Single Nucleotide Polymorphism Array Analysis in Copy Number Variant Detection: Assessment of its Feasibility in the Diagnostic Setting

by

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Abbreviations

AAIDD	American Association on Intellectual and Developmental Disabilities
ACC	Agenesis of the corpus callosum
ACC	Association of Clinical Cytogenetics Guidelines
ACMG	American College of Medical Genetics and Genomics
ADHD	Attention Deficit Hyperactivity Disorder
AGCC	Affymetrix GeneChip® Command Console
AOH	Absence of heterozygosity *also* loss of heterozygosity (LOH) *or* regions of homozygosity (ROH)
aCGH	Array Comparative genomic hybridization also CGH
ASD	Autism Spectrum Disorder
ASD/VSD	Atrial septal defect/Ventricular septal defect
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosomes
Bp	Basepairs
C	Celsius
CA	California
CFSMR	Craniofacial dysmorphism, skeletal anomalies, and mental retardation syndrome
CGH	Comparative genomic hybridization
ChAS	Affymetrix Chromosome Analysis Suite
CHOMS	Craniofacial features, Hypotonia, Childhood Obesity, Microcephaly and Substantial Speech Delay and Mental Retardation
CMA	Cytogenomic (previously chromosomal) microarray analysis
CN	Copy number
CNC	Copy number change
CNS	Central nervous system
CNV	Copy number variant *also* Copy number change (CNC)
CT	Computed Tomography
dbVar	Database of Genomic Structural Variation
DD	Developmental delay
DECIPHER	DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources
DGV	Database of Genomic Variants
DIS	Deafness Infertility Syndrome
DNA	Deoxyribonucleic acid
DS	Down syndrome
EEG	Electroencephalogram

EBR	Evidence-based Review
ECARUCA	European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations
EQA	External quality control
FASD	Fetal alcohol spectrum disorders
FISH	Fluorescence *in situ* hybridization
FRAXA	Fragile X syndrome
G-banded	Giemsa banded
GCOS	GeneChip® Operating Software
gDNA	Genomic DNA
GDD	Global developmental delay
GECCO	Genomic Classification of CNVs Objectively
GRCh	Genome Reference Consortium human assembly
HD	High density
Hg	Human genome
HIV	Human Immunodeficiency Virus
HREC	Human Research Ethics Committee
ICD-10	International Statistical Classification of Diseases and Related Health Problems 10th Revision (2010)
ICSA	International Standards for Cytogenomic Arrays
ID	Intellectual disability also Mental Retardation (MR)
IQ	Intelligence quotient
ISCN	The International System for Human Cytogenetic Nomenclature
IUFD	Intra-uterine Fetal Death
IUGR	Intra-uterine growth restriction
LCSH	Long contiguous stretches of homozygosity
Kb	Kilobase
Kbp	Kilobasepair
LCR	Low-copy repeats
LDGA	Laboratory of Diagnostic Genomic Analysis
LOH	Loss of heterozygosity *also* see ROH and AOH
MAPD	Median Absolute Pairwise Difference
Mb	Megabase
MCC	Maternal cell contamination
MCA	Multiple congenital abnormality
MLPA	Multiplex Ligation-Dependent Probe Amplification
mM	Millimole
MN	Minnesota

MR	Mental Retardation
MRI	Magnetic resonance imaging
MWS	Mowat-Wilson syndrome
NAHR	Non-allelic homologous recombination
NCBI	National Centre of Biotechnology Information
ng	Nanograms
NHLS	National Health Laboratory Services
OGT	Oxford Gene Technology
OMIM	Online Mendelian Inheritance in Man
PCR	Polymerase chain reaction
PH	Periventricular heterotopia
PKU	Phenylketonuria
QC	Quality control
QF-PCR	Quantitative Fluorescence-Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
ROH	Regions of homozygosity
rpm	Revolutions per minute
RS	Rett syndrome
RSA	Region-Specific Assay
SA	South Africa
SD	Standard Deviation
SKY	Spectral karyotyping
SNP	Single-Nucleotide Polymorphism
SNPQC	SNP Quality Control
STR	Short tandem repeats
S-XLID	Syndromic X-Linked Intellectual Disability
TAT	Turnaround time
TB	Tuberculosis
TOP	Termination of pregnancy
UCSC	University of California, Santa Cruz
UK	United Kingdom
UKNEQAS	United Kingdom National External Quality Assessment
ul	Microlitre
UPD	Uniparental Disomy
USA	United State of America
VACTERL	Vertebral anomalies, Anal atresia, Cardiac defects, Tracheoesophageal fistula and/or Oesophageal atresia, Renal and Radial anomalies and Limb defects

VOUS	Variants of Unknown Significance
VP	Variegate Porphyria
VUR	*Vesicoureteral reflux*
WHO	World Health Organization
WHS	Wolf-Hirschhorn syndrome
WHSCR	WHS critical region
WHSCR2	WHS critical region (2)
WI	Wisconsin
WS2	Waardenburg syndrome Type 2
XLID	X-Linked Intellectual Disability *also* X-Linked Mental Retardation (XLMR)

Abstract

Intellectual disability/developmental delay (ID/DD) is a significant problem in child health affecting 2 to 3% of the population worldwide. While the underlying aetiology of ID/DD in a large proportion (about 50%) of these patients is unknown, 15 to 20% of the internationally reported cases detected using microarray technologies are due to copy number variants (CNVs), whereas only 3 to 5% of ID/DD can be identified with conventional cytogenetics.

The Affymetrix® Cytoscan™ High Density (HD) Array (Affymetrix, Santa Clara, CA) containing over 2.4 million markers for copy number (CN) was used to detect genome-wide high resolution CN and single nucleotide polymorphisms (SNPs) in a cohort of 27 carefully selected patient samples. The patient selection was done based on relevant phenotypes, which included dysmorphism, ID/DD, suspected syndromes, and family history. Data analysis was performed using the Affymetrix Chromosome Analysis Suite (ChAS) (Affymetrix, Santa Clara, CA, USA software).

Seven of the patients demonstrated pathogenic CNVs. Diagnoses included Kleefstra syndrome, Mowat-Wilson syndrome, Wolf-Hirschhorn syndrome, tetrasomy 9p, and a susceptibility locus for neurodevelopmental disorders due to a deletion of chromosome 1q21.1. This indicated a 26% detection rate in this cohort. In addition, three variants of unknown significance (VOUS) were detected.

The aim of this study was to determine the potential relevance and applicability of microarray technologies for the detection of CNVs in the Western Cape ID/DD population of South Africa (SA) and in so doing, to introduce and develop molecular cytogenetics skills in the routine diagnostic cytogenetic environment. The results obtained in this study confirmed the significant improvement in the detection rate of CNVs in patients with ID/DD and thus the diagnostic utility of this technology for the detection of CNVs in ID/DD patients was confirmed.

Plan of Dissertation

This dissertation consists of five chapters. **Chapter One** presents the background of intellectual disability and cytogenomic microarrays. This chapter also outlines the aim and the study approach. **Chapter Two** includes the patient selection, molecular methodology, and description of the analysis of the data. **Chapter Three** is a detailed presentation of the findings of this study. In **Chapter Four**, the study and the resulting findings are discussed within the SA context. **Chapter Five** concludes the study with specific recommendations for the introduction of this technology into the diagnostic setting in SA. Source referencing according to the Harvard system was used throughout this dissertation. Although an attempt has been made to use terms ID and/or DD interchangeably, there are instances where each distinguishable phenotype may be referred to specifically. The terms „array comparative genomic hybridization (aCGH)" and „cytogenomic microarray analysis (CMA)" have been used interchangeably in keeping with the applicable referenced information source.

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

Cytogenetics is a component of genetics and involves the study of the structure and function of cells, specifically the chromosomes. Cytogenetic analysis is mainly concerned with the detection of gains and losses of chromosomal material, as well as structural abberations such as translocations. Conventional cytogenetics i.e. karyotyping has traditionally been the gold standard cytogenetic methodology but gradual introduction of molecular techniques for the detection of genomic copy number variations (CNVs) has created a new subdiscipline of cytogenetics i.e. molecular cytogenetics.

Standard cytogenetic techniques, such as Giemsa banding (G-banding) (Figure 1), can identify large aberrations including deletions, duplications, amplifications and unbalanced translocations, but limited resolution makes these tools unreliable for the detection of CNVs less than 5 Megabases (Mb) in size. Conventional cytogenetic methods are capable of detecting chromosome abnormalities in 10 to 15% of ID/DD patients depending on selection of the patient cohort (Ropers, 2008), though Shevell et al. (2003) reported a detection rate of only 3.7% a few years earlier. Nonetheless, a large number of patients with ID show an apparently „normal" Karyogram.

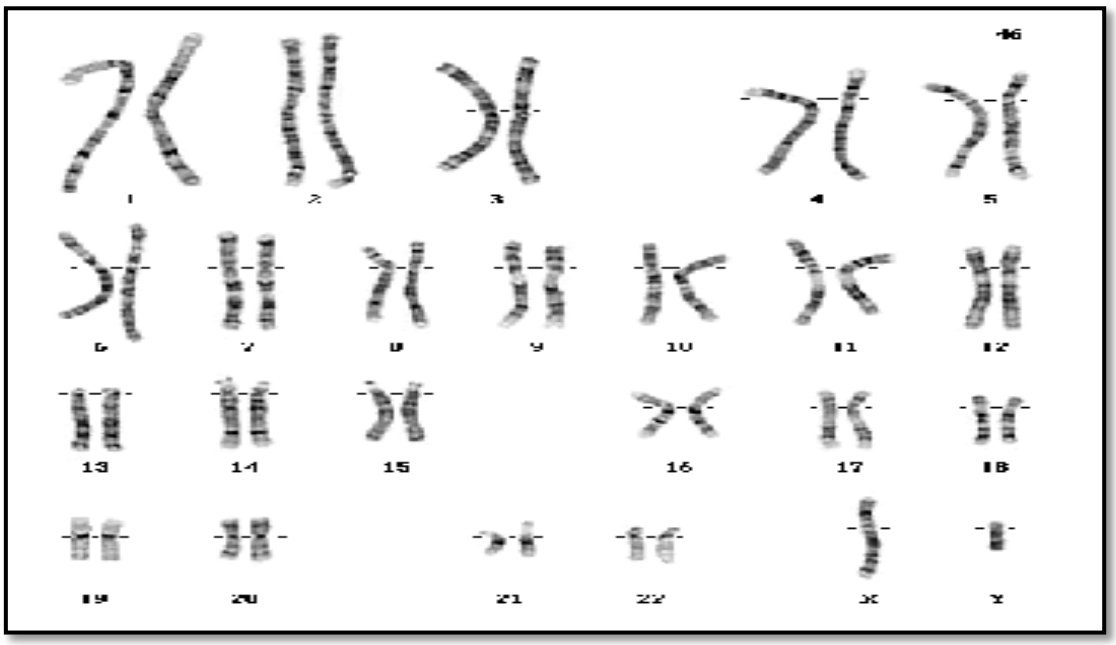


Figure 1: Depiction of a Karyogram ('normal' male - 46,XY) http://carolguze.com/text/102-14-humangenetics2.shtml

Despite the low detection rate however, chromosome analysis is still important for the detection of mosaicism and chromosomal structural rearrangements (Bi et al., 2013). Inherited balanced translocations are seen in 1 in 500 ID patients and *de novo* balanced translocations are seen in 1 in 12 000 ID patients (Jacobs et al., 1992). Chromosome analysis has further limitations: cell culture is required, an extended turnaround time (TAT), and only large abnormalities can be detected depending on the chromosome morphology. Other limitations include the labour-intensive nature of chromosome analysis, which increases staffing requirements, and work space area. Chromosome analysis is subjective and even large abnormalities can be missed, depending on the location of the abnormality and the skill of the cytogeneticist (Miller et al., 2010).

The sub-discipline of molecular cytogenetics target the actual DNA molecule to detect CNVs. These techniques include the Fluorescence *in situ* Hybridization (FISH), Multiplex Ligation-Dependent Probe Amplification (MLPA), and microarray technologies. While FISH has the advantage of a higher resolution, it is however best suited for the confirmation of known microdeletion and microduplication syndromes in patients presenting with a suggestive phenotype due to the limited number of chromosomal loci that can be analysed simultaneously (Bar-Shira et al., 2006).

Submicroscopic subtelomeric rearrangements account for approximately 5% of unexplained ID and/or Multiple congenital abnormalities (MCA) (Bar-Shira et al., 2006). These can be detected using the MLPA analysis which targets multiple sites in one test (Ahn et al., 2007). MLPA is a quick, robust and recognized method for the detection of microdeletions and microduplications (Hills et al., 2010) including in the gene-rich subtelomeric regions prone to rearrangements (Ahn et al., 2007). Cho et al. (2009) reported that MLPA can accurately assess the size of a deletion and can be used as confirmatory tests for abnormalities detected on CMA. MLPA findings can be further elucidated with the help of the available CNV databases. Detection rates vary across studies with Ahn et al. (2007) reporting a range between 0% (idiopathic ID without dysmorphism) and 10.7% (severe idiopathic ID). Limitations of the MLPA include the possibility of misinterpretation of polymorphisms which could be present in the probe annealing site as a deletion (Cho et al., 2008). The sensitivity and the incidence of false-negative results are unknown. In addition, MLPA techniques are targeted and cannot detect genome-wide alterations. The MLPA does however bridge the gap between microdeletion/subtelomeric FISH and CMA. Another molecular cytogenetic technique is Quantitative Fluorescence-Polymerase Chain Reaction (QF-PCR) which is used for the rapid determination of the common chromosomal aneuploidies. Short tandem repeats (STR) are highly polymorphic DNA regions, which are targeted using fluorescently labelled

primers, and are analysed quantitatively following capillary electrophoresis to determine the copy number of the STR markers (Mansfield, 1993; Schmidt et al., 2000). This technique has largely replaced aneuploidy FISH as it is less labour-intensive and targets more loci than FISH.

The advent of array technologies has improved the detection of submicroscopic gains and losses of the genome with Miller et al. (2010) reporting a CNV detection rate of between 15 and 20%. CNVs consist of deletions, insertions and duplications, which forms part of the genetic variation in the human genome and are implicated in ID, global developmental delay (GDD), Autism Spectrum Disorder (ASD) and MCA (Cho et al., 2008; Riggs et al., 2014).

There are several reasons for the need for a genetic diagnosis in patients with ID, GDD, ASD and MCA. These include accurate diagnostic and prognostic information to ensure appropriate genetic counselling, clinical care and educational needs, the introduction of preventative and therapeutic regimes, and the elimination of unnecessary procedures. A diagnosis may provide answers to parents‟ questions, and elucidating the genetic risk for the family may be of importance for future reproductive choices. This is important in the case of recurrence risk for a parent carrying a chromosomal rearrangement, where a positive molecular or cytogenetic result could allow for the option of prenatal or preimplantation testing (Battaglia et al., 2013; Moeschler & Shevell, 2014).

Microarray requires pre-test and post-test counselling (Darilek et al., 2008). Prenatal microarray would need extensive pre- and post-test counselling. Counselling will be supplemented with a written informed consent document, which would include a summary of the test procedure, the expected benefits, limitations and possible outcomes, and would require a signature from the patient. Pretest counselling would require discussion of the objective of the test (more detailed analysis of chromosomes than karyotyping), testing methodology (targeted vs whole genome screen and the resolution), the type of sample required and the expected results. The counsellor therefore has to prepare the patient thoroughly with regards to the range of possible findings and the resulting implications, and obtain informed consent. The possibility of testing parental samples to elucidate the microarray findings also needs to be discussed. Results of known clinical significance may provide information for recurrence risks for couples and for the extended family. A positive result can be counselled as with other types of genetic testing. Novel findings may require further testing. A negative result will be obtained in the majority of patients and may require additional testing. Counselling issues may also arise when a presymptomatic disorder is found due to the size of the genome interrogated, and patients should be informed of this

prior to testing. A VOUS, which is not present in the parents, would require a detailed discussion as this may be clinically significant to the phenotype.

1.2 ID/DD

ID/DD comprises of a „heterogeneous group of disorders" (van Bokhoven, 2011) and includes below average intellectual function and the lack of the necessary skills to perform the daily tasks of living (Rauch et al., 2006). This may involve disabilities in motor function, cognitive ability and language skills as well as a combination of these. ID/DD may be detected in infancy or early childhood but is more likely to manifest during the school years and is usually diagnosed before the age of 18 years. A formal diagnosis of ID requires more maturity in the patient to assess the intelligence quotient (IQ) accurately.

IQ continues to be used in certain classifications of ID but this focus has changed in the revision of The American Association on Intellectual and Developmental Disabilities (AAIDD) to include everyday skills (Leonard & Wen, 2002; Shevell, 2008). It should be noted that there are discrepancies in the cut-off IQ points used in different studies to define ID. The term „Intellectual Disability" (ID) has largely replaced the term „Mental Retardation" (MR) as the latter is perceived to have a negative association. Despite this change, the World Health Organization (WHO) still uses the term „mental retardation" in its „International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10)" classification system (2016) as shown in Table 1 (http://apps.who.int/classifications/icd10/browse/2016/en#).

Table 1 WHO ICD-10 Classification of Mental Retardation (2016 version)

Degree of ID	Intelligence Quotient range
Mild	50 – 69
Moderate	35 – 49
Severe	20 – 34
Profound	<20

The term DD is typically used in patients before the age of five years whereby development is compared between peers with regards to learning skills and adaptation, whereas global developmental delay (GDD) is characterized by a significant delay in two or more of the following areas: cognition, speech/language, gross/fine motor skills, social/personal skills and daily living (Shaffer, 2005; Moeschler & Shevell, 2014). Several conditions may cause DD but with no effect on intelligence.

Syndromic ID has been definitively linked to more than 400 genes, whereas approximately 50 genes have been implicated in nonsyndromic ID (van Bokhoven, 2011). van Bokhoven (2011) estimated that 90 genes could be implicated in X-linked ID (XLID). XLID can also be categorised as syndromic and non-syndromic (Ropers & Hamel, 2005), with syndromic XLID (S-XLID) patients having additional features or abnormalities. Examples of S-XLID include: (i) Fragile-X (FRAXA) syndrome which is the most common S-XLID in male patients (Chelly et al., 2006) and is due to a trinucleotide expansion in the *FMR1* gene, and (ii) Rett syndrome (RS) which is due to mutations in the *MECP2* gene or deletions/duplications of segments of chromosome Xp28 and which is the second most common cause of severe ID in females after Down syndrome (DS) (Weaving et al., 2005).

Long contiguous stretches of homozygosity (LCSH) are also implicated in ID and consist of regions of allelic homozygosity (Iourov et al., 2015). These regions are also known as regions of homozygosity (ROH) or loss of heterozygosity (LOH). Although healthy individuals can demonstrate ROH, it may be suggestive of consanguinity, Uniparental Disomy (UPD) and homozygosity in single gene recessive conditions (Gijsbers et al., 2009). Iourov et al. (2015) proved that ROH is a frequent occurrence in patients with ID, autism, congenital malformations and/or epilepsy. McQuillan et al. (2008) found that these regions were usually less than 4 Mb in size in their study of a European population, although ROHs of over 10 Mb have been reported in rare cases (Gibson et al., 2006). Grote et al. (2012) noted the variability in reporting of ROH, which was subsequently highlighted by Wang et al. (2015) stating that no consensus had been reached on a set threshold for ROH reporting by the time of their writing. Also, it is possible that ROH may make a larger contribution to ID in populations other than Europeans as reported by McQuillan et al. (2008).

ID/DD are conditions which not only affect the patient, but also their family members and care givers. The prevalence rate of DD in the general population is between 1 and 3% (Shaffer, 2005; Moeschler & Shevell, 2014). In a study on patients seen in general practice

in England, Allgar et al. (2008) reported a prevalence of ID/DD between 0.83 and 1.14%. In 1997, the WHO reported that industrialised countries have an ID prevalence rate of 3 to 4%.

Robertson et al. (2009) have noted that industrialised countries ensure detection and management of ID/DD, from an early age, by using surveillance programs. This identifies children at high-risk and the relevant support and/or treatment can be offered and therefore resources can be optimized. This is more problematic in developing countries as the resources for a screening programme may not be available and support and treatment may not be accessible to these patients. Calculating the ID prevalence rate is more complicated as reporting is not representative of all ID patients and therefore not accurate (Roeleveld et al., 1997). Furthermore, the tools used for the identification of ID in patients in industrialised countries are not always appropriate for application in the developing countries.

1.2.1 Clinical diagnosis and management

Coulter et al. (2011) reported that 55% of positive CMA cases in their retrospective study of 1 792 patients were clinically actionable. In a survey conducted by Riggs et al. (2014), in collaboration with various diagnostic laboratories which included 28 526 patients, showed hat at least 7% of cases are clinically actionable which is still higher than the detection rate that at least 7% of cases are clinically actionable. This is still higher than the detection rate of less than 1% of FRAXA in ASD patients and approximately 3.5% of FRAXA in DD/ID patients. In the study by Riggs et al. (2014), where they considered CNVs detected by a lower resolution array (ISCA Consortium 180K), 49 phenotypes were excluded as these would be unlikely to be diagnosed using CMA; only diagnoses linked to established clinical management were considered, which may account for the low detection rate. Ellison et al. (2013) calculated that 35% of abnormalities found in a study of 46 298 patients could be acted upon clinically. Henderson et al. (2014) performed CMA on 1 780 patients with neurodevelopmental disorders (DD, learning disabilities, behavioural/psychiatric disorders, seizures, ID, ASD), congenital anomalies (single or multiple), dysmorphic features, abnormal growth (failure to thrive, short stature) and hypotonia and detected positive CMA findings in 187 patients. A total of 54.5% (102/187) of these patients received medical recommendations following CMA analysis thereby verifying the clinical utility of CMA technologies.

1.3 GENETIC AETIOLOGY

The aetiology of ID/DD is complex as multiple factors such as genetic abnormalities including Mendelian disorders and chromosomal abnormalities, biochemical abnormalities and environmental factors can contribute to this phenotype (Ellison et al., 2013). Each factor may occur singly or in combination with one or more of the others. Of the genetic abnormalities, chromosomal abnormalities are the most common cause of ID, GDD, ASD and MCA (Cho et al., 2008; Riggs et al., 2014) with DS being one of the most prevalent causes with an incidence of 1 in 732 live-born infants in the United States (Sherman et al., 2007). van Bokhoven (2011) noticed that chromosomal abnormalities (structural and numerical), genomic disorders and monogenic diseases, cause up to 65% of severe ID cases.

Common genetic causes of and contributors to ID include DS, known microdeletion syndromes (for example Prader-Willi syndrome, Williams syndrome, and Angelman syndrome), and single gene mutations such as RS and Tuberous Sclerosis (Leonard & Wen, 2002). Comorbidity with ID and ASD, Attention Deficit Hyperactivity Disorder (ADHD), schizophrenia, depression and behavioural problems is common. It is reported that up to 40% of ID patients have ASD and between 50 and 80% of patients with autism/ASD have ID (van Bokhoven, 2011). Epidemiological surveys conducted in several countries found that approximately 70% of patients with autism have ID (Fombonne, 2003). van Bokhoven (2011) also stated that this adds to the evidence that ID, autism and a range of other cognitive disorders have a similar or shared molecular genetic aetiology. Betancur (2011) reported that genes and loci implicated in patients with epilepsy have also been identified in patients with ASD, indicating a shared genetic component between these two groups of neurological disorders. Several microdeletion syndromes have a combination of neurobehavioural features for example ASD, ADHD, and epilepsy, which are associated with ID (Mefford et al., 2012). The prevalence of ID due to CNVs has been reported as between 5 and 15% by Fan et al. (2007). Cooper et al. (2011) reported CNVs to be the causative factor of ID in 13.7% of patients.

CNVs can cause disease by revealing dosage-sensitive genes, disrupting genes or alter gene dosage, gene fusion or position effects, thereby impacting gene expression and phenotypic variation (Cooper et al., 2011; Zhang et al., 2009). This can lead to disorders such as microdeletion/duplication syndromes or they may contribute to risk in complex

disease. CNVs can also, through positional effects, affect gene expression or provide evolutionary chromosomal change (Redon et al., 2006). Such CNVs could cause a disruption of a coding sequence or a sequence change in a promoter region which would affect transcription and translation (Gijsbers et al., 2010). Ellison et al. (2013) stated that CNVs may be flanked by duplications which cause unequal crossing over resulting in increased mutation rates in genetic conditions which modify several varied cellular functions such as transcriptional and translational control, chromatin remodelling, protein modification, differentiation of neural and supporting cells of the nervous system and centrosome function. Understanding the different physiological pathways and interacting proteins can assist in the identification of additional contributing, potentially pathogenic, genes (Ellison et al., 2013). van Bokhoven (2011) hypothesized that between 1,500 and 2,000 genes could be implicated in ID.

A number of mechanisms have been proposed for the generation of CNVs. Homologous and non-homologous recombination mechanisms being most common. Non-allelic homologous recombination (NAHR) has a DNA repair function in dividing cells (meiosis). During NAHR a 200-300 bp segment and a RAD51 protein is required. The 3` end of ssDNA is catalysed to a duplex sequence on the sister chromatid of the homolog. The repair mechanism may however cause structural rearrangements due to low-copy repeats (LCR) (Hastings et al, 2009). LCRs are large blocks of duplicated sequence of 100 Kilobases (Kb) or more, and have been implicated in CNV regions. These repeat sequences cause instability, as in the 22q11.21 region involved with Di George syndrome (Shaikh et al., 2000). The authors postulated that recombination between LCRs cause deletions, duplications and inversions. Deletions and duplications are caused by two LCRs which are positioned in direct genomic orientation allowing for interchromatid and interchromosome exchanges. Deletions are due to interchromatid NAHR, and inversions are due to the LCRs on the same chromosome but in opposite orientation. In the same way, translocations are due to LCRs on different chromosomes (Lupski, 1998). Shaffer et al. (2007) reported 73% of the microdeletions and 82% of the microduplications in their cohort of 8 789 patients, with ID, DD, seizures and other congenital abnormalities, were caused by LCRs.

1.3.1 CNVs

A CNV is defined as „a Deoxyribonucleic acid (DNA) segment that is 1 kilobase (Kb) or larger and present in a variable copy number in comparison with a reference genome"

(Redon et al., 2006). The authors further explained that a CNV can be simple in structure, such as a tandem duplication or may involve complex gains or losses of homologous sequences at multiple sites in the genome. CNVs are common in apparently healthy individuals, which complicates interpretation of these findings, and adds to the challenge of foreseeing the clinical outcome in unique disease-causing CNVs (Koolen et al., 2009). It has been estimated that CNVs cover approximately 12% of the human genome (Redon et al., 2006). Manning & Hudgins (2010) stated that 800 or more benign CNVs could be detected in a healthy person, indicating that not all CNVs are pathogenic. Pathogenic CNVs are not only found in patients with ID/DD, ASD and MCA but also in other commonly seen conditions such as epilepsy and other neuropsychiatric disorders (Coulter et al., 2011). Frequency of detection of a CNV does not necessarily indicate whether it is pathogenic or benign (Manning & Hudgins, 2010). There is still limited knowledge concerning the pathogenicity of many CNVs with many more still continuously being discovered (Riggs et al., 2014). The reason for the variability in the phenotype of patients carrying known pathogenic CNVs is unknown but may be due to modifying alleles in the individual"s genetic background, somatic mutations, epigenetic events and environmental exposures (Ellison et al., 2013). The implication of the effects of a CNV throughout a patient"s lifetime is also not yet fully understood (Ellison et al., 2013). Many CNVs can lead to susceptibility and predisposition to various diseases e.g. HIV, lupus etc. (Canales & Walz, 2011). This is also true for a number of neurodevelopmental disorders such as ID, ASD, schizophrenia, whereby not all carrier individuals within an affected famly will present with the phenotype. Ermakova et al. (2011) proposed a “second hit” hypothesis, which can be genetic or environmental, by using a mouse model. Sahoo et al. (2011) postulated CNVs could be influenced by environmental factors and other loci which may have modifying effects. The available control population data refers to the European population, no reference is currently available for the South African population.

Despite this uncertainty, accurate diagnoses are generate which ensures appropriate genetic counselling, clinical care and educational needs, the introduction of preventative and therapeutic regimes, and the elimination of unnecessary investigative procedures. A diagnosis may provide answers to parents" questions, and elucidating the genetic risk for the family may be of importance for future reproductive choices. This is important in the case of recurrence risk for a parent carrying a chromosomal rearrangement, where a positive molecular or cytogenetic result could allow for the option of prenatal or preimplantation testing (Battaglia et al., 2013; Moeschler & Shevell, 2014).

The most common group of polymorphisms in the human genome is SNPs which are DNA sequence variations of single nucleotides. A SNP consists of a single base change, generally defined as occurring in a population at a frequency of >1%. At a frequency of <1%, it may likely be considered a mutation (especially if it is associated with a phenotype) (Human Genome Project, 1990 - 2003).

1.4 CNV DETECTION

CMA has been rapidly translated into the diagnostic setting due to the high sensitivity, specificity and reliability of the detection of CNVs in patients with DD/ID, MCA and ASD (Miller et al., 2010). CMA has also been proven to demonstrate considerable advances over conventional cytogenetics, G-banded chromosome analysis and FISH studies, by interrogating the entire genome at once. This is done by using specific probes to cover the whole genome (Shen et al., 2011). Bi et al. (2013) confirmed that nearly all the abnormalities observed on chromosome analysis in their study were also found using CMA studies and therefore supported the consensus statement by Miller et al. (2010) recommending CMA as a first tier test. Historically, chromosome analysis was the first-tier cytogenetic test for patients with ID. Since 2010, with the release of the consensus statement by Miller et al. (2010) CMA has become the first-tier test in many countries.

Bruno et al. (2012) reported that CNVs of 50 Kb in size ought to be reported, although software settings may be lowered for example on Affymetrix ChAS (Affymetrix, Santa Clara, CA, USA software). As an example of the speed of development and the subsequent introduction of this technology into routine diagnostic practice, in 2006, Moeschler & Shevell reported on aCGH as an „emerging technology", at that stage identifying subtelomeric FISH studies as being the most effective diagnostic tool for ID (Moeschler & Shevell, 2006). Two years later, Moeschler conceded to the effectiveness of array technologies with the increased detection of chromosomal abnormalities as multiple loci are interrogated in one reaction (Moeschler, 2008). The diagnostic capability of clinical cytogenetic laboratories has been significantly improved through the application of these technologies. The benefits of using CMA for CN detec-tion include: use of DNA, detection of cryptic abnormalities which would have been missed using routine chromosome analysis, customization of probes for targeting specific regions of interest, higher resolution providing a better quality result, objective data interpretation, ROH detection and the ability for data to be interfaced with

public databases. Manning & Hudgins (2010) suggested that 20% of apparently balanced translocations have a loss or gain of material which can be identified using CMA. Further advantages of using CMA in apparently balanced chromosomal rearrangements are in elucidating breakpoints, which could be disrupting genes. The detection of CNVs near the breakpoints, the detection of complex chromosomal rearrangements which cannot be detected on chromosome analysis and identification of other submicroscopic CNVs that are the cause of the phenotype, are all positive aspects of this technology (Gijsbers et al., 2010). CMA can be employed in the routine diagnostic environment as there appear to be few technical limitations (Koolen et al., 2008). Interpretation of CNVs can, however, be challenging.

Shaffer et al. (2007) tested 8 789 patients with ID, DD, seizures and other congenital abnormalities which did not reveal abnormalities with extant technologies (chromosome analysis, FISH - subtelomeric and locus specific, and/or FRAXA or other single-gene disorders). Using aCGH, abnormalities were detected in approximately 12% (1 049/8 789) of the cases: almost 7% showed CNVs with clear clinical significance, 1.2% had a benign CNV and 3.9% had VOUS. Of the VOUS, 2.5% (218) of the CNVs were detected in recurrent regions, and 1.4% (124) were single incidents. The authors postulated that the majority of the VOUS are most likely to be polymorphisms.

The diagnostic capability of clinical cytogenetic laboratories has been significantly improved through the application of these technologies. Consequently, this has resulted in the escalated discovery of novel genetic syndromes. The application of this technology for patients presenting with an ID/DD and/or MCA phenotype, has already become routine in many developed countries in patients presenting with an ID/DD and/or MCA phenotype and it has been shown that the use of high resolution microarray has increased the average diagnostic yield of genomic aberrations to between 15 and 20% (Miller et al., 2010). These authors therefore recommended that microarray analysis be performed instead of routine chromosome analysis in patients with ID/DD, ASD or MCA as a diagnostically significant yield as high as 20% (Ming et al., 2006) is possible whereas routine chromosome analysis has a diagnostic yield of approximately 3.7% (Shevell et al., 2003) and FISH has a diagnostic yield of approximately 2.6% (Ravnan et al., 2006).

Edelmann & Hirschhorn (2009) stated that „Genome-wide studies of large cohorts of patients with ID/MCA have led to the identification of chromosomal aberrations that delineate novel syndromes, some of which occur at frequencies comparable to known recurrent rearrangement disorders." The diagnosis of submicroscopic CNVs in ID/DD patients has thus been transformed with the introduction of genome-wide techniques (Mefford et al., 2012). The prevalence of ID due to CNVs has been reported as between 5 and 15% by Fan et al. (2007). Aston et al. (2008) reported an even higher detection rate, finding CNVs using SNP array analysis in 24% of ID patients. Therefore, introducing microarray analysis as a routine diagnostic test in ID/DD has launched new possibilities for identifying the cause of this „condition". With the rapid development in high-throughput technologies, it is now possible to make a genetic diagnosis in up to 65% of patients with moderate to severe ID (van Bokhoven, 2011).

1.4.1 Types of arrays

Genome-wide arrays allow for the detection of CN changes of approximately 100 Kb in size (Friedman et al., 2006). These technologies have developed rapidly with a substantial improvement in resolution which is dependent on the size of the probes and the density of the probes covering the genome (Moeschler, 2008). It is important that a microarray chip design has adequate coverage to detect different breakpoints for well documented microdeletion and duplication syndromes as atypical deletion sizes have been reported in Angelman/Prader-Willi, Williams and Smith-Magenis syndromes (Shaffer et al., 2007). To this end different array designs are available: targeted arrays which have loci of known clinical significance, whole-genome arrays which cover the whole genome and targeted arrays with probes in loci associated with chromosomal instability/rearrangements, for example pericentromeric regions (Slavotinek, 2008; Miller et al., 2010).

Two types of arrays have been developed: aCGH and SNP arrays. aCGH uses test DNA and control DNA which is differentially labelled, whereas SNP arrays use patient DNA which is directly hybridized to the array (Figure 2) and compared to a pool of normal individuals using specialised computational software. Both methods compare patient DNA to reference DNA whereby CN anomalies can be detected (Schaffer et al., 2013). CGH arrays produce better data quality with less variation and better signal-to-noise ratios when compared to SNP arrays (Shen et al., 2011). CGH platforms usually have longer probe lengths. SNP probes only bind to an exact copy of complementary DNA, and in so doing are also able to

detect UPD and LOH. SNP array analysis is considered to be the most advanced method in the assessment of genomic imbalances associated with genetic disease (Bruno et al., 2012). Array platforms which combine CN and SNP (CN+SNP) array analysis are considered to be the most advanced technologies in the assessment of genomic imbalances associated with genetic disease as it is possible to assess both CNV and copy-neutral aberrations simultaneously in one reaction (Mason-Suares et al., 2013). With the introduction of CMA into the clinical setting, arrays have been designed to be more specific for this application. Shen et al. (2011) argued that the advantages of this were: reaching a balance between sensitivity and specificity, achieving a uniform hybridization for a larger number of probes, reducing the variation in signal-to-noise ratio as experienced in higher-density arrays, testing multiple patients on a single slide when utilizing well-designed clinical arrays, and eliminating CNV fragmentation due to increased probe density. Adjacent CNVs can be merged which are then efficiently detected by lower-resolution arrays.

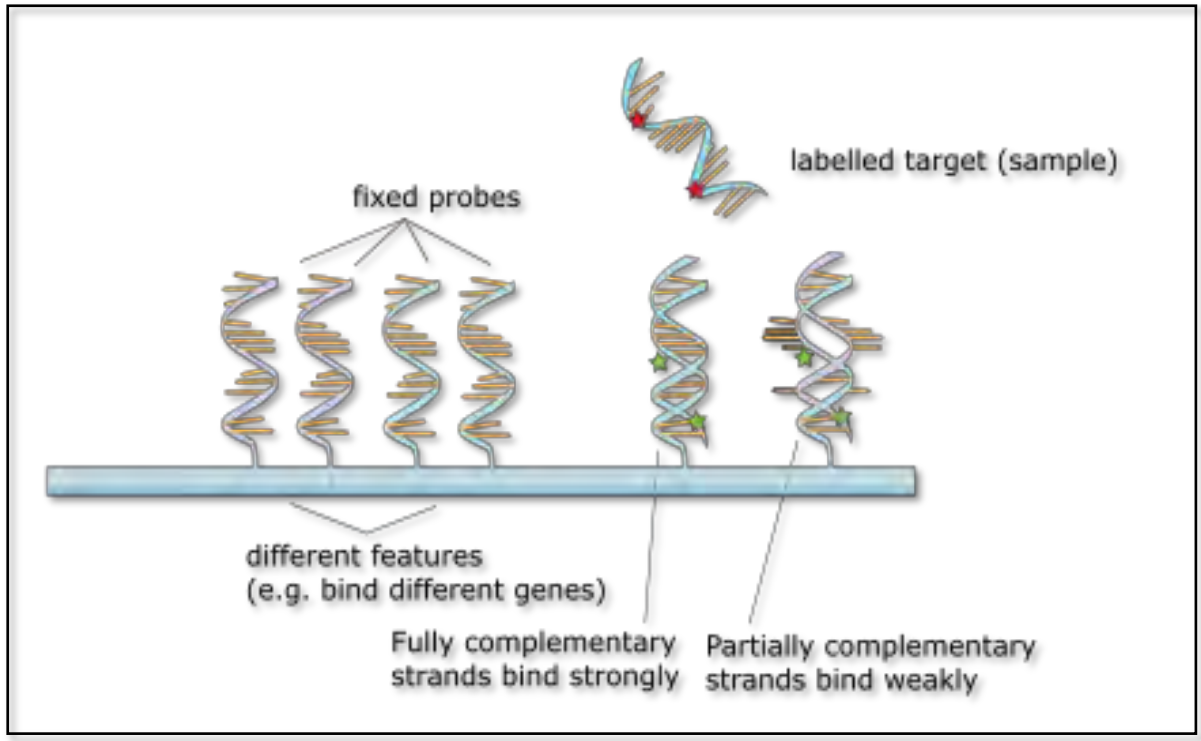


Figure 2 A presentation of the probe hybridization in microarray technology (https://en.wikipedia.org/wiki/DNA_microarray)

Important considerations for choosing a relevant array include the amount of genomic DNA required, the overall time taken to perform the assay, the amount of hands-on time required, scanner resolution, and the volume and ease of analysis of data (Huang & Crolla, 2010). The higher the resolution of the array the more difficult and time consuming the analysis becomes. Huang & Crolla (2010) reported in their array platform comparison document that

total analytical time per case is approximately 30 to 45 minutes. Each platform also has different filter settings depending on the resolution of the required analysis.

Mason-Suares et al. (2013) compared three types of array: high SNP density, mid SNP density and low SNP density. These platforms were Affymetrix Cytoscan HD (Affymetrix, Santa Clara, CA), a custom Agilent array (Agilent, Santa Clara, CA) and Oxford Gene Technology Cytosure ISCA UPD (OGT, Begbroke, Oxfordshire, UK). The aim of the study was to compare the detection rate of CNVs, mosaicism, UPD and long stretches of absence of heterozygosity (AOH) as shown in Table 2. All three platforms were able to detect the nine known CNVs. The software of the low-density platform did not calculate the percentage mosaicism. The low-density platform also detected false-positive AOH; which was resolved by adjusting the LOH score in the low-density software from 50 to 400. The authors concluded that the combination of CN and SNP arrays were able to detect CNVs larger than 100 Kb. This was more sensitive than the 400 Kb which was recommended by the American College of Medical Genetics and Genomics (ACMG) (Kearney et al., 2011). Only the high density platform was able to detect and confirm CNV calls as CNVs and SNPs were interpreted independantly, whereas the low density array software interpreted the SNP and CNV data in combination. The medium-density array also combined the oligonucleotide and SNP data for interpretation (Mason-Suares et al., 2013).

Table 2 Comparison of high SNP density, mid SNP density and low SNP density array platforms (Mason-Suares et al., 2013)

	AFFY CYTOSCAN HD	AGILENT TECH (CUSTOMISED)	OXFORD GENE TECHNOLOGY
SNP density	High	Medium	Low
Probes:	1 X 2.6 million	4 x 180 000	4 x 180 000
• **Oligo**	1.9 million	150 000	137 000
• **SNP**	750 000	30 000 (/60 000)	6 186
Backbone spacing	• 1 oligo every 2 Kb • 1 oligo every 400 bp (targeted) • 200 SNP probes/Mb • Each SNP = 6 Probes (3/allele)	• 1 oligo every 20 Kb • 10 SNP probes/Mb • 1 probe/SNP	• 1 probe every 25 Kb • Higher oligo density in ISCA regions • 3 SNP probe/Mb • Each SNP = 3 identical probes/allele
Probe length	25 bp	60 bp	60 bp
DNA	250 ng	1-1.5 ug	900 ng (pt + ctrl)

bp = basepair, ctrl = control, ng = nanogram, ISCA = International Standards for Cytogenomic Arrays, oligo = oligonucleotide, pt = patient, ug = microgram

1.4.2 Detection rates of arrays

Reported detection rates vary widely between different reports (Rauch et al., 2006). In three studies of patients with ID and dysmorphism, who had normal karyotypes, genome wide array detection rates were reported as 15%, 24% and 10%, respectively (Schoumans et al.; 2005, Shaw-Smith et al., 2004; Vissers et al., 2003). Gijsbers et al. (2010) reported on detection rates of approximately 17% in an ID cohort of 13 patients. Shoukier et al. (2013) reported a detection rate of 21.1% in a cohort of 342 ID patients which had no abnormalities detected by karyotyping and subtelomeric screening. Pathogenic CNVs were detected in 13.2% of the patients and VOUS were found in 6.4%. Bi et al. (2012) reported a detection rate of 14.5% using whole-genome arrays and 10.9% using targeted arrays in a cohort of 3 710 consecutive cases. This represented a 67% improvement when compared to the detection of abnormalities in only 8.8% of those patients on chromosomal analysis. Michelson et al. (2011) reviewed 27 studies which collectively included 6 559 GDD/ID patients, tested with whole genome arrays and found the detection rate to be between 0% and 70%, with an average of 7.8%. Syndromic and non-syndromic patients were included in these studies which may account for the lower detection rate. Coulter et al. (2011) did a retrospective chart review on 1 792 patients with ID/DD, ASD and congenital abnormalities. Abnormal variants were found in 7.3% and „variants of possible significance" were found in 5.8% of the patients tested, indicating a total detection rate of 13.1%. These authors also reported that 88% (170/194) of their cohort had had a total of 356 other tests (genetic, metabolic or neurological) which might have been unnecessary if CMA testing was performed at the outset.

