# Structural Genomic Abnormalities in Autism and Schizophrenia

*With a Focus on the 22q11.2 Deletion Syndrome*

Structurele genomische afwijkingen
in autisme en schizofrenie
*met speciale aandacht voor het 22q11.2 deletie syndroom*

(met een samenvatting in het Nederlands)

# Proefschrift

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# INTRODUCTION

## 1. Schizophrenia and autism

Mental disorders have a major impact on human well-being and represent a large economic burden to our society. The lifetime prevalence of schizophrenia and autism spectrum, the disorders that are the focus of this thesis, are respectively 1% and 0.6-0.7%[5, 6].

To date there is no curative treatment for schizophrenia and autism. In schizophrenia a combination of medication, psycho-education and rehabilitation is used in order to attenuate psychotic symptoms (hallucinations and delusions) and optimize overall functioning. Despite these efforts, the average prognosis is poor; 14 years after their first hospital admission about 60% of patients continue to suffer from psychotic symptoms; approximately 15% are symptom-free with medication while about 25% have recovered and function without medication[7].

> ***Schizophrenia*** *is a chronic psychiatric disorder characterized by the presence of hallucinations, delusions and disorganized behavior and speech (so called "positive symptoms"), as well as a reduction in the range and intensity of emotional expression and goal-directed behaviors, and poverty of speech ("negative symptoms") and a decline in overall functioning (source: DSM-IV[1])*

In autism spectrum disorders medication is sometimes used to reduce undesired behaviors such as irritation or aggressive behaviors[8, 9]. However, to date no effective medication is available against the core deficits of autism, such as social and communicative impairments. Positive effects of early intervention or behavioral training have been suggested in some studies, but have not been consistently replicated[10].

> ***Autism spectrum disorder*** *(ASD) is a group of developmental disorders characterized by impairments in social interaction, communication and the presence of restrictive or repetitive patterns of behavior. The autistic spectrum ranges from severe cases (the core syndrome called* ***autism*** *or autistic disorder) to milder forms which include pervasive developmental disorder, not otherwise specified (****PDD-NOS****) and* ***Asperger syndrome***[3]*. The prevalence of autism (approximately 0.2- 0,4%[5]) is lower than the prevalence of the broader spectrum, ASD (0.6-0.7%[5]).*

As is the case in schizophrenia, the long term prognosis of autism is unfavorable. Although there is variability, there is an average tendency for developmental regression during childhood. This means

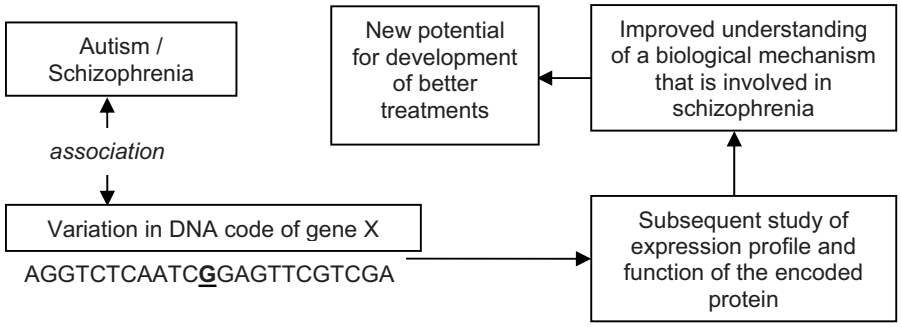
that for instance with regard to daily living skills the discrepancy increases between the actual level of abilities and what is normal given the child's chronological age[11]. Chronic poor social adjustment is seen in 60-75% of autistic individuals followed up to pre-adolescence or early adulthood, while approximately 5 – 15% of patients attain a (near) normal social life and acceptable functioning despite certain difficulties in social relationships and oddities in behavior (reviewed by Nordin et al.[12]). For the interpretation of these findings one needs to take into account that approximately 70% of autistic individuals have a variable degree of mental retardation[13]. Nevertheless, even when restricting the follow-up to autistic individuals with relatively better cognitive abilities (performance IQ>50), only about 12% acquires a high level of independence in adulthood, 10 % requires a minimal degree of support in daily living but is able to hold a job, 19% requires support and supervision but has some degree of independence outside a specialized residence, 46% needs a high level of support within a specialized residential setting and 12% is in need of high level hospital care[14].

Why is it not possible despite the huge progress medical science has made over the past few decades to cure these disorders? The main reason for the current lack of a safe and effective treatment for autism and schizophrenia is that the biological processes underlying these disorders are not well understood.

This is the motivation for the research reported in this thesis: An attempt to contribute to the ongoing international effort to improve our understanding of the biological underpinnings of autism and schizophrenia in the hope that this will open new avenues for effective treatment for individuals affected by these debilitating disorders.

## 2. Searching genes that contribute to autism and schizophrenia

One promising approach towards the improvement of our understanding of the biological mechanisms underlying autism and schizophrenia is the identification of the genes that contribute to these disorders. Once a gene is identified, subsequent studies can be undertaken to examine the timing and localization in the brain where the gene is at work ("expression profile") and the function of the protein it encodes, thus providing important information with regard to the biological pathways involved in autism and schizophrenia.




***How genetic studies can contribute to our understanding of the biology of mental disease:*** *the first step is to identify an association between a gene and a disease (left). Subsequently, studies are undertaken to elucidate where the gene is expressed (e.g. in the frontal cortex of the brain) and what the function is of the protein it encodes (for instance the breakdown of the neurotransmitter dopamine). These findings, together with results from other studies (e.g. neuro-imaging studies of the brain) contribute to a better understanding of the biological pathways that lead to schizophrenia. (In this example: that abnormalities in the metabolism of the neurotransmitter dopamine in the frontal cortex may be involved in schizophrenia). Ultimately, this knowledge may provide important directions for the development of new, more effective drugs against schizophrenia.*

In the context of the above, it is understandable that the search for genes contributing to psychiatric disorders has been an important focus of research over the last decades. Autism and schizophrenia are considered to have the strongest genetic causal contribution of all psychiatric diseases. Indeed, heritability estimates of both schizophrenia and autism are the highest amongst all mental disorders; for autism the estimated heritability is more than 90%[15], for schizophrenia between 80

and 85%[16, 17], indicative of a strong genetic causative component. In spite of this, results of the gene finding effort so far are modest at most. While an increasing number of contributing genes of (arguably) less complex phenotypes of pure somatic disorders such as blindness or bleeding disorders are identified, to date a consistent and replicable association between risk genes and autism or schizophrenia has not been demonstrated.

The main difficulties of finding the genes for autism and schizophrenia, and indeed, of finding genes for most psychiatric disorders, include the following:

1) Establishing a psychiatric diagnosis is exclusively based on clinical observations and therefore prone to errors.
2) It is likely that one psychiatric phenotype (e.g. autism) can be caused by several biological mechanisms. As a consequence different (sets of) genes may be involved from one patient to another (genetic heterogeneity).

***A gene*** *is a sequence of DNA building blocks ("base pairs") which encodes for a specific protein. The exact order of the base pairs ("DNA code") specifies the construction and thereby the function of the protein. Small changes in the DNA code, for instance a substitution of a base pair at a given position, potentially affect the characteristics and function of the resulting protein. Since all our physical features and functions are regulated by proteins at a cellular level, variations in DNA code can cause variations of our features (for instance eye color, anatomical aspects of the heart) and functions (for instance our blood's ability to coagulate). In some instances variations in DNA code results in a sub-optimal or even defective protein, leading to abnormal features (for instance congenital heart defects) or function (for instance impaired blood coagulation in bleeding disease)*[2].

With this in mind, it is important to realize that the classic study design of finding genes contributing to disease invariably implies the distribution of individuals in those with the disease ("cases") and those without ("controls") as a starting point of analysis. Subsequently, a statistical test is used to establish whether a genetic variation is more common in one of the two subgroups ("association study") or inherited together with the disease in families ("linkage study").
Thus, while a reliable allocation of individuals to the case or control group is a fundamental first step, and indeed decisive for the success of any genetic

association study, precisely this process is hampered by the complexity of the psychiatric phenotype.

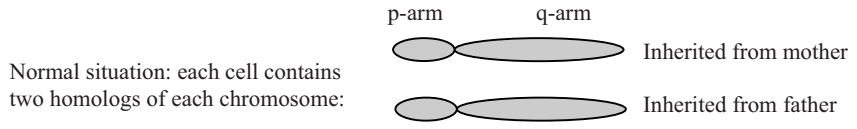
***Phenotype*** *is a term used to describe any measurable trait of an individual, again usually with regard to one specific trait. For instance, the phenotype "eye color" refers to whether a person has brown, blue or green eyes; the "schizophrenia phenotype" refers to whether or not a person suffers from schizophrenia.*

***Genotype*** *is a term used to describe the genetic characteristics of an individual, usually with regard to a specific aspect. For instance the genotypes XX and XY refer to the presence of two X chromosomes or one X and one Y chromosome, whereas the genotype* $COMT^{valmet}$ *denotes the situation of the variation of a specific gene; the individual carries one* $COMT^{val}$ *and one* $COMT^{met}$ *version ("allele") of the gene COMT.*

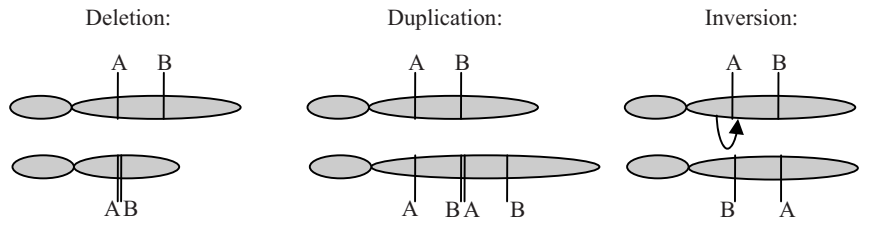
### 3. Why studying structural genomic abnormalities?

Structural genomic abnormalities are anomalies in the architecture of the chromosomes. For instance a translocation is the situation where a part of one chromosome becomes connected to a different chromosome. Other structural genomic abnormalities are deletions (a part of a chromosome is lost) and duplications (a part of a chromosome is duplicated)[18].

*With a few exceptions, the majority of human cell types contain an identical set of **chromosomes** specific for each individual. Each chromosome consists of a single continuous piece of DNA sequence which contains the code for genes and other regulatory elements. This "DNA-helix" is densely organized around protein structures. Humans cells carry 23 chromosome pairs, each pair consists of two homolog chromosomes, one of which is paternally inherited (i.e. derived from the person's father), and one is maternally inherited. Chromosomes have one long arm, referred to as "q" and one short arm, called "p"* [2].




Examples of structural genomic abnormalities:




The individual with this deletion has only one copy of the genes that are located in the affected region (between A and B).

The individual with this duplication has three copies of the genes that are located in the affected region (between A and B).

In the individual with this inversion some genes between A and B may be moved away or moved closer to regulation sites (arrow).

Since we have two copies (homologs) of each chromosome (with the exception of the sex chromosome in males), we have two copies of each gene. This implies that in the event of a deletion, genes in the deleted region are present in one copy instead of the normal situation of two copies. Carriers of a duplication have three copies of the genes that reside in the affected chromosomal region. With regard to translocations and inversions, the breakpoints of the involved regions may disrupt a code sequence for a gene and / or displace genes away from important regulatory elements. Importantly, once the extent and localization of the genomic region that is involved in a structural genomic abnormality can be characterized, the genes that reside in the affected region can be inferred.
Some structural genomic abnormalities are recurrent; i.e. they can be found in a group of individuals. When a structural genomic abnormality co-occurs with a certain disease phenotype, one can hypothesize that one or more genes in the region involved in the abnormality is causally related to the disease. For instance, an increased prevalence of autism spectrum disorders is found amongst individuals with duplications of a part of the long arm of chromosome 15[19]. Therefore, one can hypothesize that one or more genes in this region of chromosome 15 is causally related to ASD.

The advantage of studying the genes associated with disease in populations where the disease phenotype co-occurs with a structural genomic abnormality can be summarized as follows:

1) Instead of having to deal with the approximately 25,000 genes of the entire human genome (Human Genome Project; htpp://www.genome.gov), one can limit the association studies to the genes that are involved in the genomic abnormality, even though this does not exclude the influence of genes located elsewhere in the genome.
2) The first step of any study of this kind is not the identification of a psychiatric phenotype, with the associated complexities as described above, but the far more reliable identification of individuals with a certain structural genomic abnormality.

3) One can make the assumption that a psychiatric disorder in individuals with the same structural genomic abnormality is caused by the same biological pathway. In other words, the genetic (and thus to some extent the biological) heterogeneity is relatively small when compared to the heterogeneity of the same psychiatric phenotype in the general population.

The following example is an illustration of this principle. Individuals with Down syndrome have three copies of chromosome 21, instead of two. The observation that the prevalence of Alzheimer disease is increased in individuals with Down syndrome[20] suggests that one or more genes on chromosome 21 are causally related to Alzheimer disease[21]. Therefore, some investigators have concentrated their gene-finding efforts to chromosome 21, instead of searching the entire genome. Indeed, subsequent testing of this hypothesis has led to the discovery of a causative gene for Alzheimer which resides on chromosome 21[22, 23].

## 4. The 22q11.2 deletion syndrome

The 22q11-deletion syndrome is associated with a deletion of a small region (1.2 – 3 Mb) on the long arm of chromosome 22[4, 24]. This congenital malformation syndrome has an estimated prevalence of approximately 1/4,000 births, equally affecting male and female individuals[25-27]. Velocardiofacial syndrome (VCFS) and several other syndromes such as DiGeorge syndrome are associated with the same structural genetic abnormality. The term "22q11.2 deletion syndrome" (22q11DS) is now widely accepted by both clinicians and patient groups.

