probe. Another track shows the B-allele frequency which shows the genotype of each SNP locus. The B allele measures the intensity ratio of the two [54].

The SNP microarrays are a tool that have gone from genotyping a few thousands of SNPs to presently genotyping millions [64]. Genotyping is made possible by SNP arrays being developed to include SNP probes alongside copy number probes. The results of the array show the gain or loss of DNA as well as the different SNP genotypes for each SNP locus [13]. Since SNP arrays are a genotyping tool, they can detect long regions of loss of heterozygosity [65]. They can also detect low level mosaicism [14].

Two chromosomes will give three possible combinations: AA, AB and BB and the graph will show three tracks [66]. The probes are designed to hybridize two alleles (A and B alleles). Once hybridized, a scanner measures the intensity of fluorescence from the hybridized A and B probes on the array. The SNP genotypes are analysed by comparing A and B allele fluorescence intensity. A genotype of A-A is called when A fluorescence intensity is strong and B allele intensity is low; B-B is called when B fluorescence intensity is strong and A allele intensity is low. When A-B is called, that indicates that the two intensities are similar and of medium level of intensity (Levy & Burnside, 2019).

This is possible because the number of homozygous SNPs is consistent with a longer region of loss of heterozygosity in chromosomes [65]

6. Diagnostic copy number variant testing at the Division of Human Genetics

Comparative genomic hybridization (CGH) array was implemented in 2020 as the first line of testing for patients with developmental disorders and congenital abnormalities

at the Division of Human Genetics, National Health Laboratory Service and the University of the Witwatersrand. The Division uses the SurePrint G3 Unrestricted CGH ISCA v2, 8x60 kb microarray (Agilent Technologies). This microarray has 60 000 copy number probes that are concentrated at genomic regions of clinical relevance as determined by the ISCA consortium. The CNV calling is performed on Cytogenomics and visualised in Figure 1. The array has a resolution of 200kb and analyses eight samples on a single slide. It is unable to detect loss of heterozygosity LOH, UPD and low-level mosaicism (<30%).

The Division recently acquired a Affymetrix SNP- based array system to advance genomic research. The data for this study will be generated using the CytoScan Optima array (Thermo Fisher Scientific). This array consists of 18 018 copy number probes and 148 450 SNP probes. The copy number probes detect the CNVs of the test sample at the gene region the probe hybridizes. The SNP probes have a SNP site at different locations. CNV calling is performed using Chromosomal Analysis suite v4.3. The resolution offered by this array is low compared to the Agilent array currently in use in the Division. The resolution is 1 Mb for losses, 2 Mb for gains and 5 Mb for loss of heterozygosity. The resolution is low because this array is designed for prenatal testing therefore the probes are limited to specific regions with clear clinical significance. However, the availability of SNP probes makes it useful for detection of LOHs, UPDs and low-level mosaicism.

Given that CGH array has been implemented in the Division of Human Genetics, the question this project seeks to answer is whether a SNP array may add more value to the diagnostic service.