A high-resolution array, with probe resolution settings at 400 Kb or lower, is appropriate for postnatal applications to maximise the detection of potentially relevant smaller CNVs, when there is a clinical phenotype to assist in assigning relevance. In the prenatal setting however, a lower resolution (500 to 1000 Kb) is recommended for CNV detection to detect pathogenic CNVs while reducing the likelihood of VOUS (Stravropoulos & Shago, 2010). Each laboratory sets their own criteria for reporting within international guidelines while considering the patient population.

1.4.3 Mosaicism

Mosaicism is not a frequent finding as the cause of ID/DD, ASD and MCA. Affymetrix Cytoscan HD claims to detect mosaicism levels of between 30 and 70%. Bi et al. (2013) showed that a case of a two cell mosaicism resulted in a balanced net effect and a normal microarray analysis using a 180K oligonucleotide array. They also determined that mosaicism of less than 30% cannot be confidently determined using CMA. However, using a combination microarray which detects CNVs and SNPs simultaneously, mosaicism at levels as low as 5% can be detected (Mason-Suares et al., 2013). There is an increased sensitivity and detection of mosaicism when using a combination of SNP probes and CN (CN+SNP) arrays.

1.4.4 Novel genetic syndromes

The use of microarray technologies has resulted in the escalated discovery of novel genetic syndromes (Weise et al., 2012). With the improved resolution of microarray technologies, microdeletion/duplication syndromes can be delineated more accurately. As reported by Weise et al. (2012), 211 microdeletion syndromes and 79 microduplication syndromes have been found to be associated with ID and related disorders, which included 1p36 (deletion), 1q21.1 (deletion and duplication), 3q29 (deletion and duplication), 10q22-q23 (deletion), 15q11.2 (deletion and duplication), 15q13.3 (deletion and duplication), 15q24 (deletion and duplication), 16p11.2 (deletion and duplication), 16p12 (deletion), 16p13.11 (deletion and duplication), 17q12 (deletion) and 17q21.3 (deletion).

1.4.5 Limitations of arrays

Array technologies will not detect structural rearrangements such as inversions and translocations (Gijsbers et al., 2010). CMA cannot differentiate between a free trisomy and a Robertsonian translocation and is also not suitable for the urgent detection of aneuploidy as chromosome analysis and/or FISH have a shorter turnaround time (Manning & Hudgins, 2010). CMA may seem to be a short technical process but the analysis of the results can be time consuming, thereby potentially extending the turnaround time. Depending on the platform used, some aneuploidies for example XYY, some marker chromosomes, mosaicism

and triploidy may not be detected. As only CNVs are detected, the mechanism underlying the CNVs may not be clear, and only CNVs covered by the probes on the microarray will be detected (South et al., 2013). The length of the probes and the spacing in-between such as genome coverage, will affect the resolution and yield of the array used as well as the algorithms for the analysis of the data (Manning & Hudgins, 2010). Shen et al. (2011) reported that the algorithms for CNV calling were not satisfactory. The problems with the algorithms were that they were developed for specific array platforms, that probe- and locus-level variation was not considered and that difficult regions were not managed differently. South et al. (2013) stated that the inability to detect a specific defect does not exclude the diagnosis of that disorder.

1.4.6 Confirmatory testing

As multiple DNA samples are run simultaneously, confirmatory tests are a consideration for abnormal results. MLPA is an excellent assay for this purpose. It is more cost-effective than repeating the actual microarray. The TAT is also short. FISH can also be performed especially to determine the inheritance of translocations (Hills et al., 2010). Using CNV+SNP arrays, confirmatory testing is not always necessary as the SNP acts as a control and confirms the CNV detected (Mason-Suares et al., 2013), especially in the case of well-known microdeletion and microduplication syndrome which occur de novo such as Williams syndrome and Prader-Willi syndrome (CCMG Position Statement, 2009). However, to determine if a CNV is de novo or inherited, FISH or MLPA can be useful. If the CNV is inherited, it is important to assess recurrence risk for which chromosome analysis or FISH can be used for the detection of structural rearrangements (Best Practice Microarray Analysis Guidelines, HGSA, 2011).

1.5 DATA ANALYSIS AND INTERPRETATION

The technological advancement in CMA progressed at a tremendous pace with the result that data was available before laboratory staff and clinicians had the skills to interpret this information. This created a challenge for the interpretation of results and counselling of patients (Tsuchiya et al., 2009).

There are more than 15 000 CNV regions reported in the Database of Genomic Variants (DGV, http://dgv.tcag.ca/dgv/app/home). This number has been increasing continuously with time, as array technology resolution improves. Cancer research, clinical diagnostics and genome-wide association studies are some of the areas of study of CNV detection (Pinto et al., 2011). Some CNVs pose a challenge during interpretation of results, socalled VOUS and include CNVs which do not completely overlap a known genomic abnormality, CNVs which have not been previously described in the region, an abnormality of less than 1 Mb, inherited changes occurring in patients and in normal parents, a small abnormality present in an affected patient and affected parent, and a large abnormality inherited from a normal parent (Shaffer et al., 2007). Increasing the probe coverage increases the detection of VOUS adding to the complexities of result interpretation. Some sizable CNVs have been identified as benign, such as the inherited 2.7 Mb duplication at chromosomal region 14q32.32 and the inherited 1p44 duplication. Another factor to consider in CNV analysis and interpretation is imprinting effects which can have a direct effect on the genome (Shaffer et al., 2007).

Duplications of chromosome X bear special consideration. The majority of the genes on the X chromosome are involved in brain functions. As males only have one X chromosome, they are more prone to X-linked ID. The mother is often a carrier of the causal CNV on the X chromosome which may cause pathogenicity due to skewed X-inactivation. Isrie et al. (2012) proposed a workflow for the interpretation of X chromosome CNVs by using retrospective data of 2222 male patients screened over a 6-year period (see Table 3). A total of 68 patients (3.1%) had X chromosome CNVs of which 23.5% were deletions and 72.1% were duplications. Three of these patients had triplications. The severity of ID was found to be variable with some indication that environmental factors contributed. The authors conceded to the difficulty in interpretation of chromosome X duplications and emphasised that after 7 years of performing microarray assays new CNVs are still being discovered on the X chromosome. Although some of the duplications corresponded with CNVs reported by other studies, these CNVs were not recurrent in their series. Genotype-phenotype correlation was also not possible in a number of the patients as the CNVs varied in sizes and gene content and the severity of ID and contribution of environmental factors was inconsistent. A compounding factor is X-inactivation as reported by Li et al. (2010) reported on eight phenotypically normal females who had random or preferential inactivation of the X duplication CNVs while two out of seven phenotypically abnormal females showed the duplicated chromosome to be preferentially active. The authors postulated that X inactivation patterns correlated with the outcome.

Table 3: Evaluation of array results as recommended by Isrie et al. (2012)

Pathogenic	1. CNV associated with ID previously reported 2. CNV (deletion) containing known ID genes 3. CNV (duplication) containing known dosage-sensitive ID genes
Likely pathogenic	Duplication that meets all three or deletion that meets two or more of: 1. Brain expressed genes present 2. De novo CNV or skewed X-activation in mother 3. Large gene-rich region
Benign CNV	Reported in DGV or in healthy individuals
Unclassified CNV	Duplication that meets less than three of: 1. Brain expressed genes present 2. De novo CNV or skewed X-activation in mother 3. Large gene-rich region

1.5.1 Reporting

Tsuchiya et al. (2009) noted that even though different laboratories may use a similar approach when analysing a CNV, there can be discrepancies in whether it is termed benign or pathogenic. De novo CNVs would be considered more likely to be pathogenic while inherited CNVs would be considered benign when inherited from a normal parent. Miller et al. (2010) confirmed both the challenge in analysing the resulting data and the interpretation of VOUS. Tsuchiya et al. (2009) understood that it is not always possible to assess the pathogenicity of CNVs through inheritance as one or both parents are not always available for testing. The authors suggested evaluating results as set out in Table 4.

Buysse et al. (2009) stated that a common CNV should occur at least twice in the DGV with a 100% overlap, with rare CNVs having no or only one entry, as noted in Table 5. The interpretation of CNVs in this way is still a challenge, for example susceptibility loci may be present in the DGV as normal variants, and there is the possibility of unmasking recessive genes. Some approaches to the interpretation of detected CNVs are illustrated in Tables 4-6 below.

Table 4 Evaluation of array results as recommended by Tsuchiya et al. (2009)

'likely pathogenic'	1.	De novo inheritance is more likely to be pathogenic
	2.	Evidence of an abnormal phenotype or known syndrome
	3.	Known variability in expression of phenotype
	4.	Large CNVs with evidence of pathogenicity in rare case reports
'uncertain'	1.	Any CNV which falls between the „likely pathogenic" and „likely benign" categories
'likely benign'	1.	Small CNVs which lack of known genes
	2.	Lack of pathogenic evidence in databases

Table 5 Evaluation of array results as recommended by Buysse et al. (2009)

1.	Relate findings to known pathogenic/susceptibility loci to determine clinical significance
2.	Exclude common CNVs but apply caution as normal variants and pathogenic CNVs may be merged

Table 6 Evaluation of array results as recommended by Poot & Hochstenbach (2010)

1.	Evaluate each CNC using the available databases for example ECARUCA, DECIPHER
2.	Correlate the protein-encoding gene within CNCs with the phenotype
3.	Establish if the CNC is de novo or inherited

CNC = copy number change, DECIPHER = DatabasE of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources, ECARUCA = European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations

The opinion of Lee et al. (2007) is that all of a patient"s CNVs should be tabulated and the normal CNVs be removed in order to only analyse the remaining potentially pathogenic CNVs. This may simplify the analysis process to a certain extent but caution should still be exercised to confirm a true pathogenic CNV with relevant and reliable evidence. The authors further state that „the potential clinical relevance of a CNV increases with respect to the number of genes within the region of genomic imbalance." It is important to analyse any genes within a CNV especially if they are implicated in disease for example Online Mendelian Inheritance in Man (OMIM) genes, or if there may be dosage effects. Another point to take into account is that duplications appear to be less severe than deletions even though normal individuals may also carry deletions. Deletions may however uncover a

recessive mutation leading to a disease phenotype. The gene content is more relevant than the size of the CNV (Lee et al., 2007). Koolen et al. (2009) suggested a method of validation of a true CNV using the DGV and their own database. A common CNV should be described at least three times, in different databases if possible, and be similar to the patient studied. In addition, the CNV should overlap a known CNV by at least 50% and the unique segment should be less than 100 Kb in size. A large majority of their patients fell within the normal CNV category.

Riggs et al. (2012a) reported on the need for a systematic process of data analysis. Assessing the clinical significance of a specific gene can be subjective. The authors also stated that „Peer-reviewed literature is considered the gold standard for the primary evidence needed to effectively assess a particular genomic region.‟ There are several factors to consider when assessing literature: the quality of the paper, the quality of the technology used, the control population used (this is especially important in the South African context), confirmatory studies performed, and whether the CNV compares with published data with regard to size and associated phenotype? Other questions to ask include whether the mode of inheritance and the degree of phenotype presentation correlate with published data and if the patient has more than one CNV which may contribute to the specific phenotype.

These authors developed a rating system to quantify published data to standardize interpretation of results as set out in Table 7. Each genomic region would have either a rating of, (1) haploinsufficiency or (2) triplosensitivity and interpreted accordingly. Haploinsufficiency includes deletions and loss of function mutations which cause a decrease in gene dosage leading to a specific phenotype. It also includes deletions, nonsense mutations, frame-shift mutations or splicing defects causing a haploinsufficiency in a particular gene. This mutation pathogenicity must be evidence-based. Missense, silent and intronic changes should only be considered with evidence-based reported pathogenicity. Triplosensitivity includes whole gene duplications causing increased gene dosage resulting in a specific phenotype. This does not include gain of function.

Table 7: Rating scale for the interpretation of detected CNVs based on available evidence (adapted from Riggs et al., 2012a)

3	Sufficient evidence	Regions with sufficient evidence where the clinical phenotype can be due to dosage sensitivity. At least three unrelated patients would have been previously reported and in at least two different articles.	Potential clinical interpretation: *Pathogenic*
2	Emerging evidence	Cases where mutations are inherited from parents with an apparently normal phenotype. Penetrance and expressivity of loss of function mutations are not fully understood.	Potential clinical interpretation: *Uncertain, likely pathogenic* OR *Uncertain*
1	Little evidence	No clear phenotype-genotype correlation exists	Potential clinical interpretation: *Uncertain*
0	No evidence	• No evidence to prove phenotype genotype correlation exists • There is evidence that the gene is NOT involved in dosage sensitivity anomalies.	Potential clinical interpretation: *Uncertain, likely benign* OR *Benign*

X-linked conditions should only be considered with regard to dosage sensitivity in male patients. Riggs et al. (2012a) stated „the haploinsufficiency and triplosensitivity ratings for genes on the X chromosome are made in the context of a male genome to account for the effects of hemizygous duplications or nullizygous deletions". Y chromosome gene duplications are not associated with specific clinical phenotypes. Y chromosome gene deletions are however pathogenic, for example, as with the genes *SRY* and *DAZ* (Riggs et al., 2012a). Another point to consider is that one CNV may include more than one gene, all of which have to be assessed. Clinical correlation which includes phenotype and penetrance, and expressivity is of extreme importance (Riggs et al., 2012a). Large-scale case-control series can be used for both assessing the frequency of CNVs and case-control population comparisons (Cooper et al., 2011; Kaminsky et al., 2011). Case-studies can also be valuable when assessing rare CNVs. Another consideration is that some CNVs do not have any clinical relevance to the cohort being tested and would be considered „benign" as the CNV would not add value to the diagnosis in patients with DD, MCA and/or ASD.

Two meetings were held to discuss the analysis, reporting and interpretation of microarray results:

A forum called „Microarray Reporting Best Practice Workshop" was organized by the Genetics Advisory Committee of the Royal College of Pathologists of Australasia and was reported on by Bruno et al. (2012). Due to the increased detection rate of chromosomal abnormalities using CMA, a „reverse dysmorphology" or „genotype-to-phenotype" phenomenon was noted. The rapid rate of development of the microarray technology and the amount of data generated has been vast, inevitably producing inconsistencies in processing of samples, analysis, interpretation and reporting between different centres due to the incomplete knowledge of the variome (Bruno et al., 2012). The following key areas (in italics) were considered:

Reporting terminology - the „pathogenic" (well established CNVs published in peer reviewed literature), and „benign" (commonly occurring CNVs in the general population) classifications are straightforward. There should however be a clear distinction when using „unknown significance" and „uncertain significance". Patients with neurodevelopmental conditions are more likely to have so-called „susceptibility CNVs" with 2 to 4% of patients with DD, ID, ASD and MCA having been found to have „susceptibility factors" as more than one element can be involved.

Evidence-based reporting: „Critical review of literature cited in reports is a requisite competency skill for any laboratory geneticist." Interpretation of findings should be done by evaluating peer-reviewed literature with careful consideration of the cohort (size, phenotypic data, ethnicity, control samples) and the CNV calling as the interpretation of the detected CNVs is influenced by the type of array and the software used.

Comprehensive clinical information to aid analysis and interpretation: good laboratory-clinician relationships should be cultivated to ensure reliable communication with regard to patient information as phenotype information is extremely important in the interpretation of patient data.

Analysis of CNV flanking and agenic regions: as arrays offer adequate probe coverage and target relevant regions, various laboratories do not extend their analysis beyond the breakpoints whereas others may include genes upstream or downstream from the region being analysed. The same is true for agenic regions. Each laboratory has its own protocols to deal with these.

Challenges of interpretation: rare and novel CNVs are a challenge as not enough evidence is available to differentiate between pathogenic and benign variants. At present, interpretation is accomplished using case-control frequency data, CNV size and the gene content of the CNV. CN state (heterozygous vs homozygous and their respective likelihood of pathogenicity) and possible known polymorphisms should also be considered during data

interpretation. Another consideration is the overlap of a detected CNV with a potentially pathogenic gene region. Phenotypic correlation is essential. Gene function may also be disrupted by rearrangements such as duplications of a gene or insertions.

Genomic load: neurodevelopmental disorders may be caused by the aggregate effects of multiple clinically significant alleles and it is thus not always possible to arrive at a complete diagnosis. Overinterpretation in VOUS and susceptibility CNVs remain a challenge.

Homozygosity: this may indicate consanguinity, UPD or an unmasked Mendelian disorder.

Interpretation of CNV inheritance: de novo CNVs are not always pathogenic and inherited CNVs are not necessarily benign. Other factors such as imprinting and epigenetics also play a role. Also, susceptibility loci should be taken into account.

Reporting CNVs: some laboratories report all CNVs, whereas others only report clinically relevant CNVs. Consistency in reporting within each laboratory should however be maintained.

Incidental findings: including these in reports should have clinical validity and relate to treatment options.

Consanguinity: a policy should be put in place to communicate these results directly to the clinician.

Carrier status: ACMG Guidelines (Kearney et al., 2011) state that carrier status testing is beyond the scope of CMA testing.

The second meeting was an international symposium which was held by the Genetics Services Quality Committee of the European Society of Human Genetics in Amsterdam in 2011 (Sikkema-Raddatz & Sijmons, 2012). The theme was „Array in daily Practice" and five articles were published in 2012, illustrating the findings and opening further discussion. De Leeuw et al. (2012) discussed the classification and interpretation of CNVs for constitutional diagnostics. The point was made that all CNVs detected should be interpreted, although it was recognised that rare CNVs are difficult to interpret and categorize. CNVs can be classified as the following according to these authors: 1) Benign CNV/Normal genomic variant, 2) Likely benign CNV, VOUS, 3) CNV of possible clinical relevance/High-susceptibility locus or risk factor or likely pathogenic variant, and 4) Clinically relevant CNV/Pathogenic variant. These terms underscore the importance of the Genetics Advisory Committee of the Royal College of Pathologists of Australasia standardization of reporting terminology (Bruno et al., 2012). Gains (typically represented in blue) and losses (typically represented in red) give rise to considerable differences in clinical effect and should be

interpreted accordingly. De Leeuw et al. (2012) recommended that analysis of the data should involve the following steps: comparison of similarities between the CNV to in-house and national/international control datasets, comparison of similarities with affected individual datasets and comparison of gene content to literature. The more commonly consulted databases and resources are DECIPHER (https://decipher.sanger.ac.uk/), DGV http://dgv.tcag.ca/dgv/app/home, University of California, Santa Cruz Genome Browser (https://genome.ucsc.edu, UCSC), Ensembl (http://www.ensembl.org/index.html), ECARUCA (http://umcecaruca01.extern.umcn.nl:8080/ecaruca/ecaruca.jsp), ISCA (http://www.iscaconsortium.org/?viajml=1), OMIM (http://www.ncbi.nlm.nih.gov/omim), National Center for Biotechnology Information and U.S. National Library of Medicine (http://www.ncbi.nlm.nih.gov/pubmed/,PubMed) and UNIQUE (http://www.rarechromo.org/html/home.asp).

Microarray platform software offers visualization tools which demonstrate the array data, the most recent human genome reference sequence, the specific probe coverage and links the data with a number of public databases and browsers. PubMed, OMIM, UCSC and Ensembl offer genome-orientated data. DECIPHER, ECARUCA and ISCA offer a collection of individual cases. DGV offers control information with genetic and phenotypic details on the specific region studied. Cartagenia BENCH (https://cartagenia.com/cartagenia-bench-lab) is both a database and analytical platform for routine laboratory workflow and has the following components: 1) Interpretation: this involves automation of the entire work process from data retrieval, CNV interpretation to the completed report and incorporates all the commonly used databases and gene browsers, 2) phenotype database, and 3) international collaborations as well as integration with DECIPHER, ISCA and ECARUCA. A few important points to bear in mind when interpreting CNVs: it is crucial to use up-to-date sources as there is such a large amount of genetic and clinical information rapidly becoming available, databases should include the ethnicity of patients in addition to the usual demographics as population-specific genomic variants occur and there are a variety of databases, each with a different genome build according to the National Centre of Biotechnology Information (NCBI) and Genome Reference Consortium - human assembly (GRCh): for example NCBI35 [hg17], NCBI36 [hg18] and GRCh [hg19] which may cause problems when comparing data. De Leeuw et al. (2012) concluded that a single search engine (data aggregator) is needed to ensure quick and simple interpretation of data with all relevant information sourced from different platforms. At the moment UCSC Genomic Browser fulfils some of these criteria. Ideally, all data, both from research and diagnostics, should be made freely available. This data must meet strict quality criteria for submission to the relevant database. However, the

biggest challenge is still the interpretation of the data. It is of the utmost importance to link clinical information with the genetic output. De Leeuw et al. (2012) further reported that apparently normal or benign CNVs may cause or contribute to pathogenicity and should be reported. Examples of this include one allele with a deletion while the other allele carries a mutation of the gene, two identical deletions present on both alleles indicating a benign homozygous deletion, a patient inheriting two different deletions in the same gene from each parent, imprinting affecting pathogenicity, an X chromosomal CNV inherited by a male offspring, a CNV inherited from a mosaic carrier (non-affected or mildly affected), and a combination of CNVs giving rise to pathogenicity. In spite of this, the majority of CNVs must still be analysed individually for accurate interpretation as the size of the detected CNV does not necessarily correspond to the size of other reported CNVs.

There are special considerations which should be taken into account when compiling an abnormal diagnostic report. A clear, unambiguous description of the genomic imbalance, which includes the clinical interpretation, must be provided. The size, start and end positions of the probes, the gene content and references used must be reported. Follow-up studies could be recommended to elucidate or confirm the findings.

1.5.2 Incidental findings

Boone et al. (2013) demonstrated that microarray technologies not only detect disease-causing CNVs related to the patient"s phenotype but also CNVs which involve late-onset disease-causing genes such as those predisposing to certain malignancies. The authors also suggested the inclusion of incidental findings in pre-test counselling. Coulter et al. (2011) reported on two cases referred for CMA. One was a 15-year old female who was referred for learning difficulties and behavioural problems. CMA identified a 3.8 Mb deletion on chromosome 2 which included the *PROC* gene which is involved in hereditary thrombophilia due to protein C deficiency. Follow-up testing revealed a low level of functional protein C in the patient which could aid in future medical treatment. The second patient was a nine year old female with multiple abnormalities including heart defects. CMA identified a 244 Kb deletion on chromosome 22 which included the *CHEK2* gene which is implicated in Li-Fraumeni Syndrome 2, and which is associated with an increased risk of different types of cancer. This patient was therefore referred to an oncologist and her clinical management changed completely as a result of this finding.

1.5.3 Nomenclature

Karl Wilhelm von Nageli first discovered chromosomes in plants in 1842 (Kannan & Zilfalil, 2009). By 1956, optimal preparation techniques for chromosome analysis were achieved. These improvements lead to the identification of the aneuploidic basis of Down, Turner and Klinefelter syndromes, amongst others (Kannan & Zilfalil, 2009). In 1960 an international standardized nomenclature for chromosome abnormalities was adopted (Shaffer et al., 2013). Pinkel et al. (1986) reported on the use of fluorescence *in situ* hybridization (FISH) for the detection of microdeletion syndromes. In 1986, imaging systems for karyotyping were introduced (Kannan & Zilfalil, 2009). Even though the Philadelphia chromosome was first reported in 1960, the International System for Human Cytogenetic Nomenclature (ISCN) Guidelines for Cancer Cytogenetics was only released in 1991 (Shaffer et al., 2013). Shortly thereafter, Kallioniemi et al. (1992) used Comparative Genomic Hybridization (CGH) for the analysis of solid tumours and in 1998 Pinkel and colleagues used this technology for CN analysis (Pinkel et al., 1998). By 2002, Veltman and colleagues were using CGH microarrays for the detection of CNVs of telomeres in patients with ID representing a major shift from chromosome analysis, which had been the gold standard for „gross" CNV detection for decades (Veltman et al., 2003). In 2010, Miller et al. released the consensus statement stating that microarrays should be the first-tier test in patients with ID/DD, MCA and ASD. The ISCN was subsequently updated in 2013 with the removal of unused nomenclature, the addition of genome builds for microarray results, and the replacement of the MLPA section with Region-Specific Assay (RSA) which can be used for targeted arrays, MLPA, QF-PCR, etc. (Shaffer et al., 2013).

1.6 COST BENEFIT ANALYSIS

Regier et al. (2010) reported the high cost of microarray testing as compared to conventional cytogenetic analysis. They were also unsure if microarray testing was actually „good value for money". Trakadis & Shevell (2011) argued that karyotyping is labour-intensive and time-consuming and not always informative, thereby justifying the extra cost of aCGH with a significantly increased diagnostic yield. Even though microarray testing is recommended as first-tier testing for GDD and autism, this had not always been the case in practice, in part due to the perception that array testing is more expensive than traditional cytogenetics methods (such as karyotyping, FISH and MLPA).The authors concluded that with microarray testing there may be an overall decrease in TAT providing an earlier diagnosis with resulting

treatment/management, reducing parental anxiety and cutting down on additional tests and consultations which increase the overall cost (Wordsworth et al., 2007; Trakadis & Shevell, 2011). Wordsworth et al. (2007) found that the average cost of aCGH was £442 per patient (based on 25 tests per week) with the average cost of karyotyping £117 per patient (based on 61 tests per week). However if an additional multi-telomere FISH test was performed when no abnormality was detected on karyotyping, the cost rose to £400. The cost difference when compared to microarray testing is therefore negligible, with the testing yield being significantly much more extensive.

Palmer et al. (2012) in their review article, discussed the costing of CMA in Australia. In 2010, the Medicare Benefits Scheme charged the following fees: chromosome analysis: Aus$361.95, MLPA/FISH: Aus$230.95 and CMA: Aus$593.85. Performing chromosome and MLPA/FISH analysis on a sample would result in almost the same cost but with a reduced resolution than performing microarray testing.

1.7 STANDARDS AND GUIDELINES

This discussion focuses on the revolution in cytogenetic testing in patients with DD/ID, ASD and MCA with CMA superseding chromosome analysis as the gold standard. These guidelines make recommendations for technical processes and the analysis and interpretation of CNVs.

1.7.1 American College of Medical Genetics guideline on the cytogenetic evaluation of the individual with developmental delay or mental retardation (Shaffer, 2005)

In 2005 routine chromosome analysis (minimum 550 G-band resolution) was still recommended for children with unexplained MR/DD, even in the absence of other clinical features or a positive family history. Routine chromosome analysis was also recommended for children with clinical features of known chromosomal disorders such as DS as it is important to detect a possible translocation to calculate the recurrence risk. High-resolution chromosome analysis was only performed when FISH was not available. Subtelomeric FISH was only considered if chromosome analysis was normal at the 550 G-band level.

1.7.2 Microarray analysis for constitutional cytogenetic abnormalities (Shaffer et al., 2007b) – as proposed by A Working Group of the Laboratory Quality Assurance Committee of the American College of Medical Genetics

These guidelines recommended the use of 30 patient samples, in which chromosomal abnormalities had previously been identified, and which should ideally include a range of abnormalities, for the validation of a microarray platform. To establish controls, male and/or female or pooled DNA from both or opposite sex controls could be used depending on the clinical relevance. Each laboratory was meant to determine the particular platform"s mosaicism detection rate. „The possibility that a clinically significant abnormality can occur in known variant regions" should be kept in mind during analysis. They recommended that each laboratory establish an in-house database.

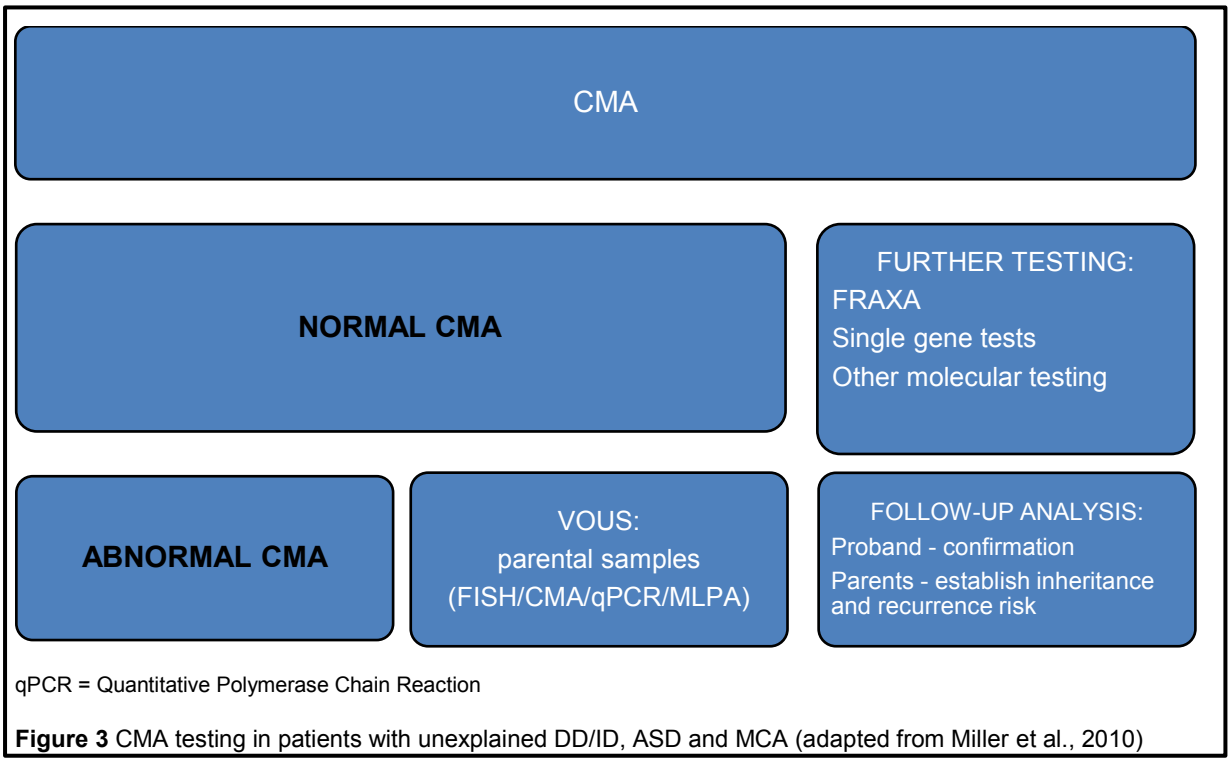
1.7.3 International Standards for Cytogenomic Arrays (ISCA)

ISCA was established as an independent group in 2007/2008 to ensure all patients receive the same quality of care by standardizing array design and by enhancing the standard of CMA testing. This group consisted of international experts in CMA from more than 75 laboratories (Manning & Hudgins, 2010). ISCA recognized the need for CNV interpretation using whole-genome analysis. It was important to interpret these results within the context of the relevant phenotype to ensure an accurate diagnosis. Many targeted regions could however not be linked to clinical evidence, contributing to challenges in data analysis/interpretation. The ISCA Consortium established a database which incorporated both phenotypic data for the interpretation of genomic variants and genomic data for the development of phenotypic profiles. Accurate and detailed phenotypic information is fundamental to clarify genotype-phenotype relationships (Riggs et al., 2012b). The ISCA Consortium formed an Evidence-based Review (EBR) Work Group which would ultimately evaluate each gene in the human genome with input from other medical geneticists. This tool could be used for the interpretation of CNVs in diagnostic laboratories (Riggs et al., 2012b).

1.7.4 Consensus statement by Miller et al. (2010) on the use of Chromosomal Microarrays on behalf of ISCA

ISCA reviewed 33 original publications which included the analysis of 21 698 patients with unexplained DD/ID, ASD or MCA using microarray technologies. At the time, guidelines still promoted G-banded karyotyping and testing for FRAXA and other single-gene disorders for

patients with these indications. At the conclusion of this review, Miller et al. (2010) released the Consensus statement „*Chromosomal Microarrays: A First-Tier Clinical Diagnostic Test for Individuals with Developmental Disabilities or Congenital Anomalies*". The diagnostic yield of CMA was much higher at 15 to 20% when compared to 3.7% with routine chromosome analysis (Shevell et al., 2003). Microarray therefore had a much higher sensitivity for the diagnosis of submicroscopic deletions and duplications. The improved resolution did however add new challenges to the interpretation of CNVs. G-banded karyotyping were still useful for the detection of low-level mosaicism and the detection of apparently balanced rearrangements. Multiple miscarriages would also still require G-banded karyotyping as the first tier test. This also holds true for suspected trisomy 13, 18 and 21, and in Turner and Klinefelter syndromes. G-banded karyotyping and/or FISH would have a shorter turnaround time, and distinguish between free trisomy and translocation. CMA was recommended as the first-tier test for patients with unexplained DD/ID, ASD or MCA instead of G-banded karyotyping, even though there is a cost consideration. G-banded karyotyping, followed by FISH (specific and subtelomeric), would be more expensive than an array which has a significantly increased yield. The clinical algorithm as shown in Figure 3 was proposed by Miller et al. (2010).



qPCR = Quantitative Polymerase Chain Reaction

Figure 3 CMA testing in patients with unexplained DD/ID, ASD and MCA (adapted from Miller et al., 2010)

1.7.5 Array-based technology and recommendations for utilization in medical genetics practice for detection of chromosomal abnormalities (Manning & Hudgins, 2010) – for the Professional Practice and Guidelines Committee of the ACMG

The ACMG updated the guidelines to advise on the use of CMA as the first-tier diagnostic test for patients with multiple abnormalities which do not fit into a specific syndrome and for nonsyndromic DD/ID and ASD.

Further recommendations included using a resolution level which is both specific and sensitive, sharing data on the established databases and performing parental studies to establish inheritance of detected CNVs. Continuous training and educational tools should be established for clinical and laboratory staff.

1.7.6 American College of Medical Genetics and Genomics recommendations for the design and performance expectations for clinical genomic copy number microarrays intended for use in the postnatal setting for detection of constitutional abnormalities (Kearney et al., 2011)

It was recommended that probes be placed at regular intervals of 400 Kb thereby covering the whole genome. Probe density of dosage-sensitive regions should be higher than in less relevant regions. The probe coverage should be equal or better than the technology which is replaced by the microarray to ensure the same or better quality results. CNV + SNP arrays are preferred even though SNP coverage is not a requirement as yet. Microarray manufacturers should specify which regions are less densely covered by probes. Furthermore, the underlying mechanisms must be well understood, for example, a deletion phenotype-associated gene may have no clinical phenotype with CN gain. Gain of function mutations resulting in dominant disorders may not be due to dosage imbalance which could give rise to a different phenotype/condition. CN gains may cause a gene disruption which should be investigated. Dominant negative mutations act antagonistically to the wild-type allele due to an altered gene product often resulting in an inactive function giving rise to a dominant or semi-dominant phenotype (Veitia et al., 2013). In a recessive disease, a single copy deletion may infer carrier status. Gene function may not be altered in the case of small intronic CNVs.

1.7.7 Best Practice Microarray Analysis Guidelines – Human Genetics Society of Australasia (2011)

CMA was recommended as best practice for patients with MCA, DD and ASD. A statistical analysis is performed as opposed to the subjective analysis of chromosomes. Appropriate

training, competence and expertise should be obtained. An external quality control (EQA) system should be subscribed to. An initial research and developmental phase must be completed before a full diagnostic service can be offered. Standard Operating Procedures must be in place and Internal Quality Assurance procedures must be applied. All processes and parameters must be validated using 20 abnormal and 10 normal samples. The quality of the DNA is crucial to the success of the CMA assay. Hardware and software must be suitable with computer, applied mathematics or bioinformatics skills being required for the understanding of the software to be able to assess the statistics and for troubleshooting. Clear reports must be written with all relevant information included: normal or abnormal, ISCN nomenclature, location and size of aberration, clinical interpretation, genome build used for analysis, confirmatory tests used, limitations of the test, if parental studies are needed, referral to or recommendation for genetic counselling services and the relevant publications or databases used for interpretation. Care should be taken not to over-interpret novel CNCs. The TAT must be between six to eight weeks from receipt of the sample.

1.7.8 ACMG Standards and Guidelines for constitutional cytogenomic microarray analysis, including postnatal and prenatal applications: revision 2013 (South et al., 2013)

Additional points were made for the introduction of microarray technology into the diagnostic laboratory. Acceptable parameters should be put in place for interpreting CNVs. Accuracy of the microarray results can be achieved by assessing 30 previously characterized samples if possible. CMA should not be used to exclude mosaicism.

1.8 THE SOUTH AFRICAN CONTEXT

The first National Disability Survey was conducted in 1999 and a 1.1% prevalence for ID was reported (Adnams, 2010). In 2001, during the national census, the prevalence of ID was reported as 0.5%. The census did not include institutionalised patients thus excluding a large portion of the affected population. Christianson et al. (2002) published the first report on the prevalence of ID in rural SA children and found the prevalence in rural SA (3.6%) to be comparable with that in Zambia. In a study in Kwazulu-Natal, Couper (2002) reported a prevalence of 1.7% for ID, 0.6% of moderate to severe perceptual disability and 0.4% of seizures. Kleintjes et al. (2006) conducted a literature review of 37 epidemiological studies to determine the prevalence of mental disorders among children, adolescents and adults in the

Western Cape Province during 2002 and 2003. This study showed that in children and adolescents, 2.5% had an IQ of 50 - 70, 0.4% an IQ between 30 and 50 and 0.1% had an IQ of below 30. ADHD was reported at a prevalence of 5%. ASD patients were not included in this study. A second National Disability Survey in 2007 reported a prevalence of 0.3% for severe intellectual or learning disability (Statistics South Africa, 2007). Kromberg et al. (2008) studied 6 692 children, between two and nine years of age, from eight rural villages in the Bushbuckridge area of Mpumalanga. The prevalence rate of ID was 3.6%. Severe ID was seen in 0.6% and mild ID was seen in 2.9% of cases. Approximately 50% of the African population consists of children below the age of 14 years and provision should be made for their future health care needs. Even though SA has an all-inclusive constitution and special policies for disabilities, including ID, these are not always taken up by or accessible to patients. Patients are entitled to grants and free primary health care. Specialized schooling is also available. Individuals living in rural areas, however, do not have ready access to these facilities. Patients with severe and profound ID are not included in the government's education benefits. ID is still not seen as a priority in the SA health and education systems (Adnams, 2010). Socio-economic factors, education level and racial segregation have a negative effect on IQ (Leonard & Wen, 2002).

SA also has the highest prevalence of fetal alcohol spectrum disorders (FASD) in the world (Bateman, 2012) which is a further contributing factor to the prevalence of ID in SA. Allison & Strydom (2009) noticed that the negative attitudes of health care workers in several studies impacted on patients with ID which resulted in their segregation from society. This attitude was described as pervasive by Njenga (2009). Cultural beliefs can also add to this negative attitude, for example in Southern Africa many traditional healers believe that ID may be caused by witchcraft. This also influences the uptake of Westernized medical care. Health care options should be designed to accommodate cultural beliefs. Njenga (2009) discussed the lack of study of ID in Africa which also highlights the lack of specially trained staff and financial and infrastructural resources, adding to the challenge of coping with large numbers of patients. The lack of resources to rectify these challenges seems to be a challenge in itself.

1.9 AIM AND OBJECTIVES OF THIS STUDY

1.9.1 Aim

The aim of this study is to investigate the relevance of CNVs in the Western Cape ID/DD population (of SA) and in so doing, to introduce and develop molecular cytogenetics skills in the routine cytogenetic environment.

1.9.2 Objectives

This aim was accomplished with the following objectives:

- to identify CNVs implicated in ID/DD
- to describe the implicated CNVs and correlate with the clinical phenotype
- to assess the diagnostic yield of CMA as compared to the current diagnostic repertoire available in SA
- to assist in planning implementation of CMA into the diagnostic laboratory in order to offer improved management and counselling of ID/DD patients and their families

Chapter 2: Methodology

2.1 STUDY SAMPLE

The study cohort comprised of patients who had been diagnosed with ID/DD and dysmorphic features, with or without structural congenital abnormalities, and had previous negative cytogenetic diagnostic tests. As part of their diagnostic work-up, the majority of these patients previously had conventional chromosome analysis and a few had microdeletion FISH and subtelomeric/microdeletion MLPA and FRAXA testing (Table 7 includes a summary of all diagnostic findings in the study cohort). One patient had a large supernumerary marker chromosome of unknown origin detected on routine karyotyping. The final cohort included 15 male and 12 female patients representing three of the four main population groups (Black, Mixed Ancestry and White) in South Africa.

Ethics approval was obtained from the Human Research Ethics Committee (HREC), University of Cape Town with annual renewal (HREC REF Number 490/2010), (Addendum I). Initially, a total of 30 patient DNA samples, which had already been stored in the DNA Registry (HREC REF Number 217/2010), in the Division of Human Genetics, University of Cape Town, were selected. Genomic DNA (gDNA) was used which was previously extracted from blood samples using various DNA isolation methods including the salting out method and using Qiagen DNA Isolation kits (Qiagen, Minneapolis, MN) and those from Gentra® and Puregene® and Maxwell® (Promega, Madison, WI, USA).