Characteristic physical manifestations include typical facial appearance, cleft palate, velopharyngeal insufficiency, lymphoid tissue hypoplasia and conotruncal heart defects, although variability is large. Already in the earliest descriptions of the phenotype, learning disabilities were also mentioned as a common feature[28, 29]. In 1992, Shprintzen, et al. reported a high prevalence of psychiatric disorders in patients with the 22q11DS, the most frequent diagnosis in his cohort being schizophrenia[30]. In subsequent studies this finding was confirmed; approximately 20-30% of adults with 22q11DS have schizophrenia[31]. However, schizophrenia is not the only psychiatric disease described in individuals with 22q11DS; amongst others, studies in 22q11DS have reported mood disorder[32], attention deficit hyperactivity disorder [33], and obsessive-compulsive disorder[34] to occur at an increased frequency.

Therefore, 22q11DS can be considered as an example of a structural genomic abnormality, in this case a deletion, with a consistent association with an increased risk of a psychiatric phenotype. Following the argument presented above, one could hypothesize that one or more genes within the deleted region on the long arm of chromosome 22 is involved in a biological pathway underlying the observed psychiatric disease. In Appendix A the genes involved in the 22q11.2 deletion are listed.

However, before the hypothesis of an association between genes at 22q11.2 and psychiatric symptoms in individuals with this syndrome can be tested, the two

components of this argument; the genotype on the one end and the phenotype on the other, need to be studied in more detail.

With regard to the psychiatric phenotype there is sufficient evidence that individuals with 22q11DS have an approximately 20 to 30 fold increased risk of schizophrenia in comparison to the general population. However, how to interpret the observation that many other psychiatric diagnoses also occur at increased rates in persons with 22q11DS?
One explanation may be that the diagnostic classification used in psychiatry, which clusters the observed psychiatric symptoms into diagnoses, may not be sufficiently adapted to the range of symptoms that can be seen in individuals with 22q11DS. Put differently, it may be that individuals with 22q11DS demonstrate combinations of psychiatric symptoms that are unusual in the general population. In this context the term "behavioral phenotype" is used. The term refers to a specific set of behavioral / psychiatric symptoms that can be observed in individuals with a genetic syndrome[35]. For instance, on average, young children with Down syndrome often demonstrate relative strengths of social-emotional skills and social relatedness, but also an inclination to stubborn and oppositional behaviors[36, 37]. These characteristics are therefore considered by some as part of the behavioral phenotype of the Down syndrome.

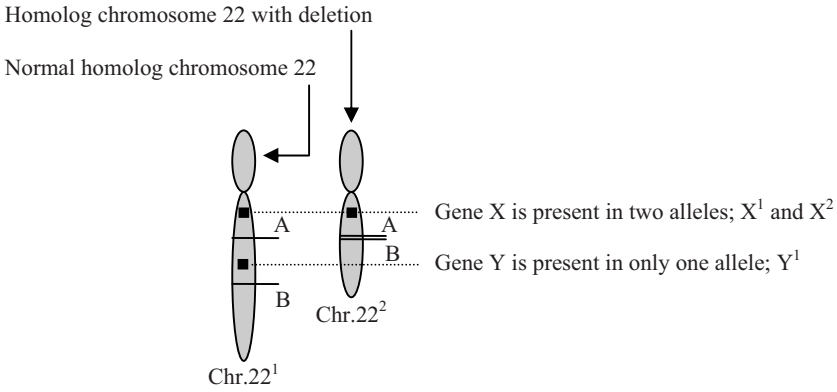
In order to examine whether it is justified to evoke the concept behavioral phenotype in relation to the 22q11.2 deletion syndrome, studies should not be limited to examine the prevalence of psychiatric diagnoses, but should also include detailed descriptions of the psychiatric observations on a symptom level. Furthermore, it is necessary to examine the extent to which the observed psychiatric symptoms are specific for 22q11DS, or alternatively, can be explained by the (non-specific) effect of the on average lower intelligence in this population.

Regarding the genotype of 22q11DS, several aspects of genetic variation between individuals with this syndrome need to be taken into account. First, it is important to be aware of the fact that individuals with 22q11DS all have a deletion at 22q11.2 in common, but differ from each other with respect to the genetic variation on the rest of the entire genome. To continue with the previously evoked example of

Alzheimer disease in persons with the Down syndrome: variations in the APOE gene, a gene *not* located on chromosome 21, influences the age of onset of Alzheimer disease in individuals with Down syndrome[38]. In a similar manner, it is likely that variations in genes outside the 22q11.2 region influence the risk of schizophrenia in 22q11DS. Although highly relevant, this aspect is beyond the scope of this thesis.

Amongst the other sources of genetic variation between individuals with 22q11DS, two variables are studied in particular in this thesis.

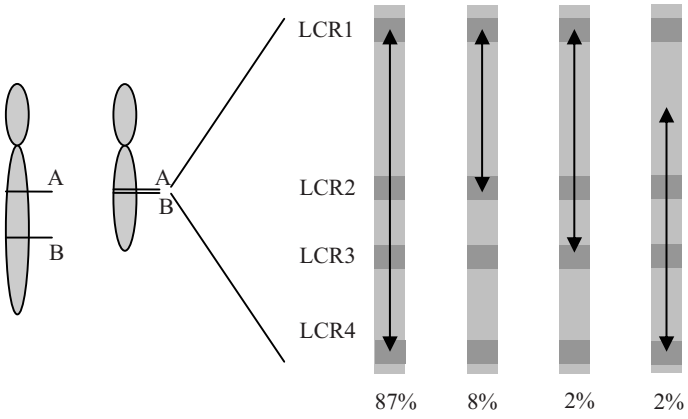
The first genetic variable is the variation of the remaining alleles (copies) of the genes that reside in the deleted region. Each of these genes is present in one allele instead of the normal situation of two alleles. Any variation in the DNA code of these genes that influences the function of the encoded protein is "functionally dominant" in this situation. Because a second, potentially compensating allele of the same gene is absent, the effect of the genetic variation at the remaining allele can be decisive.




**A functional dominant effect of the remaining allele**: normally, one allele of a gene is present on each copy of the two chromosome homologs (e.g. gene X is present in two alleles, $X^1$ and $X^2$). In case of a deletion there is only one allele of the genes that are located in the deleted region (e.g. gene Y).
A genetic variation with a functional effect on the encoded protein in the DNA sequence of one of the alleles of gene X (e.g. $X^1$) may be compensated by the normal configuration of the second allele (e.g. $X^2$). However, if there is variation in the only, "remaining" allele of gene Y ( $Y^1$), there is no compensating second allele.

For instance, there is a common variation of the gene COMT, which is associated with a significant decrease in enzyme activity. Given that COMT is located in the 22q11.2 region, this variation may have an influence on the risk of certain cognitive deficits or psychiatric symptoms in individuals with 22q11DS. This hypothesis has been tested in several studies to date, however thus far with conflicting results: in some studies a negative effect on the phenotype was associated with the $COMT^{val}$ allele[39-41], while in other studies there was no effect[42], or a negative effect associated with the opposite ($COMT^{met}$) allele[43, 44].

Another genetic variable of relevance is the size and extent of the deletion. In all cases the deletion is situated at a region on the long arm of chromosome 22q, referred to as "22q11.2". However, there is variation with regard to the exact endpoints and length of the region involved in the deletion[4]. Possibly, variation in deletion size and deletion endpoints can be associated with variation in psychiatric symptoms. Indeed, results of one study suggest that deletion characteristics can be correlated to somatic features of the syndrome, such as the typical facial features of 22q11DS and the presence of conotruncal heart defects[45], but in a different study this finding could not be replicated[46]. To our knowledge, there are no studies to date that have examined the possibility of a correlation between 22q11.2 deletion characteristics and the psychiatric symptoms.




***Different types of chromosome 22 deletions:*** *The deletions at 22q11.2 are thought to be mediated by the presence of stretches of DNA sequence that are highly similar to each other, called "low copy repeats" (LCRs). Typically, deletions at 22q11.2 occur between LCR1 and LCR4 (87%). Atypical deletions include those that are mediated by any other combination of the LCRs (e.g. LCR1 and LCR2 (8%), LCR1 and LCR3 (2%), and deletions have their endpoints outside an LCR (2%), (figure adapted from Shaikh et al.[4]).*

## 5. Rationale of the studies in this thesis

Following the line of reasoning presented in the foregoing paragraphs a rationale for the studies in this thesis can be constructed. Schizophrenia and autism are severe psychiatric disorders for which to date there is no curative treatment. The main reason is that the underlying biological pathways to these diseases are not well understood. Improving our understanding of the biology of these diseases will generate new opportunities for the development of better and more efficient treatments.
One approach to unravel the biological components involved in the causation of schizophrenia and autism is the identification of genes associated with these diseases. A gene encodes a protein of which the function and localization of action can be analyzed in subsequent studies. However, gene-finding in psychiatric diseases has proven to be a complex endeavor, with thus far modest results. A reliable identification of individuals with and without a psychiatric disease phenotype, a critical step in genetic association studies, is not easy and predisposed to inaccuracy.
An alternative approach is the study of individuals with structural genomic abnormalities concurring with a psychiatric phenotype. One can assume that one or more genes within the genomic region affected by the abnormality are involved in the biological pathway leading to the disease in these individuals. Thus, genetic research of these particular cases is an appealing strategy because it greatly limits the amount of potential candidate genes and reduces the genetic heterogeneity of the disease under study.

**Chapter 1** is a review of all studies that report the concurrence of autism and a structural genomic abnormality in individuals. This review was performed with the hypothesis that for some regions of the human genome a clustering of reported autism-related genomic abnormalities may become apparent. If true, this would suggest that these regions of overlap are particularly interesting since they may contain one or more genes associated with autism.

In **chapter2** the findings are presented of a clinical study involving 60 children, aged 9 through 18 years, all with a confirmed deletion at 22q11.2. This study was designed to improve the knowledge about the prevalence of psychiatric disorders in children with 22q11DS and to provide a description of the psychiatric phenotype on a symptom level (as opposed to limiting the description to diagnostic classes). Furthermore, it investigates to what extent the reported psychiatric disorders in children with 22q11DS are associated with the level of intelligence.

In **chapters 3 and 4** two molecular genetic studies are presented. In both studies a specific method for the detection of deletions or duplications at 22q11.2 is investigated. This method, Multiplex Ligation-dependent Probe Amplification (MLPA) is a relatively simple technique with the advantage of being more cost-effective and more informative than the existing "golden standard" of diagnosis deletions at 22q11.2 (Fluorescent In Situ Hybridization, FISH). Such a diagnostic method is important because it greatly improves the availability of a screening for structural genomic abnormalities in individuals with features of 22q11DS and because it provides specific information with regard to the extent of the genomic region involved. The latter aspect may be relevant because the precise localization and length of the deletion defines which genes are affected.
In **chapter 3** the reliability (sensitivity and specificity) of MLPA for the detection of copy number changes at 22q11.2 is tested on a set of over 50 samples with various structural genomic abnormalities all of which had been previously characterized with other molecular genetic methods. In **chapter 4** the results of an effort to improve the existing MLPA-kit are reported. In short, in this revised MLPA-kit the density of probes used to detect copy number changes at each locus is augmented in order to obtain a higher resolution of information about the 22q11.2 region.

In **chapter 5** the prevalence of 22q11.2 deletions in a sample of over 300 Dutch patients with schizophrenia is studied. Previous studies have reported that in approximately 2% of patients with schizophrenia a previously undiagnosed 22q11.2 deletion can be found. This study was set out to test the hypothesis that the prevalence of 22q11.2 deletions is higher in the subgroup of patients with schizophrenia who have enduring, idiopathic negative symptoms. This hypothesis is

funded on the observation that some of these negative symptoms have also been reported in clinical studies of 22q11DS patients (e.g. social withdrawal, lack of mental energy, bland affect and poor social skills).

**Chapters 6 and 7** describe two analyses which were carried out in line with the central premise of this thesis; i.e. that the study of patients in whom a psychiatric phenotype concurs with a structural genomic abnormality allows for the identification of genes related to this phenotype.
In **chapter 6** results are presented of a study in which the performance on three brain function paradigms is compared between children with 22q11DS and typically developing children. Only the paradigms in which the 22q11DS children perform significantly worse were carried forward as outcome measures to test the hypothesis of an interactive effect of the genes COMT and PRODH on brain function. The genes COMT and PRODH are located in the 22q11.2 region; consequently individuals with 22q11DS carry only one copy of both genes. This makes both genes vulnerable for the effect of genetic variation in the remaining alleles. Similarly, in **chapter 7** an association between the gene PIK4CA and schizophrenia in adults with 22q11DS is hypothesized. If confirmed, the findings of this study may offer part of the explanation why individuals with the 22q11.2 deletion are at increased risk of schizophrenia. The hypothesized effect of PIK4CA, located at 22q11.2, is based on a recent report of association between this gene and schizophrenia in the general population[47].

In **chapter 8** the same leading principle of this thesis is applied to an entirely different structural genomic abnormality. In this study we describe the clinical and genetic findings of a young patient with autism who carries a deletion on the long arm of chromosome 13. Since the mother of the patient carried the same deletion but did not have autism, we hypothesized that in the proband the remaining allele of one of the genes located in the deleted region was affected by a functional mutation. This “double hit” scenario could have led to a disruption of the function of the gene, making it a plausible cause of the autistic disorder diagnosed in the child.

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## CHAPTER 1

# Identification of novel autism candidate regions through analysis of reported cytogenetic abnormalities associated with autism

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**ABSTRACT:**

**Introduction:** The identification of the candidate genes for autism through linkage and association studies has proven to be a difficult enterprise. An alternative approach is the analysis of cytogenetic abnormalities associated with autism. We present a review of all studies to date that relate patients with cytogenetic abnormalities to the autism phenotype.

**Method:** A literature survey of the Medline, and Pubmed databases was performed, using multiple keyword searches. Additional searches through cited references and abstracts from the major genetic conferences from 2000 onwards, completed the search.