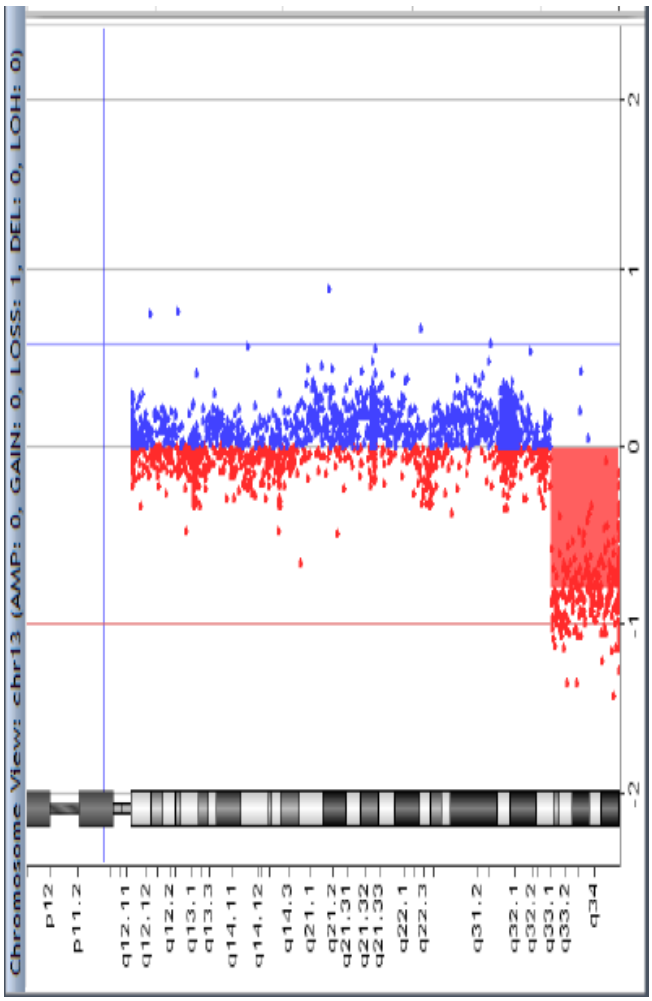

Figure 1. Cytogenomics image showing a terminal 13 deletion. The red line represents $\log_2$ ratio of 1 which indicates a deletion.

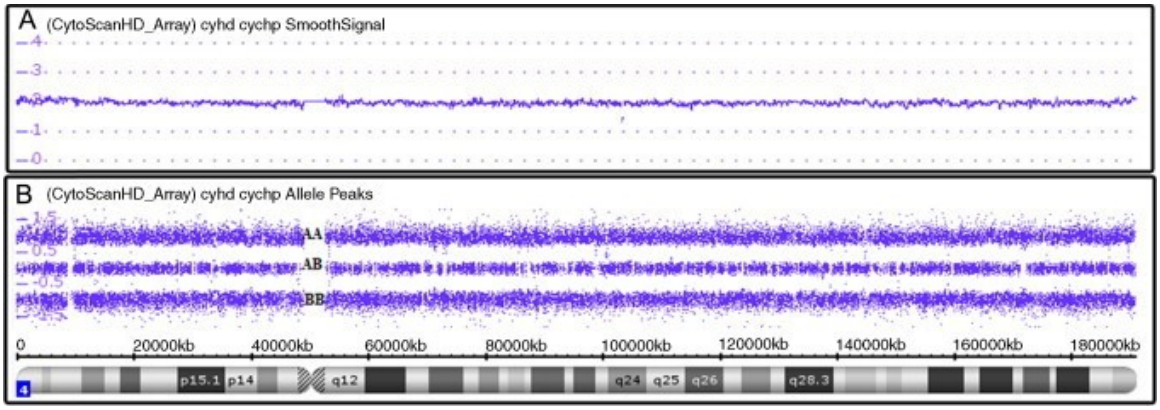

Figure 2. Affymetrix® Chromosome Analysis Suite image showing normal chromosome 4. (A) Smooth signal representing a normal copy number (purple line CN: 2.00). The information on copy number variations is from non-polymorphic probes. (B) Allele peaks representing a normal genotype with AA, AB and BB alleles (three purple lines). Information on the genotype is from polymorphic probes. (Figure adapted from Rev Bras Hematol Hemoter. 2015 Jan-Feb; 37(1): 48–54).

7. Study Aim and Objectives

The aim of this study is to evaluate the clinical utility of a SNP-based microarray in comparison to the CGH array platform for diagnostic CNV detection in the Division of Human Genetics.