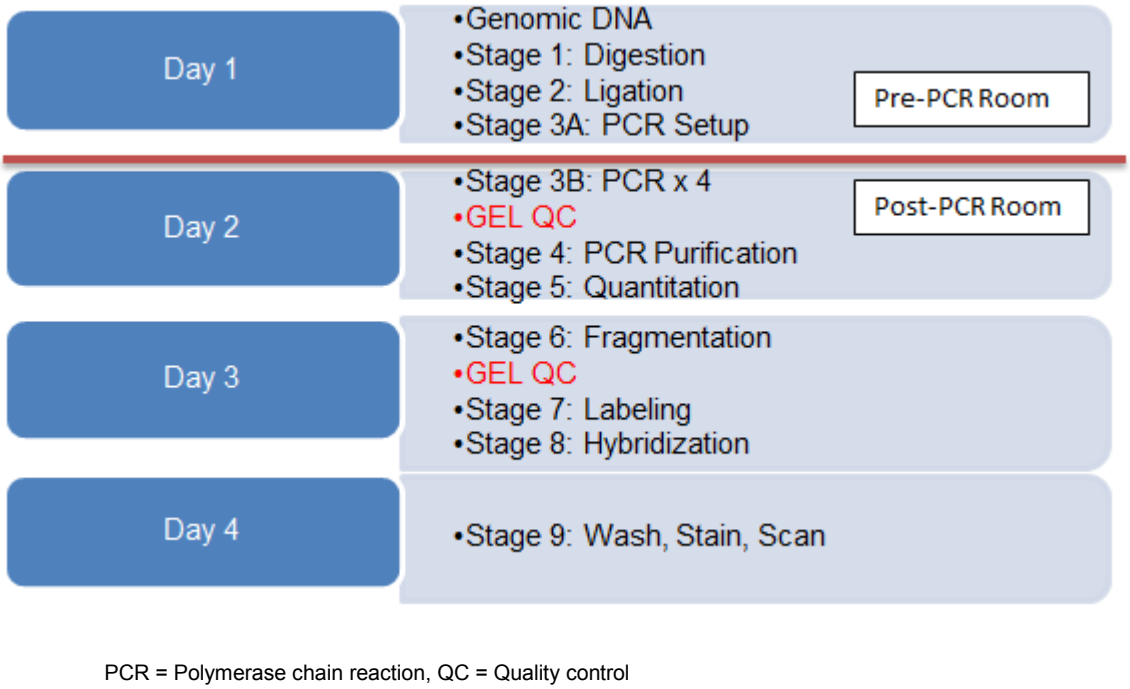
Affymetrix (Affymetrix, Santa Clara, CA, USA) made 30 Affymetrix® Cytoscan™ HD Arrays available for this study. A volume of 250 ng of double-stranded genomic DNA was required at a concentration of 50ng/ul. Due to the stringent DNA quality requirements for this array, three of the samples were excluded. The final cohort consisted of 27 patients. The small kit size excluded any follow-up parental testing with findings of VOUS. Signed consent for the use of DNA for this study was obtained from 13 of the patients. However, the remaining patients were either not contactable or the samples had been stored before consent was required for DNA banking. The latter samples were anonymised according to the requirements of the HREC.

2.2 ARRAY SPECIFICATIONS

Affymetrix® Cytoscan™ HD Arrays have been designed for the detection of genome-wide CNVs and SNPs. This platform is routinely used in various centres internationally and the array contains approximately 2.6 million markers for CN analysis which consists of approximately 750 000 SNPs and 1.9 million oligonucleotide probes. This is a targeted array with backbone spacing which covers the whole genome with a higher density of probes in regions of particular interest such as known microdeletion regions. The probe spacing consists of one oligonucleotide every 2 Kb, and one oligonucleotide probe every 400 bp in targeted regions. There are 200 SNP probes per Mb. All probes are 25 bp long. Each SNP is targeted by six probes, in other words three probes per allele (Mason-Suares et al., 2013).

2.3 PROCEDURE

The protocol using Affymetrix® Cytoscan™ HD Arrays (Affymetrix, Santa Clara, CA, USA) usually runs over a period of four days (Figure 2).



PCR = Polymerase chain reaction, QC = Quality control

Figure 4 Affymetrix Cytoscan HD Workflow (adapted)

The array was run according to the manufacturer"s instructions which consist of nine stages. The integrity of the DNA is of vital importance and should therefore not be degraded. The

average size of the gDNA was determined by electrophoresis through a 1% agarose gel. The object was to get approximately 90% of the DNA greater than 10 Kb in size; 250 ng of double-stranded genomic DNA was required and diluted to a concentration of 50ng/ul. The DNA was of a high quality and purity with the OD_{260}/OD_{280} measuring 1.8 to 2.0 and the OD_{260}/OD_{230} measuring >1.5.

All samples were taken through each step simultaneously (batched). During Digestion (Stage 1) the restriction enzyme cuts the gDNA segments within a specific nucleotide sequence (restriction site). The genomic fragments measured between 200 and 2 000 bp in length. This left overhanging nucleotides on all the fragments. The digested samples were then ligated (Stage 2) to the *Nsp* I Adaptor which recognizes the 4-bp nucleotide overhang. A unique primer sequence, which is not present elsewhere in the genome, was attached to the overhang, in the presence of the enzyme (Ligase). During the PCR step (Stage 3), which was done in quadruple, a primer which is recognized by the sequence in the adaptor (which has an artificial sequence) was used. All the fragments with different GC content and lengths were amplified equally. A QC gel was run before the PCR product was pooled to ensure optimal amplification of the DNA. The PCR magnetic bead purification step (Stage 4) eliminates salts, enzymes and buffers while concentrating the DNA. During Stage 5, the purified DNA was quantified using the NanoDrop instrument (NanoDrop Technologies, Wilmington, Delaware, USA). The acceptance criteria should be: a DNA concentration of more than 300ng/ul, the 260/280 ratio should be between 1.8 and 2.0 and the A320 should be very close to „0" (0.1). The fragmentation (Stage 6), which is critical, involved subjecting the purified samples to DNAse which fragments the DNA, which is in the size range of 200 to 2000 Kb at this stage, to approximately 50 Kb in size. The probes on the array were approximately 25 bp in size.

A second QC gel was run at this stage to assess the size of the DNA fragments. During Stage 7, the fragmented samples were labeled with Biotin, which is not fluorescent. During the hybridization step (Stage 8), the samples were denatured. The hybridization buffers ensured continued DNA denaturation, while the Oligo Control Reagent ensured successful hybridization, washing, staining and scanning of the array (Stage 9). On the GeneChip Fluidics station (Affymetrix), the fluorescently labelled stain buffer recognized the Biotin label resulting in hybridization. The non-specific bound probe was removed during several stringency washes. The fluorescent signal was scanned on the GeneChip Scanner (Affymetrix, Santa Clara, CA, USA).

For the purpose of this study, reagents for 30 samples were used. Three of the DNA samples were withdrawn from the run after the second QC gel step as DNA fragmentation was not successful.

2.4 QUALITY CONTROL

QC is imperative to the success of microarray assays. During the microarray assay QC gels were run once the DNA had been amplified to ensure optimal DNA product. Another QC gel was run after the fragmentation step to assess the size of the DNA fragments.

Before data analysis using the ChAS software, the QC of each sample was checked by assessing the following parameters: SNPQC (SNP Quality Control), Median Absolute Pairwise Difference (MAPD) and Waviness Standard Deviation (Waviness SD) metrics. These parameters are available within the ChAS software for each patient sample. The SNPQC indicated how well the genotype alleles were resolved in the data. A subset of probes measured differences in the contrast distributions for homozygote and heterozygote genotypes. The MAPD measured the variation/noise of all the probes across the genome and was reflected in the Log_2 Ratio. The median of the log_2 distance between adjacent marker pairs was calculated. A high MAPD calculation represents more noise between the array and the reference set. The Waviness SD measured the degree of waviness of the log_2 ratios among autosomal probe sets thereby capturing longer spans of variation which would not be detected in pair-wise differences. The SNPQC should be >15, the MAPD should be <0.25 and the Waviness SD should be <0.12.

2.5 ANALYSIS

Affymetrix CEL files were generated by the Affymetrix GeneChip® Command Console software (AGCC, (Affymetrix, Santa Clara, CA, USA) and analysed using the Affymetrix ChAS software program. The software compares the hybridization of patient DNA to oligonucleotide and SNP probes of normal samples. The CNVs were mapped to genome build GRCh37/hg19 for analysis and interpretation.

Initial analysis of the CNV data during 2014 used the set Standard setting filters in the ChAS software. The filters were set at a 50 marker count and a 400 kilobasepair (Kbp) CNV size which would indicate that at least 50 consecutive markers are required for a CNV call and the CNV would be larger than 400 Kbp in size. If no large gain or loss was observed, the High Resolution setting was used with the same marker count of 50 and the CNV size reduced to 100 Kbp for higher resolution detection.

The Track Files were selected for Genes, OMIM Genes, Cytobands and the ISCA Constitutional Regions library file. The data was first analysed using the Standard resolution setting and if no abnormality was found, the High Resolution setting was used. The Trak Data Types for the Standard and the High resolution analysis were CN state (Gain, Loss), Weighted Log2 Ratio, Mosaic Copy number state (Gain, Loss) and Allele peaks. The CN state indicates a gain or a loss, the Weighted Log2 Ratio would indicate no loss or gain at „0", a loss at „1" and a gain at „2", and Mosaic Copy number state would indicate a gain or a loss in a mosaic form.

The X chromosome detail view served as a control to ensure the correct patient was analysed as the sex of the patient is the only check which can be used at this stage. The X chromosome was checked for normalization. The shared X and Y markers were represented in the pink telomeric regions on the X chromosome. The X chromosome would show 3n in the pink telomeric region on the short arm of chromosome X in a male profile. A segment report was generated on each sample which contained all the CNVs detected. Each chromosome was then individually checked for gains and losses, especially on the telomeres as imbalances, for example from unbalanced translocations, will be detected on the telomeres. Telomeric regions are repetitive nucleotide regions which are more unstable (lengthening and shortening) than other chromosomal regions.

Subsequent to the initial CNV analysis, all the samples were reanalysed during a training period at the Laboratory of Diagnostic Genomic Analysis (LDGA) in Leiden, The Netherlands, in 2015. The filters for the ChAS software were set as follows: for a Gain the marker count was set at 10 and the CNV size at 20 Kbp, with a 85% confidence; for a Loss the marker count was set at 10 and the CNV size at 10 Kbp, with a 85% confidence; LOH was set at a marker count of 1,000 and a size of 2 000 Kbp. The Track Files were selected for Genes, OMIM and Cytobands. The Track Data Types selected were CN state (Gain, Loss), Weighted Log2 Ratio, Copy number change, Filter LOH, Allele peaks, Smooth signal and Genotype calls. The same checks were done for X chromosome normalization as in the initial CNV analysis.

Next the Segment Report was saved as a text file. This report contains all the CNVs detected on the sample. The Segment Report information was loaded into the Cartegenia software program. This program has been set up to the Leiden laboratory"s custom specifications an example of which take into account frequently reported benign variants reported in the DGV, as well as known population and artefactual variants detected in routine diagnostic analysis. This program also served as the in-house CNV database. The size, gene content and location were assessed in the interpretation of each genomic imbalance. DECIPHER, DGV, PubMed, OMIM and ENSEMBL were used to evaluate the detected CNVs and to assess their potential significance. Only syndromes and protein coding genes relevant to the indication for ID/DD, and the relevant clinical phenotype were reported. Variants larger than 1 Mb in size but containing no genes were also reported as this may be considered to have a structural effect leading to the phenotype. It is useful to be able to classify the detected CNVs according to known abnormalities, benign CNVs and variants of unknown significance.

The classification of CNVs was done using the following criteria as used routinely by the Leiden laboratory:

1. A known syndrome or microdeletion/duplication syndrome was reported as such
2. Susceptibility regions and a genetic abnormality associated with a clinical phenotype, not described in DGV at time of analysis, but containing the region or part of coding exon will be reported. Examples of these include the chromosome 15q11.2 abnormality which falls within the breakpoint BPI to BPII containing the genes *TUBGCP5, CYF1P1, NIPA2* and the chromosome 6 deletion of exon 3 of gene *PARK2* which is not implicated in ID.
3. Regions smaller than 150 Kb in prenatal samples, and/or inherited susceptibility regions would not be reported. This criterion was not used as all samples in this study were postnatal.
4. A genetic abnormality possibly associated with a clinical phenotype, but containing no coding genes or only containing one intron of a protein-coding gene, and which was described as a variant in DGV at the time of analysis, would not be reported.
5. If the variant is known in three or more normal controls but not associated with a known clinical phenotype (DGV excluding BAC studies), it may be classified as a polymorphism without clinical significance. The breakpoints can be within 300 Kb of a reported variant or overlapping with one probe arm area. If the variant is small, there has to be a 90% or more overlap with the patient"s CNV.
6. A genetic variant with a relevant OMIM gene for the ID/DD indication would be reported.

7. VOUS will be reported.
8. LOH with a CN of two, covered by a 1 000 probes and larger than 2 000 Kb, except chromosome X, will be reported.

As the analysis of ROH can be time-consuming, Wierenga et al. (2013) devised an analysis tool, the Genomic Oligonucleotide and SNP Array Evaluation Tool (http://firefly.ccs.miami.edu/cgi-bin/ROH/ROH_analysis_tool.cgi), which searches through OMIM, UCSC and NCBI databases, thus identifying genes and their associated recessive disorders and phenotypes. An Excel report is generated which includes the relevant OMIM genes, and disorder; the coefficient of inbreeding and consanguinity is provided as well. The coefficient of inbreeding measures the possible genetic effects due to homozygosity as a result of „breeding" between related individuals. For example, the coefficient of inbreeding is 1/16 in the offspring of first cousins, with these individuals having an increased risk for autosomal recessive disorders – these homozygous loci are typically found within the ROH (Wierenga et al., 2013). The coefficient of consanguinity measures the probability of an individual having two identical alleles at a particular locus which originate from the same ancestor"s gene – the parents therefore share the same ancestor. All the genes are then individually scrutinized for correlation to the patient"s phenotype.

Chapter 3 Results

3.1 SUMMARY OF ANALYSIS AND FINDINGS

Initial analyses during 2014 revealed large gains and losses in eight patients. However, during a follow-up training period (March – April 2015) at the LDGA in Leiden, The Netherlands, all the samples were reanalysed using the routine procedure in use in that laboratory (see section 2.4) resulting in a further five patients showing small CNVs. A summation of all of the results is provided in Table 8. Further detailed analysis of all patients in whom positive findings had been identified/detected on the arrays are provided in this section. Patients in whom no discernible rearrangements could be identified are further detailed in Appendix VIII.

In 17 of the patients (namely: 2, 3, 4, 5, 6, 7, 12, 13, 14, 15, 16, 17, 21, 22, 25, 26 and 27) only benign CNVs were detected. Seven of the patients had pathogenic CNVs: these were patients 1, 8, 9, 11, 18, 20 and 23. Three of the patients (10, 19 and 24) revealed VOUS.

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray.

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
1	F	46,XX (performed at another laboratory) PARENTS: no abnormalities detected	Not performed	del 9q34 (performed subsequent to this study)	Not performed	Microbrachycephaly, upslanted palpebral fissures, straight eyebrows, strabismus, downturned corners of the mouth, hypotonia, epilepsy, severe DD with no ambulation or speech at the age of 8 years, a small atrial and ventricular septal defect, gastro-oesophageal reflux disease	arr[hg19] 9q34.3(139,135,215-141,020,389)x1	arr[hg19] 9q34.3(139,135,215-141,020,389)x1	1 885	**Deletion**: Kleefstra Syndrome
2	M	46,XY	Not performed	No del/dupl subtel/micro-del	Not performed	DD, dysm, cleft palate, hypotonia, low set posteriorly rotated ears with a prominent crus, flat nasal bridge, a smooth philtrum with downturned corners of the mouth, long palpebral fissures, brachydactyly with one dystropic nail and rockerbottom feet with hallux valgus, overweight, able to sit at 13 months, could not walk or speak by 2 years of age.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
3	M	46,XY	Not performed	Not performed	Not performed	DD, hypotonia, cleft palate, seizures, large posteriorly rotated ears, anteverted nares, large palpebral fissures, a broad nasal root, hypertelorism and micrognathia.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected
4	M	46,XY	Not performed	Not performed	NEG	DD, dysm, brachycephaly, prominent ears, hypertelorism with mild ptosis, intermittent strabismus, a narrow nose, thin lips, smooth philtrum, pointed chin, sacral dimple, clinodactyly, single palmar creases with small nails, ligamentous laxity, *vesicoureteral reflux* (VUR) and hydronephrosis, agenesis of the corpus callosum. His mother has mild ID with similar physical signs.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
5	F	46,XX	Not performed	Not performed	Not performed	DD, ADHD, coarse facies, external eyebrow flare, prognathism, a large mouth with thick lips, no speech.	Arr[hg19](1-22,X)x2	Arr[hg19](1-22,X)x2	-	No abnormality detected
6	M	Not performed	Not performed	Not performed	Not performed	VACTERL association including truncus arteriosus and a horseshoe kidney, no fam hx	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected
7	F	Not performed	Not performed	Not performed	Not performed	Epilepsy, learning disabilities. She has two DD children. Female child: small for age with DD. Younger male child: short stature, ptosis, micropthalmia, downslanting palpebral fissures, strabismus, retrognathia, pointed ears, cryptorchidism, periventricular calcification.	Arr[hg19](1-22,X)x2	Arr[hg19](1-22,X)x2	-	No abnormality detected

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
8	F	46,XX	Not performed	Not performed	Not performed	Severe ID, dysm, colobomatous micropthalmia, microcornea, cataracts, microcephaly, bifrontal narrowing, micrognathia, clenched hands with broad distal phalanges and hypoplastic nails, Agenesis of the corpus callosum (ACC).	arr[hg19] 2q22.2q22.3(143,571,114-145,663,819)x1	arr[hg19] 2q22.2q22.3(143,571,114-145,663,819)x1	2 093	**Deletion:** Mowat-Wilson syndrome
9	M	46,XY	Not performed	No del/dupl subtel/mic-rodel	NEG	GDD, fam hx of ID, psychiatric disease (schizophrenia, depression)	arr[hg19] 1q21.1q21.2(146,101,790-147,897,962)x1	arr[hg19] 1q21.1q21.2(146,101,790-147,897,962)x1	1 796	**Deletion**: 1q21 susceptibility region
10	M	46,XY	DG NEG	No del/dupl subtel	Not performed	ID, dysm, juvenile myoclonic epilepsy, speech and hearing loss, blepharophimosis, broad nasal root with a long nose, a high arched palate, broad thumbs and tapered fingers.	arr[hg19] 1p35.2p35.1(30,476,867-33,054,650)x1	arr[hg19] 1p35.2p35.1(30,476,867-33,054,650)x1	2 578	**Deletion:** VOUS

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
11	F	46,XX	Not performed	No del/dupl subtel	Not performed	Dysm, severe DD, no speech by age 8, macrocephaly, arched eyebrows, strabismus, midface hypoplasia, upturned nares, full lower lip, lifted ear lobules, clinodactyly, broad great toes.	arr[hg19] 5q14.3q21.1(89,738,598-98,856,874)x1	arr[hg19] 5q14.3q21.1(89,738,598-98,856,874)x1	9 118	**Deletion:** VOUS
12	F	46,XX	Not performed	No del/dupl subtel	Not performed	Short stature, macrocephaly, DD, prognathism, widely spaced nipples, maxillary hypoplasia, furled eyebrows, narrow forehead, isolated growth hormone deficiency.	Arr[hg19](1-22,X)x2	Arr[hg19](1-22,X)x2	-	No abnormality detected
13	F	46,XX	Not performed	Not performed	Not performed	Severe DD, short stature, microcephaly, posteriorly rotated ears with unfolded helices, strabismus, shallow orbits, almond-shaped palpebral fissures, a tented upper lip, slightly tapered fingers, 2-3 toe syndactyly	Arr[hg19](1-22,X)x2	Arr[hg19](1-22,X)x2	-	No abnormality detected

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
14	M	46,XY	Not performed	Not performed	Not performed	Dysm, DD, multiple arterial aneurysms, cataract, prematurely aged and wasted appearance. A connective tissue disorder was suspected.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected
15	M	46,XY	Not performed	Not performed	NEG	ASD, no dysm. A brother with autism, 2 paternal nephews with ID. Paternal family history of psychiatric illness and bipolar disorder, maternal fam hx of pregnancy and neonatal loss.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected
16	M	Not performed	Not performed	Not performed	Not performed	ID, dysm, deafness, Duane anomaly (cranial nerve palsies).	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected
17	M	Not performed	Not performed	Not performed	Not performed	ID, deafness, ? dominant anaemia	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
18	F	Not performed	Not performed	Not performed	Not performed	Autosomal recessive deafness, partial sightedness, microcephaly, fam hx of ID.	Arr[hg19](1-22,X)x2	arr[hg19] 15q15.3(43,888,261-43,976,406)x1	88	**Deletion:** VOUS
19	M	Not performed	Not performed	Not performed	Not performed	ID and Variegate Porphyria, consanguineous parents	Arr[hg19](1-22)x2,(XY)x1	arr[hg19] 22q11.21(19,231,636-19,300,915)x1	69	**Deletion:** VOUS
20	F	46,XX	Not performed	Not performed	Not performed	Dysm, DD, epilepsy, microcephaly, midface hypoplasia, low set ears, flat nasal tip, low columella, epicanthic folds, posteriorly rotated ears, wide mouth.	arr[hg19] 4p16.3(68,345-2,172,555)x1	arr[hg19] 4p16.3(68,345-2,172,555)x1	2 104	**Deletion:** Wolf-Hirschhorn syndrome
21	M	46,XY	PWS NEG	Not performed	Not performed	Dysm, DD, almond shaped eyes, hypotelorism, a flat nasal bridge, tapering fingers, genu varum, subependymal grey matter heterotopia, cervical spine asymmetry, suspected mild hemimegal-encephaly.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
22	F	Not performed	Not performed	Not performed	Not performed	ID, midface hypoplasia, hypertelorism, telecanthus, epicanthus, cupped ears with preauricular tags, upslanted palbebral fissures, hypotonia and hypermobility. Fam hx ID: mother, maternal grandmother, great grandmother, maternal cousin with mild ID.	Arr[hg19](1-22,X)x2	Arr[hg19](1-22,X)x2	-	No abnormality detected
23	M	47,XY,+mar	Not performed	Not performed	Not performed	Dysm, growth restriction, unilateral cleft lip and palate, hypertelorism, strabismus, broad nasal root, prominent ears and unilateral camptodactyly of all fingers, congenital heart disease with an unbalanced atrioventricular septal defect and a double outlet right ventricle.	arr[hg19] 9p24.3q13(203,861-68,330,127)x4	arr[hg19] 9p24.3q13(203,861-68,330,127)x4	68 126	Tetrasomy 9p
24	M	46,XY	Not performed	Not performed	Not performed	Learning difficulties, dysm, hypertelorism, epicanthus, broad nose, flat nasal bridge, palmar hyperkeratosis, skin papules, multiple café au lait spots, hypopigmentation. Father and sister have similar dermatological features with no ID, no dysm.	arr[hg19] 13q33.3(109,771,548-110,072,888)x3	arr[hg19] 13q33.3(109,771,548-110,072,888)x3	301	**Duplication:** VOUS

Table 8: Copy number variants detected in this study using the Affymetrix Cytoscan HD microarray (continued).

	SEX	CHROMOSOME ANALYSIS	MICRO-DELETION FISH	MLPA	FRAXA	INITIAL CLINICAL INFORMATION	INITIAL RESULTS	CONFIRMED RESULTS, LEIDEN	SIZE (Kbp)	COMMENT
25	F	46,XX	Not performed	No microdel/ dupl	Not performed	Severe DD, epilepsy, coarse face, prominent ears, retrognathia, scoliosis, bilateral sensorineural hearing loss, microcephaly, low tone at birth with mild spastic cerebral palsy, able to sit at 9 months.	Arr[hg19](1-22,X)x2	Arr[hg19](1-22,X)x2	-	No abnormality detected
26	M	46,XY	Not performed	Not performed	NEG	ID, ASD, no dysm, seizures, absent speech.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected
27	F	46,XX	DG NEG	Not performed	Not performed	DD, moderate ID, dysm, short stature, macrocephaly, cupped pointed ears with unfolded helices, hypertelorism, telecanthus, strabismus, broad nasal root with hypoplastic alae nasi, low columella, microstomia, brachydactyly, brain white matter loss. Mother - mild ID.	Arr[hg19](1-22)x2,(XY)x1	Arr[hg19](1-22)x2,(XY)x1	-	No abnormality detected

Abn = abnormality, DD = developmental delay, Del = deletion, DG = Di George syndrome, Dupl = duplication, Dysm = dysmorphic, Fam hx= family history, Microdel = microdeletion, Misc = miscarriage, NEG = Negative, Pregn = pregnancy, PWS = Prader-Willi syndrome, Subtel = subtelomeric, T13 = trisomy 13, VACTERL = Vertebral anomalies, Anal atresia, Cardiac defects, Tracheoesophageal fistula and/or Oesophageal atresia, Renal and Radial anomalies and Limb defects, VOUS = Variant of Unknown Significance

3.2 PATIENT 1

3.2.1 Clinical features

A two-year old female patient presented with dysmorphic features and DD. Antenatally, intra-uterine growth restriction (IUGR) and polyhydramnios were detected. She was born during the third trimester with a birth weight of 2.4 kg, a head circumference of 31 cm and had to be ventilated for respiratory distress. This patient has the following clinical features: normal height and weight for age, microbrachycephaly, upslanted palpebral fissures, straight eyebrows, strabismus, pixie ears, a low nasal bridge with upturned nares and downturned corners of the mouth. She has clinodactyly with single palmar creases. Neurological features include hypotonia and epilepsy and she has severe DD with no ambulation or speech at the age of eight years. She has small atrial and ventricular septal defects and gastro-oesophageal reflux disease with recurrent chest infections. She had middle ear disease requiring ventilation tubes. Kleefstra syndrome was suspected.

3.2.2 Genetic testing

This patient had a normal karyotype done at another laboratory. Both parents had a normal karyotype. No microdeletion FISH studies were performed. Subtelomeric MLPA analysis identified a deletion at the 9q34.3 telomeric region targeted by the *EHMT1* gene probes with MLPA Analysis. FRAXA analysis was not performed.

3.2.3 CMA Analysis

CMA testing showed a submicroscopic telomeric deletion of 1.8 Mb on chromosome 9 at band q34.3 as shown in the Karyoview (Figure 5) and the detail view as depicted in Figure 6. This region was covered by 1 454 markers.

arr[hg19] 9q34.3(139,135,215-141,020,389)x1

3.2.3.1 Karyoview

Figure 5 Karyoview of Patient 1: a terminal deletion of 1.88 Mb was detected on the long arm of chromosome 9, as shown in the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

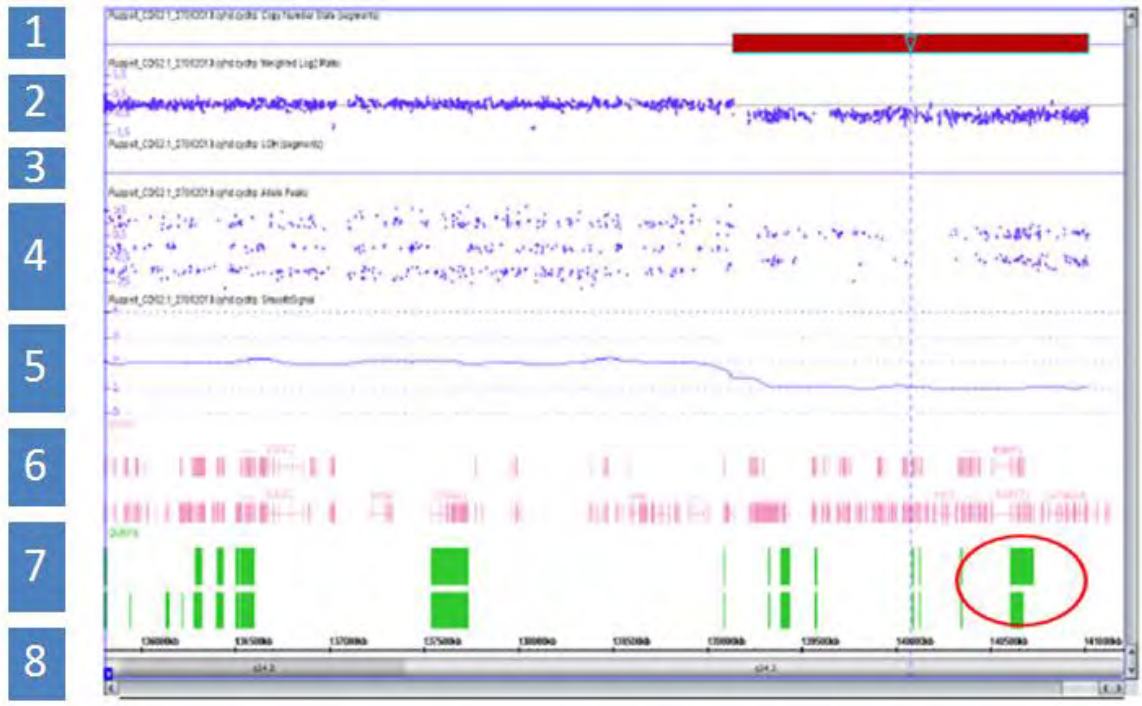


Figure 6 Detail view of Chromosome 9 of Patient 1 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) LOH, 4) allele peak deletion demonstrating the SNP markers in this region, 5) the extent of the deletion in the Smooth Signal, 6) RefSeq genes (Pink) in this region, 7) OMIM disease genes (Green), 8) coordinates and chromosome bands.

3.2.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 7) and DECIPHER (Figure 8) Genome Browsers. A deletion of 1.8 Mb in size was noted on chromosome 9 in the recurrent 9q34 microdeletion region. A total of 87 genes are included in this region.

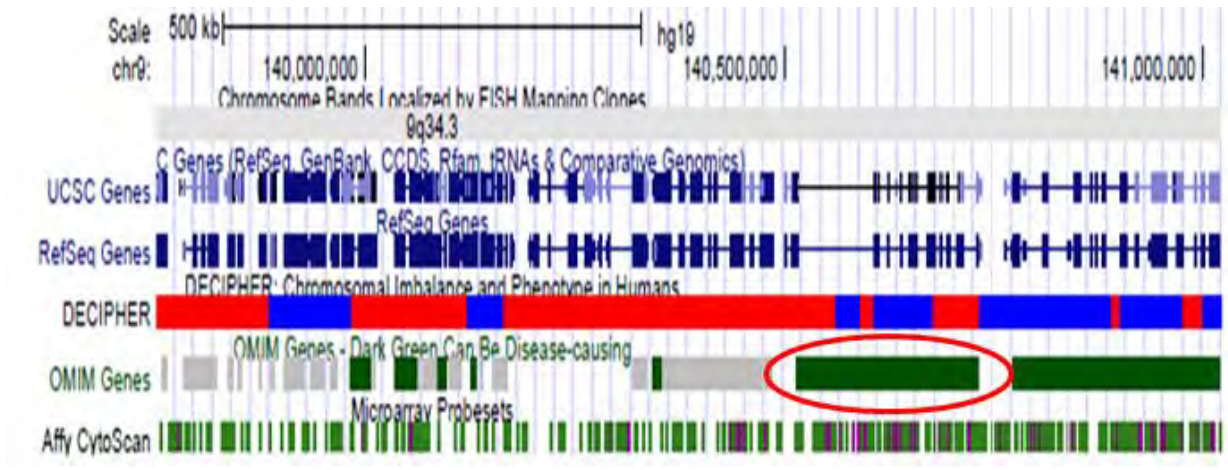


Figure 7 UCSC Browser of chromosome 9 of patient 1 (disease genes are represented in dark green, deletions in red and duplications in blue): The *EHMT1* (Euchromatic Histone Methyltransferase 1, OMIM*607001) disease-causing gene is located in the 9q34.3 region (ringed in red).

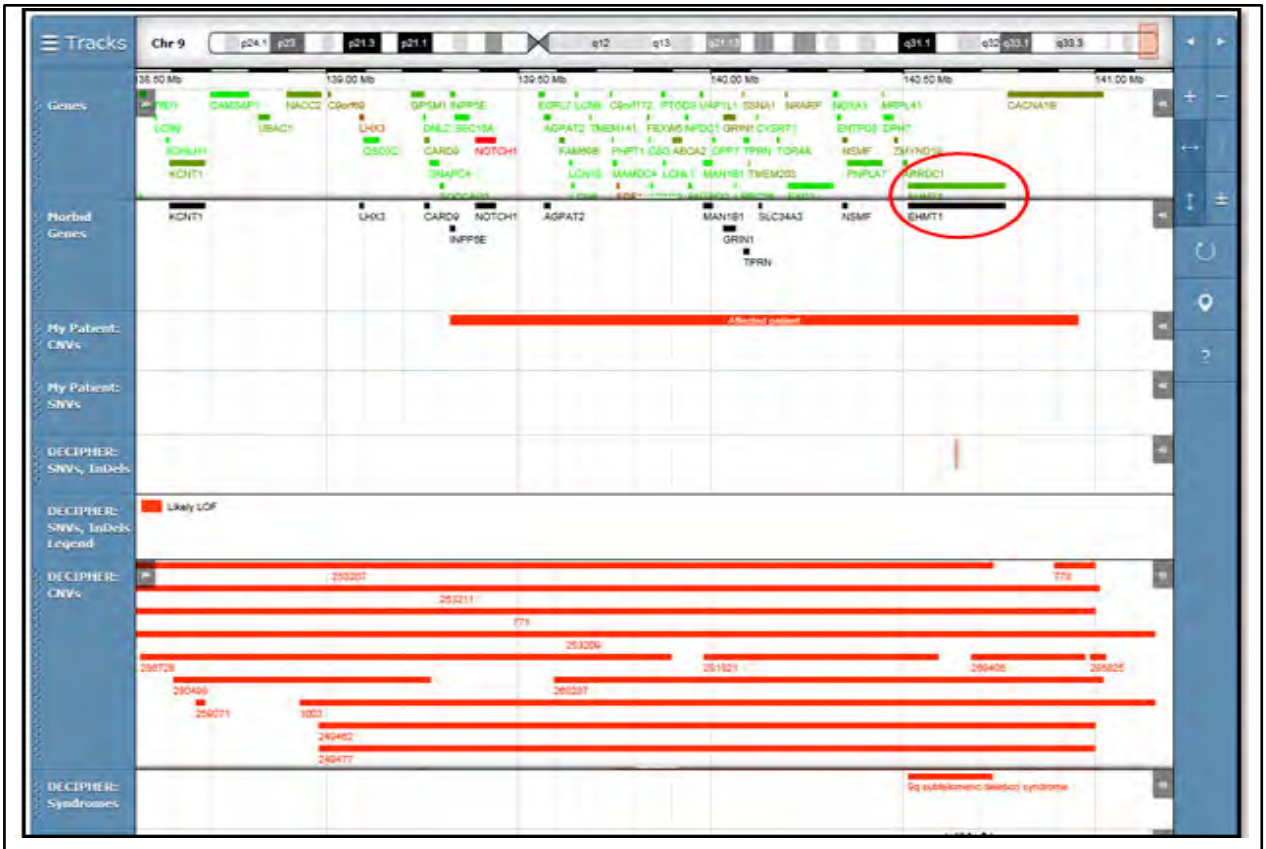


Figure 8 The 9q deletion region (DECIPHER) - the largest morbid gene in this region is *EHMT1* (ringed in red) which overlaps with the 9q subtelomeric deletion syndrome.

3.2.4 Review

Although a large number of genes (87) were involved in this 9q34 deletion region only phenotypically relevant genes were discussed in this patient, which consisted of *EHMT1, CACNA1B, ARRDC1, NOTCH1, TRAF2, COBRA1, NELF, ZMYND19* and *ARRDC1*.

EHMT1 (Euchromatic Histone Methyltransferase 1, OMIM*607001), (Genomic coordinates (GRCh37): *9:140,513,443 - 140,730,578)* is the second gene from the terminal end of the long arm of chromosome 9 and is associated with Kleefstra syndrome. Kleefstra syndrome is a recognized ID syndrome. Any rearrangement, deletions (interstitial or complete) and point mutations (for example nonsense or frameshift mutations) of *EHMT1* results in Kleefstra syndrome (Kleefstra et al., 2012). Deletions account for 75% and point mutations account for 25% of cases of Kleefstra syndrome (Kleefstra et al., 2012). The *EHMT1* gene contains 28 exons with the initiation ATG codon occurring in exon 1 (Kleefstra et al., 2012). Haploinsufficiency of the *EHMT1* gene has been proven to be the basis of the 9q subtelomeric deletion syndrome (Kleefstra et al., 2006). Kleefstra et al. (2006) originally suggested the terminal deletion of chromosome 9q34.3 is a relatively common occurrence at approximately 6% of all subtelomeric deletions in their study.

The core clinical features seen in Kleefstra syndrome include severe ID without speech development, hypotonia, and characteristic facial features which include microcephaly, brachycephaly, hypertelorism, synophrys, midface hypoplasia, protruding tongue, eversion of the lower lip, and prognathism. Seizures are also a feature of this syndrome in about 30% of cases and these can include tonic-clonic seizures, absence seizures, and complex partial epilepsy (Kleefstra et al., 2009, Verhoeven et al., 2011; Willemsen et al., 2011).

Yatsenko et al. (2009) suggested the acronym „CHOMS" for the characteristic clinical features of „craniofacial features, hypotonia, childhood obesity, microcephaly and substantial speech delay and mental retardation". Kleefstra et al. (2006) did not agree with this acronym for patients with a deletion encompassing only the *EHMT1* and *CACNA1B* genes. Childhood obesity is common in Kleefstra syndrome. Motor function is usually delayed with children starting to walk between the ages of 2 and 3 years. Patients may have congenital heart defects (50%) which include atrial or ventricular septal defects, tetralogy of Fallot, aortic coarctation, bicuspid aortic valve, and pulmonic stenosis (Kleefstra et al., 2012). Further features include micropenis (30%), cryptorchidism, vesicoureteral reflux, tracheo-/bronchomalacia, and gastroesophageal reflux – these are, however, less frequent. Brain abnormalities can be seen in some individuals. Behavioural changes may be observed during adolescence including apathy, aggressive periods, psychosis, autistic features,

catatonia, bipolar mood disorder, and regression in daily function and cognitive abilities (Verhoeven et al., 2011).

No genotype/phenotype association has been found due to the size of the deletion or between patients with deletions as compared to patients with point mutations (Kleefstra et al., 2009). The Affymetrix 500 K SNP array (Affymetrix, Santa Clara, CA) and a customised oligonucleotide array, EmArrayCyto6000_version2, FISH, MLPA and direct sequencing of the *EHMT1* gene were used to detect these deletions and mutations. However, Kleefstra et al. (2012) reported that patients with larger genomic deletions, that is greater than or equal to three Mb at 9q34, seem to experience more severe pulmonary infections and aspiration problems when compared to patients with smaller deletions or defects in *EHMT1* only. The extent and severity of clinical findings vary among individuals although penetrance seems to be 100%. There is a worldwide prevalence of this syndrome across all ethnic groups. Males and females are equally affected (Kleefstra et al., 2012). The majority of cases are de novo mutations. Willemsen et al. (2011) did, however, report on two unrelated families where the mothers carried deletions of the 9q34.3 region, with only mild learning difficulties and minor facial features, which were passed on to their affected offspring. Both mothers were found to have somatic mosaicism, detected using the Affymetrix SNP 6.0 (Affymetrix, Santa Clara, CA) in the one instance and the Agilent 105 K (Agilent Technologies, Palo Alto, CA) in the other.

Verhoeven et al. (2011) reviewed three female patients with Kleefstra syndrome. Two of these patients had a heterozygous intragenic loss-of-function mutation in the *EHMT1* gene and the other patient had a heterozygous 9q34.3 microdeletion, which included the *EHMT1* gene. All three were severely disabled and had features of neurodegenerative or regressive neurologic processes. Yatsenko et al. (2009) compared the genotype-phenotype data of 15 patients using the 44 K Agilent array (Agilent Technologies, Inc., Santa Clara, CA). They were able to define four distinct categories according to genomic rearrangements: terminal deletions (50%), interstitial deletions (25%), derivative chromosomes and complex rearrangements (25%). The distinctive characteristic features of this syndrome were present in all the patients and could not be correlated with deletion size. They were able to describe a „minimal critical region" of ~700 Kb. Two genes, *EHMT1* and *CACNA1B*, overlap in this region which extends proximally from *ARRDC1*. Both these genes are highly expressed in the brain. The authors hypothesized that both genes are dosage-sensitive and may contribute to this syndrome. This region of overlap seems to correlate with the food seeking behaviour and obesity phenotype. The core clinical features, craniofacial dysmorphism, obesity, hypotonia, microcephaly and speech impairment, map to this minimal critical region. Larger deletions are seen in patients with cardiac defects, seizures, limb and brain

abnormalities, recurrent respiratory infections, hypothyroidism and abnormal genitalia. These abnormalities can be mapped to six critical genes/regions – *NOTCH1, TRAF2, COBRA1, NELF, ZMYND19* and *ARRDC1*. Patients with deletions larger than 1.4 Mb had features of prenatal growth retardation and frequent upper respiratory and ear infections. These deletions involved *TRAF2*. The *NELF* gene is mapped to the deleted region in patients with genital abnormalities. The most severe phenotype was noted in patients with a deletion larger than 1.6 Mb involving the *NOTCH1* gene. Yatsenko et al. (2009) suggest the 9q34.3 microdeletion syndrome to be a contiguous gene syndrome. Consequently, the size of the deletion gives rise to the complexity of the phenotype. The deletion in patient 1 is larger than 1.6 Mb (1.8 Mb) and includes all of these six critical genes as shown in see Figure 9.