The quality of the phenotype (i.e. of the autism spectrum diagnosis) was rated for each included case. Available specific probe and marker information was used to optimally define the boundaries of the cytogenetic abnormalities. In case of recurrent deletions or duplications on chromosome 15 and 22 the positions of the low copy repeats that are thought to mediate these rearrangements were used to define the most likely boundaries of the implicated 'Cytogenetic Regions Of Interest' (CROIs). If no molecular data were available, the sequence position of the relevant chromosome bands was used to obtain the approximate molecular boundaries of the CROI.

**Results:** The findings of the current review indicate: 1) several regions of overlap between CROIs and known loci of significant linkage and/or association findings, and 2) additional regions of overlap among multiple CROIs at the same locus. Whereas the first finding confirms previous linkage / association findings, the latter may represent novel, not previously identified regions containing genes that contribute to autism.

**Conclusions:** This analysis not only has confirmed the presence of several known autism risk regions, but has also revealed additional previously unidentified loci, including 2q37, 5p15, 11q25, 16q22.3, 17p11.2, 18q21.1, 18q23, 22q11.2, 22q13.3 and Xp22.2-p22.3.

## INTRODUCTION

Autism is a neurodevelopmental disorder, which manifests itself in early childhood. It is characterized by impairments in social interaction, communication and the presence of restrictive or stereotyped patterns of behavior. Autism is the core syndrome of the pervasive developmental disorders as described in the fourth edition of the Diagnostic and Statistical Manual (DSM-IV)[1], which also includes other autistic like-disorders. Prevalence estimates of autism in the general population have gradually increased over the last 20 years[2-5]. Currently, the prevalence of all the pervasive developmental disorders is estimated to be 27.5 / 10,000[3]. Cumulative evidence from family and twin studies suggests that genetic factors play an important role in the etiology of autism[6, 7]. The prevalence of autism in siblings of autistic children is estimated to be 2-8%, which is much higher than can be expected on the basis of the prevalence in the normal population[8-11]. Moreover, the concordance of autism in monozygotic twins is approximately 60-91%[12, 13]. The susceptibility for autism is estimated to be more than 90% genetic, and several genes are thought to contribute to the disorder, although the mode of inheritance is unknown[14]. Apart from genetic factors, environmental factors may be involved. Viral infections during or after pregnancy have been associated with autism in some infants, typically with viruses such as Rubella[15], Hemophilus Influenzae and Cytomegalovirus[16]. Measles, Mumps and Rubella vaccination also has been related to autism[17], but no evidence for this relationship has been found in several other studies[18-20]. Intrauterine exposure to teratogenic drugs including Thalidomide and Valproate may increase the risk for autism[21, 22]. In addition, obstetric complications have been evoked as a risk factor for autism[23], although a causal relationship has not been demonstrated[24, 25]. Genetic studies have shown linkage between autism and markers located in regions on several chromosomes. However, applying a threshold of 2.2 or 3.6 for the multipoint logarithm of the odds score (MLS), the proposed thresholds for suggestive and significant findings respectively[26], only few studies met these thresholds. Based on these studies, regions that may contain genes contributing to autism include 2q[27], 3q[28], 7q[29](significant linkage), and 11p, 17q (suggestive linkage in more than one study). For an overview of linkage studies with MLS > 2.0 see table 1.

**Table 1: Linkage studies with LOD-score results > 2.0**

| Chr. | Linkage LOD > 2.0 | References |
|---|---|---|
| 1 | D1S1675 (p13.2) | Risch, Am J Hum Genet 1999; 65(2): 493-507. |
| | D1S1653 (q23.2) | Auranen M, Am J Hum Genet 2002; 71(4): 777-790. |
| | D1S1656 (q42.2) | Buxbaum JD, Mol Psychiatry 2004; 9(2): 144-150. |
| 2 | D2S2188 (q31.1) | International Molecular Genetic Study of Autism Consortium (IMGSAC). Am J Hum Genet. 2001; 69(3): 570-581. |
| | D2S364 (q31.3) | Buxbaum JD, Am. J. Hum. Genet 2001; 68: 1514-1520. |
| 3 | D3S3037 (q26.32) | Auranen M, Am J Hum Genet 2002; 71(4): 777-790. |
| | D3S3680 (p25.2) | Shao Y, Am J Med Genet 2002; 114(1) :99-105. |
| 4 | D4S1647 (q23) | Buxbaum JD, Mol Psychiatry 2004; 9(2): 144-150. |
| 5 | D5S2494 (p13.1) | Liu J, Am J Hum Genet 2001; 69(2): 327-340. Yonan AL, Am J Hum Genet 2003; 73(4): 886-897. |
| | D5S1473 (p14.3) | Buxbaum JD, Mol Psychiatry 2004;9(2): 144-50. |
| 6 | D6S283 (q16.3) | Philippe A, Hum Mol Genet 1999; 8(5): 805-12. |
| | D6S261 (q22.1) | |
| | D6S1270 (q14.3) | Buxbaum JD, Mol Psychiatry 2004; 9(2): 144-150. |
| 7 | D7S1813 (q21.2) | Barrett S, Am J Med Genet 1999; 88(6): 609-615. |
| | D7S483 (q36.1) | Liu J, Am J Hum Genet 2001; 69(2): 327-340. |
| | D7S477 (q22.1) | IMGSAC, Am J Hum Genet 2001; 69(3): 570-581 |
| | D7S2462(q36.2) | Auranen M, Am J Hum Genet 2002; 71(4): 777-790. |
| 10 | D10S1412 (p14) | Buxbaum JD, Mol Psychiatry 2004; 9(2): 144-150. |
| 11 | D11S1392 (p13) | |
| | D11S1993 (p12) | Yonan AL, Am J Hum Genet. 2003; 73(4): 886-897. |
| | D11S1392 (p13) | Buxbaum JD, Mol Psychiatry 2004; 9(2): 144-150. |
| 13 | D13S1229 (q12.3) | Barrett S, Am J Med Genet 1999; 88(6): 609-615. |
| 16 | D16S3102 (p13.13) | IMGSAC, Am J Hum Genet. 2001; 69(3): 570-581. |
| 17 | HTTINT2 (q11.2) | IMGSAC, Am J Hum Genet. 2001; 69(3): 570-581. |
| | D17S1800(q11.2) | Yonan AL, Am J Hum Genet 2003; 73(4): 886-897. |
| | SLC6A4 (q11.2) | McCauley JL, Am J Med Genet 2004; 127B(1): 104-112. |
| 19 | D19S433 (q12) | Liu J, Am J Hum Genet 2001; 69(2): 327-340. |
| | D19S714 (p13.12) | Buxbaum JD, Mol Psychiatry 2004; 9(2): 144-150. |
| X | DXS1047 (q25) | Liu J, Am J Hum Genet 2001; 69(2): 327-340. |
| | DXS7132 (q12) | Auranen M, Am J Hum Genet 2002; 71(4): 777-790. |
| | DXS6789 (q21.33) | Shao Y, Am J Med Genet 2002; 114(1): 99-105. |

Several association and candidate gene studies have been conducted, suggesting the involvement of genes of the serotonin system[30-35], genes of the gamma-aminobutyric acid system[36-39] and numerous other genes such as *PIK3CG* (7q22)[40], *RELN* (7q22)[41-43], *NRCAM*(7q22.3) and *LAMB1*(7q31.1)[44, 45], *WNT2* (7q31.2)[46] and *FOXP2* (7q31)[47]. For an overview of significant ($p<0.05$) association studies see table 2.

Although of great interest, the data from linkage studies do not directly indicate the gene(s) of interest, and candidate gene studies suffer from methodological difficulties. For example, the a priori selection of candidate genes is difficult, partly because one third of all human genes is expressed in the brain, or during brain development[48]. Further, consensus on an appropriate level of statistical correction for association tests is difficult to establish[49].

In conclusion, despite the promising results of linkage and association studies, the detection of specific genes contributing to autism has thus far been elusive. This may be due to several problems. Genetic heterogeneity of the autism phenotype, but also the likely possibility of the involvement of several interacting genes and variability in the expression of risk genes[50, 51] are examples of such problems. Also, various epigenetic mechanisms, such as parental imprinting and skewed X-inactivation, may affect the susceptibility for autism[52]. Another complicating factor is the fact that the expression of some genes is influenced by specific regulatory regions that may act from relatively large distances, even from other chromosomes[53], which further complicates the selection of candidate genes.

**Table 2: Association studies in autism with significant ($p < 0.05$) results**

| Chr. | Association | References |
|---|---|---|
| 1 | MTF1(p34.3) | Serajee FJ, J Child Neurol 2004; 19(6): 413-417. |
| 2 | INPP1(q32.2) | Serajee FJ, Journal of Medical Genetics 2003; 40: e119 |
| | SLC25A12 (q31.1) | Ramoz N, Am J Psychiatry 2004; 161(4): 662-669. |
| 6 | HLA-DRB1 (p21.32) | Torres AR, Hum Immunol 2002; 63(4): 311-336. |
| 7 | WNT2 (q31.2) | Wassink TH, Am J Med Genet 2001; 105(5): 406-413. |
| | UBE2H (7q32) | Vourc'h P, Psychiatr Genet 2003; 13(4): 221-225. |
| | Glut. receptor 8 (q31) | Serajee FJ, J Med Genet 2003; 40: e42 |
| | PIK3CG (q22), | Serajee FJ, J Med Genet 2003; 40: e119 |
| | RELN (q22) | Skaar DA, Mol Psychiatry 2005; 10(6): 563-571. |
| | | Persico AM, Mol Psychiatry 2001; 6(2): 150-159. |
| | | Zhang H, Mol.Psychiatry 2002; 7: 198 – 207 |
| | LAMB1(q31.1), | Bonora E, Eur J Hum Genet 2005; 13(2): 198-207. |
| | NRCAM(q22.3) | Hutcheson HB, BMC Med Genet. 2004 May 05;5(1):12. |
| | Engrailed2 (En2) (q36) | Gharani N, Mol Psychiatry 2004; 9(5): 474-484. |
| | FOXP2 (7q31) | Gong X, Am J Med Genet 2004; 127B(1): 113-116. |
| 15 | $GABA_A$ receptor sub- | McCauley JL, Am J Med Genet 2004; 131B(1): 51-59. |
| | units GABRB3 and | Buxbaum JD, Mol Psychiatry 2002; 7: 311-316. |
| | GABRA5 (q12) | Cook EH, Am J Hum Genet 1998; 62(5): 1077-1083 |
| | | Menold MM, J Neurogenet. 2001; 15(3-4): 245-259. |
| | | Martin ER, Am J Med Genet 2000; 96(1): 43-48. |
| | ATP10C (q12) | Nurmi EL, Mol Psychiatry 2003; 8(6): 624-634, 570. |
| | UBE3A-gene (q12) | Numri EL, Genomics 2001; 77(1-2): 105-113. |
| 16 | TSC2 (p13.3) | Serajee FJ, et al., Journal of Med Genet 2003; 40: e119 |
| 17 | Serotonin transporter | Cook EH, Mol Psychiatry 1997, 2, 247-250 |
| | (5HTT) and | Klauck SM, Hum Mol Genet 1997; 6, 2233-2238 |
| | promoter regions (q11) | Yirmiya N, Am J Med Genet 2001; 105:381-386 |
| | | Trodjman S, Molecular Psychiatry 2001, 6: 434-439. |
| | | Kim SJ, Mol Psychiatry 2002; 7(3): 278-288. |
| | | Conroy J, Mol Psychiatry 2004; 9(6): 587-593. |

An alternative and potentially fruitful approach is the analysis of cytogenetic abnormalities that have been identified in patients with autism. It is estimated that a chromosome abnormality, such as a deletion, duplication, inversion or translocation, can be identified in about 3% of these patients[54] and a vast number of such cases have been described in the literature. Several reviews evaluating cytogenetic abnormalities co-occurring with autism have been published[55-58]. These cytogenetic abnormalities can lead to functional genetic changes in several ways: 1) Dosage effects can occur in some genes as a consequence of changes in gene copy number (e.g. in a deletion or duplication). 2) Breakpoints associated with a rearrangement event may directly disrupt a gene. 3) Genes may be separated from gene-regulatory sequences as a consequence of the rearrangement (position effect). 4) Deletions may lead to the unmasking of a point mutation in a gene located in the corresponding region of the non-deleted homologous chromosome.

Our review has four aims. First, to provide an updated list of relevant publications till January 2005. Second, to provide accurate molecular positions of all relevant cytogenetic regions derived upon cross linking of all available probe and/or banding information from the case-reports with the recently nearly completed sequence database of the human genome**.** The definition of these "Cytogenetic Regions Of Interest" (CROIs) combined with data regarding expression and functions of the genes contained within these regions, may greatly aid in the identification of candidate genes for autism. Third, to present two types of overlap in the findings; namely the overlap between CROIs and loci derived from previous linkage and association studies, and alternatively, overlap among multiple CROIs at the same locus. The significance of the first is mainly a confirmation of previous findings through a different genetic approach. The importance of the latter is the potential identification of novel genomic regions not previously detected by the classic genetic approaches. The fourth aim of the current review is to provide a systematically evaluated rating of the validity of the phenotype (i.e. the clinical diagnosis of autism) of each included case report. This rating adds to the presented data in that it allows for a systematic ranking of CROIs based on the validity of the clinical diagnosis of autism.

**METHOD**

A literature survey of the Medline, and Pubmed databases was performed, using multiple keyword searches with terms such as: “autis*”, “genetic*”, “cytogenetic*, and “chromosom*” in several combinations. Additional searches through cited references and abstracts from the major genetic conferences from 2000 onwards (the annual meetings of the American Society of Human Genetics and European Society of Human Genetics), completed the search. All case-reports were investigated and summarized, with a special emphasis on the definition of the cytogenetic abnormality and the quality of the phenotype.

The quality of the phenotype (i.e. of the autism spectrum diagnosis) was rated for each included case. Ratings were based on the clinical description, the presence of a psychiatric diagnosis, preferably based on a classification system (e.g. Diagnostic and Statistical Manual of Mental Disorders, International Classification and Diagnostic systems), and the use of standard diagnostic research instruments, such as the Autism Diagnostic Interview-Revised (ADI-R)[59], the Childhood Autism Rating Scale (CARS)[60], and the Autism Diagnostic Observation Schedule-Generic (ADOS-G)[61]. The ratings were scored in several consensus meetings of psychiatrists and child psychiatrists (see table 3).