The specific objectives of this study are to:

1. To analyse CNVs detected from Affymetrix SNP-based array data for 10 selected samples.
2. To compare CNV calls/results obtained from the Affymetrix SNP-based array to the available diagnostic results from the Agilent platform.
3. To conduct a costing analysis of the Affymetrix SNP array platform

8. Methods

9. Objective 1 – Analysis of CNVs from SNP array

The CNVs detected from the CytoScan Optima array performed on the Affymetrix platform will be analysed. The dataset consists of CNV and SNP data from 10 samples with a variety of known pathogenic chromosomal rearrangements representing gains and losses. The 10 samples were selected from the diagnostic records (results) available to represent a range of structural variants of different kinds including gains, losses and large aberrations. A GeneChip Scanner 3000, using the GeneChip Command Console Software (Thermo Fisher Scientific), will be used to generate the CEL files that summarise the intensity probe signals.

The data will be analysed using a CNV-calling tool called Chromosomal Analysis Suite (ChAS) v4.3. The tool computes and plots the genome and chromosome overviews with tracks incorporated to interpret the aberrations. The ChAS software also contains algorithms for the detection of LOH and UPD. The software will be used to classify and annotate the observed aberrations. Through the databases linked to the software, the observed aberrations can be classified for pathogenicity. The databases that will be used are: Databases of Genomic Variants (DGV), DECIPHER and ClinGen. These databases contain genetic and phenotypic information of variation in the genome. They allow for storage, sharing and comparison of genetic data across research studies.

10. Objective 2 – Comparison of CNVs between the two platforms

The data generated from the CytoScan® Optima array will be compared to data that has already been generated from the Agilent platform.

The following data generated from each platform will be compared:

- The probe density at each DNA region for each platform
- The $\log_2$ ratio figures for each CNV call
- The number of calls for each sample based on a 200 kb threshold
- The segment coordinates of each CNV call that will be detected
- The gene content of the CNVs called made

The results will be validated by correlating whether the CNV with the clinical phenotype.

11. Objective 3 - Costing analysis of SNP array

A costing analysis of the Affymetrix SNP-based array will be performed using the NHLS costing model. This tool includes calculation cost of reagents, the fixed facilities and the labour cost of lab personnel. This will allow for the estimation of the total cost per sample of the SNP array. This cost will be compared to the current cost of the aCGH.

12. Ethics

A conditional ethics clearance (M220295) was obtained from the Human Research Ethics Committee (HREC) to undertake the sub study under M18056 with the following conditions: evidence of protocol approval to be submitted as soon as it is approved. The ethics clearance certificate can be found in Appendix B

13. Timeline

	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC
Literature Review												
Ethics Application												
Protocol Preparation												
Protocol Assessment												
Introduction to software												
Data Analysis												

Written Report												

14. Funding

No funding is required for this study.

15. Problems/Limitations

- The SNP-based array has a lower resolution and may not pick up some of the variants detected on a CGH array
- The sample size is small due to limited funds available for this analysis
- Neither of the microarray platforms is a gold standard, therefore results that are picked up from only one of the platforms cannot be truly validated

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17. Appendices

Appendix 1

NATIONAL HEALTH LABORATORY SERVICE
School of Pathology, University of the Witwatersrand

DIVISION OF HUMAN GENETICS

Hospital Street, Johannesburg, 2001 I PO Box 1038, Johannesburg, 2000
[T] +27 11 489 9223 I [M] +27 78 080 8841 I [F] +27 11 489 9226 I [E] human.genetics@nhls.ac.za

Date: 25 February 2022

I wish to apply for permission to use data/samples from the Division of Human Genetics covered under the Ethics Clearance Certificate M180506:

Using anonymised residual diagnostic samples and –file data for audit, research and development:

Name of Staff member/Student:	Minenhle Mayisela
Staff/ Student Number:	1880919
Degree/Non-degree purposes	MSc Genomic Medicine
E-mail address:	1880919@students.wits.ac.za
Contact Number:	0787888219

Name of Supervisor (if PI is a student): Nkateko Mayevu

Staff/ Student Number:	NHLS: 6247
E-mail address:	nkateko.mayevu@nhls.ac.za
Contact Number:	(011) 489 9237

My study title is: Evaluating a SNP based array for diagnostic copy number variation detection - a comparative study

I require access to the following samples/patient files (Give numbers and description):

I require 10 samples with complex structural chromosomal rearrangements identified using CGH microarray (Agilent). The data from the samples will be compared with newly generated data from the SNP array platform (Affymetrix). The resolution of the two microarray platforms will be assessed.