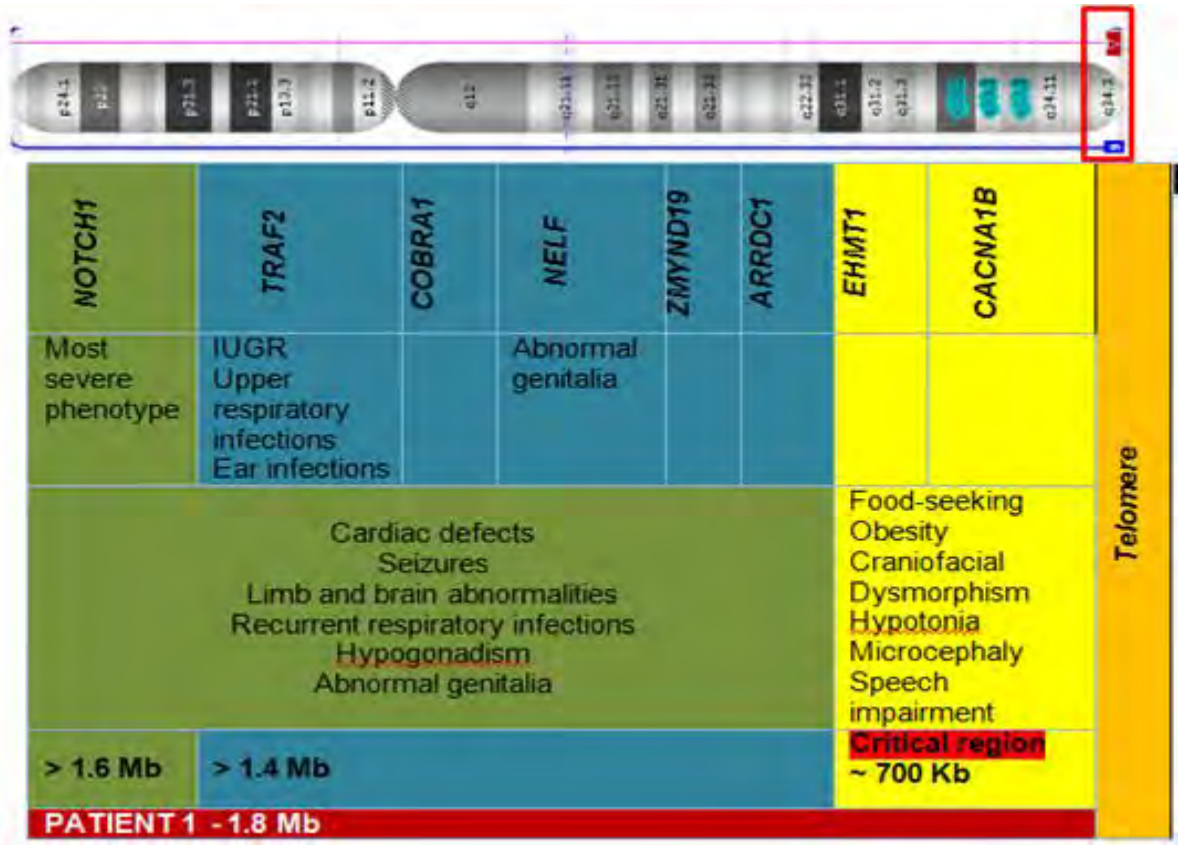


Figure 9 A depiction of the gene positions and the phenotypic effect in patients with Kleefstra syndrome: Orange – 9q telomeric region, Yellow – the critical region which includes the *EHMT1* and *CACNA1B* genes (~700 Kb in size), Blue – the 1.4 – 1.6 Mb deletion and Green – 1.6 Mb and larger deletions which are the most severe.

3.2.5 Clinical correlation

As discussed, several of the clinical features in this patient correlate with the features of Kleefstra syndrome. The main features being: IUGR, Atrial Septal Defect/Ventricular Septal Defect (ASD/VSD), brachycephaly and microcephaly, hypotonia, recurrent infections, gastro-

oesophageal reflux disease, epilepsy and severe DD, not walking or talking by the age of eight years. The deletion in this patient correlates with the most severe form of the syndrome, as it is larger than 1.6 Mb in size.

3.2.6 Conclusion

This patient"s result is consistent with a diagnosis of Kleefstra Syndrome. The large deletion involving additional genes may explain the severity of her phenotype.

3.3 PATIENT 8

3.3.1 Clinical features

This female patient presented with severe ID, dysmorphism and opthalmological abnormalities including colobomatous micropthalmia, microcornea and cataract. Further dysmorphic features included microcephaly, bifrontal narrowing, small dysplastic ears, a flat nasal bridge with low columella, micrognathia and clenched hands with broad distal phalanges and hypoplastic nails and broad great toes and thumbs. Antenatally, polyhydramnios was detected and dysmorphism was suspected. She was born with a birth weight of 3.5 kg and a head circumference of 36 cm. Brain CT scan revealed agenesis of the corpus callosum (ACC). Renal ultrasound was normal. There is a maternal niece with learning difficulties.

3.3.2 Genetic testing

Chromosome analysis demonstrated a normal female karyotype. No microdeletion FISH studies were performed. MLPA Analysis was not performed. FRAXA analysis was not performed.

3.3.3 CMA Analysis

CMA testing showed a submicroscopic interstitial deletion of 2.1 Mb on chromosome 2 at band q22.2 to q22.3 as shown in the Karyoview (Figure 10) and detail view depicted in Figure 11. This region is covered by 2 019 markers.

arr[hg19] 2q22.2q22.3(143,571,114-145,663,819)x1

3.3.3.1 Karyoview

Figure 10 Karyoview of Patient 8: an interstitial deletion of 2.09 Mb was detected on the long arm of chromosome 2 as indicated in the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

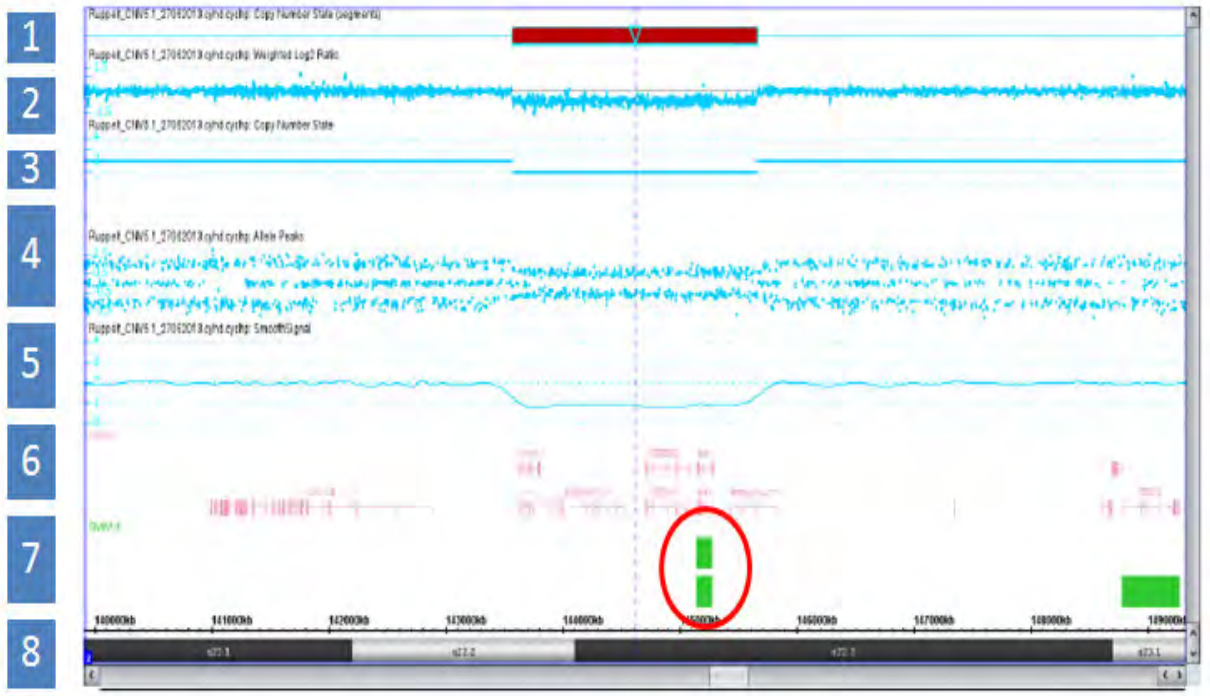


Figure 11 Detail view of Chromosome 2 of Patient 8 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) LOH, 4) allele peak deletion demonstrating the SNP markers in this region, 5) the extent of the deletion in the Smooth Signal, 6) RefSeq genes (Pink) in this region, 7) OMIM disease genes (Green), 8) coordinates and chromosome bands.

3.3.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 12) and DECIPHER (Figure 13) Genome Browsers. A deletion of 2.1 Mb in size was noted on chromosome 2 which spans the recurrent 2q22 microdeletion region. Four genes are included in this region: *KYNU, ARHGAP15, GTDC1* and *ZEB2. ZEB2* is the suspected morbid gene in this deletion region.

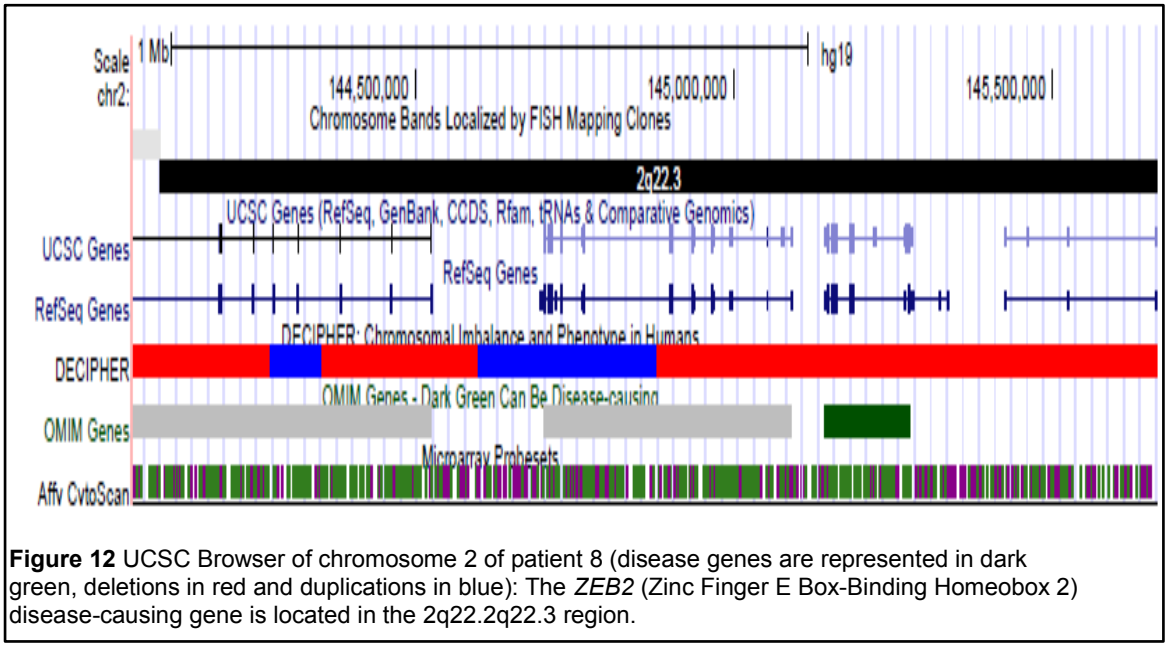


Figure 12 UCSC Browser of chromosome 2 of patient 8 (disease genes are represented in dark green, deletions in red and duplications in blue): The *ZEB2* (Zinc Finger E Box-Binding Homeobox 2) disease-causing gene is located in the 2q22.2q22.3 region.

Figure 13 The 2q22 deletion region - the morbid gene in this region is ZEB2 (as depicted in DECIPHER), ringed in black.

3.3.4 Review

ZEB2 (Zinc Finger E Box-Binding Homeobox 2, OMIM*605802) has genomic coordinates at *(GRCh37): 2:145,141,941 - 145,277,957*), and has alternative titles and symbols: *ZFHX1b* (Zinc Finger Homeobox 1b), *SMADIP1* (Smad interacting protein 1) and *SIP1* (Smad interacting protein 1). This gene consists of ten exons and nine introns and spans about 70Kb of DNA. *ZEB2* mRNA is expressed in nearly all tissues and it encodes the protein product, SIP1. A deletion of this gene was first described by Mowat et al. (1998) in six patients, who presented with ID and were characterised by specific facial features. Four of the patients also presented with Hirschsprung disease. This syndrome was named Mowat-Wilson syndrome (MWS). MWS has been reported in patients from Europe, Australia, the USA and Korea with the size of deletion varying from 300 Kb to 11 Mb (Park et al., 2013). Park et al. (2013) supported the *ZEB2* haplo-insufficiency hypothesis as the cause of MWS rather than this being a contiguous gene syndrome.

Espinosa-Parrilla et al. (2002) reported on the *ZEB2* gene in the neural retina and expression in the anterior epithelium of the lens, in a patient with clinical features of strabismus. By 2003, 45 patients had been reported with either a de novo deletion or intragenic heterozygous mutations of the *ZEB2* gene (Mowat et al., 2003). The male to female ratio of MWS was 31/14. Garavelli & Mainhardi (2007) also reported an increased male to female ratio of 1,42:1 and a prevalence across ethnic groups. All 45 patients reported by Mowat et al. (2003) had the typical facies and moderate to severe ID, with seizures or an abnormal electroencephalogram (EEG) in 90% of the patients and microcephaly in 84% of the patients. Milestones were delayed with children walking at about four years of age while a number of patients did not achieve walking. Speech was absent or consisted of a few words, 50% of the patients had short stature and 47% had a congenital heart anomaly. Interestingly, 62% of patients had Hirschsprung disease although this may have been underdiagnosed. ACC and renal anomalies were seen in 42% and 38% of patients, respectively. Male genitourinary anomalies were reported in 60% of patients.

The distinctive facies in MWS change from infancy through childhood, adolescence and adulthood (Mowat et al., 2003). In infancy, the patient generally presents with a prominent chin which appears narrow and triangular resulting in an angular shaped face, hypertelorism, deep set large eyes, broad nasal bridge, saddle nose, prominent rounded nasal tip, open mouth, full or everted lower lip, posteriorly rotated ears which are uplifted with a central depression. In childhood, the face elongates with the chin being more prominent. These patients often have wide, horizontal eyebrows. The upper lip often has an „M" shape tapering to the sides. Prognathism with a long chin is evident in adulthood. The nasal tip overhangs the philtrum. It was subsequently recognised that this condition can occur with or without Hirschsprung disease in the presence of the distinctive syndromic features (Wilson et al., 2003).

Dastot-Le Moal et al. (2007) discussed different mutations found in patients with MWS. These ranged from whole gene deletions to truncating mutations (nonsense or frameshift). The authors postulated that haploinsufficiency of the *ZEB2* was responsible for MWS. Even though more than 100 mutations of *ZEB2* had been identified by 2007, no clear genotype-phenotype correlation could be established for the less common abnormalities. The disparity in size and breakpoints of the reported deletions is also not consistent with a specific phenotype. Dastot-Le Moal et al. (2007) further suggested that some, for example ocular abnormalities are underreported. Tantales et al. (2013) reported on two Cypriot patients with structural ocular abnormalities: iris and chorioretinal colobomata and partial aniridia. One patient had an exonic deletion and the other had a point mutation. The authors suggest that structural ocular abnormalities be included in the MWS features even though the pathogenesis is unclear. They also proposed that ocular abnormalities are underreported in MWS. In addition, during their literature review they found the following eye abnormalities: iris, choroidal, chorioretinal and optic disc colobomata, microphthalmia, an Axenfeld anomaly, strasbismus, cataracts and ptosis.

Zweier et al. (2005) recommended the inclusion of structural eye anomalies in the clinical features of MWS. In their study of 28 patients, 14.3% were found to have eye abnormalities. These most commonly included microphthalmia, with iris coloboma and cataract in one patient and an Axenfeld anomaly in another patient. They also reported that many patients have a strabismus even though this is not necessarily reported by parents. Ariss et al. (2012) did a literature review of 170 MWS cases of which seven had an eye abnormality. The abnormalities included microphthalmia iris, retinal and optic disc colobomata, cataracts, ptosis, strabismus, iris heterochromia and an Axenfeld anomaly. The authors further reported on a nine month old female patient who had no light perception, microphthalmia (right eye), optic nerve hypoplasia (left eye), severe optic nerve pallor, retina and choroid colobomas, extensive chorioretinal and retinal pigment epithelium atrophy and extensive retinal atrophy. The right eye had extensive lens opacities and korectopia. This patient was found to have a deletion mutation. The authors postulated the ZEB2/SIP1 protein is implicated in neural patterning, and mutations affecting this gene result in ocular abnormalities. Ariss et al. (2012) concurred with Dastot-Le Moal et al. (2007) and Zweier et al. (2005) that eye abnormalities were underreported in MWS. Espinosa-Parrilla et al. (2002) demonstrated *ZEB2* gene expression in human embryos in the eye, specifically in the neural retina and the anterior epithelium of the lens, indicating a pleiotropic role in embryogenesis. Bassez et al. (2004) postulated that MWS does not fit the contiguous gene syndrome hypothesis as the phenotype may vary significantly from patient to patient.

3.3.5 Clinical correlation

This patient has severe ID and GDD, microcephaly, bifrontal narrowing, a flat nasal bridge with low columella, and agenesis of the corpus callosum which correlates with features described in MWS. She has severe abnormalities of the eyes including cataracts, coloboma and micropthalmia, resulting in blindness. All of these clinical features have been reported in MWS. This patient did not, however, have some of the other well-described features of MWS such as seizures detected in a large number of MWS patients [90% described by Mowat et al. (2003)], Hirschsprung disease or cardiac abnormalities.

3.3.6 Conclusion

This patient"s phenotype is consistent with the diagnosis of MWS. The deletion encompassing the *ZEB2* gene contributes to the clinical presentation in this child.

3.4 PATIENT 9

3.4.1 Clinical features

A male patient presented with GDD, and a family history of ID and psychiatric disease (schizophrenia, depression).

3.4.2 Genetic testing

Chromosome analysis demonstrated a normal male karyotype. No microdeletion FISH studies were performed. No chromosomal microdeletion/duplication was identified with MLPA Analysis. No expansion mutation was detected on FRAXA analysis.

3.4.3 CMA Analysis

CMA testing revealed a submicroscopic interstitial deletion of 1.8 Mb on chromosome 1 at band q21.1 to q21.2 as shown in the Karyoview (Figure 14) and the detail view as depicted in Figure 15. This region is covered by 1 358 markers.

arr[hg19] 1q21.1q21.2(146,101,790-147,897,962)x1

3.4.3.1 Karyoview

Figure 14 Karyoview of Patient 9: an interstitial deletion of 1.8 Mb was detected on the long arm of chromosome 1 as indicated in the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

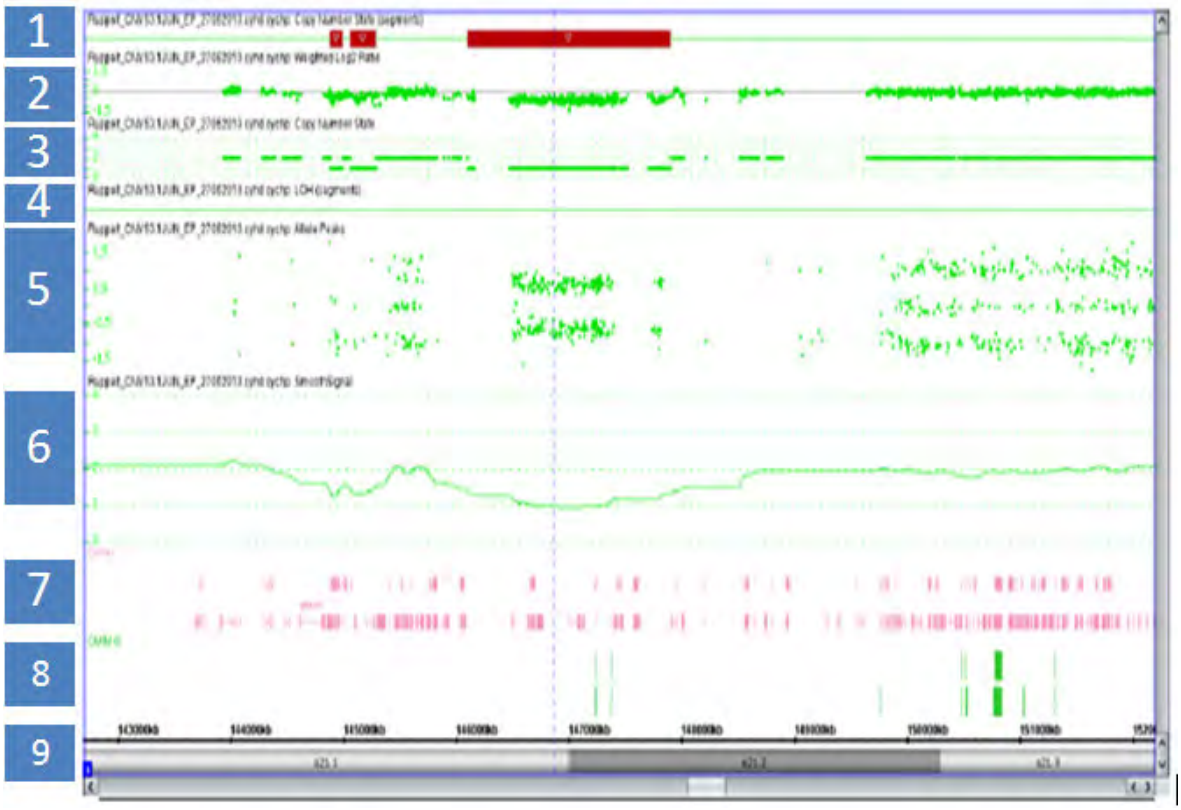


Figure 15 Detail view of Chromosome 1 of Patient 9 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) the copy number state, 4) LOH, 5) allele peak deletion demonstrating the SNP markers in this region, 6) the extent of the deletion in the Smooth Signal, 7) RefSeq genes (Pink) in this region, 8) OMIM disease genes (Green), 9) coordinates and chromosome bands

3.4.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 16) and DECIPHER (Figure 17) Genome Browsers. A deletion of 1.8 Mb was noted on chromosome 1 which spans the recurrent 1q21.1 microdeletion region. A total of 14 genes are included in this region.

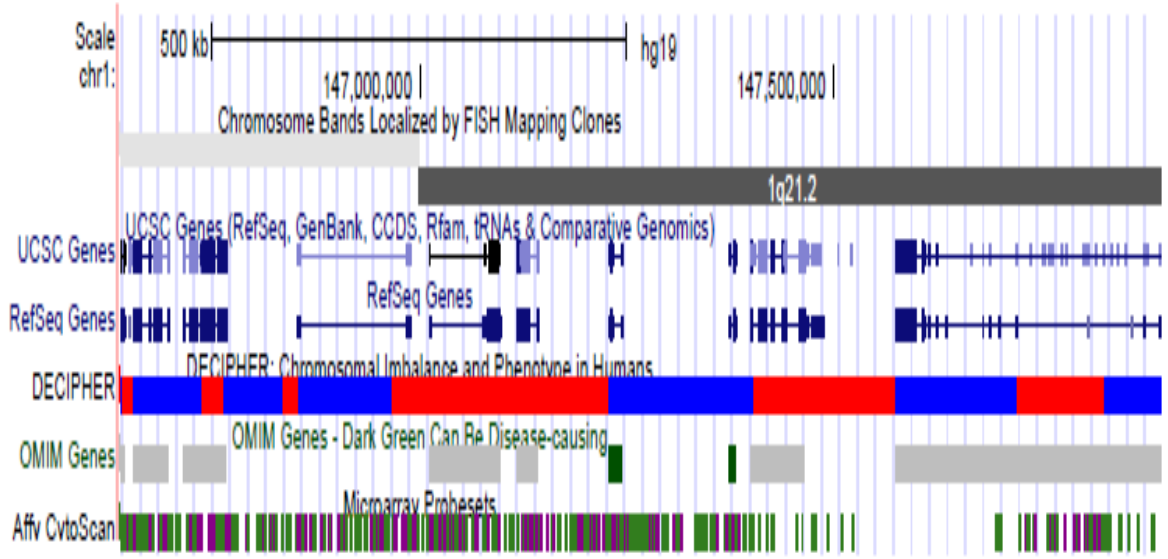


Figure 16 UCSC Browser of chromosome 1 of patient 9 (disease genes are represented in dark green, deletions in red and duplications in blue).

Figure 17 Illustration of the1q21.1 deletion region containing the *GJA8* Morbid gene (DECIPHER)

3.4.4 Review

The location of the recurrent 1q21.1 microdeletion region is 146,533,376-147,883,376 on the distal chromosome 1q21.1 deletion region, and measures approximately 1.35 Mb (Stefansson et al., 2008). The deletion in patient 9 (146,101,790-147,897,962) spans this entire deletion region, and is larger in size (1.8 Mb). Stefansson et al. (2008) described the prevalence of this deletion to be approximately 11 in 4 718 (0.23%) in patients with DD, ID and/or congenital abnormalities, and most of whom had schizophrenia or related neuropsychiatric disorders. The authors reported that the *GJA8* gene is associated with schizophrenia. Brunetti-Pierri et al. (2008) identified 27 patients with this microdeletion using targeted BAC aCGH. The clinical features noted were facial dysmorphism such as frontal bossing, deep-set eyes and a bulbous nose. DD and/or learning disabilities were noted in most cases. Behavioural abnormalities for example ADHD, autism, anxiety/depression, antisocial behaviour and aggression were observed in some cases. Another finding was that some unaffected parents carried the same CNV and the authors speculated that this may be due to these CNVs being benign or pathogenic with incomplete penetrance. However, this deletion was not found in large population studies and is seen more commonly in patients referred for CMA, and is therefore unlikely to be benign. Mefford et al. (2008) showed that the clinical features in patients with 1q21.1 deletions are variable. They identified CNVs in 25 patients with features of mild to moderate ID, microcephaly, cardiac abnormalities and cataracts using BAC aCGH, a customised NimbleGen Systems oligonucleotide array and the HumanHap 300, the HumanHap 550 and HumanHap 650Y Beadchips (Illumina) to assess the patients.

Rodriquez-Murillo et al. (2012) reported in their review article that deletions of 1q21.1 have been reported in other neurodevelopmental and neurological disorders for example ID, autism and seizure disorders. More recently, Luo et al. (2014) identified CNVs which affect genes which are implicated in schizophrenia. Eight genes were identified of which three are within the 1q21.1 microdeletion susceptibility region: *BCL9, GJA5, GJA8.* These genes are expressed in the central nervous system.

3.4.5 Clinical correlation

Unfortunately there is limited clinical information available for patient 9 but GDD is consistent with the finding that most patients with this deletion have ID. The family history of ID and psychiatric illness including schizophrenia would suggest that the deletion may be present in other family members.

3.4.6 Conclusion

In light of this patient"s result and the strong family history of psychiatric disease a diagnosis of the 1q21.1 recurrent microdeletion (susceptibility locus for neurodevelopmental disorders) is most likely the cause of the phenotype. It is recommended that family studies should be performed.

3.5 PATIENT 10

3.5.1 Clinical features

This 12-year old male patient presented with ID, juvenile myoclonic epilepsy, speech delay and hearing loss and dysmorphic features. The dysmorphic features included blepharophimosis, a broad nasal root with a long nose, a high arched palate, broad thumbs and tapered fingers. There is no family history of neurodevelopmental disorders or epilepsy. This patient had a birth weight of 2.8 kg with a height on the 50th percentile and head circumference on the 25th percentile.

3.5.2 Genetic testing

This patient had a normal karyotype. Microdeletion FISH for Di George syndrome was negative. No microdeletion or duplication was noted using subtelomeric MLPA analysis. FRAXA analysis was not performed.

3.5.3 CMA Analysis

CMA testing showed a submicroscopic interstitial deletion of 2.6 Mb on chromosome 1 at band p35.2 as shown in the Karyoview (Figure 18) and the detail view depicted in Figure 19. This region is covered by 1 934 markers.

arr[hg19] 1p35.2p35.1(30,476,867-33,054,650)x1

3.5.3.1 Karyoview

Figure 18 Karyoview of Patient 10: an interstitial deletion of 2.5 Mb was detected on the short arm of chromosome 1 as indicated by the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row.) The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

Figure 19 Detail view of Chromosome 1 of Patient 10 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) LOH, 4) allele peak deletion demonstrating the SNP markers in this region, 5) the extent of the deletion in the Smooth Signal, 6) RefSeq genes (Pink) in this region, 7) OMIM disease genes (Green), 8) coordinates and chromosome bands

3.5.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 20) and DECIPHER (Figure 21) Genome Browsers. A deletion of 2.5 Mb was noted on chromosome 1. A total of 44 genes are included in this region of which *EPB41, CCDC28B* and *LCK* are the reported morbid genes.

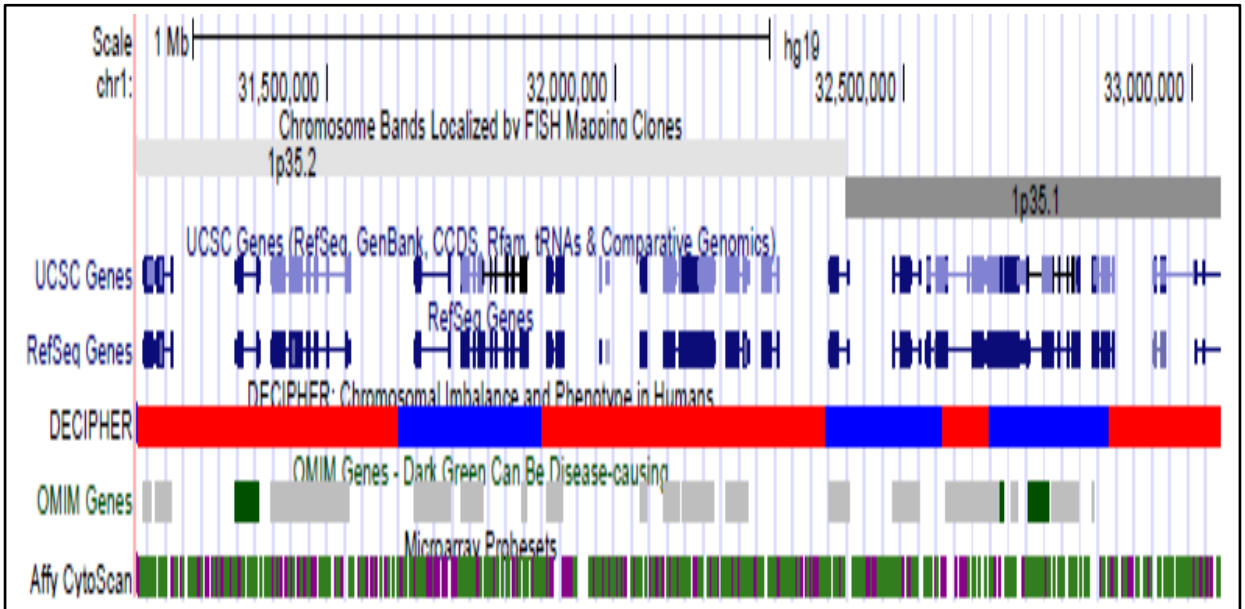


Figure 20 UCSC Browser of chromosome 1 of patient 10 (disease genes are in dark green, deletions in red and duplications in blue): *EPB41* (Erythrocyte Membrane Protein Band 4.1, OMIM*130500), *CCDC28B* (Coiled-Coil domain-Containing Protein 28B, OMIM*610162) and *LCK* (Lymphocyte-Specific Protein-Tyrosine Kinase*153390) are morbid genes in this region.

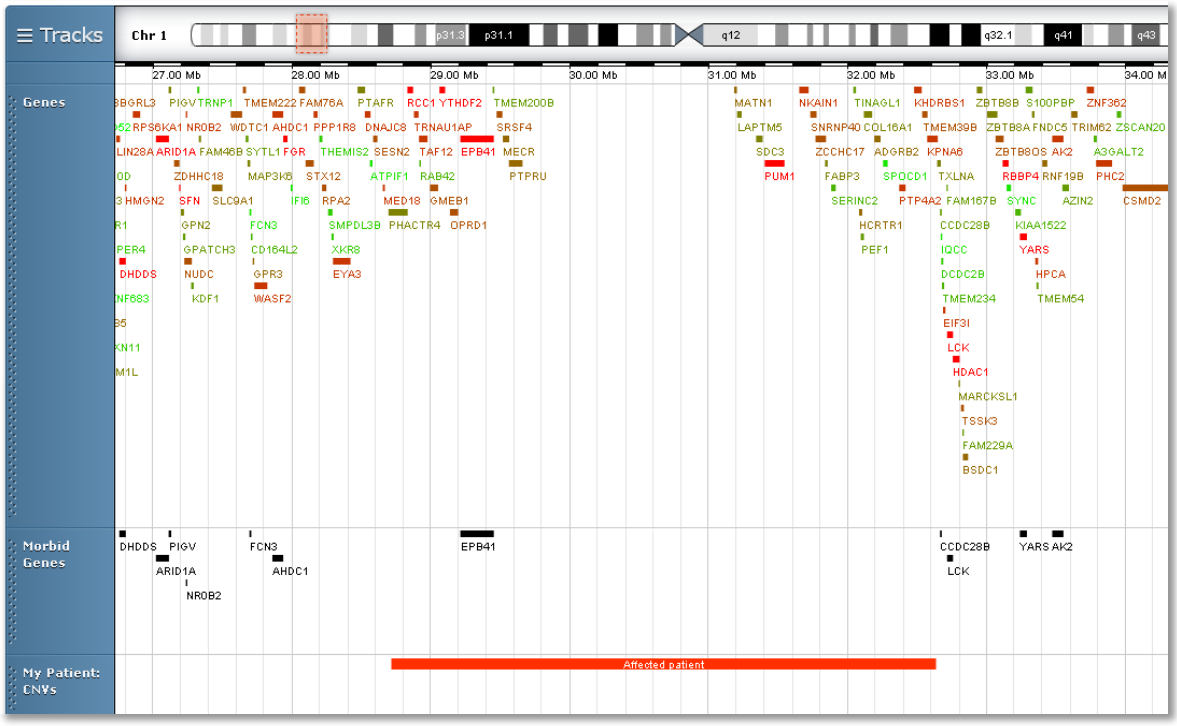


Figure 21 The 1p35.1 deletion region - the largest morbid gene in this region is the *EPB41* gene (DECIPHER).

3.5.4 Review

EPB41 (Erythrocyte Membrane Protein Band 4.1, OMIM*130500), *CCDC28B* (Coiled-Coil domain-Containing Protein 28B, OMIM*610162) and *LCK* (Lymphocyte-Specific Protein-Tyrosine Kinase*153390) are morbid genes in this region, as seen in Figure 4 (DECIPHER). These genes have not previously been reported to be associated with any phenotype relevant to this patient.

The DGV did not report a CNV in this region. However, during a DECIPHER database search, this patient"s deletion corresponded with a previously reported patient (258365). The reported patient"s phenotype included behavioural/psychiatric abnormality, cryptorchidism, hypertelorism, ID, slender build, tapered finger and widely spaced teeth. A Pubmed search did not yield any articles relevant to this region.

3.5.5 Clinical correlation

Two clinical features overlapped between this patient and the patient described in DECIPHER: ID and tapered fingers.

3.5.6 Conclusion

Although no previously reported disease-causing genes could be identified in this region, the size of the deletion (2.5 Mb) should be considered significant. This variant is classified as a VOUS at the time of reporting. Parental studies may be useful to further elucidate this finding.

3.6 PATIENT 11

3.6.1 Clinical features

A female patient presented with dysmorphism and severe DD with no speech at the age of eight years. There is a background history of early failure to thrive and gastroesophageal reflux. Dysmorphic features included a relative macrocephaly, arched eyebrows and strabismus with midface hypoplasia. She has upturned nares with a full lower lip and lifted ear lobules. Skeletal features include clinodactyly and broad great toes. Central nervous system (CNS) imaging was done, however no definite abnormality was reported.

3.6.2 Genetic testing

Chromosome analysis demonstrated a normal karyotype. No microdeletion FISH studies were performed. No subtelomeric deletions or duplications were detected on MLPA Analysis. FRAXA analysis was not performed.

3.6.3 CMA Analysis

CMA testing revealed a submicroscopic interstitial deletion of 9.1 Mb on chromosome 5 at band q14.3 to q21.1 as shown in the Karyoview (Figure 22) and the detail view in Figure 23. This region is covered by 7 092 markers.

arr[hg19] 5q14.3q21.1(89,738,598-98,856,874)x1

3.6.3.1 Karyoview

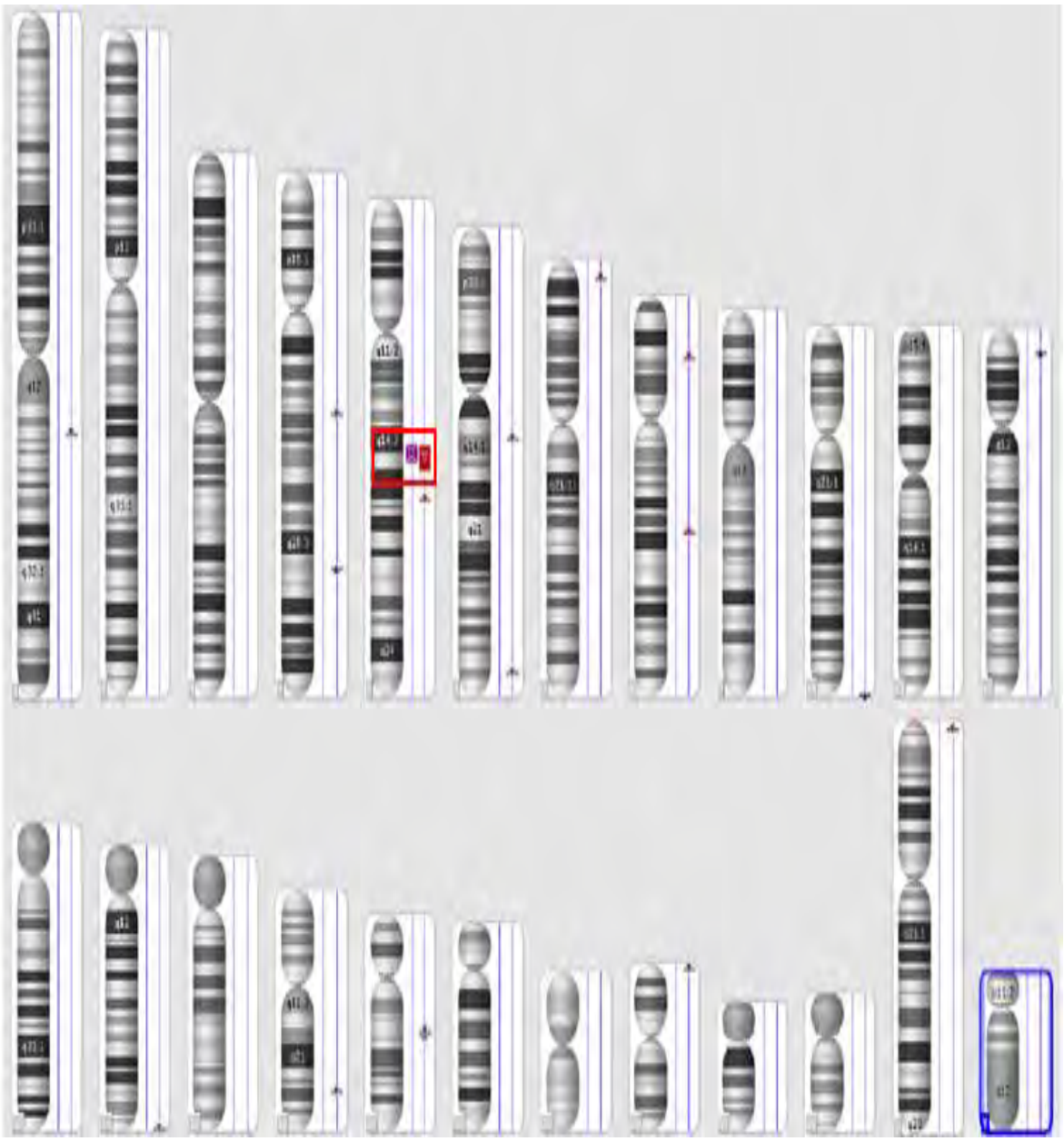


Figure 22 Karyoview of Patient 11: an interstitial deletion (red) of 9.1 Mb was detected on the long arm of chromosome 5 as indicated by the red square. An artefactual region of homozygosity (purple) was also reported in the analysis software. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

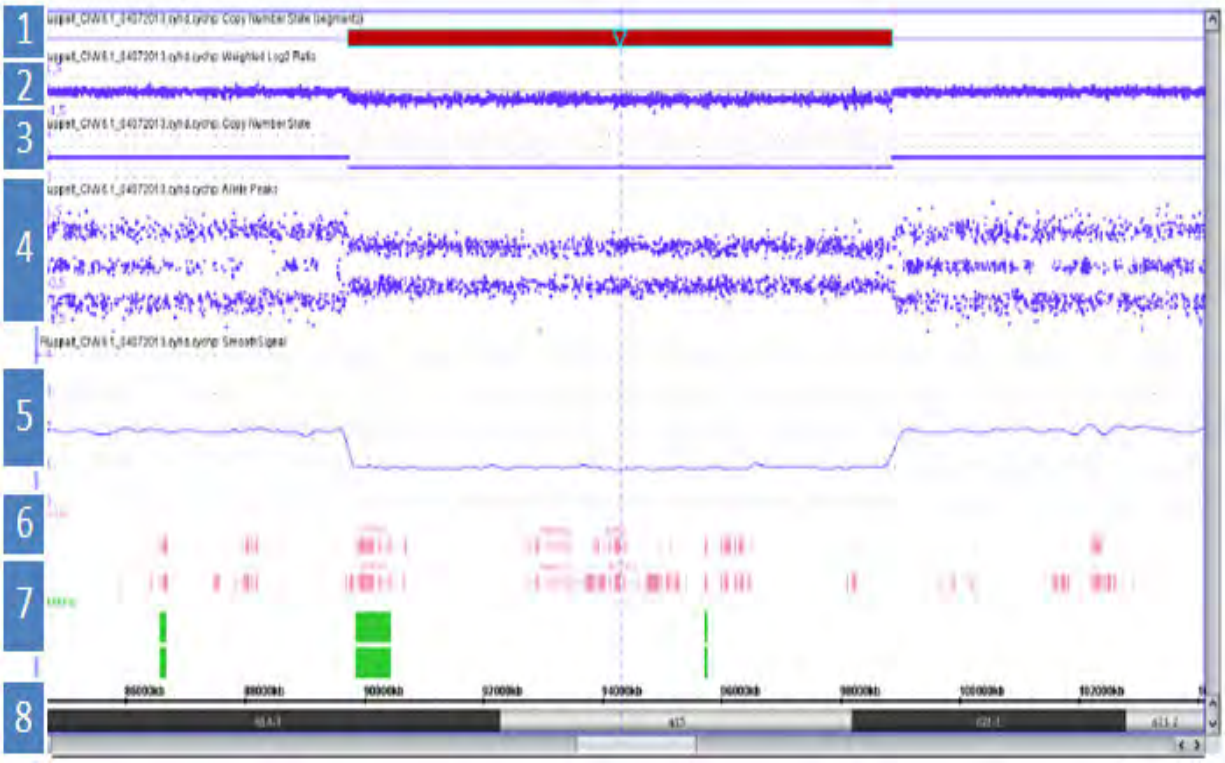


Figure 23 Detail view of Chromosome 5 of Patient 11 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) the copy number state, 4) allele peak deletion demonstrating the SNP markers in this region, 5) the extent of the deletion in the Smooth Signal, 6) RefSeq genes (Pink) in this region, 7) OMIM disease genes (Green), 8) coordinates and chromosome bands.