Cases involving chromosomal mosaicism were excluded. Cases with a gene mutation as the most likely genetic cause for autism were excluded as well, for example patients with fragile-X syndrome caused by FMR1 mutations. All karyotype descriptions are presented according to the International System for human Cytogenetic Nomenclature 1995 (ISCN 1995).

Cases were scrutinized for specific probe/marker information to optimally define the boundaries of the cytogenetic abnormalities. In case of recurrent deletions or duplications on chromosome 15 and 22 the positions of the low copy repeats that are thought to mediate these rearrangements were used to define the most likely boundaries of the CROIs. Positions of the low copy repeats were defined based on the available literature[62-65].

Several genome databases were used (see table 4). If molecular cytogenetic information was lacking, the CROIs could only be defined based on the karyotype descriptions. In such cases the sequence position of the relevant chromosome bands were used to obtain the approximate molecular boundaries of the CROI. In several cases marker positions appeared inconsistent with the initial karyotype provided. In those cases karyotypes were corrected according to the marker information. Finally, in some cases authors were contacted in an attempt to obtain more updated information (for instance on marker results or methods used to obtain the autism spectrum diagnosis).

**Table 3: Quality rating of clinical diagnosis of autism spectrum disorder**

| **Quality of phenotype** | **Criteria** |
|---|---|
| ***** (5) | Validated checklist and diagnosis according DSM or ICD classification |
| **** (4) | Validated checklist or diagnosis according DSM or ICD classification and clinical description |
| *** (3) | Diagnosis with clinical description without classification. |
| ** (2) | Clinical description highly suggestive for autism but without formal diagnosis. |
| * (1) | Some clinical autistic like features – no formal diagnosis |

**RESULTS**

The findings from the cytogenetic cases and the data from linkage and association studies are integrated in figure 1. This figure is a largely condensed visualization of the overview. The complete graphic overview can be found at the repository WebPages of Molecular Psychiatry (http://www.nature.com/mp). In this diagram all CROIs are represented in scale on each chromosome, along with their sequence coordinates, the phenotype validity ratings, additional comments and references.

Overlap between several CROIs and loci from linkage and association studies are found amongst others on multiple locations on chromosome 7, on 15q11-q13, and 5p14.

Possible novel regions are indicated by overlap between multiple CROIs without linkage or association findings at the same locus. Such regions are found on 2q37, 5p15, 11q25, 16q22.3, 17p11.2, 18q21.1, 18q23, 22q11.2, 22q13.3 and Xp22.2-p22.3 (red boxes, figure 1).

**DISCUSSION**

This review is intended to provide researchers in the field of autism with an overview of potentially interesting regions for positional candidate genes. Cytogenetic abnormalities related to autism are provided with well defined positions of these regions on the corresponding chromosomes whenever possible, and we also included a critical evaluation of the diagnosis of autism in all cases. The results of this literature survey confirm several known loci and indicate several novel CROIs associated with the autism phenotype (see figure 1 and the repository WebPages of Molecular Psychiatry).

The CROIs are not equally distributed across the human genome, but rather tend to cluster on specific regions. These regions include: Chromosome 2 band q37, chromosome 5 band p14-p15, several locations on the long arm of chromosome 7, chromosome 15 band q11-q13, chromosome 11 band q25, chromosome 16 band q22.3, chromosome 17 band p11.2, chromosome 18 bands q21.1 and q23, chromosome 22 bands q11.2 and q13.3 and chromosome X band p22. From these regions, most cases were reported on chromosomes 7, 15 and 22. The frequent occurrence of autistic patients with 15q11-q13 and 22q11.2 rearrangements may be partly due to the particular molecular organization of these chromosomal regions, as these rearrangements arise by unequal meiotic recombination between low copy repeat clusters[66].

The large number of cases with rearrangements of chromosome 15 is consistent with previous reports, and includes deletions, interstitial duplications and isodicentric supernumerary marker chromosomes 15 leading to tetrasomy of the involved region. The latter, encompassing the Prader Willi/Angelman Syndrome critical region, are usually maternally inherited. The Prader Willi/Angelman critical region is gene rich and includes the gamma-aminobutyric acid receptor genes. These genes are expressed on neurons throughout the CNS, at both pre- and postsynaptic sites. Indeed, several association studies of the GABA receptor genes have produced significant results[36, 39, 67, 68]. Association studies of other potentially interesting genes for this region include *ATP10C*[69] and *UBE3A*[70]. However, no linkage with LOD scores larger than 2.0 was found to this region.

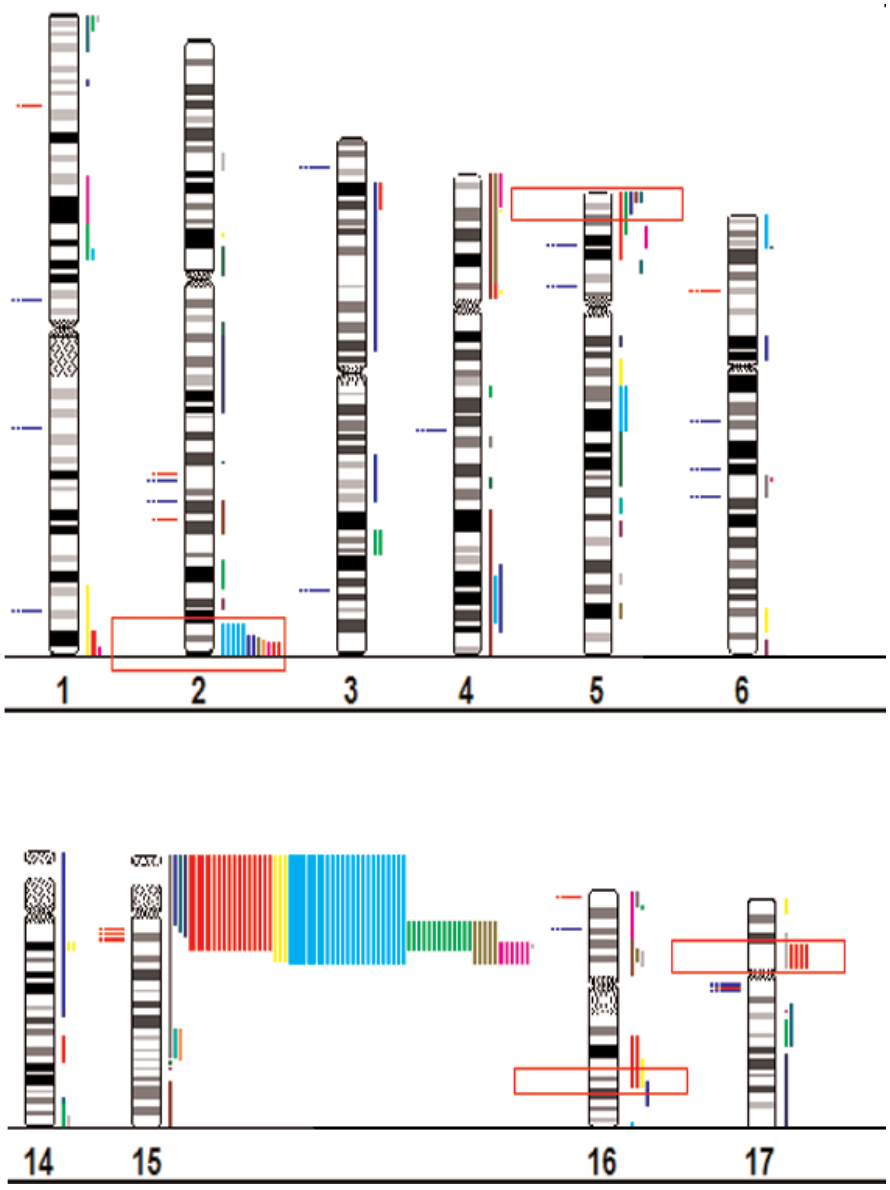
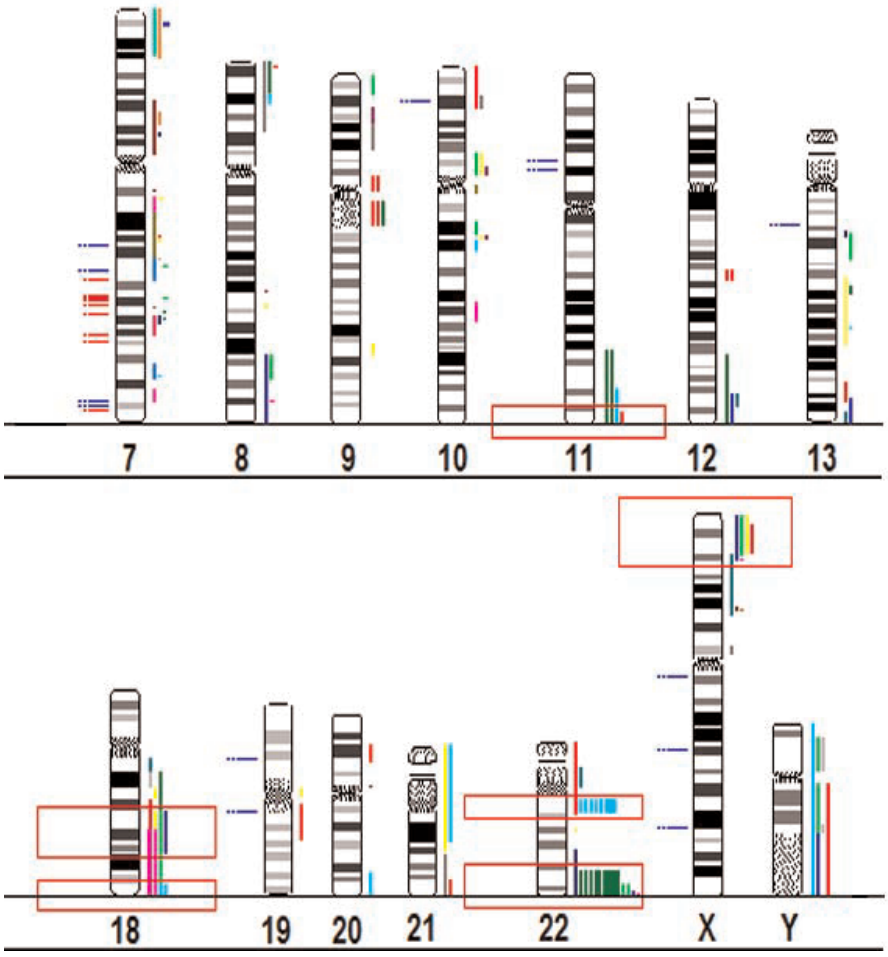



**Figure 1:** Overview of all Cytogenetic Regions Of Interest (CROIs) associated with the autism phenotype across the human genome. At the left side of each chromosome, linkage findings (LOD>2.0) are indicated by blue two-dotted lines and significant association findings ($p<0.05$) by red one-dotted lines. At the right side the CROIs are represented by colored bars.




Bars with the same color next to each other indicate the same CROI reported in more than one case report, thicker bars represent more than 2 cases in the same case report. Red boxes indicate potential novel regions where more than 4 case reports overlap at the same locus, without previous linkage and /or association findings

The relatively large number of cases that was identified for chromosome 22 is somewhat remarkable. Most of these cases are deletions of the q11.2 and q13.3 region of chromosome 22. Cumulative evidence indicates that both deletions are associated with specific phenotypes, both of which include autistic features[71, 72]. Deletions of the terminal 22q13 distal band may represent a clinically definable syndrome that includes pervasive behaviors and impaired language development[73]. The 22q11.2 region is associated with the Velo-Cardial-Facial Syndrome, with characteristic physical manifestations including typical facial appearance, anatomical and/or functional abnormalities of the palatal shelves such as cleft palate and velopharyngeal insufficiency, lymphoid tissue hypoplasia and conotruncal heart defects[74-76]. Although patients with this syndrome are considered to be at high risk to develop psychosis[77, 78], the relationship between this syndrome and autism has only recently been recognized[79]. This may explain why no association studies for this region have been published so far. Comparable to the Prader Willi/Angelman critical region on chromosome 15, no linkage was found for this region.

It could be speculated that the lack of linkage findings for 15q11-13 and 22q11.2 and 22q13.3 is caused by genetic heterogeneity of the autism phenotype or by the fact that genetic subtypes of autism are specific for the chromosome 15q and 22q rearrangement syndromes respectively, and that a gene within these regions does not contribute significantly to autism in cytogenetically normal individuals.

For chromosome 7, the CROIs are located across the entire long arm of the chromosome. Chromosome 7 encompasses over 150 million nucleotides and 1917 gene structures[80]. A large collection of published and unpublished cases of autism with cytogenetic abnormalities of chromosome 7 is presented on the website of The Centre for Applied Genomics (www.chr7.org). Based on elaborate research regarding possible imprinted genes, fragile sites and duplications, candidate genes for several developmental disorders have been suggested, including candidate genes for autism[81]. Interestingly, for this chromosome there is a considerable overlap of the cytogenetic regions of interest and the results from linkage[82, 83] and association studies[43, 84-88] (see figure-1, table-1 and table-2). Of special interest are the regions 7q21-q22 and 7q31-q32. Several candidate genes for autism are located within

these regions, including PIK3CG (7q22)[89], RELN (7q22)[43, 90, 91], RCAM(7q22.3) and LAMB1(7q31.1)[92, 93], WNT2 (7q31.2)[94] and FOXP2 (7q31)[95], GRM8 (7q31)[96] and UBE2H (7q32)[97].

Apart from the large number of case reports of chromosome 7, 15 and 22, interesting results were also found for chromosome 2 band q37, chromosome 5 band p14-p15, chromosome 11 band q25, chromosome 16 band q22.3, chromosome 17 band p11.2, chromosome 18 bands q21.1 and q23 and chromosome X band p22.