I will use samples/data covered under the following Section of the above mentioned Ethics clearance:

Category D/E

Retrospective database of clinical patient records and residual diagnostic samples:

> **Category A**: Existing patient clinical records from 1970s to 1 September 2018, for which no consent has been obtained
>
> **Category B**: Existing residual diagnostic samples from mid 1980s for which no consent has been obtained

Prospective database of clinical patient records and residual diagnostic samples (after 1 September 2018)

> **Category C**: Clinical records of patients who presented to Human Genetics Clinics after 1 September 2018 from whom written consent is obtained.
>
> **Category D**: Residual Diagnostic samples from patients who presented to Human Genetics Clinics after 1 September 2018 from whom written consent is obtained.
>
> **Category E**: Residual Diagnostic samples from patients referred to the Division of Human Genetics from whom consent has not be obtained I attach a summary of my main project objectives.

I am aware that if I may still need to apply to the Human Ethics Research Committee for approval for my research project

Signature: M.M

Name: Minenhle Mavisela

Supervisor signature:

Name: Nkateko Mayevu

Approved:

Amanda Krause, MBBCh, PhD
Medical Geneticist/Associate Professor
Head of Division

2

Appendix 2

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO:

Ms M Mayisela
School of Pathology
Division of Human Genetics
Medical School
University

E-mail: 1880919@students.wits.ac.za

CC:

Supervisor: Ms N Mayevu and Dr F Baine-Savanhu
<Nkateko@Mayevu@nhls.ac.za>
and <HREC-Medical Research Office@wits.ac.za>

FROM:

Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2022/03/16

REF: R14/49

PROTOCOL NO: **M220295** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *Chromosomal re-arrangements investigated on two microarray platforms: comperative study*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain2007/Clearcon.wps

Appendix B. Ethics clearance

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Ms M Mayisela

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220295

NAME: (Principal Investigator)	Ms M Mayisela
DEPARTMENT:	School of Pathology Division of Human Genetics Medical School University
PROJECT TITLE:	*Chromosomal re-arrangements investigated on two microarray platforms: comparative study*
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under M180506 Prior condition (Faculty protocol approval) satisfied on 2022/07/13
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Ms N Mayeyu and Dr F Baine-Savanhu
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2022/03/16