3.6.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 24) and DECIPHER (Figure 25) Genome Browsers. A submicroscopic interstitial deletion of 9.1 Mb in size was noted on chromosome 5. A total of 37 genes are included in this region.

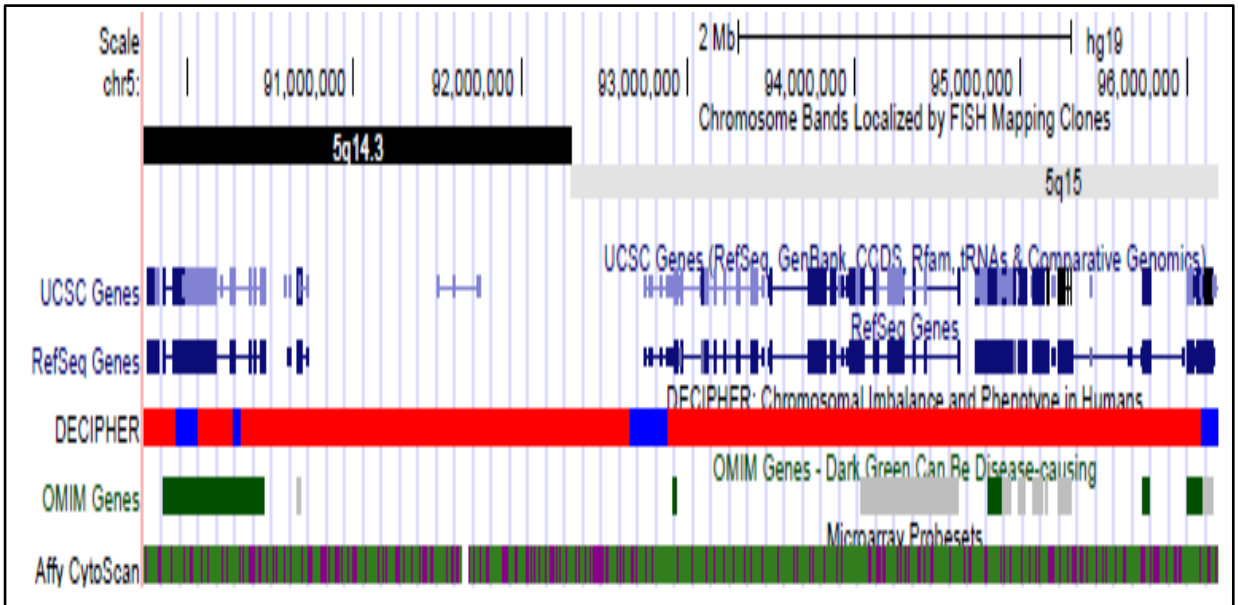


Figure 24 UCSC Browser of chromosome 5 of patient 11

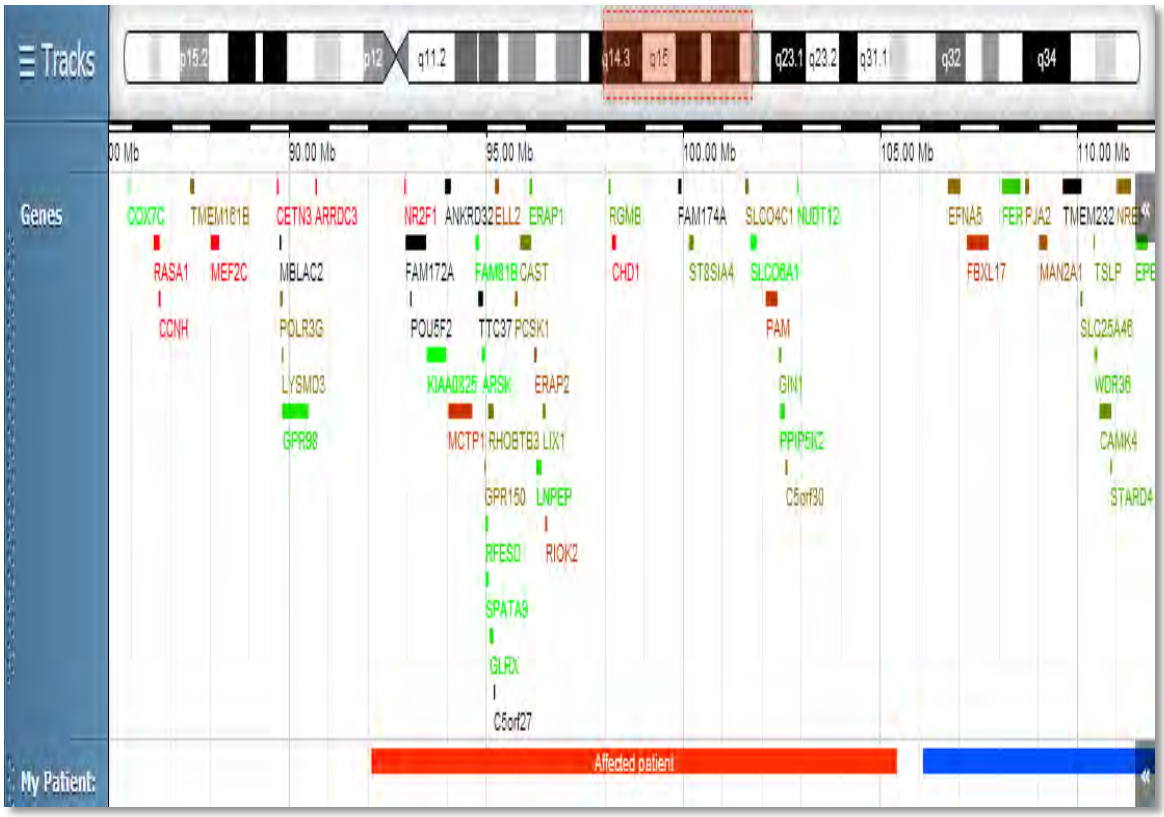


Figure 25 5q14.3q21.1 deletion region – a representation of the genes in the deleted region on chromosome 5 (DECIPHER).

3.6.4 Review

This is a large interstitial deletion of 9.1 Mb on chromosome 5q14.3 to q21.1. This deletion is distal to the previously described 5q14 deletion syndrome region which includes the *MEFC2* gene.

Cardoso et al. (2009) reported on three patients with interstitial deletions in the 5q14 to q21.1 region. All three patients had features of periventricular heterotopia (PH), ID and epilepsy. Further features, which overlapped with patient 11, included: minor facial dysmorphism (high arched eyebrows), delayed ability to walk (at five years, five years and three years of age respectively) and no language skills by five years of age in all three patients. The authors further proposed a 5.8 Mb critical region for PH containing the following genes: *CETN3, POLR3G, LYSMD3, GPR98, ARRDC3, NR2F1, C5ORF21, POU5F2, KIAA0825, C5ORF36, ANKRD32, MCTP1, FAM81B* and *KIAA0372*. Four of these genes are thought to be the most likely cause of PH: *GPR98* which has an important role in brain development, *CETN3* which may have a role in neuronal migration, *MCTP1* which plays a role in controlling neuronal motility and *NR2F1* which plays a role neurogenesis, cellular differentiation and migration during embryonic development (Cardoso et al., 2009). These authors postulated that a loss of a combination of these genes may lead to this phenotype as single gene haploinsufficiency may not lead to this phenotype. A total of ten genes overlapped with the deletion in patient 11: namely *POLR3G, LYSMD3, GPR98, ARRDC3, NR2F1, POU5F2, C5ORF36, ANKRD32,*

MCTP1, FAM81B (See Figure 26). Al-Khateb et al. (2013) reported on a patient with a 582 Kb deletion which overlaps with the genomic coordinates in patient 11 extending from 92,742,875 to 93,324,350 bp. Included in this region are the following genes*: FLJ42709, NR2F1, FAM172A, MIR2277, POU5F2.*

Cardoso et al. (2009) noted the association with PH, but drew attention to the variability in onset and extent of epilepsy in their three patients. Patient 11 and the patient reported by Al-Khateb et al. (2013) both do not have epilepsy or PH. The lack of speech in these patients may indicate widespread cortical impairment (Cardoso et al., 2009). Of the nine clinical features assessed in Table 10, the majority were seen in two or more of the five patients. Although CNS imaging was previously done in this patient, careful review for PH is indicated in the light of these findings.

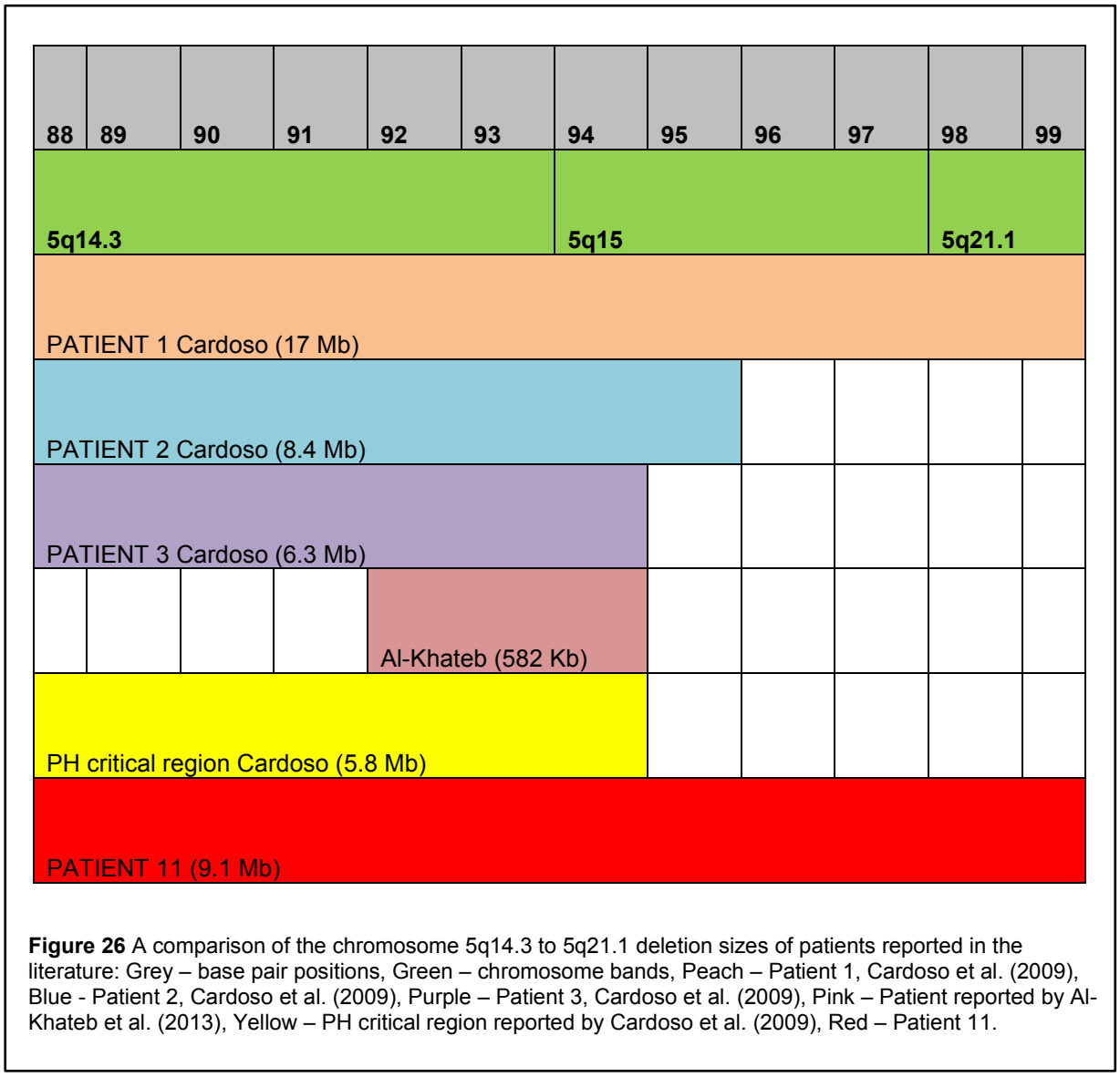


Figure 26 A comparison of the chromosome 5q14.3 to 5q21.1 deletion sizes of patients reported in the literature: Grey – base pair positions, Green – chromosome bands, Peach – Patient 1, Cardoso et al. (2009), Blue - Patient 2, Cardoso et al. (2009), Purple – Patient 3, Cardoso et al. (2009), Pink – Patient reported by Al-Khateb et al. (2013), Yellow – PH critical region reported by Cardoso et al. (2009), Red – Patient 11.

3.6.5 Clinical correlation

Patient 11 has the following clinical features in common with the reported patients:

Table 9 Comparison of the clinical features of Patient 11 and patients reported in the literature.

	Present study	Cardoso et al (2009)			Al-Khateb et al. (2013)
	Patient 11	**Patient 1**	**Patient 2**	**Patient 3**	
Feeding difficulties, gastroesophageal reflux as infants	Yes	No	No	No	Yes
Flaring nares	Yes	No	Yes	No	Yes
Strabismus	Yes	Yes (exotropia)	No	No	Yes
Arched eyebrows	Yes	Yes	Yes	No	Yes
Developmental delay	Yes	Yes	Yes	Yes	Yes
Late onset walking	Yes	Yes	Yes	Yes	Yes
No speech	Yes	Yes	Yes	Yes	Single words by age 3 years
PH	Not reported	Yes	Yes	Yes	No
Epilepsy	No	Yes	Yes	Yes	No
Macrocephaly	Yes	No	No	Yes	No

3.6.6 Conclusion

This patient"s result is partially consistent with the cases reported in the literature by Cardoso et al. (2009) and Al-Khateb et al. (2013). It seems likely that the large deletion of 9.1 Mb containing 37 genes contributes to the clinical presentation in this child.

3.7 PATIENT 18

3.7.1 Clinical features

A female patient presented with presumed autosomal recessive deafness, partial sightedness, microcephaly and craniosynostosis. A family history of ID was reported. This patient has an affected brother with similar features.

3.7.2 Genetic testing

Chromosome analysis and microdeletion FISH studies were not performed; neither MLPA nor FRAXA analysis were performed.

3.7.3 CMA Analysis

CMA testing revealed a submicroscopic interstitial deletion of 88 Kb on chromosome 15 at band q15.3 as shown in the Karyoview (Figure 27) and the detail view in Figure 28. This region is covered by 68 markers.

arr[hg19] 15q15.3(43,888,261- 43,976,406)x1

3.7.3.1 Karyoview

Figure 27 Karyoview of Patient 18: an interstitial deletion of 88 Kb was detected on the long arm of chromosome 15 as indicated in the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

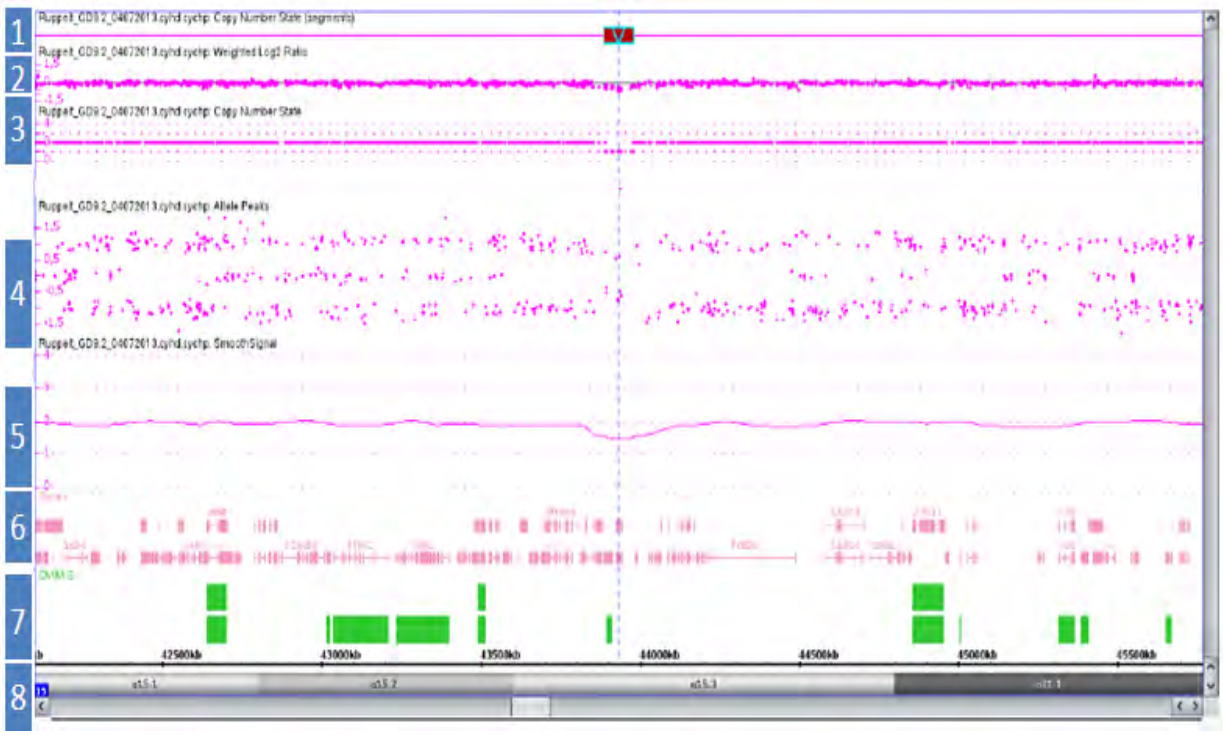


Figure 28 Detail view of Chromosome 15 of Patient 18 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) LOH, 4) allele peak deletion demonstrating the SNP markers in this region, 5) the extent of the deletion in the Smooth Signal, 6) RefSeq genes (Pink) in this region, 7) OMIM disease genes (Green), 8) coordinates and chromosome bands

3.7.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 29) and DECIPHER (Figure 30) Genome Browsers. A deletion of 88 Kb was noted on chromosome 15. Three genes are included in this region of which *STRC* has been listed as a morbid gene.

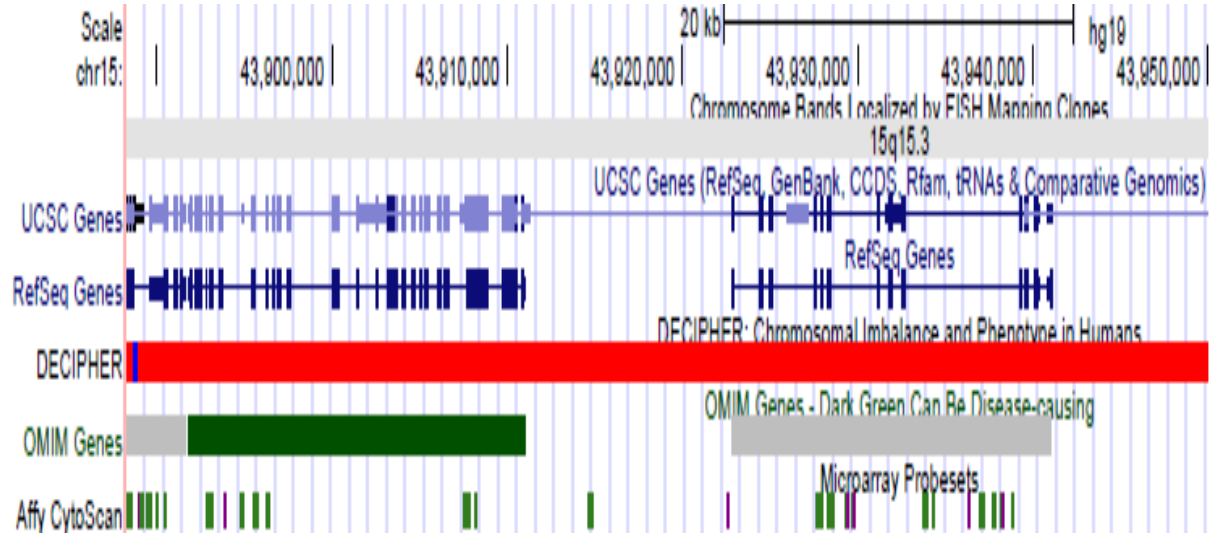


Figure 29 UCSC Browser of chromosome 15 of patient 18 (OMIM disease genes are represented in dark green, deletions in red and duplications in blue): The *STRC* (Stereocilin, OMIM*606440) disease-causing gene is located in the 15q15.3 region.

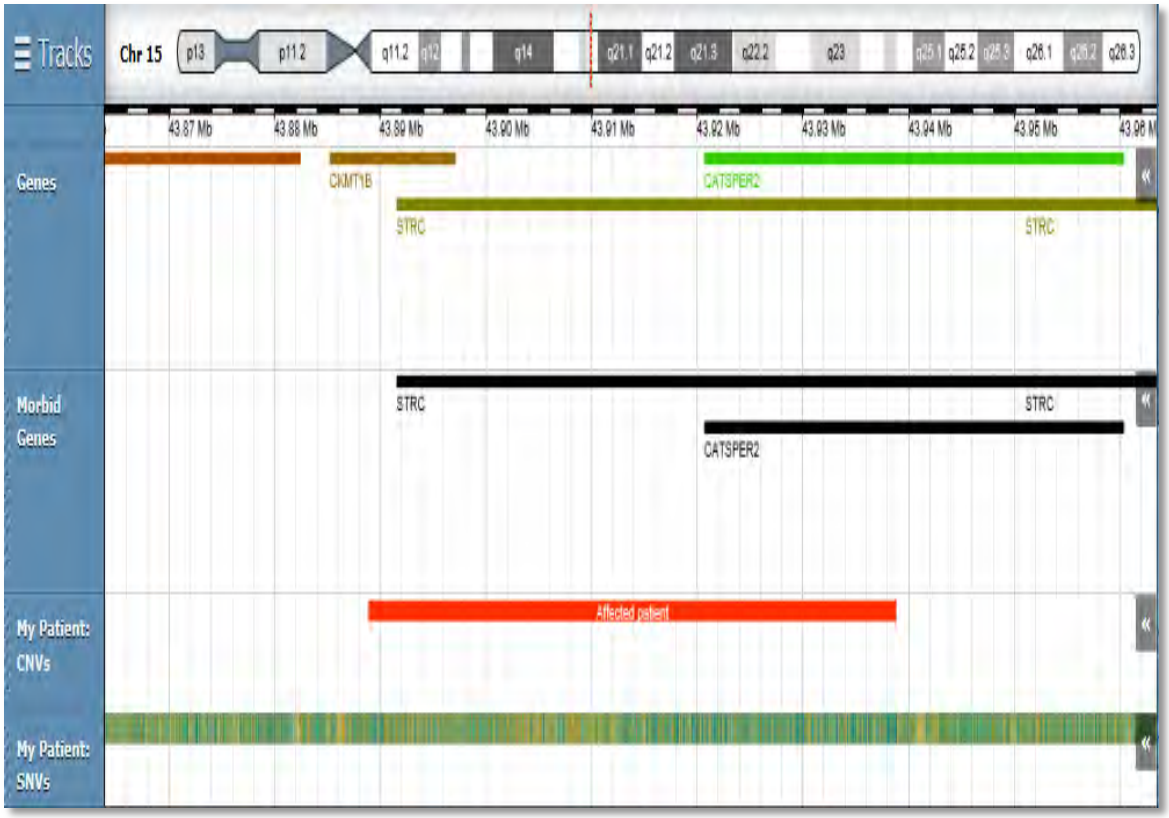


Figure 30 The 15q15.3 deletion region- the largest morbid gene in this region is the *STRC* gene (DECIPHER). The *CATSPER2* gene also intersects this region.

3.7.4 Review

Three genes were included in this region - *CKMT1B, STRC, CATSPER2. STRC* is the largest reported morbid gene in this region, as shown in Figure 30 (DECIPHER). *CKMT1B* and *CATSPER2* partially overlap the deleted region.

STRC (Stereocilin, OMIM*606440), genomic coordinates (GRCh37): 15:43,891,684-44,002,285 is about 19 Kb in size and contains 29 exons (Verpy et al., 2001). The gene is expressed in the outer hair cells of the inner ear. Stereocilin is a large extracellular structural protein and is important for the proper functioning of the stereociliary tips (Verpy et al., 2001). The *stereocilin* (*STRC*) gene is implicated in nonsyndromic autosomal recessive sensorineural hearing loss (Francey et al., 2012; Zhang et al., 2007).

CATSPER2 (Cation Channel, Sperm-Associated, 2, OMIM*607249), Genomic coordinates (GRCh37): 15:43,922,771-43,941,038 is only expressed in sperm (Zhang et al., 2007).

*CKMT1B (*Creatine Kinase, Mitochondrial 1B, OMIM*123290), genomic coordinates (GRCh38): 15:43,592,856-43,599,405, catalyzes the reversible transfer of high-energy phosphate from Adenosine triphosphate (ATP) to creatine and is observed in patient serum with profound shock. Although this gene overlaps the deleted region, its contribution to this phenotype is unclear.

Genetic deafness is estimated to occur at 1 in 200 patients in developed countries. Hearing loss is genetically heterogeneous and can be classified as either syndromic or non-syndromic. (Francey et al., 2012). Syndromic deafness has other phenotypic features in addition to the deafness (Zhang et al., 2007). Contiguous gene deletions are only rarely the cause of syndromic hearing loss (Zhang et al., 2007). Up to 80% of non-syndromic hereditary deafness is due to recessive genes (Knijnenburg et al., 2009). More than 1% of mixed deafness populations are due to *STRC* deletions (Hoppman et al., 2013; Francey et al., 2012). Knijnenburg et al. (2009) reported that the incidence of hearing loss caused by *STRC* deletions is 1 in 16 000.

Avidan et al. (2003) described the Deafness Infertility Syndrome (DIS), a contiguous gene deletion syndrome, with the *CATSPER2* gene implicated in infertility and the *STRC* gene implicated in deafness in male patients. Female patients homozygous for this deletion have hearing loss but are fertile (Hoppman et al., 2013).

3.7.5 Clinical correlation

The deletion of the *STRC* gene and the clinical feature of hearing loss may indicate a further putative mutation on the second *STRC* allele in this patient. The other clinical features could not be readily explained. The *CATSPER2* gene deletion will not affect this female patient as it is only expressed in sperm cells.

3.7.6 Conclusion

It seems likely that the deletion encompassing the *STRC* gene in this patient could contribute to the clinical presentation. Family studies may be useful to establish inheritance of this deletion. Sequencing for *STRC* gene mutations may reveal the putative mutation on the second *STRC* allele. Nevertheless, it is important to bear in mind that the other deleted genes in the region may well be contributing to the phenotype and require further investigation or following up on emerging genotype/phenotype correlations in the various databases. Furthermore, it should also be noted that there could be variants in other parts of the genome that may be contributing to the complex phenotype.

3.8 PATIENT 19

3.8.1 Clinical features

A male patient presented with ID and variegate porphyria (VP). This patient had two male siblings with ID, of whom one also had porphyria. One of his sisters, who had sapphire blue eyes, had a son who was deaf. The parents of these siblings were related. As only male siblings seemed to be affected with ID, FRAXA was initially considered as the ID could be X-linked. Waardenburg syndrome was considered the likely diagnosis in the abovementioned sister and her child.

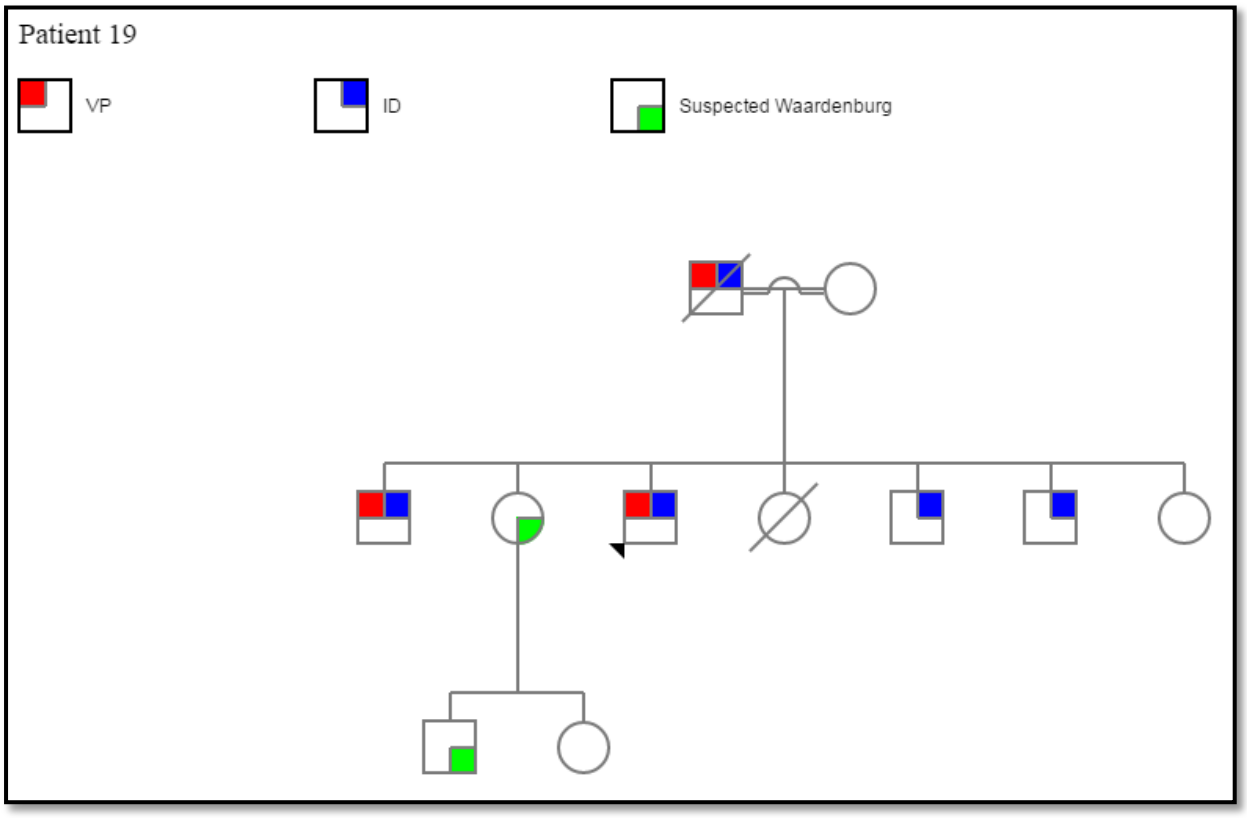


Figure 31 Pedigree for patient 19

3.8.2 Genetic testing

No previous genetic testing was performed.

3.8.3 CMA Analysis

CMA testing revealed a submicroscopic interstitial deletion of 69 Kb on chromosome 22 at band q11.21 as shown in the Karyoview (Figure 32) and the detail view in Figure 33. This region is covered by 140 markers. In addition, seven regions of homozygosity were noted, as listed in Table 10.

arr[hg19] 22q11.21(19,231,636-19,300,915)x1

3.8.3.1 Karyoview

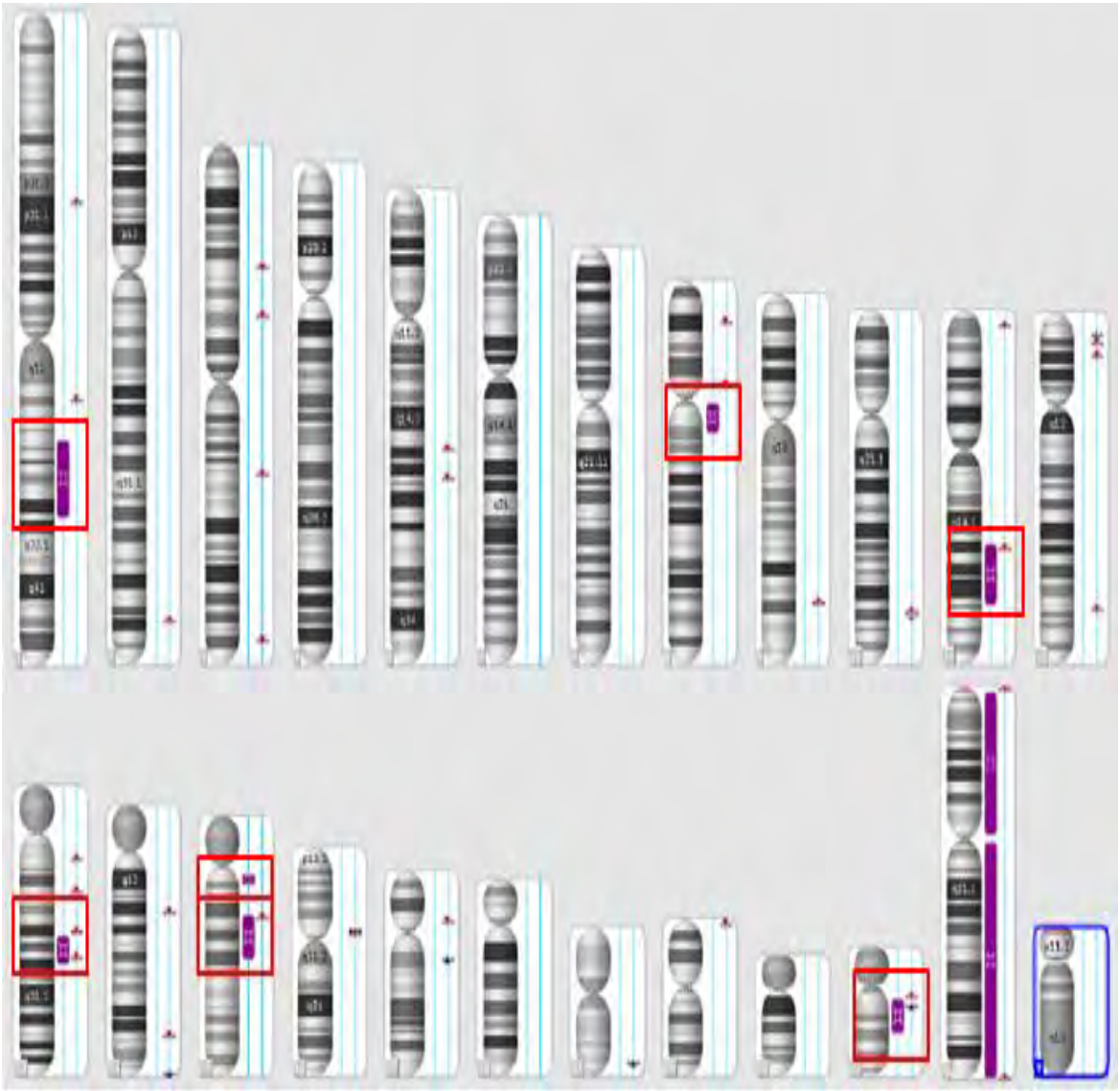


Figure 32 Karyoview of Patient 19: Seven regions of homozygosity were detected in this patient as indicated by the red squares. The deletion on chromosome 22 is indicated with the ROH in the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row.) The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

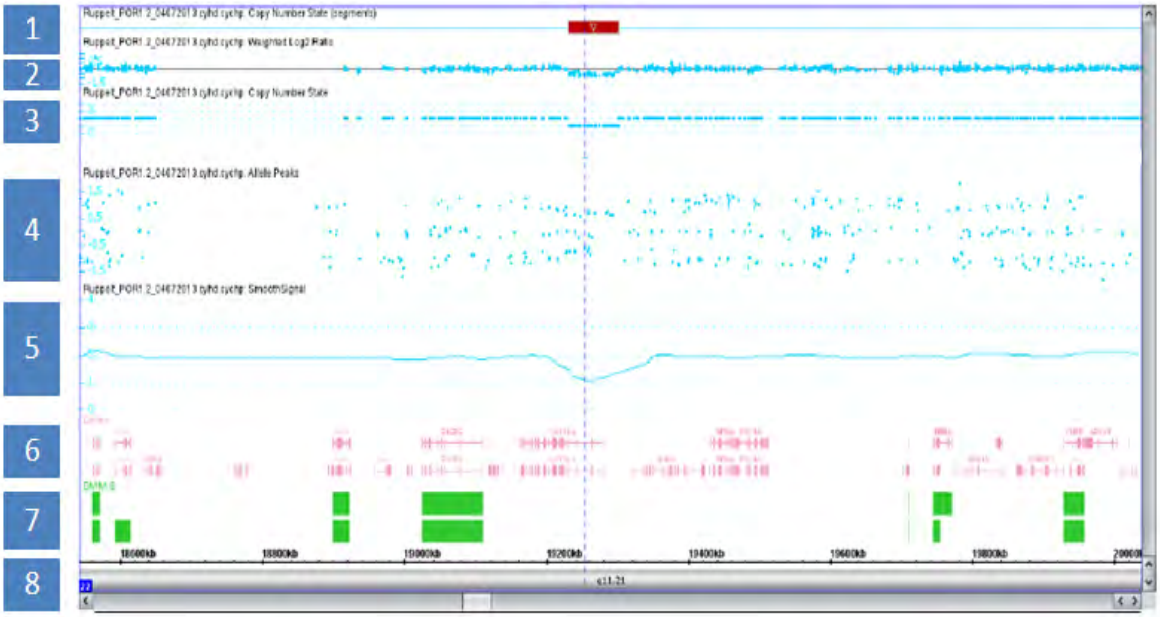


Figure 33 Detail view of Chromosome 22 of Patient 19 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) LOH, 4) the allele peak deletion demonstrating the SNP markers in this region, the extent of the deletion in the Smooth Signal, 6) RefSeq genes (Pink) in this region, 7) OMIM disease genes (Green), 8) coordinates and chromosome bands.

Table 10 The regions of homozygosity detected in Patient 19

	Chromosomal region	Number of genes	Length (bp)
1.	Chr **1**:163833566-192259492	112	28,425,927
2.	Chr **8**:46913605-57659691	26	10,746,087
3.	Chr **11**:90152430-112341574	79	22,189,145
4.	Chr **13**:59835620-70527825	5	10,692,206
5.	Chr **15**:23107347-26811301	15	3,703,955
6.	Chr **15**:38799586-55846849	121	17,047,264
7.	Chr **22**:20950327-33886848	121	12,936,522

3.8.3.2 Database search

The chromosome coordinates of the deletion were entered into the interactive UCSC Genome Browser (Figure 34). One gene was included in this region.

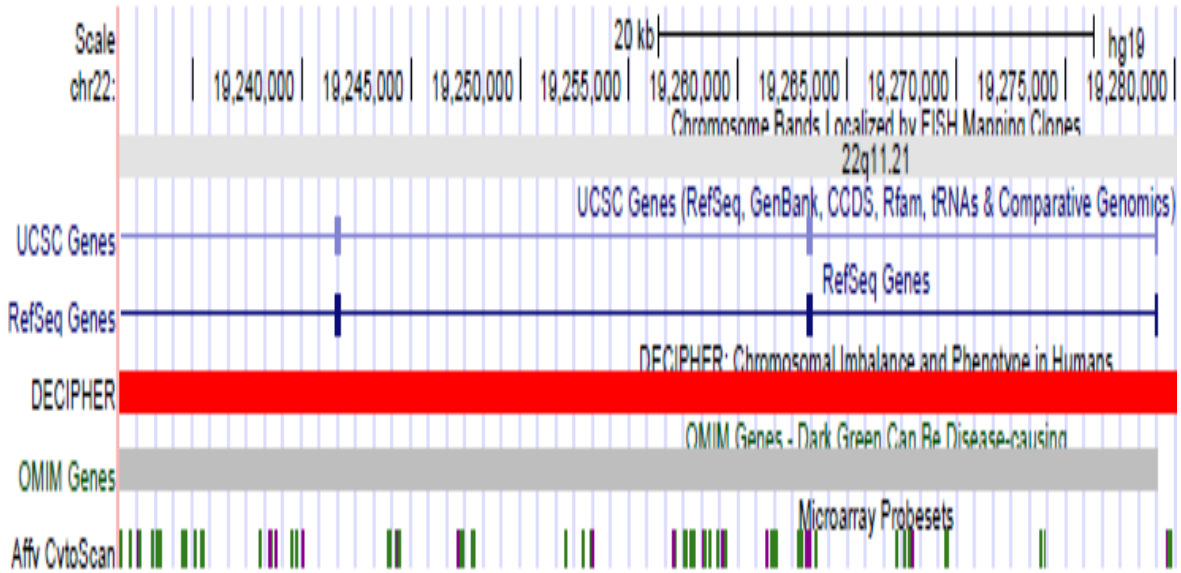


Figure 34 UCSC Browser of chromosome 22 of patient 19

The seven ROH were run through the Genomic Oligonucleotide and SNP Array Evaluation Tool and the genes scrutinised for phenotypic effect. A total of 497 genes were found in these regions which were subsequently filtered to include only autosomal recessive phenotypes (76 genes).