Additionally, some regions appear to strengthen results from linkage and association studies. For instance, overlap of several CROIs coincides with linkage / association findings at the same locus on 5p14[98], 10p14[99] and 16p13.3[100]. 5p14 is a region of overlap with linkage findings, whereas 5p15 may represent a novel region. The estimated distance between both regions is 15 Mb. However, given the possibly rather low resolution of both linkage and cytogenetic findings, an alternative possibility of only one locus of interest at 5p14-15 indicated by both findings may also be valid. Important in this respect, the basepair coordinates of the CROIs refer to their most likely physical location. This, however, does not exclude a functional effect that reaches beyond the boundaries. Positional effects due to rearrangements up to 1.3 Mb away from the breakpoints have been reported[101-103].

New high resolution methods of detection of copy number changes across the genome, such as array based Comparative Genomic Hybridization (arrayCGH)[104-106], Representational Oligonucleotide Microarray Analysis (ROMA)[107] and SNP-based arrays[108] are increasingly applied in clinical and research studies. In the data collected for the current review a small number of arrayCGH findings is included, for instance at 1p36.33[109] and at 6q21[110]. The increasing resolution of these methods improves accuracy of CROI delineation and will enable the detection of increasingly small copy number changes in patients with autism that would have remained undetected by the older methods.

It is important to note that segmental copy number changes in the human genome do not occur exclusively in patients, but appear to be present in unaffected subjects as well[111, 112]. Although the prevalence in the human population of such large-scale

copy number polymorphisms (CNPs) is unknown, it is estimated that some of these CNPs occur up to 10% in the general population. It is possible that these genomic imbalances underlie certain human phenotypic variation and susceptibility to disease, for example autism, and may partially explain the difficulties to identify candidate genes using linkage studies. For instance, Yu et al. reported small deletions on chromosomes 7 and 8 in multiple families with two or more sibs with autism[113]. While some of the detected deletions appeared to be specific for the affected families, others were found to be also prevalent in a control sample (and therefore not included in the present review). The increasing use of high resolution methods for detection of segmental aneuploidies will provide essential information with regard to the prevalence of (small) regions with a gain or loss of copy number in the general population, and their potential significance for the autism phenotype.

One could question the value of studying CROIs associated with autism that lack support from linkage and / or association studies. In these regions, genes are likely to contribute to autism in the individuals with the cytogenetic abnormality, but appear to lack a significant effect at the population level. Indeed, if otherwise the function of such genes is not or only rarely affected (e.g. by a functional polymorphism), it is unlikely that these genes represent candidate genes for autism in the general population. However, whereas such genes may not be involved in autism in the general population, it is highly likely that they can provide essential information with regard to biological pathways and genetic networks involved in the causation of autism in the general population. This viewpoint demands a shift from a narrow focus on individual candidate genes towards a broader view involving the integration of available information into candidate gene networks or pathways[114].

This study has several limitations. First, despite the large number of cases that have been identified, cases may have been missed in the literature search. This may be due to the fact that some studies have a specific interest in either autism or cytogenetics but not in both. For instance a case may be presented with a well described cytogenetic abnormality but without a detailed description of the behavioral phenotype. In addition, it is likely that many clinical cases have not been

published while at the same time a publication bias may exist in favor of the clinically most interesting cases.
A second limitation is caused by the fact that for some regions a broad availability of probes exists, in contrast to other regions. It is obvious that, if probes are widely available, the corresponding region will be detected more frequently. Thus, overrepresentation in the current overview may in some cases represent availability of probes instead of significant association with the autism phenotype. Inversely, other regions may be associated with autism but are underrepresented in this review because of low availability of probes. In addition to the frequent de novo occurrence of aberrations, this may explain the finding of large numbers of cases for the 15q11-q13 region and the 22q11.2 and 22q13 regions. Nevertheless, this phenomenon only involves the chance of detection of a cytogenetic abnormality and does not affect the chance that abnormalities of such a region are related to autism. Therefore, it can still be concluded that these regions are of potential interest to autism. With the increasing use of molecular methods providing genome-wide information on copy number changes (e.g. arrayCGH, ROMA, discussed above), this 'detection bias' is likely to disappear.

A third limitation of the present study is inherent to the fact that molecular data were not available in cases from the older literature which potentially may have produced false interpretations of the genomic region of interest. This risk, however, seems limited because the assumption that a region is potentially interesting is based on the overlap of multiple cases, often independently detected in different research laboratories.

Fourth, we can not exclude the possibility that genes with an important contribution to the pathogenesis of autism are located in regions of the genome for which cytogenetic abnormalities have not been reported so far. Although it cannot be ruled out that such a region exists, the large number of cases included in this review seems to decrease this possibility. Certainly, at present a potential relevance to autism of some of the less frequently or not reported regions in this overview can not be excluded.

Fifth, our presentation of overlapping results from cytogenetic studies, linkage studies and exclusively those results from association studies that were reported as significant may lead to over interpretations, since significant results from some association studies have been contradicted by other studies (e.g. the serotonin system[115, 116] reelin[117-119] and the GABA receptor genes[120]). However, irrespective of whether association findings agree on a certain locus, the value of the presented findings is the confirmation of a locus of interest through two different and independent approaches.

Despite these limitations, the present study offers several methodological advantages. First, to our knowledge, a complete list of relevant publications is provided. The inclusion of all relevant case reports that related cytogenetic abnormalities to the autism phenotype, provides unbiased research data since no exclusion criteria (other than mosaicism) were used a priori.

Second, using the nearly completed DNA sequence of the human genome[121] the sequence positions of all relevant cytogenetic regions were derived. This allows researchers in the field to easily combine our data with their own data regarding possible candidate genes or genomic regions.

Third, the clinical diagnosis of autism has been systematically evaluated. This permits a systematic ranking of CROIs based on the validity of the clinical diagnosis of autism.

The study of a specific phenotype, such as autism, in relationship to cytogenetic abnormalities is frequently met with skepticism. In the case of autism this is often based on the assumption that many patients with cytogenetic abnormalities suffer from mental retardation and that in such cases autism can not be diagnosed. It is therefore important to note that patients with mental retardation usually do not suffer from autism[122] and can be reliably distinguished from mentally retarded patients with autism[123]. Furthermore, mental retardation can be found in 70% of patients with autism and may to be part of the phenotype. Indeed, in most of the identified cases of this literature study mental retardation was present.

In conclusion, the data presented in this review 1) confirm the association with autism of several genomic regions previously identified through linkage and or association studies, and 2) reveal possible novel, not previously identified genomic regions associated with autism. A next step for future research could be an integral analysis of available information on the genes residing in the CROIs to investigate whether specific biological pathways emerge from this combination. From a clinical perspective, it is imperative to include a cytogenetic evaluation of patients with an autism spectrum diagnosis, to which purpose the data provided by this review may be helpful.

**Table 4: Genome databases screened for this review**

| Database | Version | WebPages |
|---|---|---|
| UCSC Genome Browser | May 2004 | http://www.genome.ucsc.edu |
| Ensembl Genome Browser | July 2004 | http://www.ensembl.org |
| NCBI Mapviewer | April 2004 | http://www.ncbi.nlm.nih.gov/mapview |
| Marshfield Clinic Research Foundation. Center for Human Genetics | | http://research.marshfieldclinic.org/genetics |
| The Human Genome Organization | | http://www.gene.ucl.ac.uk/hugo |

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## CHAPTER 2

# The 22q11.2 deletion in children: high rate of autistic disorders and early onset of psychotic symptoms

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**ABSTRACT**

**Introduction:** To examine psychopathology and influence of intelligence level on psychiatric symptoms in children with the 22q11.2 deletion syndrome (22q11DS).
**Method:** 60 patients aged 9 through 18 yr. were evaluated. Assessments followed standard protocols, including structured and semi-structured interviews of parents, videotaped psychiatric interview and intelligence assessment of the child. Intelligence level, psychiatric symptoms and DSM-IV classification provided the main outcome.
**Results:** High rates of Autism Spectrum Disorders (30 out of 60, 50.0%), and psychotic symptoms (16 out of 60, 26.7%), were found in this sample. In 7 out of 60 (11.7%), the psychotic symptoms interfered with behavior and caused considerable distress. In these cases the diagnosis of a psychotic disorder was applied. The average age of the children with psychotic symptoms at time of assessment was 14;2 yr. While it is likely that the high rate of psychopathology in this sample is to some extent associated with the lower level of cognitive function, a major effect of the degree of cognitive impairment on psychiatric morbidity was not found.
**Conclusion:** Autism Spectrum Disorders and sub threshold autistic symptomatology are common in children with 22q11DS. Furthermore, a high rate of psychosis and psychotic symptoms is found in this childhood sample, suggesting an early onset of psychosis in 22q11DS patients. Autistic and psychotic disorders should be considered as main elements of the behavioral phenotype of 22q11DS children.

## INTRODUCTION

The 22q11.2 deletion syndrome (22q11DS) is a congenital multi system disorder with an estimated prevalence at birth of approximately 1/4,000, equally affecting male and female individuals[1-3]. Velo-Cardio-Facial Syndrome (VCFS) and several other syndromes such as DiGeorge syndrome and Cayler syndrome can be caused by this genetic abnormality, characterized by a deletion of a small region (1.2 – 3 Mb) on the long arm of chromosome 22[4, 5].
Characteristic physical manifestations include characteristic facial appearance, anatomical and/or functional abnormalities of the palatal shelves like cleft palate and velopharyngeal insufficiency, lymphoid tissue hypoplasia and conotruncal heart defects. In the earliest descriptions of the phenotype, learning disabilities were also mentioned as a common feature[6-8]. In 1992, Shprintzen, et al. reported a high prevalence of psychiatric disorders in patients with the 22q11DS, the most frequent diagnosis in his cohort being schizophrenia[9].

Since then, a growing number of studies on the psychopathology in 22q11DS patients has been published. The majority of these studies was undertaken in samples of adult 22q11DS patients and some in samples including both children and adult patients. To date, a limited number of clinical studies specifically report on the psychiatric profile of 22q11DS children[10, 11]. Recently a study involving 25 adolescents and young adults with 22q11DS (age range 13 to 25 yr.) was published[12].
In several studies of adults with 22q11DS a high rate (20 - 30%) of psychotic disorders is reported[13, 14]. In addition, 22q11DS patients, including patients without psychosis, were found to exhibit significantly more schizotypic traits in comparison to their first-degree relatives and a healthy non-related control group, suggesting an increased predisposition to psychosis associated with the presence of the deletion[13].
Reports on the prevalence of mood disorders (major depressive disorder and dysthymia) in 22q11DS patients (children and adults) range from 11.5 % to 40%[11, 13, 15, 16]. High prevalence rates of Attention Deficit Hyperactivity Disorder (ADHD) are also reported, ranging from 35% to 46%[10, 15, 17]. Obsessive-Compulsive Disorder (OCD) has been described in several studies ranging from 8% to 33%[15, 18].

Observation of autistic-like behavior is mentioned in several studies. Gerdes et al.[19] describe self-directed behavior, preference for independent play, diminished motivation by external appreciation, noncompliance, high activity and poor social skills. One clinical study[17] specifically included assessment of Autistic Spectrum Disorders (ASD) and established a prevalence of ASD of approximately 30% in a sample of 32 22q11DS patients. Moreover, more than 50% of the subjects who did not meet sufficient criteria for ASD were nevertheless found to exhibit symptoms in the three core domains of autism (communication, social interaction, stereotypical behavior). In another more recent study by Fine et al., the question whether social and communicative deficits in this population qualify for the diagnosis of an ASD was further evaluated. A standardized screening interview by phone was performed in a subset of 22q11DS children (n=20) that fulfilled strict diagnostic criteria for ASD based upon scores from an initial mailed questionnaire survey (n=98, age range 2 – 12 yr.). This study reported a prevalence of ASD among 22q11DS children of approximately 14%[20].

Summarizing the results of psychiatric studies in 22q11DS patients, a high prevalence of psychosis (20-30%) among adults with 22q11DS appears to be the most consistent result across the different reports, whereas “psychosis-like” symptoms are reported in adolescents and young adults (age range 13 – 25 yr.)[12]. In children, variable rates of a range of psychiatric disorders are reported, including affective disorders, attention deficit disorders and ASD, but not psychosis. The question that surfaces from these findings is whether the psychiatric disorders reported in children with 22q11DS should be considered as independent phenomena or alternatively, as markers of an increased vulnerability for schizophrenia in adulthood. The present study was set up as a prospective longitudinal study in order to address this question.

In this article we report the cross sectional psychiatric findings in a sample of 60 children with 22q11DS, using appropriate assessment tools to encompass a broad range of psychiatric disorders, including autism and psychosis. A second question that is addressed concerns the extent to which psychiatric morbidity is associated with the level of cognitive functioning within this population.

## METHOD

*Subjects*

All children (n=60, inclusion criterion age 9 – 20 yr.) were presented by their parents through publicity on the website and newsletter of the parents' network of 22q11DS children in the Netherlands. An estimated 40 – 50 % of Dutch parents with school-aged children with 22q11DS is affiliated to this association. Furthermore, the information on the website is accessible to everyone. In order to further minimize the effect of selection bias, the information provided to the parents stated that children were invited to participate regardless of the presence of behavioral problems.

The assessment protocol is part of a larger ongoing longitudinal behavioral and genetic study on 22q11DS patients that has been approved by the Dutch Central Committee on Research Involving Human Subjects (C.C.M.O.). Written informed consent was obtained from participants and their parents or guardians.

*Sample*

The sample (n=60) consisted of 23 males and 37 females (difference in sex distribution not significant; p=0.092, Binomial Test). Distribution of total IQ levels in the entire sample as well as in male and female subgroups followed a normal distribution. Average total IQ was 65.2 (st.dev. 13.9), with a significant difference in mean IQ level between males and females (p=0.035, t-test), see table 1. All children were carriers of a 22q11.2 deletion, as confirmed with a positive Fluorescent In Situ Hybridization (FISH) test with adequate probes for the 22q11.2 region carried out in different genetic centers. At the time of assessment 3 children were treated with psychiatric medication (atypical antipsychotics). Other medication used by some participants included thyroid stimulating medication (n=2), calcium / Vit. D supplement (n=2), anticonvulsive medication (n=2) and bronchial anti-inflammation inhalation aerosol (n=1).