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

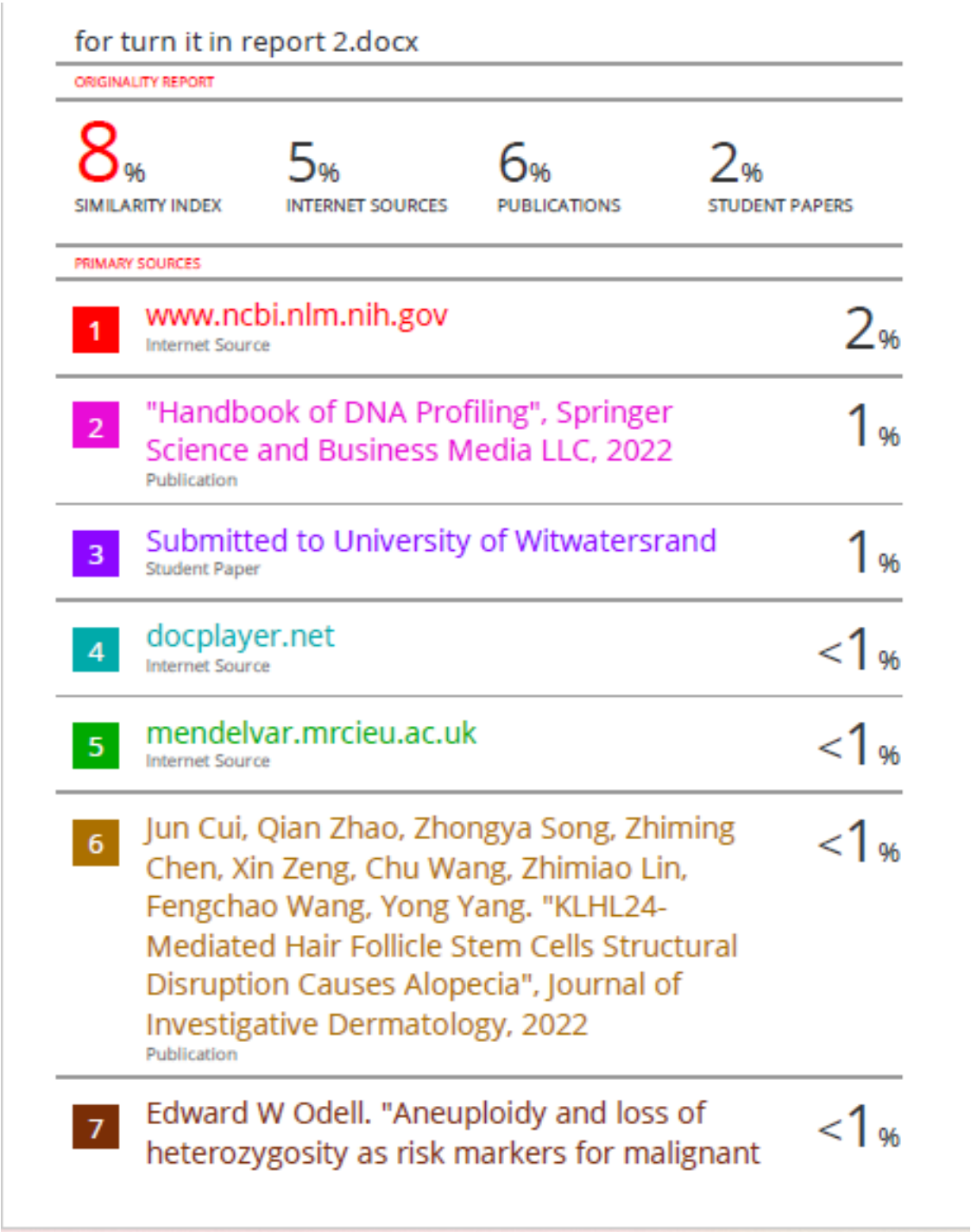
To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. I agree to submit a yearly progress report. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **February** and therefore reports and re-certification will be due in the month of Feb. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

18 August 2022

Signature of Principal Investigator — Date

Appendix C. Turn-it-in report



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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

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Appendix D. Author guidelines for the Journal of Molecular Diagnostics

The Journal of Molecular Diagnostics *(JMD)*, the official journal of the Association for Molecular Pathology (AMP), co-owned by the American Society for Investigative Pathology (ASIP) and published by Elsevier, Inc., seeks to publish high-quality original papers on scientific advances in the field of molecular diagnostics, the translation and validation of molecular discoveries in medicine into the clinical diagnostic setting, and the description and application of technical advances in the field of molecular diagnostic medicine. The editors welcome for review manuscripts that contain: novel discoveries with direct application to clinical diagnostics or clinicopathologic correlations including studies in oncology, infectious diseases, inherited diseases, predisposition to disease, clinical informatics, or the description of polymorphisms linked to disease states or normal variations; the application of diagnostic methodologies in clinical trials; or the development of new or improved molecular methods which may be applied to diagnosis or monitoring of disease predisposition.