3.8.4 Review

The deleted region at 22q11.21 is covered by 140 markers on the Affymetrix Cytoscan HD. One gene, *CLTCL1*, was included in this region, which is highly expressed in skeletal muscle. This gene has been classed as a VOUS by the OMIM database but a report by Nahorski et al. (2015) identified a homozygous c.988G-A transition in exon 7 of the *CLTCL1* gene in a consanguineous family with children affected with severe DD and pain insensitivity, with five ROH detected using the Affymetrix GeneChip® Human Mapping 250 K Nsp Array. These authors proved that the *CLTCL1* gene is involved in early prenatal neurodevelopment.

A total ROH of 106 Mb was found in patient 19. The coefficient of consanguinity was 1/16 and the coefficient of inbreeding was 1/32. The following genes of interest were found in the ROH:

Homozygous mutation in the *TMCO1* gene (Genomic coordinates (GRCh38): 1:165,724,290-165,768,921, OMIM*614123) on chromosome 1q24 cause Craniofacial dysmorphism, Skeletal anomalies, and Mental retardation syndrome (CFSMR, OMIM#213980). This condition has

autosomal recessive inheritance and has been reported in consanguineous families (Xin et al., 2010).

Two genes implicated in autosomal recessive deafness were detected in the ROH, the *STRC* gene (OMIM*606440) on chromosome 5q15.3 which is implicated in Autosomal Recessive Deafness 16 (OMIM#603720), and the *RDX* gene (OMIM*611022) on chromosome 11q23 which is implicated in Autosomal Recessive Deafness 24 (OMIM#611022).

Another ROH, at chromosome 8q11, contained the gene implicated in Waardenburg syndrome Type 2D (WS2D), *SNAI2*. WS is characterised by piebaldism and deafness (Read & Newton, 1997). WS most frequently occurs in a dominant inheritance pattern (Read & Newton, 1997), although the authors postulated that it may also occur in a recessive form. Sanchez-Martin et al. (2002) suggested that a recessive form of the WS2 syndrome is caused by a homozygous deletion of the *SNAI2* gene.

3.8.5 Clinical correlation

The number and extent of ROHs is consistent with an individual from a consanguineous mating. The phenotype of CFSMR patients is more severe than that described in patient 19 with significant skeletal abnormalities not present in this patient. Despite his positive family history, this patient also has none of the features of Waardenburg syndrome or deafness.

3.8.6 Conclusion

The deletion on chromosome 22q is a VOUS (OMIM database). Due to the large total size of the ROH many genes may each be implicated in the phenotype of this patient. Additional phenotype information would be valuable. Molecular *PPOX* gene testing starting with common SA/Afrikaner R59W mutation could be considered to assist in cascade screening and counselling in this family.

3.9 PATIENT 20

3.9.1 Clinical features

A female patient presented with dysmorphic features and DD with no family history of note. Epilepsy developed at the age of one year. This patient had the following additional clinical features: microcephaly, midface hypoplasia, low set ears, a flat nasal tip, low columella, epicanthic folds, posteriorly rotated ears and a wide mouth. A brain Computed Tomography (CT) scan and renal ultrasound were both normal.

3.9.2 Genetic testing

Chromosome analysis demonstrated a normal karyotype. No microdeletion FISH studies were performed. MLPA Analysis was not performed. FRAXA analysis was not performed.

3.9.3 CMA Analysis

CMA testing revealed a submicroscopic subtelomeric deletion of 2.1 Mb on chromosome 4 at band p16.3 as shown in the Karyoview (Figure 35) and the detail view in Figure 36. This region is covered by 2 436 markers. A region of homozygosity of 4.87 Mb was noted on chromosome 10 at band q25.1 to q25.3.

arr[hg19] 4p16.3 (68,345-2,172,555)x1

3.9.3.1 Karyoview

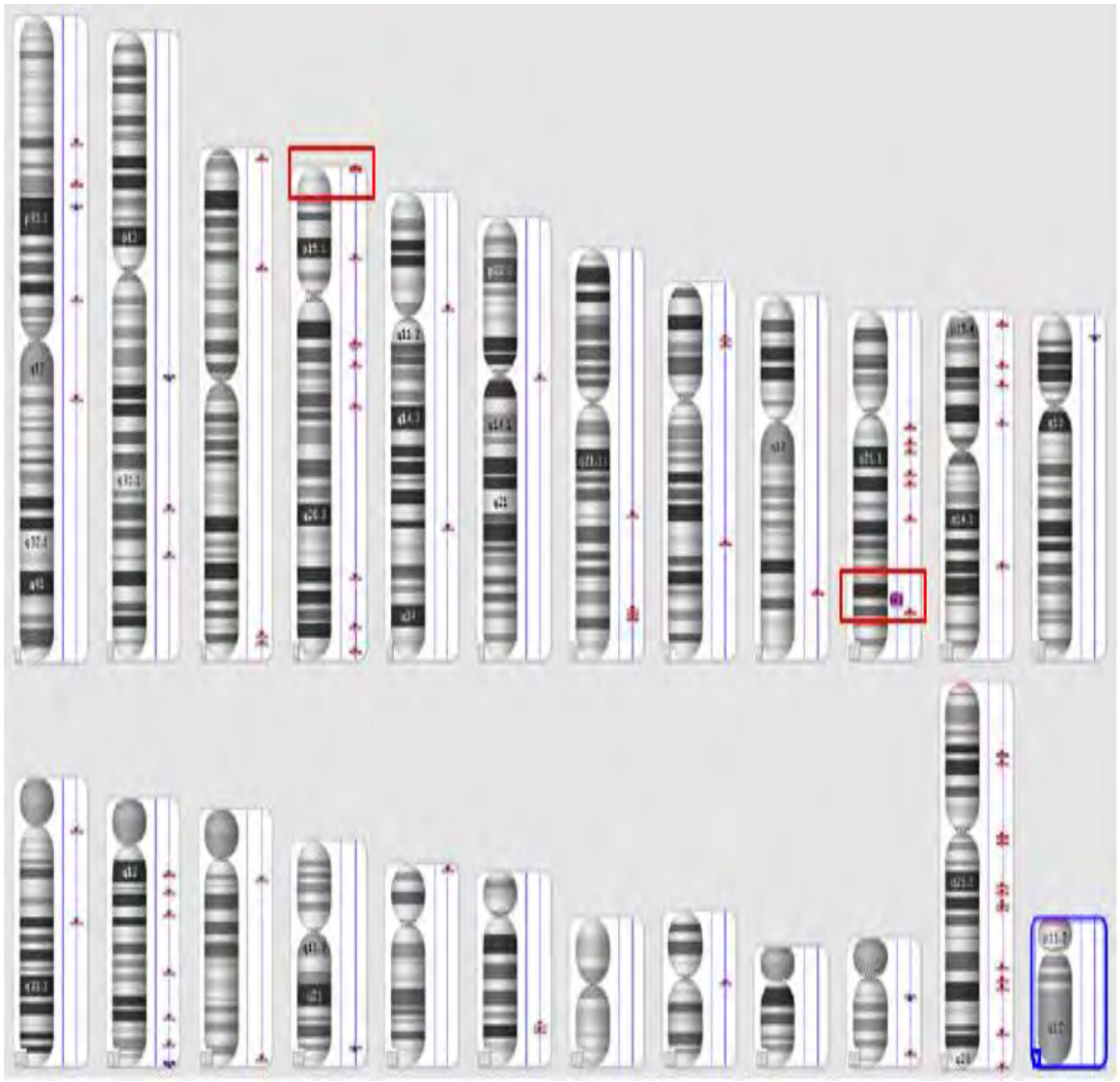


Figure 35 Karyoview of Patient 20: a terminal deletion of 2.1 Mb was detected on the short arm of chromosome 4 as indicated in red. A ROH of 4.87 Mb was detected on the long arm of chromosome 10, as shown in the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

Figure 36 Detail view of Chromosome 4 of Patient 20 showing: 1) the copy number state: deletion (Red), 2) the Weighted Log2 Ratio, 3) the copy number state, 4) 4) LOH, 5) allele peak deletion demonstrating the SNP markers in this region, 6) the extent of the deletion in the Smooth Signal, 7) RefSeq genes (Pink) in this region, 8) OMIM disease genes (Green), 9) coordinates and chromosome bands

3.9.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 37) and DECIPHER (Figure 38) Genome Browsers. A deletion of 2.5 Mb in size was noted on chromosome 4 which spans the Wolf-Hirschhorn (WHS) syndrome region. A total of 44 genes are included in this region of which *LETM1*, *WHSC1,* and *WHSC2* are morbid genes.

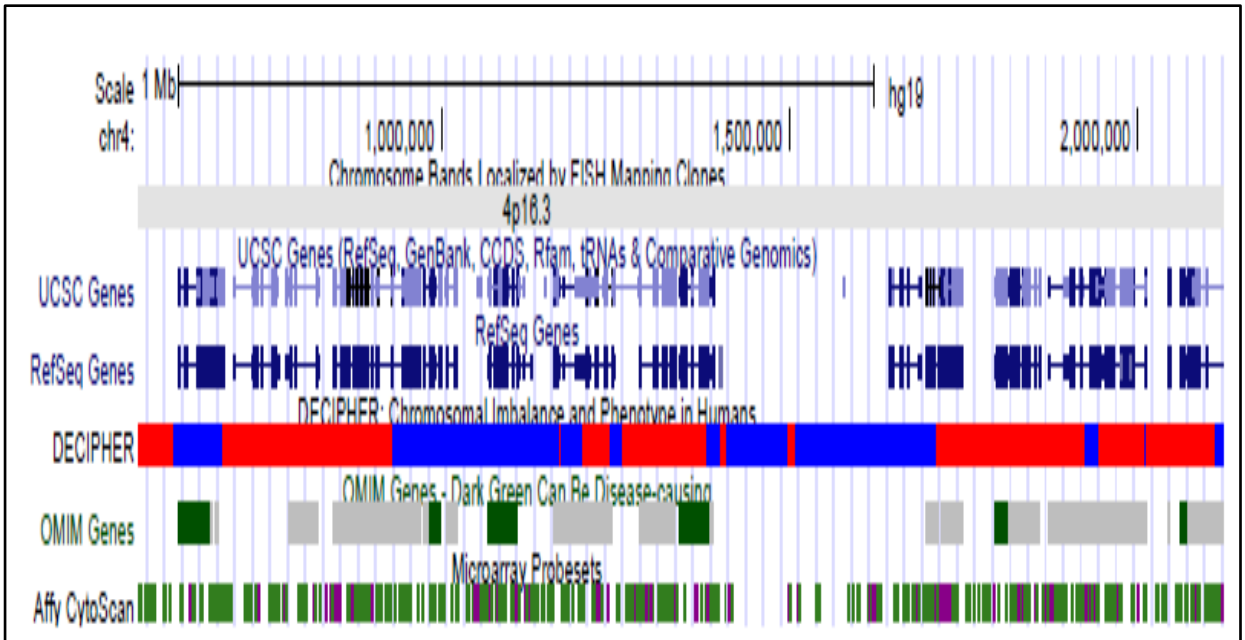


Figure 37 Illustration of the UCSC Browser of chromosome 4 of patient 20 (disease genes are represented in dark green, deletions in red and duplications in blue): The *WHSC1*, *WHSC2* (OMIM*606026), and *LETM1* disease-causing genes are located in the 4p16.3 region.

Figure 38 The 4p16.3 deletion region - the morbid genes in this region are the *WHSC1*, *WHSC2* (not depicted), and *LETM1* genes WHS critical region (DECIPHER).

3.9.4 Review

WHS is a contiguous gene deletion syndrome (OMIM#194190), Genomic coordinates (GRCh37): 4:0–4,500,000. This syndrome was first described by Cooper & Hirschhorn in 1961 (Battaglia & Carey, 1998). It is characterized by a hemizygous deletion on the short arm of chromosome 4 at band 16.3. Three genes have been found to be involved in WHS: *WHSC1* (Wolf-Hirschhorn Syndrome Candidate 1 gene, OMIM*602952), *WHSC2* (Wolf-Hirschhorn Syndrome Candidate 2 gene, OMIM*606026), and *LETM1* (Leucine Zipper/EF-Hand-Containing Transmembrane Protein 1, OMIM*604407). See Figure 39 below.

Wright et al. (1997) described the WHS critical region, WHSCR1, with a size of 165 Kb which is approximately 2 Mb from the telomere. This region is gene dense. Van Buggenhout et al. (2003) identified the WHS critical region further using a high resolution chromosome 4p16 oligonucleotide array and found that the typical WHS facial appearance is due to hemizygosity of the *WHSC1* gene. The other features may be the result of haploinsufficiency of more than one gene in the region. Zollino et al. (2003) described a new, more distal, WHS critical region (WHSCR2) which falls entirely within the greater WHS critical region (WHSCR). This was found to be a 300- to 600-Kb interstitial deletion on 4p16.3. The *WHSC1* gene overlaps both the WHSCR and WHSCR2 regions. *LETM1,* which is a calcium channel gene, flanks the WHSCR and is believed to be involved with the „seizures" phenotype (van Buggenhout, 2004).

Endele et al. (2011) demonstrated that *C4orf48* (Chromosome 4 Open Reading Frame 48, OMIM*614690) is expressed in cortical and cerebellar development. The authors therefore postulated that *C4orf48* encodes a novel neuropeptide which may be involved in the neurological features of WHS if deleted.

Kerzendorfer et al. (2012) implicated the *SLBP* (Stem-Loop Binding Protein, OMIM*602422) gene in impaired DNA replication which may contribute to the growth retardation and microcephaly in WHS.

WHS has been subclassified into a „classical" and „mild" form. Clinical features of WHS include severe growth deficiency (both pre- and postnatal), variable developmental disability, severe intellectual disability, microcephaly, characteristic cranio-facial features known as the „Greek warrior helmet" appearance (broad bridge of the nose, high forehead, prominent glabella, hypertelorism, high-arched eyebrows, protruding eyes, epicanthal folds, short philtrum, distinct mouth with downturned corners and micrognathia), closure defects (cleft lip or palate, cardiac septal defects), poorly formed ears with pits/tags and seizures (Battaglia et al., 2008). Seizures occur in 50 to 100% of children. The seizures may lessen with age. Skeletal abnormalities, other congenital heart defects, hearing loss, urinary tract malformations and structural brain abnormalities may also be seen in WHS.

It has been reported that 50 to 60% of WHS is ascribed to a de novo deletion with the rest being due to a more complex rearrangement such as an unbalanced translocation or a structural rearrangement (Zollino et al., 2003). Zollino et al. (2003) found the deletions of the patients they studied varied in size and breakpoint positions. A deletion of less than 3 Mb resulted in a milder phenotype and the absence of malformations (Zollino et al., 2003). WHS is also frequently due to abnormal segregation from a reciprocal translocation carried by a parent. Giglio et al. (2002) suggested that this kind of translocation may be the second most common translocation after t(11;22) which may cause Emanuel syndrome and is the most common reciprocal translocation in humans. Translocations, mosaicism and unbalanced translocations resulting in a derivative chromosome 4 had also been described as the cause of WHS by Wieczorek (2000). Battaglia et al. (1999) reported the incidence of WHS as 1 in 20 000 to 1 in 50 000 births with a female predominance of 2 to 1.

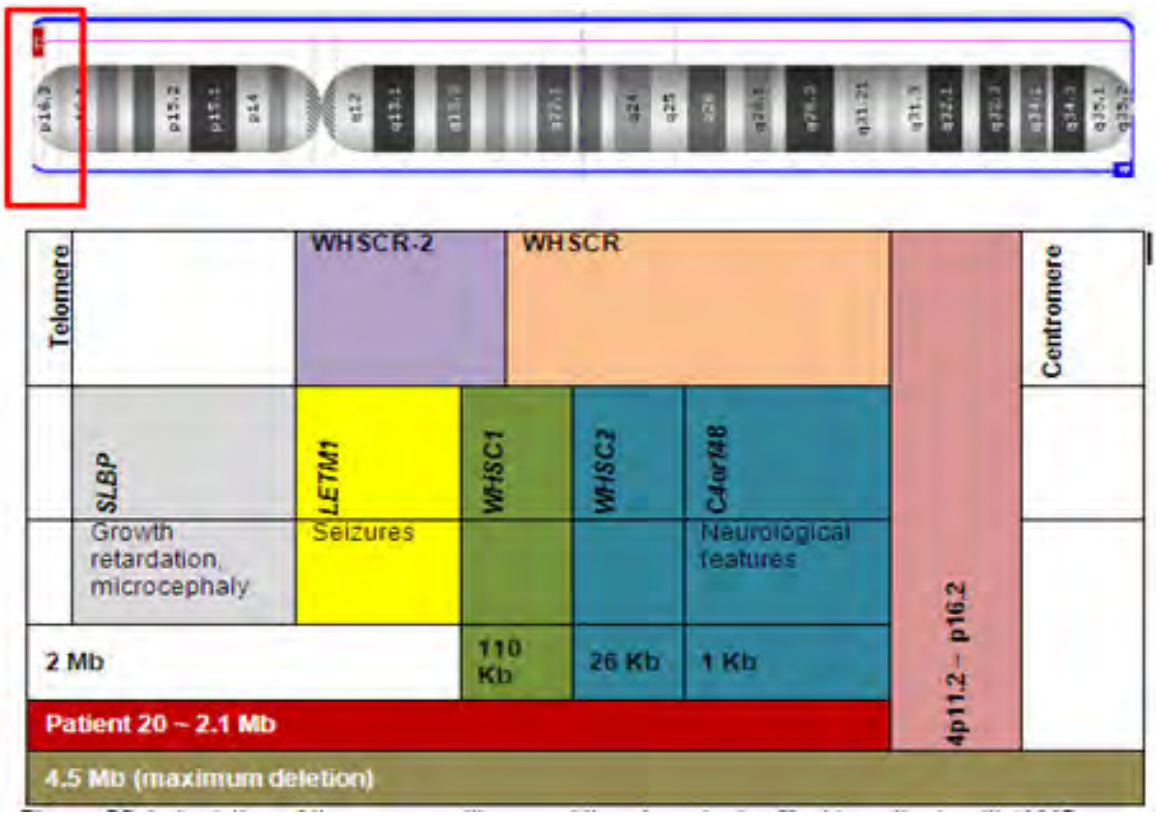


Figure 39 A depiction of the gene positions and the phenotypic effect in patients with WHS: White – 4p telomeric region, and chromosome 4 centromere, Peach – WHSCR, Purple – WHSCR2.

3.9.5 Clinical correlation

Three of the clinical features in this patient specifically correlated with the features of WHS - DD, microcephaly and epilepsy with some possible overlap of craniofacial dysmorphism. The lack of more severe clinical features may correlate with the findings of Zollino et al. (2003) which indicated a milder phenotype when the deletion is less than 3 Mb in size.

3.9.6 Conclusion

This patient"s result is consistent with a diagnosis of WHS. The deletion size may explain the milder phenotype in this patient.

3.10 PATIENT 23

3.10.1 Clinical features

A newborn male patient presented with dysmorphic features and structural congenital abnormalities. Trisomy 13 was suspected. Clinical findings included growth restriction, unilateral cleft lip and palate, hypertelorism, strabismus, a broad nasal root, prominent ears and unilateral camptodactyly of all fingers. Congenital heart disease with an unbalanced atrioventricular septal defect and a double outlet right ventricle was confirmed on echocardiography.

3.10.2 Genetic testing

Chromosome analysis demonstrated a male patient with a supernumerary marker. The marker was the size of an E group chromosome (Figure 40). Neither microdeletion FISH nor MLPA analyses were performed.

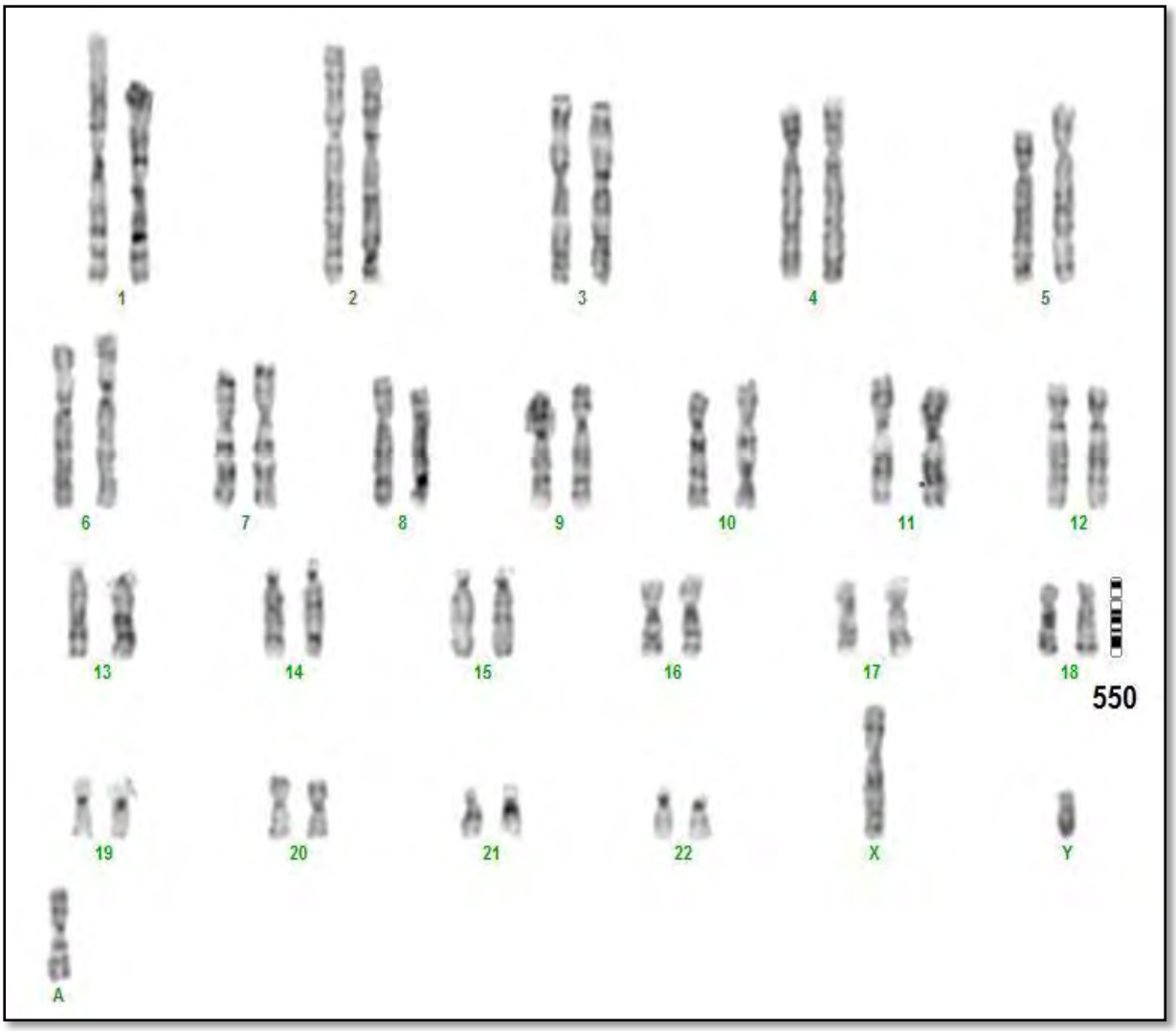


Figure 40 Karyogram of patient 23 depicting the marker chromosome detected (A).

3.10.3 CMA Analysis

CMA testing was performed. Four copies of a segment of chromosome 9, from band p24.3 to band q13, and which was 68.1 Mb in size, were detected as shown in the Karyoview (Figure 41) and the detail view in Figure 42. This segment included the entire short arm and extended into the long arm of chromosome 9. This region was covered by 37 168 markers.

arr[hg19] 9p24.3q13(203,861-68,330,127)x4

3.10.3.1 Karyoview

Figure 41 Karyoview of Patient 23: 4 copies of a segment of chromosome 9 were detected, as indicated in red. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

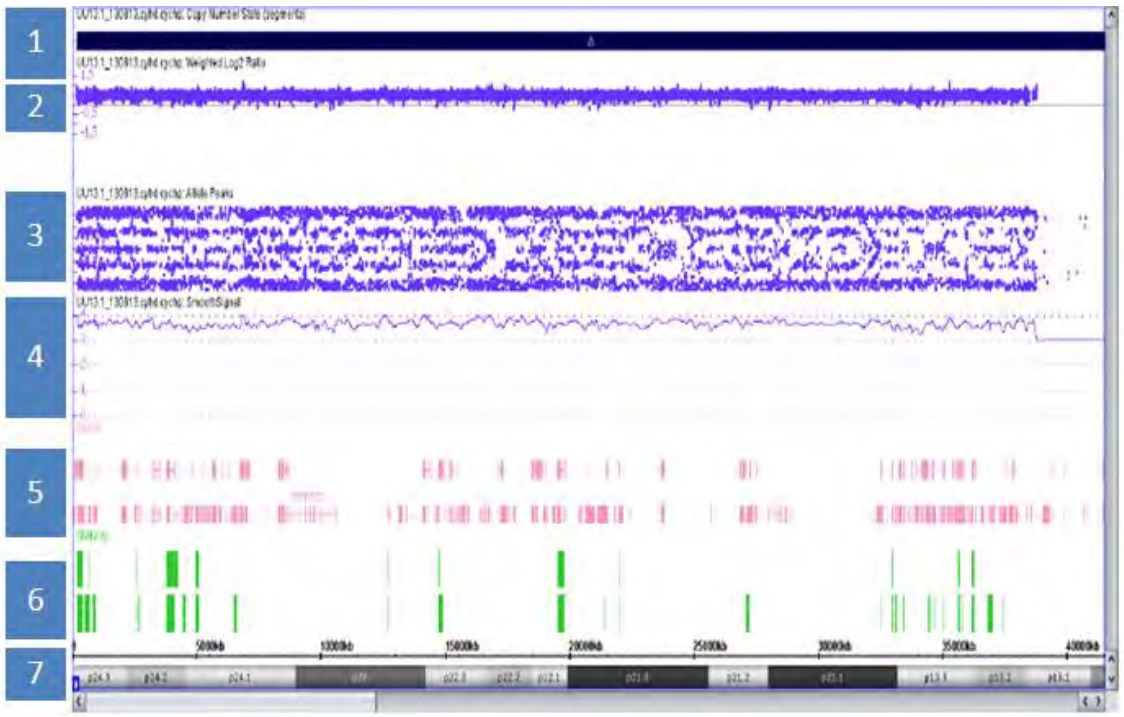


Figure 42 Detail view of Chromosome 9 of Patient 23 showing: 1) the copy number state: tetrasomy (Blue), 2) the Weighted Log2 Ratio, 3) LOH, 4) the allele peak increase demonstrating the SNP markers in this region, 5) the extent of the tetrasomy in the Smooth Signal, 6) RefSeq genes (Pink) in this region, 7) OMIM disease genes (Green), 8) coordinates and chromosome bands.

3.10.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 43) and DECIPHER (Figure 44) Genome Browsers. Four copies of a segment of chromosome 9, 68.1 Mb in size, were noted. A total of 252 genes is included in this region.

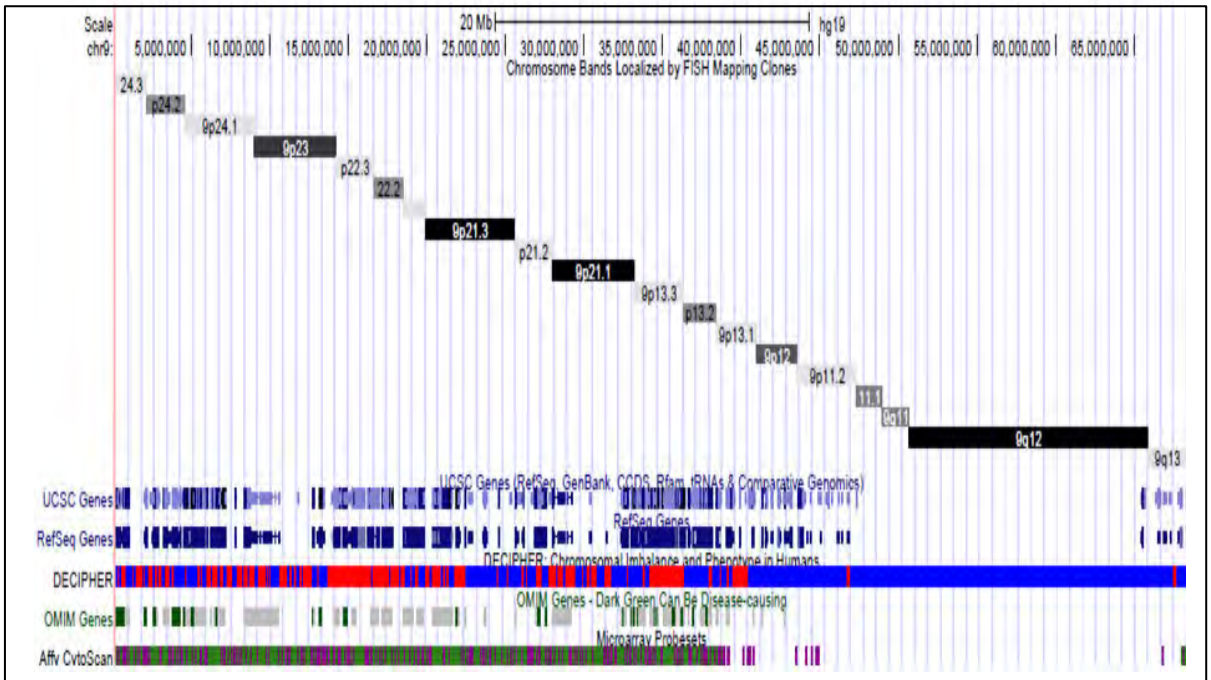


Figure 43 Illustration of the UCSC Browser of chromosome 9 of patient 23 (disease genes are represented in dark green, deletions in red and duplications in blue)

Figure 44 A representation of the genes in the duplicated region of chromosome 9, as seen through DECIPHER.

3.10.4 Review

The first case of tetrasomy 9p was described in 1973 (Ghymers et al., 1973 cited in Dhandha et al., 2002). Dhandha and colleagues (2002) reported on three tetrasomy 9p cases and did a literature review. Variable survival rates were reported, with mosaic cases having a longer survival rate. Mosaicism could only be excluded if more than one type of tissue is analysed, typically lymphocyte culture would not detect mosaicism. Dhandha et al. (2002) found the following clinical features in the literature: hypertelorism, broad nasal root or bulbous nose, cleft lip or palate, ear abnormalities and micrognathia. Further clinical features included DD, central nervous system abnormalities, limb defects, pre and postnatal growth retardation, congenital heart disease, IUGR, renal abnormalities, large fontanelle, abnormal genitalia in male patients, and a short neck with an excess of nuchal skin. Less common findings were downturned corners of the mouth, gastrointestinal abnormalities, epicanthal folds, early death, vertebral abnormalities and brachycephaly. These authors also stated „this pattern of ultrasound findings is also suggestive of trisomy 13" as these diagnoses are usually made prenatally (Dhandha et al., 2002). Cazorla Calleja et al. (2003) described an infant with tetrasomy 9p at the breakpoint q13. This patient had a Dandy-Walker malformation, ventricular septal defect, global delay and other congenital abnormalities. El Khattabi et al. (2015) reported a further 12 cases of tetrasomy 9p, who were diagnosed using microarray technology. Tetrasomy 9p is the second most common supernumerary isochromosome and is usually seen in lymphocytes and occasionally in fibroblasts (El Khattabi et al., 2015). Tetrasomy 9p is usually seen in mosaic form although a low-level mosaicism can be missed. The most common associated abnormalities are Dandy-Walker malformation, cleft lip/palate and intra-uterine growth restriction. El Khattabi et al. (2015) found a correlation between involvement of the 9q region and cardiac malformations, ID and survival period.

3.10.5 Clinical correlation

The following features noted in this patient corresponded with the findings in the literature: Growth restriction, unilateral cleft lip and palate, hypertelorism, strabismus, a broad nasal root, limb defects (unilateral camptodactyly of all fingers) and congenital heart disease (an unbalanced atrioventricular septal defect and a double outlet right ventricle). In addition the suspicion of Trisomy 13 in these patients on clinical grounds has also been described. Although this abnormality is called tetrasomy 9p, the abnormality extended into the long arm of chromosome 9 as in the case described by Cazorla Calleja et al. (2003).

3.10.6 Conclusion

This patient"s result is consistent with a diagnosis of tetrasomy 9p and is the cause of the clinical presentation in this child.

3.11 PATIENT 24

3.11.1 Clinical features

This male patient presented at 13 years of age with learning difficulties, dysmorphic features and dermatological abnormalities. The dysmorphic features noted were hypertelorism, epicanthus and a broad nose with a flat nasal bridge. The skin findings included palmar hyperkeratosis, skin papules, multiple café au lait spots and areas of hypopigmentation. The boy"s father and sister had similar dermatological features but were of normal intellect and did not have dysmorphic features.

3.11.2 Genetic testing

Chromosome analysis demonstrated a normal male karyotype. Microdeletion FISH studies, MLPA, and FRAXA analysis were not performed.

3.11.3 CMA Analysis

CMA testing was performed and a submicroscopic interstitial duplication of 301 Kb was noted on chromosome 13 at band q33.3 as shown in the Karyoview (Figure 45) and the detail view in Figure 46. This region is covered by 336 markers.

arr[hg19] 13q33.3(109,771,548-110,072,888)x3

3.11.3.1 Karyoview

Figure 45 Karyoview of Patient 24: duplication on the long arm of chromosome 13 was detected as indicated in the red square. (Chromosomes are not numbered but are aligned conventionally: that is chromosomes 1 – 12 in the first row, and chromosomes 13 – 22, X and Y in the second row). The blue lines to the left of the chromosomes represent the ROH and CN tracks respectively.

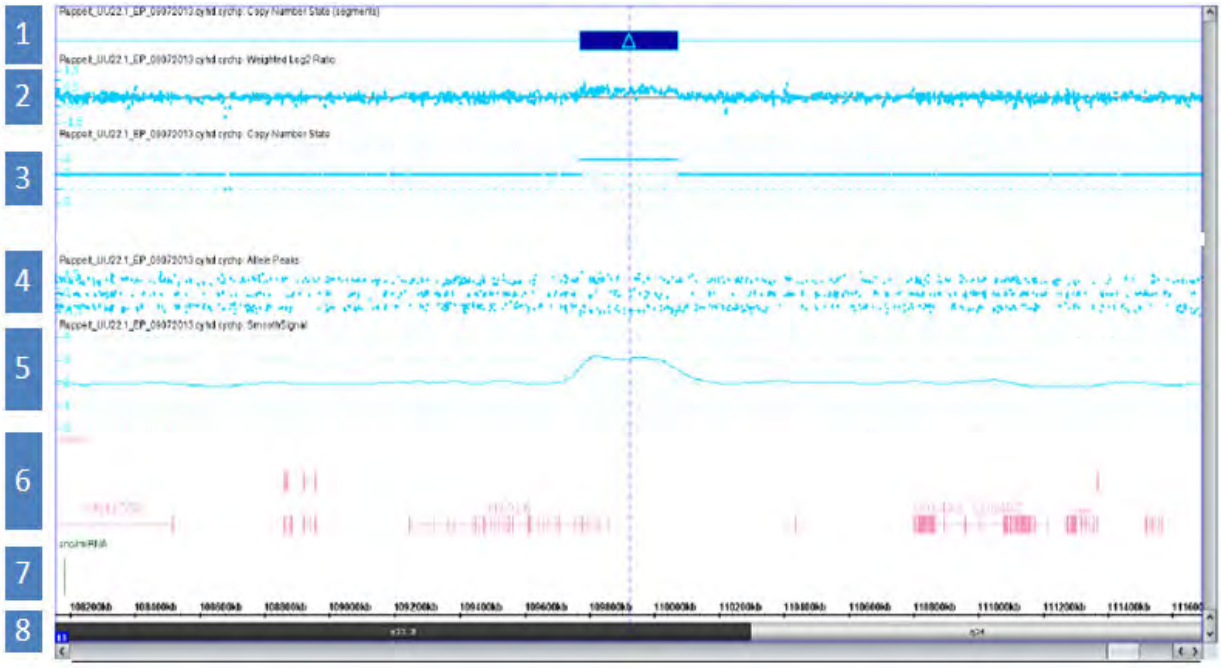


Figure 46 Detail view of Chromosome 13 of Patient 24 showing: 1) the copy number state: duplication (Blue), 2) the Weighted Log2 Ratio, 3) the extent of the copy number duplication, 4) LOH, 5) the allele peak duplication demonstrating the SNP markers in this region, 6) the extent of the deletion in the Smooth Sign, 7) RefSeq genes in this region, 8) coordinates and chromosome bands.

3.11.3.2 Database search

The chromosome coordinates were entered into the interactive UCSC (Figure 47) and DECIPHER (Figure 48) Genome Browsers. A duplication of 301 Kb was noted on chromosome 13. The *MYO16* gene was included in this region.

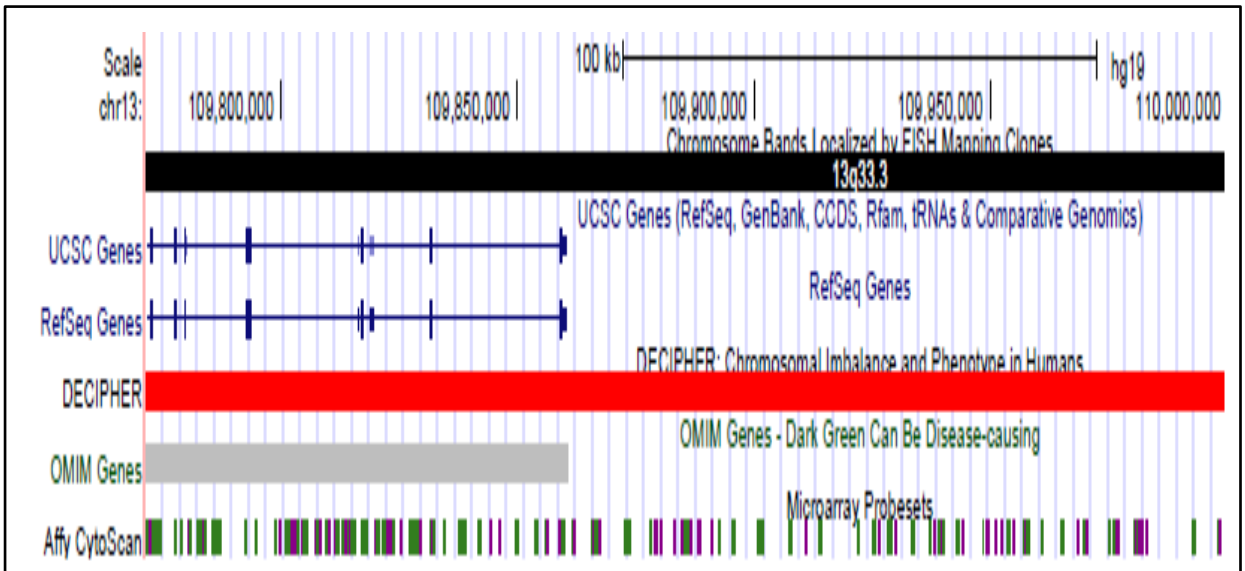


Figure 47 Illustration of the UCSC Browser showing a section of chromosome 13 of patient 24 (disease genes are represented in dark green, deletions in red and duplications in blue)

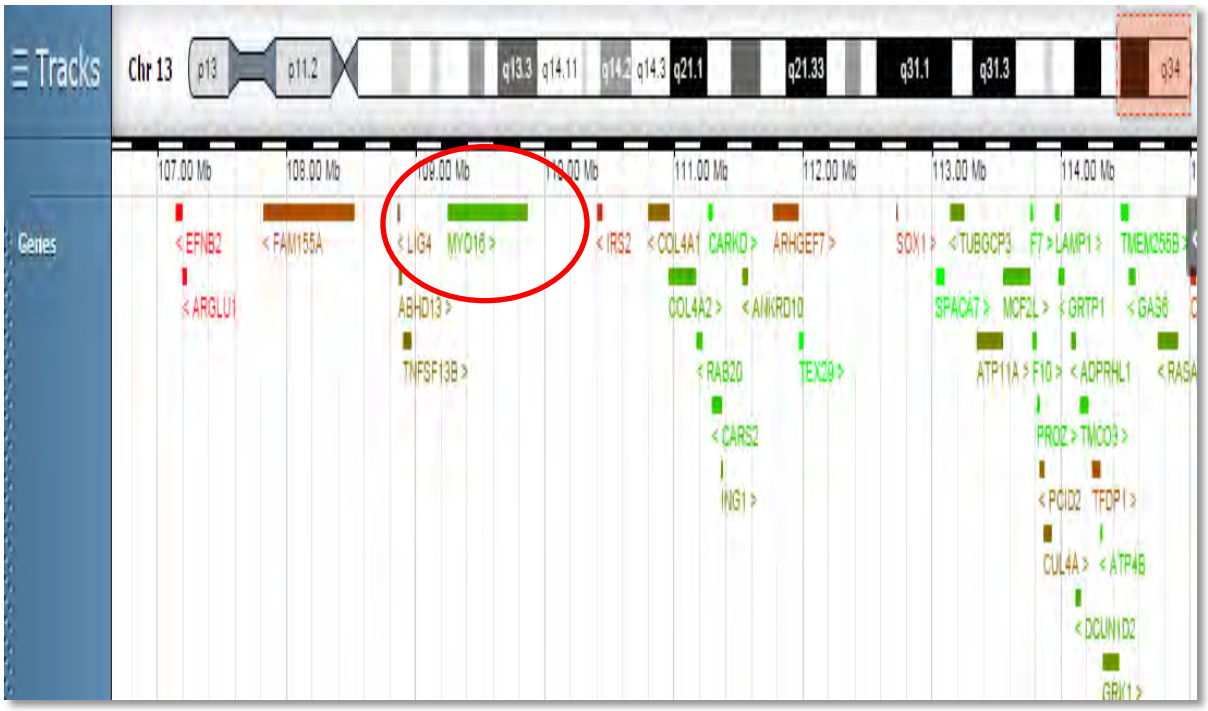


Figure 48 Illustration of the location of the *MYO16* gene on chromosome 13q33 (DECIPHER)

3.11.4 Review

Faletra et al. (2012) described a patient with a duplication of chromosome 13q in the form of a marker chromosome. This duplication of 44 Mb was much larger in size than the duplication seen in patient 24 which was 301 Kb in size. The duplication in Patient 24 does however fall within the reported region and involves band q33.3 (109,771,548-110,072,888). The duplication of the patient in the case report of Faletra et al. (2012) involved bands q21.22-q34 (71,024,411-115,103,529). Their patient had features of hypopigmentation (phylloid hypomelanosis) and moderate ID. Liu et al. (2015) found an association of the *MYO16* gene with ID/DD suggesting it plays a role in neurodevelopment.