**Table 1: sample characteristics**

| | Males | Females | p-value | Whole sample |
|---|---|---|---|---|
| **N** | 23 | 37 (35)[1] | 0.092[B] | 60 (58)[1] |
| **Mean TIQ ± SD** | 60.5 ± 13.5 | 68.3 ± 13.5 | 0.035 [T,2] | 65.2 ± 13.9 (range 42 – 98) |
| **Mean Age ± SD** | 13;4 ± 2;7 | 13;10 ± 2;8 | 0.442 [T] | 13;7 ± 2;7 (range 9;1 – 18;7) |

TIQ= Total Intelligence Quotient, [B] = Binomial Test, [T] = t-test.
[1] Two female patients with severe mental retardation were removed from this analysis.
[2] The difference in IQ level is not significant (P=0.15, T-test) when including estimated IQ levels of 25 and 35 for the two removed female cases with severe mental retardation.

*Measures*

All examinations were performed at the Child and Adolescent Psychiatry Department of the University Medical Centre Utrecht. All psychiatric assessments, including interview of the caregiver(s) and interview and psychiatric evaluation of the child were performed by the same investigators. Psychiatric assessment followed standard protocols, with structured and semi-structured interviews of the parents and children, including DSM-IV semi-structured interviews (Attention Deficit Hyperactivity Disorder (ADHD), Oppositional Defiant Disorder, Conduct Disorder, Pervasive Developmental Disorder, Separation Anxiety Disorder, Generalized Anxiety Disorder, Obsessive Compulsive Disorder, Eating Disorders and the criteria for the research diagnosis of Multiple Complex Developmental Disorder (MCDD)[21, 22]). The rationale for the inclusion of MCDD criteria was based upon the fact that the presence of autistic features, disturbances of emotion and affect regulation, psychosis-like symptoms and anxiety are all reported in individuals with 22q11DS, while the co-occurrence of these symptom clusters may be suggestive for MCDD.

In addition, the sections mood disorder and psychosis of the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL)[23], the Autism Diagnostic Interview-Revised (ADI-R)[24], and two parental questionnaires: Conners' Rating Scales -Revised [25] and the Child Behavior Checklist (CBCL)[26] were used.

With regard to the semi-structured DSM-IV interview and the K-SADS-PL, in all cases both the child and the caregivers were interviewed. When direct information from the child was limited as a result of mental retardation or language impairment, the final scoring was essentially based on the caregiver's information. The semi-structured DSM-IV interview was constructed using all criteria for each disorder (see above). During the interview all criteria were systematically probed and uniformly scored with scores varying between 0 (absent), 1 (questionable), 2 (present, mild) and 3 (present, severe).

The ADI-R was obtained and scored by certified interviewers. The ADI-R focuses on the three core domains of autism (i.e. social interaction, communication and stereotyped behaviors) yielding algorithmic scores which can be interpreted using the cut-off value for each domain[27]. Reliability of the ADI-R in a population with mild to moderate mental retardation has been established[28]. In this study, the diagnoses Autism and Pervasive Developmental Disorder, Not Otherwise Specified (PDD-NOS) will be collectively referred to as Autism Spectrum Disorders (ASD).

Furthermore, information from school was obtained using the Teacher Rating Form (TRF)[26], and a teachers' version of the Conners' Rating Scale[25]. In many cases additional information from school or institutional settings was obtained by telephone after permission of the participant and caregivers. Medical history including previous pediatric or psychiatric examinations, previous cognitive assessments and observational records from child developmental / educational centers were obtained in all participants and summarized.

In addition, intelligence level in every child was assessed using the Dutch version of the Wechsler Intelligence Scales: WISC-III[29]. In three cases the WISC-R was used, in three cases the adult scale (WAIS-III). In one additional case intelligence

assessment was not performed, however IQ data from a Wechsler assessment for preschool children (WPSSI-R) were available. In two cases intelligence level was estimated to be below 40 upon clinical impression. Therefore IQ assessment was not performed with a Wechsler Scale. However, scores from a nonverbal intelligence test (Snijders-Oomen Nonverbal intelligence test Revised, or SON-R 2.5-7)[30, 31] indicated IQ levels below the range of moderate mental retardation in both cases (age equivalent 3;7 yr. in one patient at a calendar age of 9;6 yr. and age equivalent 4 yr. at a calendar age of 16;3 yr. in the second patient). Results from the used psychiatric assessment instruments are likely to be less reliable in individuals with severe or profound mental retardation. Therefore, we excluded data pertaining to these two lower functioning individuals from all statistical analyses. Only the results from the consensus DSM-IV diagnostic process from these two patients, - they were both found not to have a psychiatric disorder,- were included in the overview of DSM-IV diagnoses presented in table 2.

A videotaped psychiatric assessment of the child and all collected information were subsequently presented in a multidisciplinary consensus meeting headed by an experienced child psychiatrist (H.v.E.) leading to a best estimate diagnosis according to the DSM-IV criteria. In this diagnostic process the information from the direct clinical interviews and observations of the child were considered most important. Thus, data from the diverse questionnaires (such as the Conners' Rating Scales and the CBCL) were used as additional information, subordinate to the data from the direct clinical interviews. All children with a lifetime history of a depressive disorder, including Major Depressive Disorder, Dysthymia, Depressive Disorder associated with Amphetamine use and Depressive Disorder, Not Otherwise Specified are included in one diagnostic subgroup "Depressive Disorder", see table 2.

*Data analysis*

All statistical analyses were conducted with SPSS version 11.5 statistical analysis software. Normal distributions of intelligence level in the entire sample and in male and female subgroups were assessed with the Kolmogorov-Smirnov test. Two statistical approaches were used for the assessment of the relationship between IQ and psychiatric morbidity. First, unpaired t-tests were used to test the presence of significant differences in mean intelligence level between diagnostic subgroups. Second, the total score from the ADI-R algorithm was used as an indication for severity of autistic symptoms. Similarly, the total number of psychotic symptoms was calculated from the K-SADS-PL items pertaining to the presence of hallucinations, delusions and thought disorder. Then, utilizing these symptom scores as dimensional variables the Pearson correlation coefficient was used to test correlations between IQ level and the severity of autistic symptoms and psychotic symptoms respectively. In order to avoid multiple testing, statistical analyses were limited to three diagnostic groups that were sufficiently represented in this sample, i.e. the subgroup of patients without any psychiatric diagnosis, and the subgroups with ASD and / or psychosis respectively. For all calculations the Wechsler IQ levels of the entire sample, except for the two cases lacking these data, were used (n=58). Possible differences in psychopathology rates between male and female subgroups were assessed using the Fisher's Exact Test.

## RESULTS

*DSM-IV classification and frequency of symptoms*

Table 2 summarizes the diagnostic findings of the current study. The ASD group consisted of 30 patients (50.0%); 27 patients were diagnosed PDD-NOS, 3 patients were diagnosed as having autism. With respect to the symptoms as measured by the Autism Diagnostic Interview-Revised (ADI-R), 20 patients (33.3%) had ADI-R scores beyond the cut-off values in the three core domains (social interaction, communication, stereotyped behaviors). Of the remaining patients, 11.7% were found to have scores above cut-off in 2 domains and 20.0% in one of the three ADI-R domains. Remarkably, many patients *without* ASD displayed a significant amount of autistic symptoms (see figure 1). Given the high prevalence of autistic symptomatology in this sample a diagnosis of psychotic disorder was exclusively considered when hallucinations and / or delusions were reported. This was the case in 16 Patients (26.7%).

The symptoms reported included mostly auditory (voices) as well as visual hallucinations and paranoid delusions. In 9 patients no DSM-IV diagnosis was made because these symptoms appeared neither to be causing distress nor to be influencing the patient's behavior. In the remaining 7 patients (11.7%), however this was the case and consequently a diagnosis of psychotic disorder was justified. In 2 (3.3%) out of these 7 patients, a clear decline in general functioning was observed. These 2 patients thus fulfilled DSM-IV criteria for schizophrenia, paranoid subtype. In the remaining 5 (8.3%) patients the psychotic symptoms either caused distress or influenced the child's behavior, however, a clear decline in functioning was not observed. Therefore they were diagnosed as suffering from psychosis NOS. In none of the cases consistent co-occurrence of psychotic symptoms with affective disturbances was noted. The average age of the children with psychotic symptoms at time of assessment was 14;2 yr. (st.dev. 2;4 yr., range 9;1 to 18;7 yr.). Disregarding mental retardation, phonological disorder and language disorders as psychiatric disorders, 20 participants (33.3%) were free of any psychiatric DSM-IV diagnosis. This subgroup included the two individuals with severe mental retardation. There were no significant differences in the rate of psychiatric disorders between male and female participants.

**Table 2: Diagnostic findings and comparison by intelligence level**

| Consensus DSM-IV diagnoses | With diagnosis | | Without diagnosis | | P-value Unpaired t-test[1] | Prevalence rate in general population |
|---|---|---|---|---|---|---|
| | n (%) | Mean IQ (Range) | n (%) | Mean IQ[1] (Range) | | |
| Autism Spectrum Disorder[2] | 30 (50.0) | 64.7 (42-91) | 30 (50.0) | 65.7 (45-98) | 0.791[T] | 0.275%[8] |
| Psychotic disorder[3] | 7 (11.7) | 58.1 (50-74) | 53 (88.3) | 66.2 (42-98) | 0.046[Tυ] | 0.14 - 0.46%[9] |
| Depressive disorder[4] | 5 (8.3) | 64.2 (55-72) | 55 (91.7) | 65.3 (42-98) | ∅ | 9.5%[10] |
| ADHD – IS[5] | 3 (5.0) | | 57 (95.0) | | ∅ | |
| ADHD – HypS[6] | 1 (1.7) | | 59 (98.3) | | ∅ | |
| ODD | 3 (5.0) | | 57 (95.0) | | ∅ | |
| Conversion disorder | 1 (1.7) | | 59 (98.3) | | ∅ | |
| Any psychiatric disorder[7] | 40 (66.7) | 64.3 (42-91) | 20 (33.3) | 67.2 (45-98) | 0.477[T] | |

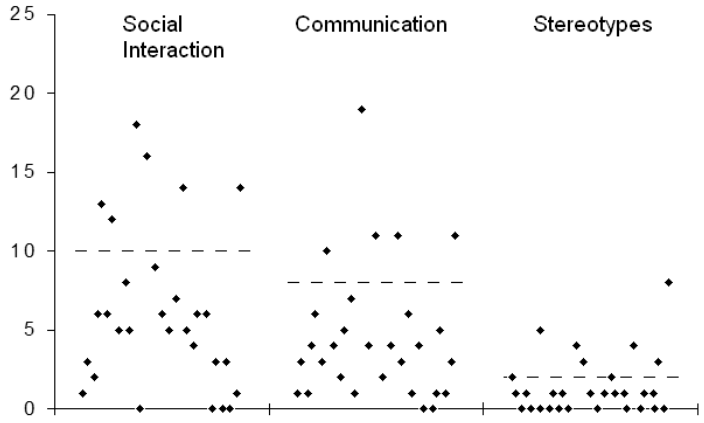
[T] = t-test, [Tυ]= t-test, uneq. var., [∅] = P-values not calculated. [1] Two female patients with severe mental retardation (MR) were removed from this analysis. [2] Autism and Pervasive Developmental Disorder-NOS, [3] Schizophrenia, paranoid type and psychosis-NOS, [4] Lifetime diagnosis: Major Depressive Disorder, Dysthymia, Depressive Disorder associated with Amphetamine use and Depressive Disorder-NOS, [5]Attention Deficit Hyperactivity Disorder (ADHD), Inattentive Subtype, [6] ADHD, Hyperactive / Impulsive Subtype, [7] MR, phonological disorder and language disorders were not counted as psychiatric disorders.

[8] Fombonne E: *J Autism Dev Disord.* 2003;33:365-382

[9] Prevalence of schizophrenia in the general population. Jablenksy A: *Eur Arch Psych Psychiatry Clin Neurosci.* 2000;250:274-285

[10] Cumulative prevalence of any depressive disorder at age 16. Costello EJ et al.: *Arch Gen Psychiatry*. 2003;60:837-844.

**Figure 1: ADI-R Symptom domain scores in patients without ASD**




Dotted lines represent cut-off values in each symptom domain of the ADI-R

*Relationship between intelligence level and diagnostic subgroups / symptoms.*
No difference in intelligence level between those with and those without ASD was found (p=0.791, t-test). However, when comparing mean intelligence level between those with and those without a psychotic disorder a significant difference was detected (p=0.046, t-test), suggesting that a low IQ is a risk factor for psychosis in children and adolescents with 22q11DS. Further, comparing the subgroups with and without any psychiatric disorder no significant difference was found (p=0.477, t-test).
Finally, the Pearson correlation coefficient was used to test correlations between IQ level and severity of autistic symptoms and psychotic symptoms respectively. In both cases no relevant correlations were found; IQ level and autistic symptoms ($r = -0.093$, $p = 0.486$), and IQ level and psychotic symptoms ($r = -0.060$, $p = 0.656$). Results from these correlation analyses remained essentially unaltered when performed in male and females separately.

## DISCUSSION

*Psychopathology*

The primary finding of the current study is a high rate of autism spectrum disorders (50.0%) and psychosis (11.7%), including psychosis-NOS and schizophrenia.Taken together, 66.7% of participants had one or more psychiatric disorders according to DSM-IV criteria.