Manuscript Categories. In addition to **Regular Articles**, the Journal also publishes **Technical Advances**, which are intended to report breakthroughs in methodology or analytical tools applied to molecular diagnostics. Submissions to this category should include a detailed description of the methodological design and discussion of how this technique improves the practice of molecular diagnostics. There are no official limits to word count or number of tables and figures that can be included in for Regular Articles or Technical Advances; authors should consider their budgetary restrictions when preparing their manuscript. The Editors reserve the right to request reduction of text length or removal of data.

The Journal also features **Reviews, Mini-Reviews, Commentaries, Special Articles, Practice Advances, Guest Editorials,** and **Perspectives** solicited by the Editor-in-Chief, as well as AMP meeting abstracts and other articles of interest to the membership of AMP. Authors who wish to publish a **Review** or **Mini-Review** should send their curriculum vitae along with an outline of the proposed article for prior approval by the Editor-in-Chief; proposals should be emailed to jmd@asip.org. Reviews have a maximum of 5,000 words, 75 references, and 4 tables or figures (at

least one of which is mechanistic). Mini-Reviews have a maximum of 3,000 words, 40 references, and 2 tables or figures (one of which is mechanistic). Supplemental data should not be overly utilized to expand beyond these listed parameters. **Practice Advances** are submitted from AMP Education programs to highlight webinar offerings from the same group. This article format is not currently open to external submissions. **Commentaries** are submitted by invitation only and relate to a concurrently published manuscript. **Guest Editorials** and **Perspectives** are published by invitation only.

Review Process. The Editors perform an initial evaluation on all submissions to determine whether they believe the manuscript is within the scope of *JMD* and will also achieve a sufficient priority score to warrant publication. Priority is determined by the Editors' assessment of the manuscript relative to other papers being considered. The aim is that expeditious treatment will enable authors whose manuscripts do not meet these criteria to submit their manuscript elsewhere as soon as possible without unnecessary delays. For manuscripts accepted for external review, the Editor-in-Chief assigns an Associate Editor according to their expertise. The Associate Editor will solicit reviewers (typically, two external reviews are sought). Authors are encouraged to propose potential reviewers during the submission process. Authors may also request that specific reviewers not be used due to prior collaborations, known conflicts of interest, or direct competition. The Editors will make every effort to respect requests that are well-founded; however, the Editors do have the authority to utilize such a reviewer if it is necessary for expert peer review. It is the practice of the Journal to conduct a blinded peer-review process. The peer-review process is kept completely confidential; it is considered a violation of this confidentiality for authors to identify or attempt to communicate directly with peer reviewers or Associate Editors regarding their manuscript. All editorial communications should be directed through the Editorial Office at jmd@asip.org. The reviewer comments and Associate Editor's recommendation are evaluated by the Editor-in-Chief for disposition and transmittal to the authors. Every effort is made to complete the review process within 35 days of the date received.

Review Decisions. Only a portion of manuscripts will be accepted for publication. A number of worthy manuscripts will be rejected based on priority or for being out of scope. The Journal will advise authors whether the manuscript is accepted, eligible

for resubmission after minor or major revisions, or rejected. A manuscript may be returned to the authors without outside review if the Editors find it inappropriate for publication in this Journal. **Appeals** (rebuttals) to rejection decisions must be submitted within 60 days to be considered.

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Authors

Authorship. Authorship is defined as 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the manuscript or revising it critically for important intellectual content; 3) final approval of the version to be published; and 4) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Authors should meet all four conditions. When a group serves as a coauthor, the list of members (individuals or institutions) should be included in the Acknowledgements. When submitting a manuscript to the Journal, the corresponding author takes responsibility on behalf of all authors for the authorship, authenticity, and integrity of the research being reported. A current and functional email address needs to be provided for each author at submission, professional and institutional email addresses are strongly preferred. All coauthors will receive a notification email at the time of submission that requests their confirmation to be affiliated with the manuscript. Authors added at revision also need an email address provided in the system and will be sent the confirmation email request. Any change made to the list of authors (addition, removal, change in order) after manuscript acceptance requires consent of all authors and editorial approval.