3.11.5 Clinical correlation

Patient 24 had generalised pigmentary abnormalities with multiple café au lait spots as compared to the phylloid hypomelanosis reported by Faletra et al. (2012). The duplication of the *MYO16* gene may explain the milder phenotype in this patient as compared to deletion of this gene which is associated with ID/DD.

3.11.6 Conclusion

It seems possible that the duplication of 301 Kb containing the *MYO16* gene could contribute to the clinical presentation of learning disability in this child as the deletion of this gene has been associated with ID; this may pertain to dosage sensitivity (Froyen et al., 2007). The skin disturbances can however not be readily explained. At this stage, however, this duplication

should be considered a VOUS, and it would be worth considering family studies to further elucidate the finding.

CHAPTER 4 DISCUSSION

ID/DD comprises of a „heterogeneous group of disorders" (van Bokhoven, 2011) characterised by below average intellectual function and the lack of the necessary skills to perform the daily tasks of living. Conventional chromosome analysis (karyotyping) has been the gold standard for the detection of chromosomal abnormalities in patients with ID/DD, ASD and MCA. Microarray technologies are a high-resolution technology which interrogates the whole genome and is used for the detection of CNVs. Microarray has largely replaced karyotyping, which can only detect chromosomal abnormalities larger than 5 Mb in size, as microarray, with its increased resolution, can detect CNVs as small as 50 Kb in size. Although karyotyping can still be useful for the detection of balanced translocations and low-level mosaicism, CMA is the recommended first-tier test for the detection of submicroscopic aberrations in patients with ID/DD, ASD and MCA since the release of the consensus statement by Miller et al. (2010) who reported on a CNV detection rate of between 15 and 20% in 21 698 patients with ID/DD/MCA across 33 studies. This is a significant improvement on the 3.7% chromosome analysis detection rate in a review of six studies which included 3 672 patients with GDD or mild to moderate ID, some of which had DS, sex chromosome abnormalities and unbalanced translocations (Shevell et al., 2003).

In South Africa, chromosome analysis, microdeletion and/or subtelomeric FISH and MLPA are still the only tests available for the detection of CNVs in the public sector. The current testing repertoire for ID/DD/MCA/ASD in the NHLS Groote Schuur Hospital Human Genetics diagnostic laboratory includes Fragile X testing and MLPA (subtelomere, microdeletion/duplication and X-linked assays), karyotyping/chromosome analysis and microdeletion/duplication FISH for CNV detection. Over a recent 18 month period (January 2015 to June 2016), the detection rates using these technologies were 5%, 7%, 1% and 11%, respectively. The cost of sequential testing can approximate the total cost of microarray CNV analysis.

CMA is not performed routinely in SA despite it being in routine diagnostic use in the majority of developed countries. This gap in technical expertise and analytical knowledge was identified at the outset of the current study. This study was initiated to investigate the relevance of CNV detection in a small cohort of ID/DD patients in SA. Upon successful completion of the project the CMA technology would be introduced into the routine diagnostic laboratory. Included in the study were 30 patients diagnosed with ID/DD who had a high likelihood of CNV-related pathogenesis and from whom blood/DNA was readily available. The workplan was developed with the Affymetrix representatives and the Centre for Proteomic and Genomic Research (CPGR), who host the platform and are a service provider for genomic

and proteomic technologies. This service included QC of samples, running of arrays and data processing. Three samples had to be excluded due to poor DNA quality. A total of 27 patient samples were ultimately used for CMA and further analysis. The samples were assayed at the CPGR and the CEL files were released for analysis. The Cytoscan HD process was run over four days as per the manufacturer‟s instructions. This is a long workflow but consists of basic molecular techniques. As mentioned previously, QC is crucial for the success of this assay. The DNA was therefore initially scrutinized for quality, and QC gels were run after the PCR step to assess adequate amplification and again after the fragmentation step to assess adequate fragmentation.

The CEL files were imported into the Affymetrix ChAS software for analysis. Training in data analysis with the Affymetrix product specialist in Cape Town provided a valuable introduction to the use of the software. Data analysis and interpretation proved to be a challenge as a large number of CNVs were detected on each patient sample and a workflow for analysis had to be established. Microarray data analysis is time-consuming even to trained scientists. No local diagnostic support was available as microarray work is not routinely done in SA. An „internship‟ at the Laboratory of Diagnostic Genomic Analysis (LDGA) in Leiden, with high throughput CMA competency and data analysis was undertaken. This not only facilitated confirmation of the initial findings and assisted in the detection of further CNVs but also provided hands-on training in a centre of excellence for CMA.

Personal correspondence with three international laboratories gave further insight into data analysis in other centres. Two laboratories, in Cardiff (Wales) and in Sydney (Australia) used the Agilent Cytochip microarray platform routinely and the third laboratory in Manchester (UK) used the OGT ISCA platform routinely. Both these platforms are medium resolution arrays which have the seeming advantage of detecting fewer CNVs (than the Cytoscan HD arrays used in the present study) and thus less „noise‟. This is an important consideration when implementing CMA into routine diagnostic use. Although the Affymetrix Cytoscan HD has the best resolution at 2.6 million probes, it generates a large amount of CNV data as demonstrated in this study. This said, the Affymetrix Cytoscan HD is in routine diagnostic use in the Leiden laboratory and is used for prenatal and postnatal samples. The filter settings in the Affymetrix ChAS software are important, as this can eliminate some of the noise as CNVs as small as 10 Kb in size can be detected. Although each laboratory had their designated workflow for analysis, all the laboratories used the same CNV databases and genome browsers such as DGV, DECIPHER and UCSC, and literature searches for the analysis and interpretation of the CNVs detected.

This pilot investigation successfully identified large well-delineated CNVs, as well as other significant CNVs, with a detection rate of 26%. This high detection rate in a small cohort was also an indication of accurate phenotyping and patient selection. During analysis, causative

CNVs of well-known microdeletion syndromes such as Kleefstra, Wolf-Hirschhorn and Mowat-Wilson syndrome were detected. One susceptibility region, the 1q21 recurrent microdeletion (susceptibility locus for neurodevelopmental disorders), was also identified. Other significant CNVs such as a 9.1 Mb deletion on the long arm of chromosome 5 which corresponds with previously reported cases, and an 88 Kb deletion on chromosome 15 containing a significant gene, were revealed. An OMIM described VOUS on chromosome 22 was detected in one patient who also demonstrated seven ROHs indicating consanguinity as seen in the family pedigree. Another two VOUS were a 2.5 Mb deletion on the short arm of chromosome 1 and a duplication of 301 Kb containing a gene involved in ID, previously reported with a deletion. A large supernumerary marker chromosome, which was previously detected on chromosome analysis, was identified as a tetrasomy of the short arm of chromosome 9. The majority of the CNVs detected corresponded with CNVs in the Leiden laboratory"s Cartagenia database. Numerous benign polymorphisms and known Cytoscan variants, such as the well-known duplication 14q32.33, were also identified. Three of the benign CNVs were detected in this cohort which were not previously reported in the Leiden database: an 18 Kb deletion at 6q14.3q15, a 31 Kb duplication at Xq13.1 and a 10 Kb deletion at 2q32.3. These CNVs may be specific to the SA population and it is likely that more population specific CNVs would be detected in routine testing as well as pathogenic CNVs and VOUS. All the benign and pathogenic CNV regions detected had a dense probe coverage including the duplication region of patient 24 (13q33.3) which has a previously described deletion syndrome associated with the same region. Family studies and other modes of analysis, for example in exploring ROH, may also be needed. VOUS require careful clinical follow-up and consultation with the clinical geneticist for interpretation.

Despite the proven utility of microarray it is costly. Cost is a problem in all settings with the majority of laboratories providing CMA on a cost neutral basis. An increase in probe resolution is directly related to an increase in the cost of the array. A balance between cost and utility is crucial in the resource-constrained public sector in SA. It would therefore be important to ensure that clinicians are educated in the appropriate use of this technology and work with medical genetic services to set up appropriate referral pathways for patients with ID/DD. An important limiting factor of performing microarray testing in South Africa is that all costs are dependent on the currency exchange rate. In 2011, the Cytoscan HD array was priced at R4 031 per (array) chip and consumables. By 2013, this price had increased to R7 846. The price stabilized in 2015 to R7 798 per array. Referral to laboratories in the United Kingdom (UK) for microarray CNV testing cost between £350 and £550. This is between R7 000 - R11 000 with the August 2016 exchange rate (R1 = £20).

Subsequently to commencing this study, Affymetrix released two additional arrays (see Table AT 2 in Addendum V for comparison): the 750 K which is a medium density array and developed for routine diagnostic use for patients with ID/DD, and the Optima array which has

a lower resolution at approximately 315 000 probes (which was developed for prenatal samples).

Table 11 lists the estimated **cost per microarray per patient,** as per the prices quoted in February 2016 for each of the microarrays offered by Affymetrix® Cytoscan™ HD Array (Affymetrix, Santa Clara, CA, USA).

Table 11 Comparison of the cost of Affymetrix array kits available (February 2016)

	Affymetrix HD	**Affymetrix 750K**	**Affymetrix OPTIMA**
ARRAY	R7 790	R4 592	R3 187
TAQ	R323	R323	Not required
CPGR SERVICE FEE	R1 400	R1 400	R1 400
TOTAL	**R9 513**	**R6 315**	**R4 587**

The cost in Table 11 only includes consumables and the outsourced service provider fee. It does not include the cost of time required to analyse, interpret and report the result by fully trained professionally registered laboratory staff. A realistic price estimate must take into account the cost of labour and other laboratory overheads. A price proposal received from the CPGR in June 2016 for the Affymetrix Optima option included R4 172.54 for reagents and consumables and a service fee of R1 047.46, amounting to a total of **R5 220 per sample (excluding VAT)**. This price is based on a minimum batch of 12 samples. A final charge of R7 000 would need to be raised in a local diagnostic environment in order to cover expenses if the lowest resolution array is used. Microarray is an expensive diagnostic test in developed countries, and is even more so in the resource-limited SA public sector health care system; however, the improved CNV detection rate, with improved health care management of the patient, should offset this cost.

These advantages may not always be perceived as worthwhile and there are doctors, hospitals and governments in certain countries who are doubtful about using CMA as a first-tier test or second-tier test, taking into account that additional follow-up investigations may still be needed, as is the case with the additional testing of parental samples to determine the disease association of VOUS in probands (Wordsworth et al., 2007). Despite the higher diagnostic yield, direct clinical utility of CMA may be viewed as limited, since many patients still need on-going support and hospital care. It is true that the majority of patients will test CMA negative and further testing will be required. However, the benefits of a diagnosis remain important to clinicians and patients/parents and there is a clearly established role for CMA testing in the investigation of ID/DD. One might argue that FRAXA testing which is generally accepted as a routine diagnostic test has a small diagnostic yield of less than 3.5% in patients with ID/DD/ASD, compared to the diagnostic yield of CMA at between 15 to 20% for the same indications (Riggs et al., 2014).

Lynch (2011) stated that the benefits of array testing outweigh its cost as „array testing has such a huge diagnostic yield that its introduction is inevitable whether or not the economics make sense." Doctors are motivated to give their patients specific results, which will be of „emotional benefit" to the family (Lynch, 2011). The switch from conventional cytogenetics to CMA is inevitable and protocols should be established to ensure the most appropriate diagnostic service is available for patients. Lenhard et al. (2005) observed that „the value of genetic diagnosis of infantile disabilities encompasses significant and long-lasting emotional relief for the parents". Earlier diagnosis may eliminate the necessity of further follow-up tests such as neuroimaging and invasive tests, like muscle biopsies (Wordsworth et al., 2007). Lynch (2011) also stated that there are „hidden benefits" which are difficult to cost and include accurate diagnosis which may lead to customized treatment regimes, appropriate genetic counselling, clinical care and educational aspects required for the understanding of the recurrence risk for future reproductive choices.

The way forward

This pilot study was initiated in collaboration with the CPGR to investigate the 750 K array and the feasibility of using the Optima array for routine postnatal samples. It proved the value of both the Affymetrix 750 K and the Optima arrays for use in our diagnostic setting as it confirmed the results obtained on the Affymetrix Cytoscan HD, subtelomeric/microdeletion MLPA results, the OGT array and revealed a new finding. The new finding was made using the Optima and 750 K arrays (the HD was not performed); this finding revealed a 7.9 Mb gain on chromosome 11p15 and a 4.49 Mb loss on chromosome 14q32 indicating a possible unbalanced translocation. The parent did not show any aberration. Both these arrays have suitable probe coverage of the subtelomeric regions to detect gains and losses, which may be due to unbalanced translocations.

In establishing a CMA diagnostic service, a stepwise approach should be considered. Although an increase in CMA referrals will increase the DNA extraction workload, CMA will largely replace the currently used MLPA assays, microdeletion FISH assays and a sizable amount of the conventional chromosome analysis requests. The role of microdeletion for confirmation of CMA findings would need consideration. It is expensive to keep FISH probes on hand in the laboratory, especially if these are infrequently used.

A referral process to the CPGR for sample processing will initially be established. This will allow the NHLS diagnostic laboratory staff to focus on training and interpretation of the data analysis. Once staff competency is established, and depending on resources and referral numbers, a dedicated instrument for the diagnostic environment will be considered, with NHLS laboratory staff performing all steps in processing CMA samples.

After consultation with the local Medical Geneticists, the consensus was that the Optima array was compatible, with regard to coverage and cost, with the currently available testing repertoire (karyotyping, microdeletion and subtelomeric MLPA). The Optima array offers a minimum resolution of 1 Mb for losses, 2 Mb for gains and 5 Mb for AOH/LOH. This resolution will be used for the diagnostic reporting of postnatal results, thereby also accounting for VOUS, which would be reported in line with the Leiden laboratory criteria (> 1 Mb in size). The 750 K array would be the preferred array for patients with ID/DD. However, in order to establish the microarray diagnostic service within the NHLS, a tiered approach will be necessary. The recommendation for the introduction of microarray is therefore to implement the Optima microarray assay and once this has been successfully established, the 750 K array will be introduced. Strict gatekeeping criteria of this costly test will be instituted to prevent test requests for inappropriate phenotypes and to direct the requests to carefully selected patients.

Diagnostic analysis and reporting criteria need to be established according to international guidelines but taking into account the local context. These criteria will include the size of CNVs reported, reporting of VOUS and incidental findings such as cancer predisposition genes, when and which follow-up studies should be recommended, and when parental studies will be required. These criteria will be set in consultation with the relevant clinicians.

As the laboratory is in close contact with the clinicians referring the samples (and with resident Medical Geneticists within the Division of Human Genetics), requests for additional information and the discussion of recommendations should be easily facilitated. The reporting structure will be set according to international guidelines and recommendations set by external quality control programs.

CHAPTER 5 CONCLUSION

ID adds a significant burden to the family of an affected individual. This burden also extends to society as a whole. Performing CMA as the first-tier diagnostic test in patients with ID/DD, MCA and ASD would increase diagnostic yield and contributes to improved management in a number of patients. Receiving a diagnosis is of value to the patient/family, and contributes to treatment options, prognosis and allows for accurate recurrence risks counselling in both the index family and the extended family.

This study of CMA in a selected group of ID/DD patients showed a high diagnostic yield (26%) and confirmed that CNVs is a significant cause of ID/DD in the SA population. In addition, the skills and experience gained during this study will facilitate the introduction of microarray technologies in the routine diagnostic laboratory for the investigation of ID/DD, ASD and MCA in SA. In selected patients CMA should be offered as the first tier test replacing conventional cytogenetics.

5.1 Recommendations

1. Further research into ID to establish the incidence in SA as well identifying CNVs relevant to the SA population.
2. Training of laboratory staff on the processing, analysis and reporting of CMA data is required.
3. Training of clinical staff on the advantages and limitations of CMA, and in the clinical relevance of CMA results is required.
4. A referral service should be established with a partner such as the CPGR for the routine processing of samples. The raw data can then be analysed and reported on by trained staff in the relevant laboratories.
5. A national SA database should be established to reveal population specific CNVs and for consensus of reporting. Data from the current study can be used initially.
6. Communication through platforms reaching the entire SA genetic community such as the Genetics Expert Committee (GEC), that has been established within the NHLS should drive the implementation of CMA in the public health sector in SA.
7. Referral pathways and strict gate-keeping criteria should be defined to optimise access to CMA testing through Genetic clinics in the larger centres.

INTERNET RESOURCES

Affymetrix http://www.affymetrix.com/estore/

Cartagenia BENCH (https://cartagenia.com/cartagenia-bench-lab

DECIPHER https://decipher.sanger.ac.uk/

DGV http://dgv.tcag.ca/dgv/app/home

DNA Microarray – Wikipedia https://en.wikipedia.org/wiki/DNA_microarray

Ensembl http://www.ensembl.org/index.html

ECARUCA http://umcecaruca01.extern.umcn.nl:8080/ecaruca/ecaruca.jsp

Genetics Home Reference http://www.ghr.nlm.nih.gov/

Genomic Oligonucleotide and SNP Array Evaluation Tool

http://firefly.ccs.miami.edu/cgi-bin/ROH/ROH_analysis_tool.cgi

Human Genetics II – Biology 102 Course

http://carolguze.com/text/102-14-humangenetics2.shtml

ICD-10 Version:2016 http://apps.who.int/classifications/icd10/browse/2016/en#

ISCA/ICCG http://www.iscaconsortium.org/?viajml=1

Karger Medical and Scientific Publishers http://www.karger.com/Book/Home/257302

OMIM http://www.ncbi.nlm.nih.gov/omim

PubMed http://www.ncbi.nlm.nih.gov/pubmed/

UCSC https://genome.ucsc.edu/

UKNEQAS http://www.uknegas.org.uk/

UNIQUE http://www.rarechromo.org/html/home.asp

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ADDENDUM I ETHICS APPROVAL 2010

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6626 • Facsimile [021] 406 6411
e-mail: hrec@uct.ac.za

18 October 2010

HREC REF: 490/2010

Dr R Goliath
Human Genetics
Suite 3.14 Werner & Beit Building North
Medical school

Dear Dr Goliath

PROJECT TITLE: SNP ARRAY ANALYSIS IN CNV DETECTION: ASSESSMENT OF ITS FEASIBILITY IN THE DIAGNOSTIC SETTING

Thank you for submitting your new study to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the FHS HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until 28 October 2011.

We note that the above-mentioned study is a follow-up/sub-study to project 466/2005 entitled: Unravelling the molecular basis of a cohort of affected boys with X-linked mental retardation and their families previously approved by the HREC.

Please send us an annual progress report (website form FHS 016) if your research continues beyond the approval period. Alternatively, please send us a brief summary of your findings so that we can close the research file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

lemjosh

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Signed

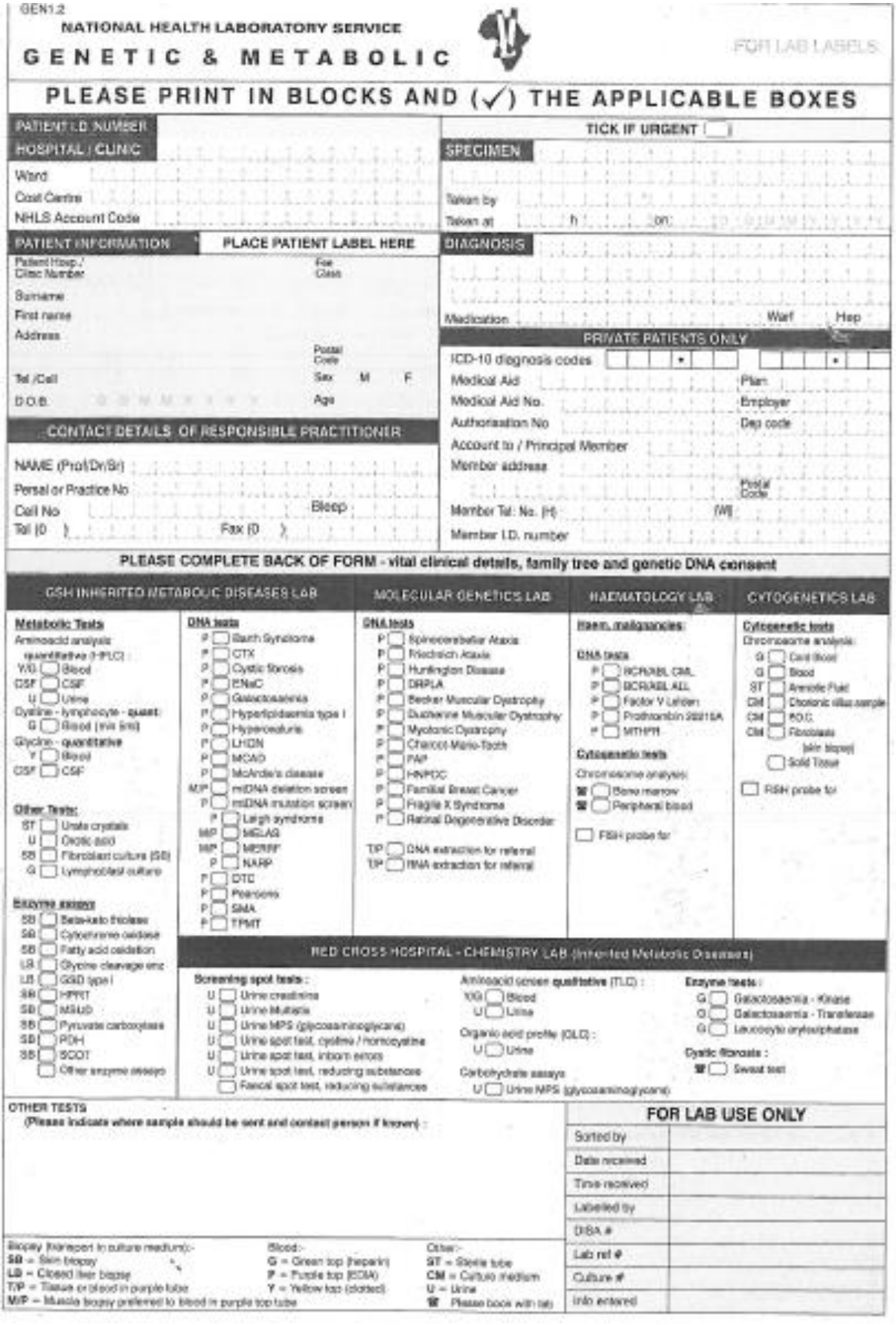
Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Initial

ADDENDUM II GENETICS REQUEST FORM WITH CONSENT



GEN1.2

NATIONAL HEALTH LABORATORY SERVICE

GENETIC & METABOLIC

FOR LAB LABELS

PLEASE PRINT IN BLOCKS AND (✓) THE APPLICABLE BOXES

PATIENT I.D. NUMBER

HOSPITAL / CLINIC

Ward

Cost Centre

NHLS Account Code

TICK IF URGENT

SPECIMEN

Taken by

Taken at h on

PATIENT INFORMATION

PLACE PATIENT LABEL HERE

Patient Hosp./ Clinic Number

Fax Class

Surname

First name

Address

Postal Code

Tel /Cell

Sex M F

D.O.B.

Age

DIAGNOSIS

Medication

Warf Hep

PRIVATE PATIENTS ONLY

ICD-10 diagnosis codes

Medical Aid

Plan

Medical Aid No.

Employer

Authorisation No

Dep code

Account to / Principal Member

Member address

Postal Code

Member Tel: No. (H) (W)

Member I.D. number

CONTACT DETAILS OF RESPONSIBLE PRACTITIONER

NAME (Prof/Dr/Sr)

Persal or Practice No

Cell No Bleep

Tel (0) Fax (0)

PLEASE COMPLETE BACK OF FORM - vital clinical details, family tree and genetic DNA consent

GSH INHERITED METABOLIC DISEASES LAB

Metabolic Tests

Aminoacid analysis quantitative (HPLC):

Y/G Blood

CSF CSF

U Urine

Cystine - lymphocyte - quant:

G Blood (min 5ml)

Glycine - quantitative

Y Blood

CSF CSF

Other Tests:

ST Urate crystals

U Orotic acid

SB Fibroblast culture (SB)

G Lymphoblast culture

Enzyme assays

SB Beta-keto thiolase

SB Cytochrome oxidase

SB Fatty acid oxidation

LB Glycine cleavage enz

LB GSD type I

SB HPRT

SB MSUD

SB Pyruvate carboxylase

SB PDH

SB SCOT

Other enzyme assays

DNA tests

P Barth Syndrome

P CTX

P Cystic fibrosis

P ENaC

P Galactosaemia

P Hypertriglidaemia type I

P Hyperoxaluria

P LHON

P MCAD

P McArdle's disease

M/P mtDNA deletion screen

P mtDNA mutation screen

P Leigh syndrome

M/P MELAS

M/P MERRF

P NARP

P OTC

P Pearsons

P SMA

P TPMT

MOLECULAR GENETICS LAB

DNA tests

P Spinocerebellar Ataxia

P Friedreich Ataxia

P Huntington Disease

P DRPLA

P Becker Muscular Dystrophy

P Duchenne Muscular Dystrophy

P Myotonic Dystrophy

P Charcot-Marie-Tooth

P PAP

P HNPCC

P Familial Breast Cancer

P Fragile X Syndrome

P Retinal Degenerative Disorder

T/P DNA extraction for referral

T/P RNA extraction for referral

HAEMATOLOGY LAB

Haem. malignancies:

DNA tests

P BCR/ABL CML

P BCR/ABL ALL

P Factor V Leiden

P Prothrombin 20210A

P MTHFR

Cytogenetic tests

Chromosome analysis:

Bone marrow

Peripheral blood

FISH probe for

CYTOGENETICS LAB

Cytogenetic tests

Chromosome analysis:

G Cord blood

G Blood

ST Amniotic Fluid

CM Chorionic villus sample

CM P.O.C.

CM Fibroblasts (skin biopsy)

Solid Tissue

FISH probe for

RED CROSS HOSPITAL - CHEMISTRY LAB (Inherited Metabolic Diseases)

Screening spot tests :

U Urine creatinine

U Urine Multistix

U Urine MPS (glycosaminoglycans)

U Urine spot test, cystine / homocystine

U Urine spot test, inborn errors

U Urine spot test, reducing substances

Faecal spot test, reducing substances

Aminoacid screen qualitative (TLC) :

Y/G Blood

U Urine

Organic acid profile (GLC) :

U Urine

Carbohydrate assays

U Urine MPS (glycosaminoglycans)

Enzyme tests :

G Galactosaemia - Kinase

G Galactosaemia - Transferase

G Leucocyte arylsulphatase

Cystic fibrosis :

Sweat test

OTHER TESTS

(Please indicate where sample should be sent and contact person if known) :

FOR LAB USE ONLY

Sorted by	
Date received	
Time received	
Labelled by	
DBA #	
Lab ref #	
Culture #	
Info entered	

Biopsy (transport in culture medium):-
SB = Skin biopsy
LB = Closed liver biopsy
T/P = Tissue or blood in purple tube
M/P = Muscle biopsy preferred to blood in purple top tube

Blood:-
G = Green top (heparin)
P = Purple top (EDTA)
Y = Yellow top (clotted)

Other:-
ST = Sterile tube
CM = Culture medium
U = Urine
Please book with lab

Additional information needed for cytogenetic and molecular genetic samples

Relevant clinical findings and family history: _____

_____

_____

_____

_____

Indicate ethnic origin of patient's mother _____ father _____

If prenatal referral, please give age of mother _____ gestational age _____ donor # _____

Abnormalities noted on ultrasound scan _____

_____

Is patient : affected ☐ at risk ☐ carrier ☐ spouse ☐ query ☐ unaffected ☐

Have samples for genetic studies been sent to the lab before? Yes / No / Don't know

Family tree

Consent is mandatory for tests performed in the Molecular Genetics Laboratory, and if DNA storage is required.

Consent for DNA analysis and storage

1. I _____, request that an attempt be made using genetic material to assess the probability that I / my child / my unborn child (DELETE WHERE NOT APPLICABLE) might have inherited a disease-causing mutation in the gene for: _____

2. I understand that the genetic material for analysis is to be obtained from: blood / skin sample / other (specify) (DELETE WHERE NOT APPLICABLE)

3. I request that no portion of the sample be stored for later use. ☐(MARK IF APPLICABLE)
   OR
   I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):
   (a) possible re-analysis
   (b) analysis for the benefit of members of my immediate family
   (c) research purposes, subject to the approval of the Research Ethics Committee, provided that all information will remain confidential.

4. The result of the analysis will be made known to me, via my doctor(s), in accordance with the relevant protocol, if and when available.

5. If clinically relevant, I authorise that the results may be made known to family members.

6. I have been informed that:
   (a) the analysis procedure is specific to the genetic condition and cannot determine the complete genetic makeup of an individual.
   (b) the genetics laboratory is under an obligation to respect medical confidentiality.
   (c) genetic analysis may not be informative for some families or family members.
   (d) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
   (e) where biological material is used for research purposes, there may be no direct benefit to me.

7. I understand that I may withdraw my consent for any aspect of the above at any time without this affecting my future medical care.

8. ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY

Doctor/Consultant _____ Date _____

Patient / Parent Signature _____ Date _____

CTS = Cardiofaciocutaneous syndrome
DRPLA = Dentatorubral pallidoluysian atrophy
EIEE = Epileptic infantile encephalopathy
FAP = Familial adenomatous polyposis coli
GSD = Glycogen storage disease type I
HNPCC = Hereditary non-polyposis colorectal cancer
HPRT = Hypoxanthine phosphoribosyl transferase
LHON = Leber hereditary optic neuropathy
MCAD = Medium chain acyl coenzyme A dehydrogenase
MSUD = Maple syrup urine disease
mtDNA deletion screen = KSS and CPEO
mtDNA mutation screen = Leigh's syndrome, MELAS, MERRF and NARP
MTHFR = Methylene tetrahydrofolate reductase
OTC = Ornithine transcarbamylase
PDH = Pyruvate dehydrogenase
SMA = Spinal muscular atrophy
SCOT = Succinyl CoA acyl transferase
TPMT = Thiopurine methyl transferase

ADDENDUM III QUALITY CONTROL

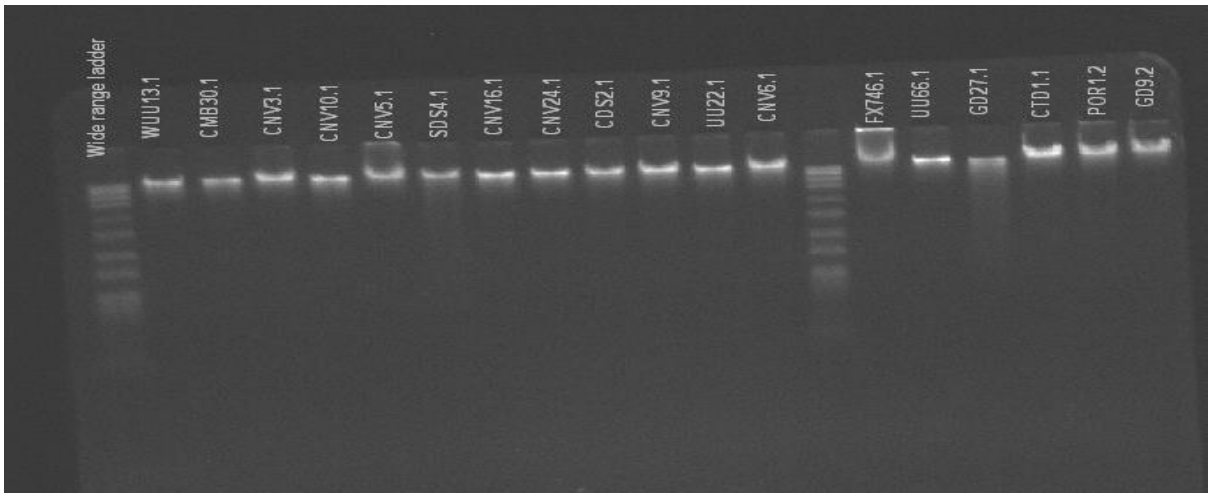


Figure A1 Gel electrophoresis QC of a subset of the cohort

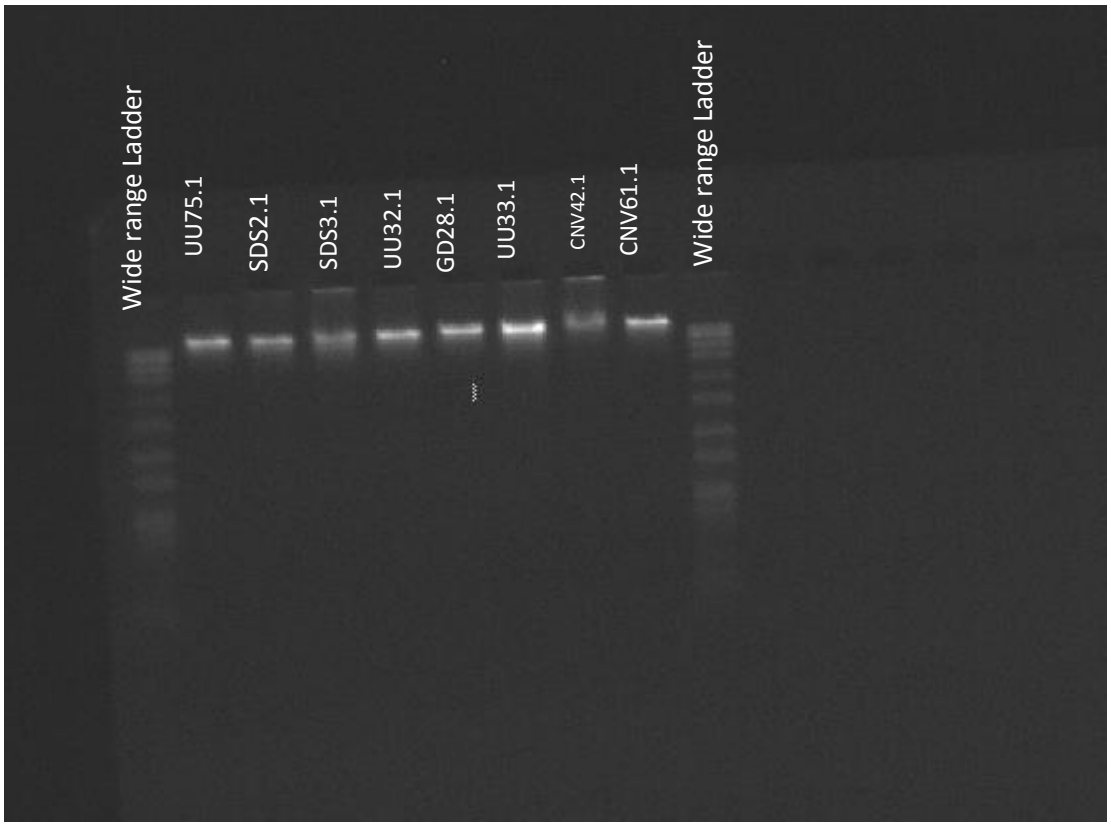


Figure A2 Gel electrophoresis QC of a second subset of the cohort

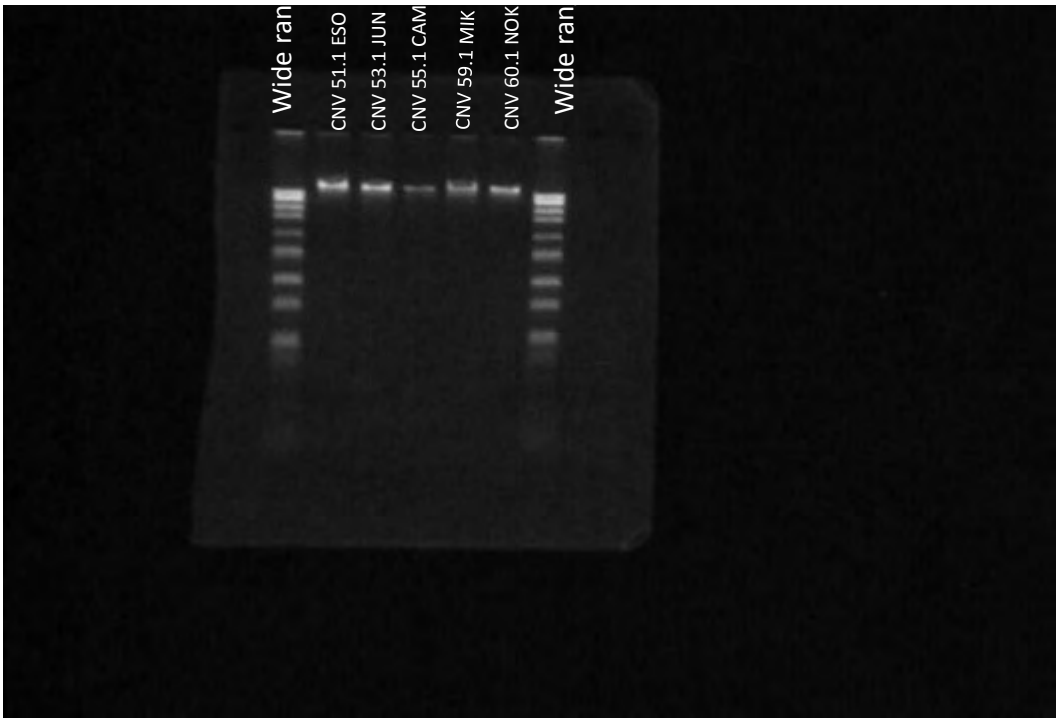


Figure A3 Gel electrophoresis QC of a third subset of the cohort

AT1 DNA QUALITY OF COHORT

	ng/ul	A260	A280	260/280	260/230
1	266.49	5.33	2.858	1.87	1.9
2	150.72	0.754	0.4	1.89	1.6
3	175.64	3.513	1.904	1.85	2.29
4	162.02	3.24	1.697	1.91	1.72
5	123.14	2.463	1.285	1.92	1.58
6	273.79	5.476	2.901	1.89	1.58
7	592.4	11.848	6.547	1.81	2.32
8	350.69	7.014	3.871	1.81	1.57
9	287.83	5.757	3.06	1.88	2.21
10	372.15	7.443	4.131	1.8	1.67
11	405.54	8.111	4.417	1.84	1.94
12	93.16	1.863	0.997	1.87	1.74
13	312.14	6.243	3.337	1.87	1.98
14	532.64	10.653	5.844	1.82	2.13
15	1465.24	29.305	15.925	1.84	2.04
16	61.13	1.223	0.672	1.82	2.02
17	118.49	2.37	1.266	1.87	1.5
18	1381.67	27.633	c	1.86	2.32
19	860.77	17.215	9.153	1.88	2.28
20	60.91	1.218	0.651	1.87	2.22
21	287.79	5.756	3.219	1.79	1.59
22	346.02	6.92	3.717	1.86	1.99
23	152.47	3.049	1.614	1.89	1.52
24	201.85	4.037	2.159	1.87	2.36
25	108.14	2.163	1.146	1.89	1.48
26	172.93	3.459	1.862	1.86	1.76
27	159.24	3.185	1.734	1.84	2.39

ADDENDUM IV Standard Operating Procedure: METHODOLOGY

Detailed methodology

The Affymetrix® Cytoscan™ HD Array (Affymetrix, Santa Clara, CA, USA) was used to perform genome-wide high resolution copy number and SNPs detection in this cohort. This array contains approximately 2.6 million markers for CN consisting of approximately 750 000 SNP and 1.9 million oligonucleotide probes. This is a targeted array with backbone spacing of one oligonucleotide every 2 Kb and one oligonucleotide probe every 400 bp in targeted regions. There are 200 SNP probes per Mb. All probes are 25 bp long. Each SNP is targeted by 6 probes, 3 per allele (Mason-Suares et al., 2013).

Data analysis software specifically for this analysis is available. The ChAS software requires a 25-probe call for CNVs and uses the Bayesian robust linear modeling with Mahalanobis distance perfect match algorithm to call SNPs (Mason-Suares et al., 2013). The Cytoscan HD provides information on sample heterogeneity and UPD.

Affymetrix Cytoscan™ HD Procedure

This array consisted of a square glass substrate mounted in a plastic cartridge. The oligonucleotides were contained on the inner glass surface of the array. Washing and hybridization occurs in a chamber reservoir directly under the glass.

GENERAL REQUIREMENTS:

CONTROLS

Positive and negative controls should be used in each run – control DNA is supplied in the kit and TR Buffer can be used for the negative control in the PCR gel QC stage.

EQUIPMENT

1. Only calibrated equipment should be used.
2. The equipment should be maintained according to recommended schedules.
3. One-directional flow should be maintained to avoid contamination.
4. Use filtered pipette tips throughout.
5. Heat the thermal cycler lid before each step.

DNA REQUIREMENTS

1. The DNA must be of a high purity:

OD_{260}/OD_{280}	1.8 – 2.0
OD_{260}/OD_{230}	>1.5

2. DNA should not be degraded. Using 1% agarose gel the average size of the gDNA can be determined. Approximately 90% of the DNA must be greater than 10 Kb in size.

2. 250 ng of double-stranded genomic DNA is required.
3. DNA dilutions must be made to a concentration of 50ng/ul.
4. Defrost the gDNA when needed, mix before use.

REAGENTS AND PLATES

1. All reagents were kept on ice throughout.
2. The plates were covered with adhesive film to prevent sample loss and cross-contamination during vortexing.
3. The plates were spun at room temperature at 1500 revolutions per minute (rpm) for 1 minute.
4. The reagent vials were spun for 3 seconds.
5. The reagents were vortexed 3 times for 1 second each.
6. The plates were vortexed for 1 to 2 seconds in each of the 5 sectors (Figure 45).