When focusing on the presence of hallucinations and / or delusions, as a finer measure of psychopathology, a total of 16 children (26.7%) of the participants reported at least one of these psychotic symptoms. The young age at assessment of these symptoms is striking. Based on the fact that several children reported having psychotic symptoms for a significant amount of time, we expect the age of onset of psychotic symptoms in 22q11DS children to be significantly younger than 14 years. Previous reports vary in regard to whether schizophrenia in individuals with 22q11DS starts at a typical age of onset[32, 33], or later[13], or earlier[34]. The findings of the current study are in support with an earlier age of onset. A young age of onset also appears to be in keeping with the high number of 22q11 deletions (5.3%) found in Childhood Onset Schizophrenia patients[35].

All three children treated with antipsychotics were diagnosed with ASD. In addition, one was found to suffer from schizophrenia. The treatment with antipsychotics is likely to have attenuated the intensity of psychotic symptoms in these cases. When re-analyzing the sample without the 5 children who were treated with psycho-active medication (atypical antipsychotics or anticonvulsives), the main findings of this report remain essentially unchanged (data not shown).

With regard to autistic symptoms it appears that children without ASD nevertheless display a significant amount of autistic symptoms. This finding is concordant with previous studies[17, 20].

In the current study final DSM-IV classification resulted from best estimate diagnosis in a multidisciplinary consensus meeting. As a result, there is some discrepancy between the number of patients with symptom scores above the cut of values in all three domains of the ADI-R (33.3%) and the number of patients

allocated a consensus diagnosis of ASD (50.0%), illustrating the complexity of some of the cases.

Five out of the seven patients with a psychotic disorder were co-morbid for ASD. One could argue that autistic symptoms overlap with (prodromal) features of schizophrenia. Indeed, many studies have shown that individuals with schizophrenia are more likely to display aberrant developmental features including abnormal social behaviors during childhood[36-39]. Thus, while the observed symptoms in the present study are currently best described with the term "Autism Spectrum Disorders", they may in fact be indicative of aberrant neurodevelopment and as such represent pre-morbid deficits of psychosis. Indeed, evidence for this notion has been recently reported in a study on children with Childhood Onset Schizophrenia[40]. In this context it is noteworthy that ASD occurs as often in male as in female 22q11DS patients, given the well established 4:1 ratio of ASD in males and females in the general population[41]. While the equal sex distribution in those affected by ASD in the current study could be due to sampling error, it could also be viewed as supportive of the notion that ASD symptomatology in this population may be considered as prodromal features of psychosis. Indeed, a 1:1 sex ratio for ASD such as found in the present study is more reminiscent of the sex ratio found for schizophrenia in the general population (male:female ratio median: 1.40[42]. Longitudinal follow-up of the currently reported sample and other study samples will likely help to clarify this issue.

A willingness to make social approaches was present in the majority of the children with ASD or with autistic traits. However, the quality of these social approaches could generally be described as inappropriate, maladapted or peculiar. Also, the child was often reported to preferentially initiate social interaction with adults over peers. This type of social relatedness appears to match the ASD subgroup "active but odd" as proposed by Wing et al[43]. However, firm conclusions regarding this issue cannot be drawn as appropriate questionnaires based on Wing's subtyping scheme (e.g. Wing Subgroups Questionnaire (WSQ)[44]), were not included in the current study.

Following DSM-IV guidelines a separate diagnosis of ADHD was not made when occurring in the context of an ASD, unless ADHD symptoms were too severe to be only accounted for as part of ASD. This was the case in 2 out of a total of 4 children with ADHD in this sample. Additionally, 10 children (16.7%) fulfilled 6 or more out of 9 DMS-IV criteria for ADHD, inattentive subtype. In 7 of these children a diagnosis of ASD was made. Of the remaining 3, two were diagnosed with ADHD and one with a depressive disorder. The co-occurrence of inattention symptoms and ASD symptoms in this population may partly explain why in most previous studies study high rates of ADHD are found while no ASD is reported in the absence of appropriate ASD assessment methods.

In the present study no mania or hypomania was diagnosed. Irritability and sometimes temper tantrums were however reported by a number of parents, often in the context of resistance to change, and therefore in this study interpreted as behavioral disturbances commonly associated with ASD.
Finally, previous reports of OCD in 22q11DS patients were not replicated in the present study, while assessment of these disorders was included in the standard methods. A possible explanation may be the fact that OCD and ASD share phenotypic characteristics. Indeed, it has been proposed that OCD is part of the broader phenotype of ASD as was suggested by the aggregation of OCD in relatives of children with autism[45].

*Influence of intelligence level*
It has been shown in many previous studies that the rate of psychiatric disorders is increased in individuals with impaired cognitive function. The overall high rate of psychopathology in this study is consistent with these observations. One could hypothesize that the high rate of psychopathology is to some extent related to the same disruptions in neurodevelopment that also have resulted in a lower cognitive function. In the present study a further analysis of the effect of the severity of the cognitive impairment on psychiatric morbidity in patients with the 22q11.2 deletion was performed.

On the whole, the results suggest that the degree of cognitive impairment within this sample does not have a major effect on psychopathology, with the exception of a small (approximately 8 IQ points) but significant difference in the mean IQ between children with a diagnosis of psychotic disorder and those without. However, when using the number of psychotic symptoms as a continuous variable no significant correlaton with IQ level was found.

There are two possible explanations for this discrepancy. First, the subgroup of patients with psychotic disorder (n=7) in this study may have been too small and not representative of 22q11DS patients with psychosis in the general population, thus skewing the results. In this respect it is also important to acknowledge that it is likely that additional participants of this study will eventually develop psychosis in the coming years, during the more typical ages of onset of schizophrenia. Indeed, Murphy et al. report no significant difference in IQ levels between 22q11DS adult patients with psychosis (n=15) and those without (n=35)[13]. Also in keeping with the presented results are the findings by Baker et al. in a group of adolescents with the 22q11.2 deletion and an IQ matched control group. The 22q11DS group showed a higher rate of psychiatric morbidity without association between the degree of cognitive impairment and the presence or severity of psychopathology. In particular, ratings on a schizotypy scale were significantly higher in the 22q11DS group and not correlated with IQ level within this sample[12].

The alternative explanation holds that in 22q11DS patients a relatively higher IQ is associated with a more favorable course of psychotic illness, at least during adolescent years. Thus, all 22q11DS patients, irrespective of their level of cognitive impairment, may be at increased risk for the emergence of psychotic symptoms. However, one could speculate that in some patients a relatively lower IQ as well as the earlier onset of psychotic symptoms are both indicative of a greater neurodevelopmental impact.

*Limitations*

Several limitations of this study should be taken into account when interpreting its results. First, while specific care was given with regard to the inclusion of the participants, it is nevertheless likely that some sort of selection bias has played a role. For instance, parents of the more severely affected children with 22q11DS may have been more inclined to join the Parent's Network than parents of less affected children. Therefore, prevalence of psychiatric disorders and psychiatric symptoms of the current study should not be generalized to the entire 22q11DS population.

Secondly, the absence of an IQ-matched control group precludes the analysis of a specific effect of the 22q11.2 deletion on the rate of psychiatric morbidity. However, analysis of the effect of the level of cognitive impairment on psychiatric morbidity within the studied sample was feasible. Further, while such an effect was testable in the largest subgroups, remaining subgroups were insufficiently large to allow reliable statistical testing.

With regard to the diagnosis of ASD, the administration of a protocolized observation method such as the Autism Diagnostic Observation Schedule (ADOS) would have strengthened the assessment procedures.
Finally, in the present sample females were slightly overrepresented, though not significantly, while IQ level between the sex subgroups was significantly different. We assume this to be a consequence of sampling error, given the fact that there are no indications of an uneven sex or intelligence per sex distribution among 22q11DS patients in other reported study samples. Accordingly, statistic analysis revealed no significant differences between rates of psychopathology between males and females.

*Clinical implications*

Mental health care professionals should be aware of an increased a priori possibility of ASD when confronted with a child with 22q11DS. Even in the absence of a formal diagnosis of ASD, mild subclinical impairments in the areas of communication, socialization and repetitive behaviors may be present and may necessitate monitoring. This is all the more important because it appears that not so

much the drive to engage in social contact in these children is impaired, but more the manner in which these interactions are initiated or (fail to be) maintained. This, together with often relatively good (but rather superficial) verbal skills may lead the clinician into disregarding the possibility of a diagnosis of ASD in a too early stage of the psychiatric assessment. Our clinical experience with this population suggests that specifically social abilities of these children tend to be overestimated. This leads to a chronic exposure to a too demanding environment, which in turn may be associated with secondary psychopathology.

Conversely, when should a clinician start to consider the possibility of a 22q11.2 deletion in a child with psychiatric problems? Without providing a complete review, the most common medical characteristics that typify this syndrome are cardiac abnormalities and pharyngeal anomalies including cleft palate and milder expressions of disturbed pharyngeal development leading to velopharyngeal insufficiency. Furthermore, lymphoid tissue hypoplasia, hypocalcemia and congenital renal deformations are also frequently observed[46-49].
Within the setting of a child psychiatry practice the following hallmarks are often observable and should trigger health care providers to pursue further genetic consultation: characteristic facies (including a small face with malar flatness, hooded eyelids, a bulbous nose tip and abnormally formed ears), and nasal speech, sometimes with substantial speech impairment. Further, medical history should be checked for any of the above-mentioned medical disorders as well as for the presence of: feeding problems in infancy (notably nasal regurgitation, reflux, constipation and /or poor suck), hypotonia and recurrent or chronic middle ear infections. Regarding cognitive development, a variable degree of cognitive impairment can be present, often with a significant discrepancy between verbal and performance skills[50, 51]. Finally, expressive language is often more delayed than what can be expected based upon the general cognitive impairment[19]. For a more complete overview of associated symptoms see for example[46-49, 52]. Importantly, none of the listed symptoms are obligatory present in any individual with the syndrome[53].

Finally, one could ask whether all children with 22q11DS should be examined by a mental health professional. This may be a provocative thought to some. On the other hand, the systematic screening of all 22q11DS patients for main associated physical abnormalities such as hypoparathyroid function or heart abnormalities, is common practice. Based upon this and other studies we recommend psychiatric examination of all children with 22q11DS, preferably before the age of ten years. The early detection of psychiatric problems, some of which may be treated with medication or positively influenced by other interventions, may have positive effects on the clinical outcome and quality of life of 22q11DS patients.

In summary, we report a high rate (50.0%) of Autism Spectrum Disorders. Psychotic symptoms (26.7%) are found to be present at a young age, in 11.7 % qualifying for the diagnosis of Psychotic Disorder. The high rate of psychopathology in this sample is likely to some extent related to the low cognitive level. However, with regard to the degree of cognitive impairment, no major effect on psychiatric morbidity was found, indicative of a specific effect of the underlying genetic disorder. In order to improve the quality of clinical care for 22q11DS patients, the behavioral phenotype of 22q11DS should be further examined with a special focus on the longitudinal course of symptoms. At the same time our understanding of the biological underpinnings of psychosis and autism in the general population may greatly benefit from the genetic research involving patients with 22q11DS.

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## CHAPTER 3

# MLPA: A Rapid, Reliable and Sensitive Method for Detection and Analysis of Abnormalities of 22q

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**ABSTRACT**

**Introduction:** In the current study essential test characteristics of the recently described Multiplex Ligation-dependent Probe Amplification (MLPA) method are presented using chromosome 22 as a model. This novel method allows the relative quantification of ~ 40-45 different target DNA sequences in a single reaction.
**Method:** For the purpose of this study, MLPA was performed in a blinded manner on a training set containing over 50 samples, including typical 22q11.2 deletions, various atypical deletions, duplications (trisomy and tetrasomy) and unbalanced translocations. All samples in the training set have been previously characterized by FISH with cosmid or BAC clones and / or cytogenetic studies.
**Results:** MLPA findings were consistent with cytogenetic and FISH studies, no rearrangement went undetected and repeated tests gave consistent results. At a relative change in comparative signal strength of 30% or more, sensitivity and specificity values were 0.95 and 0.99, respectively. Given that MLPA is likely to be used as an initial screening method, a higher sensitivity, at the cost of a lower specificity was deemed more appropriate. A Receiver Operator Curve (ROC) analysis was performed to calculate the most optimal threshold range, with associated sensitivity and specificity values of 0.99 and 0.97 respectively. Finally, performance of each individual probe was analyzed, providing further useful information for the interpretation of MLPA results.
**Conclusion:** MLPA has proven to be a highly sensitive and accurate tool for detecting copy number changes in the 22q11.2 region, making it a fast and economic alternative to currently used methods. The current study provides valuable and detailed information on the characteristics of this novel method.

## INTRODUCTION

The presence of chromosome specific, low-copy repeats (LCRs) on the proximal part of the long arm of chromosome 22 predisposes this region to cytogenetic rearrangements[1, 2]. To date, the most prevalent abnormality mediated by LCRs in this region is the 22q11.2 deletion, leading to syndromic disorders that include Velocardiofacial Syndrome (VCFS), DiGeorge Syndrome and Conotruncal Anomaly Face Syndrome. This deletion-based disorder is increasingly referred to as "22q11.2 deletion syndrome" (22q11DS). Clinical characteristics of 22q11DS include significant cardiac defects, thymic, parathyroid, craniofacial, developmental, neurological and behavioral manifestations[3-8].

The typically deleted region occurs between LCR-A and LCR-D and spans a ~3 Mb genomic region. The most common distal endpoints of smaller, atypical deletions are LCR-B or LCR-C[9]. In addition, consistent with the expected influence of the LCRs, internal duplications and even a triplication of 22q11.2 have recently been described[10, 11]. The internal duplication is associated with a variable clinical phenotype including some of the features of 22q11DS[12]. Further, the breakpoints implicated in generating the Cat Eye Chromosome, a supernumerary inverted duplication of proximal 22q, frequently matches the proximal LCR or one of the more distal LCRs of the 22q11.2 region[13-15]. The only recurrent non-Robertsonian translocation in humans, the recurrent t(11;22)[16] (Emanuel Syndrome) has LCR-B as its chromosome 22 breakpoint. Finally, several translocations occur between 22q11.2 and other chromosomes, including a recurrent t(17;22)[17], a t(20;22)[18], a t(4;22)[19], a t(1;22)[20] as well as numerous others[21, 22]. The various rearrangements of 22q11.2 have been extensively reviewed[2, 15].