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Manuscript Preparation. Manuscripts should be prepared in the style of the Journal and in accordance with The Uniform Requirements for Manuscripts Submitted to Biomedical Journals (see *http://www.icmje.org*). The required file format for text is Microsoft Word. Standard abbreviations can be found in the CSE Style Manual: *Scientific Style and Format* (8th ed., 2014). All pages of the manuscript should be double-spaced and numbered (including references, tables and figure legends); line numbers should also be included to assist reviewers in making comments. Other formatting specifications (eg, font size and type, margin settings, etc) are left to the authors' discretion, as papers accepted for publication will be reformatted according to the print specifications of the Journal. Manuscripts not prepared in accordance with the submission guidelines detailed below may be returned to the authors. Authors are encouraged to include a list of nonstandard abbreviations to aid reviewers; however, the Journal does not publish abbreviations or keywords as part of the final article. In addition, authors should be ready to comply with Editors' requests for copies of any similar works in preparation, copies of cited manuscripts that are submitted or in press, and/or supporting manuscript data (eg, data not shown, but summarized in the manuscript) that may aid the review process.

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Abstract. An abstract of 220 words or less should be prepared on a separate page and should be intelligible to the general reader without reference to the text. The abstract should clearly summarize the background, methodology, results, and significance of the study. Structured abstracts are not Journal style and will need to be revised to eliminate section headings. Abbreviations, external website links, and citations should be avoided.

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In Regular Articles and Technical Advances first-person personal pronouns (I, me, my, we, us, our) should be kept to a minimum. Replace active verbs with passive verbs, especially with regard to eliminating first-person personal pronouns. Use

present tense for figure legends since the data is static and is viewable now (except for describing what was done in an experiment) but past tense in the Results because this describes results that WERE obtained in the past. Headings in the Results section should be in present tense.

For **Materials and Methods,** authors should describe experimental and statistical methods in enough detail that other researchers can replicate results and evaluate claims. enough detail that other researchers can replicate results and evaluate claims. Authors are encouraged to review the NIH Principles and Guidelines for Reporting Preclinical Research (see *http://www.nih.gov/about/reporting-preclinical-research.htm*). Inclusion nclusion of method or reagent details as supplementary material is not acceptable. In addition, linking to protocols online is not permissible as there is no guarantee that the information will remain unchanged and accessible in perpetuity. The sequences of oligonucleotides, if not previously published, should be provided. Novel DNA or protein sequences should be deposited to an appropriate database (eg, Genbank, EMBL, SWISS-PROT), with the accession numbers included in the manuscript. When providing supplier information for materials sources, company name and location (city and state, or city and country) should be provided. When describing reagents such as antibodies, cell lines, animal strains, bacteria, and viruses, authors should include the source, characteristics, dilutions, strain, species, sex, authentication, etc. as necessary for repetition of the experiments. Website references to company or commercial information are not permitted. All novel materials and the procedures to prepare them should be described in sufficient detail to allow their reproduction (eg, DNA constructs, analytical software). Materials that are approved for investigational-use only should be clearly indicated.

Methods should state whether sample size was determined statistically prior to experimentation, whether samples were randomized (and how), whether data acquisition was blinded (particularly for subjective scoring methodologies), and what criteria were used to include/exclude data points or subjects. Experimental procedures should include the number of replicates performed and the number of samples in each experimental condition. A careful power analysis undertaken at the beginning of work can help assure the significance of study findings. "Discrepant analysis" is regarded as statistically biased and should not be employed for

assessment of assay sensitivity and specificity. Reports of assay sensitivity and specificity should include appropriately calculated confidence intervals. Special care should be taken to assure that statistical methods are appropriate, with clearly defined statements of the statistical test(s) used, sample size, and measures reported (eg, mean, median, SD, SEM, confidence intervals). Statistical methods may be assessed by a professional statistician as part of the peer review process.