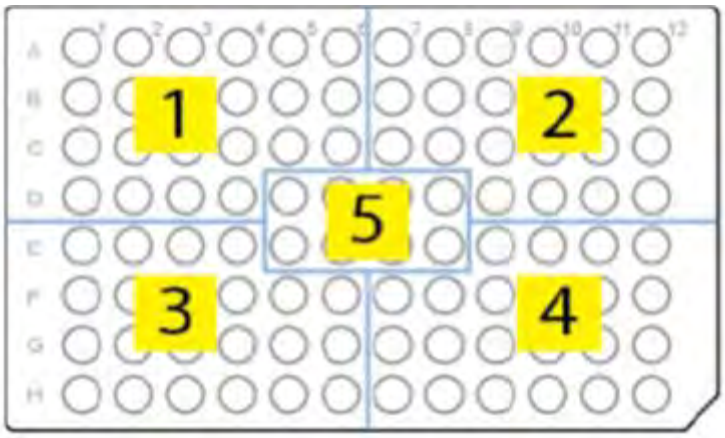
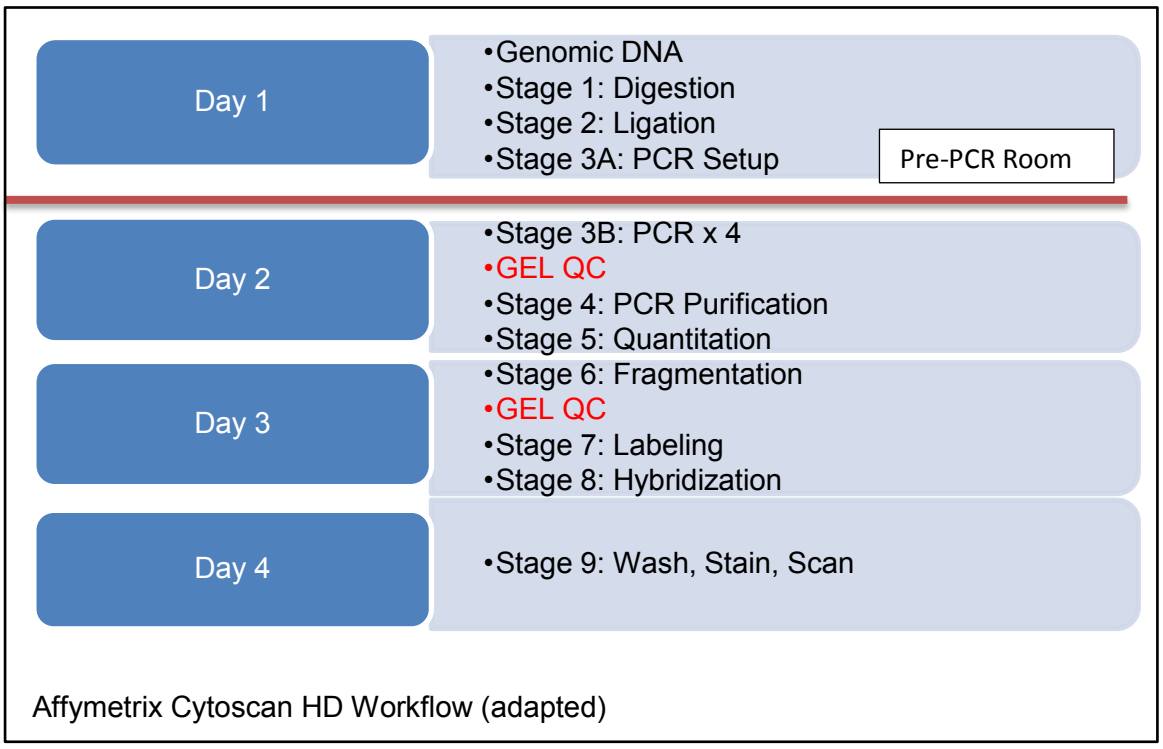


Figure A4 5-Sector vortex scheme

7. Scratches and contamination on the outside of the array may lead to inaccurate results.
8. Oils and other substances from skin can fluoresce giving inaccurate results.
9. The array were stored at 2° to 8°C.
10. All work was done on an aluminium cooler throughout.
11. The enzyme was kept in the freezer until needed, and placed on a -20°C cooler once out of the freezer.
12. Samples can be stored after each stage of the procedure.



Affymetrix Cytoscan HD Workflow (adapted)

DAY 1

STAGE 1 - RESTRICTION DIGEST

Required time: 3 hours including 30 minutes hands-on

1.1 In the Pre-PCR area, the cycler lid was preheated.

1.2 5ul of the diluted gDNA (see DNA Step 4) was aliquoted into the tubes in a tray.

1.3 The buffer and BSA were thawed, vortexed, spun and stored on ice.

1.4 The Digestion Master Mix was prepared:

REAGENT	1 SAMPLE	30 SAMPLES
Chilled Affymetrix® Nuclease-Free Water	11.55ul	346.6ul
10 x Nsp I buffer	2.00ul	60.0ul
100 x BSA	0.20ul	6.0ul
Nsp I	1.00ul	30.0ul
Total	**14.75ul**	**442.5ul**

1.5 The Digestion Master Mix was vortexed and spun down.

1.6 The Digestion Master Mix was added to each gDNA sample:

Reagent	Volume
gDNA (50ng/ul)	5ul
Digestion Master Mix	14.75ul
Total	**19.75ul**

1.7 The plate was covered, vortexed and spun.

1.8 The following Digest program was run on the thermal cycler:

Digest Thermocycler program:

Temperature	Time
37°C	2 hours
65°C	20 minutes
4°C	Hold

1.9 The plate was covered, vortexed and spun.

1.10 The samples were placed on an aluminium cooler on ice in preparation for Stage 2.

STAGE 2 – LIGATION

Required time: 4 hours including 30 minutes hands-on

1.1 The T4 DNA Ligase Buffer and 50uM Adaptor, Nsp I were thawed at room temperature.

1.2 The T4 DNA Ligase Buffer was vortexed to suspend any precipitate.

1.3 The T4 DNA Ligase was kept frozen until needed.

1.4 The Ligation Master Mix was prepared:

REAGENT	1 SAMPLE	30 SAMPLES
10 x T4 DNA Ligase Buffer	2.50ul	75.0ul
50uM Adaptor, Nsp I	0.75ul	22.5ul
T4 DNA Ligase	2.00ul	60ul

Total	**5.25ul**	**157.5ul**

1.5 The Ligation Master Mix was vortexed and spun down.

1.6 The Ligation Master Mix was added to each sample:

Reagent	**Volume**
Digested DNA	19.75ul
Ligation Master Mix	5.25ul
Total	**25.0ul**

1.7 The plate was covered, vortexed and spun.

1.8 The following Ligate program was run on the thermal cycler:

Ligate Thermocycler program:

Temperature	**Time**
16°C	3 hours
70°C	20 minutes
4°C	Hold

1.9 The plate was closed, vortexed and spun.

1.10 The samples were placed on an aluminium cooler on ice in preparation for Stage 3.

DAY 2

STAGE 3A – PCR SETUP

Required time: 1 hour hands-on

1.1 The plate was covered and spun.

1.2 The ligated sample was diluted:

Reagent	**Volume**
Ligated DNA	25ul
Nuclease-Free Water	75ul

Total	**100ul**

1.3 The plate was covered, vortexed and spun.

1.4 The samples were placed on an aluminum cooler on ice.

1.5 Four PCR reactions were required: four times 10ul of each sample was aliquot to the PCR plate. The remaining sample was kept in the freezer.

1.6 The Titanium Taq PCR Buffer, dNTP Mixture and PCR Primer 002 were thawed.

1.7 The Nuclease-free water and GC-Melt Reagent were kept on ice.

1.8 The 50 x Titanium™ Taq DNA Polymerase was kept frozen until needed.

1.9 The PCR Master mix was prepared:

REAGENT	**1 SAMPLE**	**30 SAMPLES**
Chilled Affymetrix® Nuclease-free water	39.5ul	1,185.0ul
10 x Titanium™ Taq PCR Buffer	10.0ul	300.0ul
GC-Melt Reagent	20.0ul	600.0ul
dNTP Mixture (2.5 Millimole (mM) each)	14.0ul	420.0ul
PCR Primer 002	4.5ul	135.0ul
50 x Titanium™ Taq DNA Polymerase	2.0ul	60.0ul
TOTAL	**90.0ul**	**2,700.0ul**

1.10 The PCR Master Mix was vortexed.

1.11 The PCR Master Mix was poured into a reservoir.

1.12 90ul of the PCR Master Mix was added to the samples:

Reagent	**Volume**
Diluted Ligated DNA	10ul
PCR Master Mix	90ul
Total	**100ul**

1.13 The samples were vortexed twice and spun.

1.14 The plate was kept on ice and loaded on a preheated thermal cycler in the Post-PCR room.

1.15 The following PCR program was run:

PCR Thermocycler program:

Temperature	**Time**	
94°C	3 minutes	X 1
94°C	30 seconds	X 30
60°C	45 seconds	
68°C	15 seconds	
68°C	7 minutes	X 1
4°C	Hold	

STAGE 3B – PCR SETUP

1.1 5ml nuclease-free water and 2ul loading dye were loaded Into 24 new well gel strip tubes.

1.2 3ul of the PCR product was added to the gel strip tubes.

1.3 The tubes were sealed, vortexed and spun.

1.4 8ul of the sample mix was loaded on a 2.5% agarose gel using a 100 bp ladder and run at 120V for 90 to 210 minutes.

1.5 The majority of the product should be between 150 and 2000 bp.

STAGE 4 – PCR PURIFICATION

Required time: 4 hours hands-on

1.1 All 4 PCR products for each sample were pooled into a 1.5ml Eppendorf tube.

1.2 45ml absolute ethanol was added to the Purification Wash Buffer.

1.3 The Purification Beads were inverted to ensure a homogeneous mixture.

1.4 720ul of the Purification Beads were added to each pooled sample. The mixture was inverted 10 times.

1.5 The mixture was left at room temperature for 10 minutes.

1.6 After centrifuging for 3 minutes at maximum speed the tube hinges were opened to face outward and placed on a magnetic stand.

1.7 Once the pellet had moved to the magnet, the supernatant was carefully pipetted without disturbing the pellet and discarded.

1.8 1ml of Purification Wash Buffer was added to each tube.

1.9 The tubes were vortexed at maximum for 2 minutes, followed by 3 minute spinning with the hinges facing out.

1.10 The tubes on placed on the magnetic stand, all of the Purification Wash Buffer was removed and the tubes were left uncapped at room temperature for 10 minutes.

1.11 52ul Elution Buffer was dispensed onto the beads using a 200ul pipette

1.12 The tubes were vortexed for 10 minutes at maximum speed to resuspend the beads.

1.13 The tubes were centrifuged with the hinges facing outward.

1.14 The tubes were returned to the magnetic stand. 47ul of the eluted sample was transferred to a new 96-well plate once the beads formed again.

1.15 The plate was vortexed and spun.

STAGE 5 – QUANTITATION

Required time: 30 minutes hands-on

1.1 18ul aliquots of Affymetrix® Nuclease-free water was aliquoted into new Eppendorf tubes.

1.2 2ul of each purified sample was added to each of the Eppendorf tubes, vortexed and spun.

1.3 The NanoDrop was used to determine the concentration (ng/ul), 260/280 ratio and the A320 of each sample. The acceptance criteria should be:

DNA Concentration	>300ng/ul
260/280 ratio	1.8 – 2.0
A320	Very close to „0" (0.1)

1.4 The remainder of the samples were transferred to the aluminum cooler block on ice.

DAY 3

STAGE 6 – FRAGMENTATION

Required time: 1.5 hours including 30 minutes hands-on

- **This is a critical step – work quickly and cold at all times.**
- **The enzyme is very sensitive to temperatures.**

1. The thermal cycler lid was heated.
2. The sample plate was cooled on ice prior to use.
3. The centrifuge was cooled to 4°C before use.
4. All the reagents were kept on ice while making up the Fragmentation Master Mix.
5. The Fragmentation Master Mix was prepared according to the Fragmentation Reagent:

REAGENT	**2.0U/ul**	**2.25U/ul**	**2.5U/ul**	**2.75/ul**	**3.0U/ul**
Chilled Affymetrix® Nuclease-free water	122.4ul	123.2ul	123.8ul	124.4ul	124.8ul
10 x Fragmentation Buffer	158.4ul	158.4ul	158.4ul	158.4ul	158.4ul
Fragmentation Reagent	7.2ul	6.4ul	5.8ul	5.2ul	4.8ul
TOTAL	288.0ul	288.0ul	288.0ul	288.0ul	288.0ul

6. 10ul of the Fragmentation Master Mix was added to the Purified PCR product:

Reagent	**Volume**
Purified PCR Product	45ul
Fragmentation Master Mix	10ul

Total	**55ul**

7. The Fragmentation Master Mix was vortexed at high speed in the 5 sector format, 1 second per sector.

8. The mixture was spun in a pre-cooled centrifuge at 2000 rpm for 1 minute.

9. The plate was loaded on a preheated thermal cycler and the following PCR program was run:

Fragment Thermocycler program:

Temperature	**Time**
37°C	35 minutes
95°C	15 minutes
4°C	Hold

10. The plate was vortexed and spun.

11. The samples were kept on ice.

12. 4ul of the fragmented sample was added to new Eppendorf tubes.

13. 28ul nuclease-free water was added to each tube.

14. The tubes were sealed, vortexed and spun.

15. 2ul of loading dye was added to 8ul of sample.

16. 8ul of the sample mix was loaded on a 2.5% agarose gel using a 25 bp ladder and run at 120V for 90 to 180 minutes.

17. The average fragment distribution should be between 25 and 125 bp.

18. The remaining aliquots were stored in the freezer.

19. Once the QC was within range, Stage 8 could be initiated.

STAGE 7 – LABELING

Required time: 5 hours including 30 minutes hands-on

1. The thermal cycler lid was preheated.
2. The 5 x TdT Buffer and 30mM DNA Labeling Reagent was thawed.
3. The TdT enzyme was kept in the freezer until use.
4. The Labeling Master Mix was prepared on ice:

REAGENT	**1 SAMPLE**	**30 SAMPLES**
5 x TdT Buffer	14.0ul	420.0ul
30mM DNA Labeling Reagent	2.0ul	60.0ul
TdT	3.5ul	105.0ul
TOTAL	19.5ul	585.0ul

5. The samples were vortexed and spun.
6. 19.5ul of the Labeling Master Mix was added to each sample:

Reagent	**Volume**
Fragmented DNA	51ul
Labeling Master Mix	19.5ul
Total	**70.5ul**

7. The tubes were vortexed in 5 sector format and spun.
8. The samples were placed in the thermal cycler and run:

Labeling Thermocycler program:

Temperature	**Time**
37°C	4 hours
95°C	15 minutes
4°C	Hold

9. The samples were vortexed and spun.
10. The samples were place on ice.

STAGE 8 – HYBRIDIZATION

Required time: 16 to 19 hours including 45 minutes hands-on

1. The arrays were allowed to reach room temperature.
2. The hybridization ovens were heated with the rotation turned on at 50°C for 1 hour.
3. The arrays were labelled with the sample identifiers.
4. The Hybridization Master Mix was prepared in a 15ml conical tube:

REAGENT	**1 SAMPLE**	**30 SAMPLES**
Hyb Buffer Part 1	165.0ul	4,950.0ul
Hyb Buffer Part 2	15.0ul	450.0ul
Hyb Buffer Part 3	7.0ul	210.0ul
Hyb Buffer Part 4	1.0ul	30.0ul
Oligo Control Reagent 0100	2.0ul	60.0ul
TOTAL	190.0ul	5,700ul

5. The mixture was vortex after the addition of each reagent (viscous).
6. 190ul of Hybridization Master Mix was added to each sample:

Reagent	**Volume**
Labeled DNA	70.5ul
Hybridization Master Mix	190ul
Total	**260.5ul**

7. The plate was sealed, vortexed and spun.
8. The following program was run on the thermal cycler:

Hybridization Thermocycler program:

Temperature	**Time**

95°C	10 minutes
49°C	1 minute
49°C	Hold

9. 200ul of sample (leave on the cycler) using a P200 pipette was loaded onto the array.

   Only 4 arrays can be hybridized at a time.

10. Any excess fluid must be cleared off and the septa sealed.
11. The array was placed in the hybridization tray.
12. The arrays were loaded immediately onto the hybridization oven at 50°C and 60 rpm for 16 to 18 hours.
13. A work list was created in order to register the arrays on the scanner.

DAY 4

STAGE 9 – WASH, STAIN, SCAN

Required time: Wash and stain: 3 hours including 30 minutes hands-on

Scan: 15 minutes hands-on, ~32 minutes on the scanner per array

1. Samples must remain in the hybridization oven until the fluidics station is ready.
2. The following reagents were prepared:

Position	Reagent	Volume	Tube Colour
1	Stain Buffer 1	500ul	Amber
2	Stain Buffer 2	500ul	Clear
3	Array Holding Buffer	800ul	Blue

3. The Fluidics Station was primed with the Wash buffers.
4. The stain solutions were loaded.
5. The Fluidics Protocol was started with the cartridge lever down.
6. The septa on the arrays were opened and placed in the Fluidics Station.
7. Check for bubbles during the process.

8. The septa were covered before scanning.
9. Once all the washes had been completed, the Shutdown protocol was run.
10. The scanner was warmed up for 20 minutes.
11. The array window was cleaned and placed in the autoloader.
12. Scanning was started.

ADDENDUM V STANDARD OPERATING PROCEDURE: ANALYSIS

The Affymetrix Chromosome Analysis Suite (ChAS) used the following specifications:

- Affymetrix Chromosome Analysis Suite (ChAS) 2.0 User Manual Rev 6
- The GRCh37/hg19 genome build was used for the analysis in this study
- Algorithm: CytoScanHD_Array.single_sample.def.NA32.3.v1.chasparam
- CytoScanHD_Array.na32.3.annot.db, CytoScanHD_Array.na32.3.v1.REF_MODEL

Initial analysis

In 2014, the initial analysis of the CNV data generated using the ChAS software was performed as follows:

1. The filters were set to the Standard setting:

Marker count	50
Size (Kbp)	400

2. If no large gain or loss was observed, the High Resolution setting was used:

Marker count	50
Size (Kbp)	100

Large gains and losses were observed in 8 patients. However, during a training period (March – April 2015) at the Laboratory of Diagnostic Genomic Analysis (LDGA) in Leiden, Netherlands, all the samples were reanalyzed using the routine procedure in use in this laboratory using the following criteria:

1. The Standard filters were set as follow:

Gain

Marker count : 10
Size (Kbp) : 20
Confidence : 85

Loss

Marker count : 10
Size (Kbp) : 10
Confidence : 85

LOH

Marker count : 1,000
Size (Kbp) : 2,000

2. The Tracks were set as follow:

a) Files

i. Genes

ii. OMIM

iii. Cytobands

b) Data types

i. CN state (Gain, Loss)

ii. Weighted Log2 Ratio

iii. Copy number change

iv. Filter LOH

v. Allele peaks

vi. Smooth signal

vii. Genotype calls

3. Check the QC and save as a text file:

SNPQC	> 15
MAPD	<0.25
Waviness SD	< 0.12

If the waviness SD >0.12, adjust the confidence to 90%. Assess if analysis is possible taking into account the type of sample and the clinical indication.

4. Save X chromosome Detail View which includes the complete chromosome X. This representation acts as a control to ensure the correct patient is being analysed.
5. Save Segment Report as a text file. This report contains all the CNVs detected on the sample. If there are more than 100 segments, adjust the confidence to 90%. This is due to poor QC. A result can still be obtained in the case of large rearrangements.
6. Check each chromosome individually for gains and losses, especially on the telomeres.
7. Check the X chromosome normalization. The shared X and Y markers are represented in the pink telomeric regions on the X chromosome. The X chromosome will show 3n in the pink telomeric region on the short arm of chromosome X.
8. The segment report information is loaded into the Cartegenia software program. This program has been set up to the Leiden laboratory"s specifications for example frequently reported benign variants reported in DGV, known Cytoscan variants. This program also serves as the in-house CNV database.
9. The size, gene content and location are assessed in the interpretation of each

imbalance.

10. Use the DECIPHER Genome Browser, Database of Genomic Variants (DGV), PubMed, OMIM and ENSEMBLE to evaluate the detected CNVs and their significance. An in-house database can also be used if available.
11. Only syndromes and protein coding genes relevant to the indication such as intellectual disability, and the clinical phenotype is reported.
12. Variants larger than 1 Mb in size but containing no genes should be reported as this may have a structural effect leading to the phenotype.
13. The classification of CNVs was done using the following criteria:
    a) A known syndrome or microdeletion/duplication syndrome was reported as such
    b) Susceptibility regions and a genetic abnormality associated with clinical phenotype, not described in DGV at time of analysis, but containing the region or part of coding exon would be reported. Examples of these include the chromosome 15q11.2 abnormality which falls within the breakpoint BPI to BPII containing the genes *TUBGCP5, CYF1P1, NIPA2* and the chromosome 6 of deletion exon 3 of gene *PARK2* which is not implicated in ID.
    c) Regions smaller than 150Kb in prenatal samples, and/or inherited susceptibility regions would not be reported. This criterion was not used as all samples in this study were postnatal.
    d) A genetic abnormality possibly associated with a clinical phenotype, but containing no coding genes or only containing one intron of a protein coding gene, and which was described as a variant in DGV at the time of analysis, would not be reported.
    e) If the variant is known in 3 or more normal controls but not associated with a known clinical phenotype (DGV excluding BAC studies), it may be classified as a polymorphism without clinical significance. The breakpoints can be within 300 Kb of a reported variant or overlapping with 1 probe arm area. If the variant is small, there has to be a 90% or more overlap with the patient"s CNV.
    f) A genetic variant with a relevant OMIM gene for the ID/DD indication would be reported.
    g) LOH with a CN of 2, covered by a 1000 probes and larger than 2000 Kb, except chromosome X, would be reported.
    h) Variants of unknown significance (VOUS) will be reported.
14. Regions of homozygosity/loss of heterozygosity can be entered into a web-based SNP array evaluation tool (www.ccs.miami.edu/ROH). This tool correlates these regions with OMIM, UCSC and NCBI databases reporting on relevant candidate disorders.

REPORT WRITING

There are minor differences in the reporting criteria of the external quality control (EQA) scheme and the Association of Clinical Cytogenetics (ACC) Guidelines.

1. United Kingdom National External Quality Assessment (UKNEQAS - EQA scheme) Requirements.

Reports should include the following information:

- Patient identifiers
- Sample type
- Referral reason
- ISCN nomenclature
- Written description including male or female profile
- Report criteria – platform, type, version, practical resolution and genome build
- Clinical interpretation
- Confirmation or follow up including testing parental samples
- Referral to genetic counselling services if applicable
- Limitations of the test

2. Association of Clinical Cytogenetics (ACC) Guidelines

a. A report where no significant imbalance was observed should include the summary statement and the karyotype using ISCN nomenclature, a description of the array which would include the manufacturer, array version, analysis software used, minimum resolution reported, and the limitations of the test.

b. An abnormal report should include the following:

- the summary statement and the karyotype using ISCN nomenclature including the genome build
- a description of the array which would include the manufacturer, array version, analysis software used, minimum resolution reported
- a clear explanation of the imbalance detected
- the start and end positions of the informative markers of the imbalance
- the size of the imbalance
- references substantiating and correlating with the finding, including databases used
- recommendations for specific follow-up studies for example subtelomeric FISH, chromosome analysis

- the clinical interpretation of the result should include the gene content of the imbalance or the specific gene relevant to the syndrome/phenotype, correlation between the findings and the clinical features

- follow-up studies which would include future risk of recurrence in the family and referral for genetic counselling
- limitations of the test

Appendix VI Comparison of available Affymetrix arrays according to probe resolution

Affymetrix, of which the Affymetrix Cytoscan HD (Affymetrix, Santa Clara, CA) was used for the current study, subsequently released the Affymetrix 750 K and the Affymetrix Optima. The 750 K is intended for routine diagnostic use in postnatal ID/DD patients but at a much lower resolution than the Affymetrix cytoscan HD. The 750 K array offers a medium probe resolution but will eliminate the amount of variants, benign and VOUS, detected, thereby reducing the „noise" of the higher resolution Cytoscan HD array. Low-level mosaicism may not be detectable with the 750 K array. The Affymetrix Optima is for the use in Prenatal and Product of Conception samples for the detection of aneuploidy, submicroscopic aberrations, mosaicism, maternal cell contamination (MCC), triploidy and copy neutral regions such as AOH and UPD.

AT 2 Comparison of available Affymetrix arrays according to probe resolution

	CYTOSCAN HD	CYTOSCAN 750K	OPTIMA
SNP density	High	Medium	Low
Probes:	1 X 2.6 million	1 X 750,436	1 X 315,608
• **Oligo**	1.9 million	550,000	18,018
• **SNP**	750,000	200,436	148,450
Application	• CNV • Copy-neutral LOH • UPD • Low-level mosaicism	• CNV • Copy-neutral LOH • UPD	PRENATAL AND POC: • Aneuploidy • Submicroscopic aberrations • Mosiacism • Maternal cell contamination (MCC) • Triploidy • Copy neutral events e.g. AOH and UPD
Coverage • ISCA Constitutional (340) • Cancer genes (526) • OMIM Morbid genes (2,640) • X Chromosome OMIM Morbid genes (177) • RefSeq genes (31,121)	• 100% • 100% • 98% • 100% • 96%	• 100% • 100% • 83% • 93% • 80%	• 396 regions of prenatal interest

Addendum VII Reportable CNVs detected

Summary of the relevant CNVs found in this study cohort:

Patient 1 is a female patient diagnosed with Kleefstra Syndrome as she had a submicroscopic telomeric deletion of 1.8 Mb on chromosome 9 at band q34.3 - arr[hg19] 9q34.3 (139,135,215-141,020,389)x1. The deletion in this patient correlated with the most severe form of the syndrome as it is larger than 1.6 Mb in size. The additional genes involved may explain the severity of her phenotype.

Patient 8 is a female patient diagnosed with MWS as she had a submicroscopic interstitial deletion of 2.09 Mb on chromosome 2 at band q22.2 to q22.3 - arr[hg19] 2q22.2q22.3(143,571,114-145,663,819)x1. This patient had severe ID and GDD, microcephaly, bifrontal narrowing, a flat nasal bridge with low columella, and agenesis of the corpus callosum which correlates with features described in MWS. This patient did not however have some of the other well-described features of MWS such as seizures (detected in 90% of Mowat et al., (2003) patients), Hirschsprung disease or cardiac abnormalities. Patient 8 has structural eye abnormalities including cataracts, coloboma and microphthalmia leaving her blind. These have been reported in MWS.

Patient 9 is a male patient with a deletion in the 1q21.1 microdeletion susceptibility region (susceptibility locus for neurodevelopmental disorders). A submicroscopic interstitial deletion of 1,79 Mb was noted on chromosome 1 at band q21.1 to q21.2 - arr[hg19] 1q21.1q21.2(146,101,790-147,897,962)x1. This patient"s deletion (146,101,790-147,897,962) was larger in size at 1.76 Mb than the typical 1.35 Mb size of the deletion syndrome region. The 1q21.1 recurrent microdeletion (susceptibility locus for neurodevelopmental disorders) is most likely the cause for the ID phenotype in this patient and possibly the psychiatric illness in his family. It is recommended that family studies should be done to confirm this.

Patient 11 is a female patient with a submicroscopic interstitial deletion of 9.1 Mb on chromosome 5 at band q14.3 to q21.1 - arr[hg19] 5q14.3q21.1(89,738,598-98,856,874)x1. This patient"s result was partially consistent with the cases reported in the literature by Cardoso et al. (2009) and Al-Khateb et al. (2013). A 5.8 Mb critical region for PH was proposed. Although PH was reported in the other patients, it has not been reported in this patient. Follow-up should be done to establish a possible diagnosis of PH. It seems likely that the large deletion of 9.1 Mb containing 37 genes contributes to the clinical presentation in this child.

Patient 18 is a female patient with a submicroscopic interstitial deletion of 88 Kb on chromosome 15 at band q15.3 - arr[hg19] 15q15.3(43,888,261- 43,976,406)x1. Three genes are included in this region of which the *STRC* gene encodes stereocilin which is located in the outer hair cells in the inner ear. The *STRC* gene is implicated in nonsyndromic autosomal

recessive sensorineural hearing loss (Francey et al., 2012; Zhang et al., 2007). The deletion of the *STRC* gene and the clinical feature of hearing loss may indicate a further putative mutation on the second *STRC* allele in this patient. Although the other clinical features could not be readily explained, it seems likely that the deletion encompassing the *STRC* gene in this patient contributes to the clinical presentation.

Patient 20 is a female patient was diagnosed with WHS. A submicroscopic subtelomeric deletion of 2.1 Mb on chromosome 4 at band p16.3 - arr[hg19] 4p16.3(68,345-2,172,555)x1. Only three of the clinical features in this patient specifically correlated with the features of WHS - DD, microcephaly and epilepsy with some possible overlap in craniofacial dysmorphism. Zollino et al. (2003) described a milder phenotype when the deletion is less than 3 Mb in size and may explain the milder phenotype in this patient.

Patient 23 is a male patient with a supernumerary marker on chromosome analysis. The marker was the size of an E group chromosome. Four (4) copies of a segment of chromosome 9 from band p24.3 to band q13, and which was 68.1 Mb in size, was detected. This segment included the entire short arm and extended into the long arm of chromosome 9 - arr[hg19] 9p24.3q13(203,861-68,330,127)x4. This patient"s result is consistent with a diagnosis of tetrasomy 9p and is the cause of the clinical presentation in this child.

Patient 19 is a male patient with ID and VP who demonstrated a submicroscopic interstitial deletion of 69 Kb on chromosome 22 at band q11.21 - arr[hg19] 22q11.21(19,231,636-19,300,915)x1. One gene, *CLTCL1*, was included in this region. This gene has been classed as a VOUS by the OMIM database although Nahorski et al. (2015) identified a homozygous c.988G-A transition in exon 7 of the *CLTCL1* gene in a consanguineous family with children affected with severe DD and pain insensitivity. Seven ROH totally 106 Mb in size were detected in this patient. The Genomic Oligonucleotide and SNP Array Evaluation Tool was used for the analysis and interpretation of ROH (Iourov et al., 2015; Wierenga et al., 2012). Upon analysis genes involved in the CFSMR syndrome, two genes involved in autosomal recessive deafness and a gene implicated in WS2D was found. The ROH confirmed the consanguinity in the family. Despite these findings it is not clear that this explains the patient"s phenotype.

Patient 10 is a 12-year old male patient with a submicroscopic interstitial deletion of 2.5 Mb on chromosome 1 at band p35.2 - arr[hg19] 1p35.2p35.1(30,476,867-33,054,650)x1. *EPB41*, *CCDC28B* and *LCK* are morbid genes in this region which did not seem to contribute to this patient"s phenotype. The DECIPHER genomic database reported a patient (258365) with a similar-sized deletion to patient 10. Two of the clinical features overlapped between this patient and the patient described in DECIPHER: intellectual disability and tapered fingers. Although no relevant disease-causing genes could be identified in this region, the size of the deletion (2.5 Mb) may be considered significant. This variant was classified as a VOUS.

Patient 24 is a male patient with a submicroscopic interstitial duplication on chromosome 13 at band q33.3 which was 301 Kb in size - arr[hg19] 13q33.3(109,771,548-110,072,888)x3. One gene (*MYO16)* was included in this region. It seems possible that the duplication of 301 Kb containing the *MYO16* gene could contribute to the clinical presentation in this child as the deletion of this gene has been associated with ID. This duplication should be considered a VOUS. Family studies may be useful to elucidate the finding.

Patients 15, 21 and 22 demonstrated a benign CNV of between 17 and 19 Kb in size on chromosome 6 at bands q14.3 to q15 (genomic coordinates: 6:87,998,958-88,016,639). Patients 12 and 22 demonstrated a benign CNV of 31 Kb in size on chromosome X at band q13.1 (genomic coordinates: 69,111,882-69,143,094). Patients 12 and 25 demonstrated a benign CNV of 10 Kb in size on chromosome 2 at band q32.3 (genomic coordinates: 192,880,111-192,891,920). These CNVs were classified as benign as they were small and did not contain any relevant genes.

A total of 10 patients revealed ROH with patient 19 demonstrating seven such regions. Another observation was a ROH call which was in fact an artifact due to a deletion in the same region (chromosome 6 in patient 11).

Addendum VIII Patients with normal CMA results

1. PATIENT 2

1.1 Clinical features

A male patient presented with dysmorphic features and developmental delay. This patient was born at term with a birth weight of 3.65 kg. There was no family history of significance. This patient has the following craniofacial features: cleft palate, hypotonia, low hairline, low set posteriorly rotated ears with a prominent crus, flat nasal bridge, gum hypertrophy, full nasal tip, a smooth philtrum with downturned corners of the mouth and long palpebral fissures. Skeletal features included brachydactyly with one dystropic nail and rockerbottom feet with hallux valgus. He is overweight with weight on the 90th centile but height just above the 3rd centile for age. He was able to sit at 13 months but could not walk or speak by 2 years of age. A CT brain scan was normal.

1.2 Genetic testing

This patient had a normal karyotype and no microdeletion FISH studies were performed. No microdeletion/microduplication or subtelomeric rearrangement was detected on MLPA analysis. FRAXA analysis was not performed.

1.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

1.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. One LOH was detected although no disease-related genes relevant to the patient"s phenotype were found.

2. PATIENT 3

2.1 Clinical features

A male patient presented with developmental delay, hypotonia, cleft palate and seizures. He also had large posteriorly rotated ears, anteverted nares, large palpebral fissures, a broad nasal root, hypertelorism and micrognathia. A female sibling with similar features including cleft palate and epilepsy died at 18 months. His mother had one miscarriage.

2.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies, MLPA or FRAXA analysis were performed.

2.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

2.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. One LOH was detected although no disease-related genes relevant to the patient‟s phenotype were found.

3. PATIENT 4

3.1. Clinical features

A male patient presented with developmental delay and dysmorphic features including brachycephaly, prominent ears, hypertelorism with mild ptosis and intermittent strabismus. He also had a narrow nose, thin lips with a smooth philtrum and a pointed chin. Further features include a sacral dimple, clinodactyly and single palmar creases with small nails. He also had ligamentous laxity, vesicoureteral reflux (VUR) and hydronephrosis. This patient had a birth weight of 2.26 kg and a head circumference of 33 cm. An magnetic resonance imaging (MRI) detected agenesis of the corpus callosum. His mother has mild intellectual disability with similar physical signs.

3.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies or MLPA analysis were performed. Negative for FRAXA.

3.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

3.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

4. PATIENT 5

4.1 Clinical features

A female patient presented with moderate developmental delay and ADHD. Clinical features reveal a coarse facies, external eyebrow flare, prognathism and a large mouth with thick lips. She has no speech. She had tuberculous meningitis at 6 months. There is no family history of note.

4.2 Genetic testing

This patient had a normal karyotype. No other genetics tests were performed.

4.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

4.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. One LOH was detected although no disease-related genes relevant to the patient"s phenotype were found.

5. PATIENT 6

5.1 Clinical features

A male patient presented with features suggestive of Vertebral anomalies, Anal atresia, Cardiac defects, Tracheoesophageal fistula and/or Oesophageal atresia, Renal and Radial anomalies and Limb defects (VACTERL) association including truncus arteriosus and a horseshoe kidney with no significant family history.

5.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies, MLPA or FRAXA analysis were performed.

5.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

5.4 Conclusion

The result is normal. Only benign CNVs were detected in this patient. However, a ROH was noted on chromosome 5 and chromosome 6. The ROH on chromosome 6 does however contain the *APC* gene which is implicated in Familial Adenomatous Polyposis. This gene is listed in Boone et al. (2013) as relevant to CNVs involved in dominant cancer predisposition genes. It is unlikely to be of relevance in a dominant cancer phenotype.

6. PATIENT 7

6.1 Clinical features

This patient has epilepsy and learning disabilities. She had a child which was stillborn at term and has two developmentally delayed children. The female child is small for her age and has developmental delay. The younger male child also has short stature but with the following additional findings. Facial dysmorphic features include ptosis, micropthalmia, downslanting

palpebral fissures, and strabismus together with retrognathia and pointed ears. He has cryptorchidism and CNS imaging shows periventricular calcification.

6.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies or FRAXA analysis were performed. No microdeletion/microduplication or subtelomeric rearrangement was detected with MLPA Analysis.

6.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

6.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

7. **PATIENT 12**

7.1 Clinical features

A female patient presented with short stature, prenatal onset of macrocephaly and developmental delay. Other clinical features included prognathism, widely spaced nipples, maxillary hypoplasia, furled eyebrows and a narrow forehead. She also had a choledochal cyst. She has isolated growth hormone deficiency.

7.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies or FRAXA analysis were performed. No microdeletion/microduplication or subtelomeric rearrangement was detected with MLPA Analysis.

7.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

7.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

8. PATIENT 13

8.1 Clinical features

A female patient presented with severe developmental delay. She has the following clinical features: short stature, microcephaly, posteriorly rotated ears with unfolded helices, strabismus, shallow orbits, almond-shaped palpebral fissures, upturned nares and a tented upper lip. She has a narrow chest, hypoplastic labia majora, clinodactyly, with slightly tapered

fingers and 2-3 toe syndactyly. A brain MRI showed deep cortical sulci. There was no family history of note.

8.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies, MLPA or FRAXA analysis were performed.

8.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

8.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. One LOH was detected although no disease-related genes relevant to the patient"s phenotype were found.

9. PATIENT 14

9.1 Clinical features

A male patient presented with dysmorphism, DD, multiple arterial aneurysms, cataract and a prematurely aged and wasted appearance. A connective tissue disorder was suspected.

9.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies, MLPA or FRAXA analysis were performed.

9.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

9.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

10. PATIENT 15

10.1 Clinical features

A male patient presented with ASD. He had no dysmorphic features and a head circumference on the 50th percentile. He has a brother with autism and 2 paternal nephews with ID. There is a paternal family history of psychiatric illness and bipolar disorder and a maternal family history of pregnancy and neonatal loss.

10.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies or MLPA analysis were performed. No expansion mutation was detected with FRAXA analysis.

10.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

10.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

11. PATIENT 16

11.1 Clinical features

This male patient presented with intellectual disability, dysmorphism and deafness. He had Duane anomaly (cranial nerve palsies).

11.2 Genetic testing

Chromosome analysis demonstrated a normal karyotype. No microdeletion FISH studies, MLPA or FRAXA analysis were performed.

11.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

11.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

12. PATIENT 17

12.1 Clinical features

A male patient presented with intellectual disability, deafness and possible dominant anaemia.

12.2 Genetic testing

Chromosome analysis was not performed. No microdeletion FISH studies, MLPA or FRAXA analysis were performed.

12.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

12.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

13. PATIENT 21

13.1 Clinical features

A male patient presented with dysmorphic features and developmental delay. This patient has the following craniofacial features: a low posterior hairline, his ears appear to have „cleft lobules" with an unusual crus, almond shaped eyes, hypotelorism and a flat nasal bridge. Skeletal features included tapering fingers and genu varum. The patient had a brain MRI at 3 years of age which showed subependymal grey matter heterotopia, normal occipital lobe morphology, cervical spine asymmetry and a suspected mild hemimegalencephaly. He was obese with a height on the 50th percentile and a head circumference on the 75th percentile.

13 .2 Genetic testing

Chromosome analysis demonstrated a normal male karyotype. Microdeletion FISH studies for Prader-Willi syndrome were negative. No MLPA or FRAXA analysis were performed.

13.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

13.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

14. PATIENT 22

14.1 Clinical features

A female patient presented with ID. The following craniofacial features were observed: midface hypoplasia, hypertelorism, telecanthus, epicanthus, cupped ears with preauricular tags and upslanted palbebral fissures. She also had hypotonia and hypermobility. She had a low birth weight. Her growth was around the 3rd percentile. There is a family history of ID: her mother, maternal grandmother and great grandmother and a maternal cousin had features of mild ID. Two maternal uncles had features of FASD.

14.2 Genetic testing

Chromosome analysis, microdeletion FISH studies and MLPA analysis were not performed. No expansion mutation was detected with FRAXA analysis.

14.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

14.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. Two LOH was detected although no disease-related genes relevant to the patient"s phenotype were found.

15. PATIENT 25

15.1 Clinical features

A female patient presented with severe developmental delay and epilepsy. She had the following clinical features: coarse face, prominent ears, retrognathia, scoliosis, bilateral sensorineural hearing loss, microcephaly and thin corpus callosum with white matter loss on MRI. This patient had placental insufficiency in utero and was born between 34 and 36 weeks with a birth weight of 1.5 kg. She also had an inguinal hernia and low tone at birth with mild spastic cerebral palsy but was able to sit at 9 months. There was no family history of note.

15.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies or FRAXA analysis were performed. No microdeletion or duplication was detected with MLPA Analysis.

15.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

15.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

16. PATIENT 26

16.1 Clinical features

A male patient presented with intellectual disability and autism spectrum disorder. He did not have obvious dysmorphology but had seizures and absent speech. There was no family history of note.

16.2 Genetic testing

This patient had a normal karyotype. No microdeletion FISH studies or MLPA analysis were performed. No expansion mutation was detected with FRAXA analysis.

16.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22)x2,(XY)x1

16.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. No LOH was detected.

17. PATIENT 27

17.1 Clinical features

This female patient has developmental delay and moderate intellectual disability with multiple dysmorphic features. She is short with her height on the 3rd centile with macrocephaly, a

widow"s peak, a low anterior hairline, cupped pointed ears with unfolded helices as well as hypertelorism and telecanthus with strabismus. Additional dysmorphic features include a broad nasal root with hypoplastic alae nasi, a low columella, microstomia and brachydactyly. Brain CT scan showed non-specific white matter loss. Her mother was mildly intellectually disabled.

17.2 Genetic testing

Chromosome analysis demonstrated a normal female karyotype. Di George microdeletion FISH using the *TUPLE (HIRA)* probe was performed and no microdeletion was detected. MLPA and FRAXA analysis was not performed.

17.3 CMA analysis

CMA testing showed only benign CNVs in this patient.

arr[hg19](1-22,X)x2

17.4 Conclusion

Only benign CNVs were detected in this patient. The result is normal. One LOH was detected although no disease-related genes relevant to the patient"s phenotype were found.