Publication in the Journal implies that the authors agree, upon reasonable request, to share any materials or data that are integral to the results presented in the article, including whatever would be necessary for a skilled investigator to verify or replicate the claims. This may include original software code used in the data analysis. Agreement to share reagents or software code does not preclude the authors from implementing a Data Use Agreement. Authors must disclose upon submission any restrictions on the availability of materials or information, such as for patented or dual-purpose materials. Reporting guidelines for specific study designs (eg, randomized controlled trials) can be found online via the Enhancing the QUAlity and Transparency Of health Research (EQUATOR) network (see *http://www.equator-network.org/reporting-guidelines*). Authors must affirm that the research protocol was approved by the appropriate institutional review boards or ethics committees for human (including use of human cells or tissues) or animal experiments and that all human subjects provided appropriate informed consent and/or that regulations concerning the use of animals in research were adhered to. If race/ethnicity is reported, authors should state who determined race/ethnicity, how the options were defined, and why race/ethnicity was important in the study. Authors should be prepared to provide study protocol number(s) if requested.

Manuscripts that meet the criteria set forth by **STARD** (Standards for Reporting of Diagnostic Accuracy) will be annotated with a special footnote on the title page. Authors who wish their manuscripts to receive this designation should submit a completed checklist (available at *http://www.equator-network.org/reportingguidelines/stard*) at the time of submission for evaluation by the Editors at the time of acceptance. Further information about STARD can be found in Bossuyt PM et al, STARD 2015: an updated list of essential items for reporting diagnostics accuracystudies. BMJ 2015; 351: h5527.

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***Figure Legends*.** Figure legends should be submitted in a list as part of the manuscript (separate from the figure files) and should adequately describe all descriptors: arrows, arrowheads, scale bars, insets, asterisks, boxes/circles/etc. in line graphs, and any other notations. Histology images should have all staining methods described, and either the magnification or the measure of the scale bar in the image should also be included. Descriptive text, rather than graphics inserted into the text, should be used when possible (eg, closed circles, open boxes, etc.); alternatively, a legend key should be included in the figure. Statistical significance (eg, *P* values) should be clearly defined by asterisks (*, **, ***) or by other sequential symbols: *, †, ‡, §, ¶, ||; these may be doubled up if needed. *P* values should be labeled by symbol in the image, with definitions appearing only in the legend (eg, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Labeling of data as not significant (NS) is unnecessary. All abbreviations should also be defined.

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Appendix E. Exchange rates indicators for South Africa

Exchange Rates selected indicators

South Africa

Source: International Financial Statistics (IFS)

Metadata by Country (IFS)

Concept	Unit	Code	Scale	2017	2018	2019	2020	2021
National Currency per SDR, End of Period	Rate	ENSE_XDC_XDR_RATE	Units	17.54	19.99	19.39	21.15	22.26
National Currency per SDR, Period Average	Rate	ENSA_XDC_XDR_RATE	Units	18.47	18.74	19.96	22.93	21.05
Domestic Currency per U.S. Dollar, End of Period	Rate	ENDE_XDC_USD_RATE	Units	12.32	14.38	14.03	14.69	15.91
Domestic Currency per U.S. Dollar, Period Average	Rate	ENDA_XDC_USD_RATE	Units	13.32	13.23	14.45	16.46	14.78
Nominal Effective Exchange Rate, Trade Partners by Consumer Price Index	Index	ENEER_IX	Units	65.27	65.31	62.08	55.67	60.03
Real Effective Exchange Rate, based on Consumer Price Index	Index	EREER_IX	Units	79.65	80.83	78.27	71.02	77.62