The current estimate with regard to the prevalence at birth of 22q11.2 deletions is approximately 1/4,000 –6,000[23-26]. While no information is available on the frequency of duplications and triplications, recent reports suggest a higher prevalence than previously assumed[27]. Taken together, the existing findings imply a high rate of occurrence of different cytogenetic rearrangements involving 22q11.2 that are associated with various ranges of mild and severe clinical manifestations.

Thus, proper diagnosis has implications related to clinical outcome, reproductive options and treatment modalities.

Although standard karyotyping and occasionally Multiplex Fluorescence In Situ Hybridization (M-FISH)[28] are used to identify translocations involving 22q, the most widely used genetic diagnostic procedure for the detection of copy number changes at 22q11.2 is Fluorescence In Situ Hybridization (FISH). Despite the high reliability of such methods, they are laborious and do not have the sensitivity to detect small deletions or duplications (<40kb) within 22q11.2. Given the high prevalence of 22q rearrangements in the population a reliable rapid, sensitive and less expensive method is necessary to detect these frequent 22q rearrangements.

Recently, a novel commercially available PCR based technique, Multiplex Ligation-dependent Probe Amplification (MLPA), has been favorably described with regard to its use in the detection of typical deletions in the 22q11.2 region[29]. MLPA is a recently presented method[30] that concurrently permits the relative quantification of ~ 40 - 45 different target DNA sequences. The current analysis extends previous reports in that it provides an in depth evaluation of test characteristics, including a sensitivity and specificity assessment of individual probe results, an analysis of probe reliability and a calculation of the optimized threshold range for the most likely purposes of the test. Furthermore, specific emphasis has been placed on determining its utility with regard to the following criteria: 1) cost effectiveness 2) ease of use, and 3) suitability for the detection of various types of copy number differences within the 22q11.2 region. We present the results of MLPA on a training set of previously characterized samples derived from individuals with a variety of rearrangements of the 22q11.2 region. These include typical 22q11.2 deletions, a variety of atypical deletions, 22q11.2 duplications (trisomy and tetrasomy) and unbalanced translocations. The analyses were based upon the determination of the concordance of individual MLPA probe results with the previously performed FISH results for each sample in the training set. Thus, this study was designed to provide further detailed information with regard to essential characteristics of the currently available MLPA kit for the detection of copy number changes in the 22q11.2 region.

## METHODS

*Sample set*

Over the past 20 years the Clinical Genetics Center at The Children's Hospital of Philadelphia (CHOP) has enrolled numerous patients with a variety of deletions and rearrangements of 22q such as those described in the introduction in IRB approved research protocols. Characterization of 22q11 rearrangements by FISH analysis with cosmid or BAC clones had been previously performed in our research laboratory. A group of 62 well characterized samples, including typical 22q11.2 deletions, atypical deletions, duplications (trisomy and tetrasomy) and unbalanced translocations, as well as samples without a known rearrangement were selected to create a training set for the purpose of this study. MLPA was performed in the research laboratory blinded to the molecular genetic status of the samples in the training set. Shown in Table 1 is a detailed description of the cell lines utilized as the training set. The cell lines are designated by "in-house" identifier numbers assigned by the tissue culture core facility. The training set includes a group of standard 22q11.2DS deletions (34 cases), several recurrent smaller 22q11.2DS deletions (8 cases), several unique 22q11.2DS associated deletions (3 cases), a group of unbalanced translocations to 22q (9 cases), several interstitial duplications of 22q11.2 (2 cases), several supernumerary der(22)t(11;22)(q23;q11) cell lines (3 cases), a Cat Eye Syndrome cell line, and several balanced translocations to 22q (2 cases).

*Laboratory Methods*

Genomic DNA (20 – 500 ng) was denatured (5 min at 98$^{o}$ C) and subsequently hybridized to the MLPA probe set according to the manufacturer's protocols (P023 Kit: DiGeorge Syndrome; MRC-Holland; Amsterdam, The Netherlands, www.mrc-holland.com). The P023 kit contains sets of hemi probes for 39 loci, including 11 on the long arm of chromosome 22. They are strategically distributed in and around the region flanked by LCRs A to D. The remaining 28 loci are located on chromosomes 4 (6 loci), 7 (1 locus), 8 (5 loci), 10 (10 loci), 17 (4 loci) and 18 (2 loci). Each PCR amplifiable locus in this kit consists of two hemi probes each of which contains half of the target sequence. The entire target sequence contains ~ 40 - 75 nucleotides that can be PCR amplified subsequent to hemi-probe ligation. In one hemi probe of

each probe set the target specific sequence at one end is separated from the universal primer at its other end by a variable length "stuffer" fragment of 19-370 nucleotides. Detailed methodology for MLPA is described elsewhere[30].
In the current study PCR amplification was carried out on an ABI 9700 thermal cycler (Applied Biosystems, Foster City, CA) and electrophoresis was performed using the ABI 3700 DNA analyzer (Applied Biosystems, Foster City, CA) with Rox 500 size standard and GeneMapper software (GeneMapper® Software v3.0, Applied Biosystems, Foster City, CA).

*Statistical Methods*
Analysis of results was carried out upon the transfer of GeneMapper results to an Excel spreadsheet. Failed samples were deleted and primer-dimer / control peaks were removed. Uncorrected MLPA results display a negative correlation between probe size and signal strength. This artifact probably results from preferential injection of smaller fragments through the capillaries of the DNA analyzer. Prior to the normalization of the data, a correction of the artificial down sloping of signal strengths was accomplished using a binomial regression model. Each signal value was divided by its predicted value obtained from the regression model. This method has been described by Mavriogiannis et al. (*http://leedsdna.info*).

Normalization was achieved by division of signal values by the sum of the signal values of all probe sets (loci) for each sample. Finally, normalized signal values were compared to signal values from normal controls by dividing the normalized signal values with the average calculated from the results derived from 4 – 7 control samples; the product of this calculation was termed the dosage quotient. When a target locus in a test sample is disomic (i.e. present in a normal copy number, or n=2), the normalized signal value for that locus will be equal to that of the control sample, and thus the dosage quotient will be close to 1.0. A deletion of a locus in a test sample (n=1) should result in a reduction of relative signal strength of >30%, whereas a duplication should lead to an increase in relative signal strength of >30% (i.e. the delta value is 0.3). Thus, a normal or disomic copy number should generate a normalized signal value of 1, in the case of a deletion or duplication, the normalized signal should be less than 0.7 (1-0.3) or greater than 1.3 (1+0.3)

respectively. A delta value of 0.3 is a commonly accepted empirically derived threshold value for genetic dosage quotient analysis (e.g.[31]).

Confirmation of each of the results obtained in the MLPA-derived dataset, could be accomplished by comparison with existing data derived from previous studies. These studies included extensive FISH mapping of breakpoints, cytogenetic G-band analysis (in cases of translocations), or a combination of both. Under the assumption that the existing data were accurate, each locus signal was then individually labeled as false or true negative / positive respectively.
For each locus signal in a given run 1) the variation of the signals from that particular locus in the control samples and 2) the variation of the combined locus signals of that particular sample were calculated and expressed as standard deviations. These values will be referred to as "Probe (locus) SD" and "Sample SD".

*Assessment of probe set (locus) reliability*
Differences in reliability of signal outcome are likely to exist between the individual probe sets, as a result of probe-inherent characteristics such as kinetics of hybridization, ligation and amplification. Therefore, the mean Probe SD for each individual probe set was calculated from the results of the normal samples. The Levene test for homogeneity of variance was used to assess whether the variability of these values was statistically significant. Subsequently, a correlation between mean Probe SD values and the sum of all spurious results was tested using the Pearson regression analysis. If both suppositions can be confirmed, the mean Probe SD for a probe provides useful information with regard to the reliability of that probe.

*Assessment of the optimal cut-off values*
Receiver operating characteristic (ROC) analysis was used to determine the optimal cut-off values. The ROC curve is determined by plotting the sensitivity versus 1 minus the specificity. Using this approach the Y-axis represents the fraction of correctly recognized deletions and duplications (i.e. sensitivity), while the X-axis represents the fraction of false positives (i.e. the fraction of loci with a normal copy number which are incorrectly labeled as deletions or duplications). The threshold

range around the expected value of 1.0, in the case of a normal copy number, can be varied by altering the delta value. The normal threshold range lies between 1.0 plus and minus the delta value. The sensitivity and specificity characteristics were computed for a series of delta values and used to generate the ROC curve. By lowering the delta value, the threshold width decreases and thus the rate of false positives increases and the rate of false negatives decreases. When the delta value is increased the opposite occurs.

Two methods were used to determine the optimal cut-off. The first used an empirical method of selecting a point at which the sensitivity was 0.99. Given the most likely purpose for utilizing the MLPA P023 test, sensitivity was considered more important than specificity. The second method for determining the optimal cut-off utilized the slope of the tangential line to the ROC curve. The slope represents the weight of a false positive versus the weight of a false negative. When both outcomes are considered equally important, a delta value corresponding to the point where the tangential slope to the ROC curve is 45 degrees should be selected. This point is situated in the elbow of the ROC curve. However, since sensitivity was considered to be more important than specificity, -(i.e. the weight of a false negative is greater),- a point to the right of the elbow of the ROC curve, where the tangential slope is less than 45 degrees, should be selected. The associated delta value will generate relatively fewer false negatives (high sensitivity) than false positives (relatively lower specificity). Conversely, the delta value associated with the point where the tangential slope is greater than 45 degrees is associated with a high specificity whereas the sensitivity is relatively lower.

**RESULTS**

An overview of results from the MLPA studies on a selection of the samples from the training set (Table 1) is shown in Figure 1. Taking the combined results of all 22q11.2 probes into consideration, MLPA findings were concordant with the prior FISH studies; none of the rearrangements went undetected and in replicates using the same samples, consistent results were achieved. In two cases, MLPA findings revealed an atypical proximal breakpoint centromeric of LCRA, unsuspected based upon previous FISH studies. This was subsequently confirmed by additional FISH and molecular studies (see samples CH01-242 and CH02-008 in Figure 1). Further, the MLPA findings revealed a variant distal breakpoint in one of the same samples (CH02-008) that was also subsequently confirmed by additional FISH studies.

Examination of the individual results for any of the 39 probes in the kit when limited to probes with a Probe SD and Sample SD below 0.20 indicates that the outcomes are very robust; at a delta value of 0.3, sensitivity and specificity are 0.952, 95% Confidence Interval (95%C.I.) 0.928 – 0.969 and 0.986, 95% C.I. 0.984 – 0.988, respectively.

**Table 1: description of the training set.**

| Class[a] | Karyotype | Category of rearrangement | n |
|---|---|---|---|
| 1 | 46,XX or XY,del(22)(q11.2) | Standard 3Mb 22q11.2 Deletion (LCR A-LCR D) | 34 |
| 2 | 46,XX or XY,del(22)(q11.2) | Recurrent Nested 22q11.2 Deletion (LCR A – LCR C) | 3 |
| 3 | 46,XX or XY,del(22)(q11.2) | Recurrent Nested 22q11.2 Deletion (LCR A – LCR B) | 5 |
| 4 | 46,XX,del(22)(q11.2) | Unique Variant 22q11.2 Deletion | 1 |
| 5 | 46,XY,del(22)(q11.2) | Unique Variant 22q11.2 Deletion | 1 |
| 6 | 46,XY,del(22)(q11.2) | Unique Variant 22q11.2 Deletion | 1 |
| 7 | 46,XY,-22,+der(22)t(?;22)(?;q13) | Translocation to 22qter of Acrocentric Short Arm | 1 |
| 8 | 45,XX,-11,-22, +der(11)t(11;22)(11q25;q11.2) | Unbalanced 22q11DS-AssociatedTranslocation[b] | 1 |

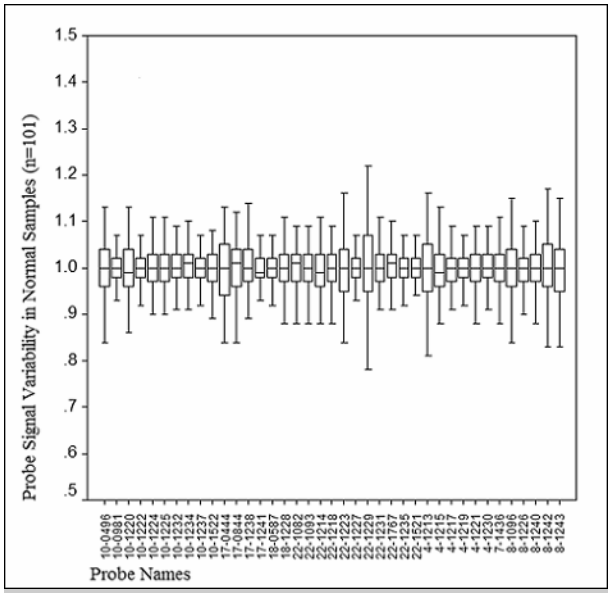
| Class[a] | Karyotype | Category of rearrangement | n |
|---|---|---|---|
| 9 | 45,XY,-1,-22, +der(1)t(1;22)(p36;q11.2) | Unbalanced 22q11DS-AssociatedTranslocation | 1 |
| | 45,XX,-4,-22, +der(4)t(4;22)(q35.1;q11.2)mat | Unbalanced 22q11DS-AssociatedTranslocation[c] | 1 |
| | 45,XY,-4,-22, +der(4)t(4;22)(q35.2;q11.2) | Unbalanced 22q11DS-AssociatedTranslocation | 1 |
| | 45,XX,-12,-22, +der(12)t(12;22)( p13.3;q11.2) | Unbalanced 22q11DS-AssociatedTranslocation | 1 |
| | 45,XX,-17,-22, +der(17)t(17;22)(p13.3;q11.2) | Unbalanced 22q11DS-AssociatedTranslocation | 1 |
| | 45,XX,-17,-22, +der(17)t(17;22)(q25.3;q11) | Unbalanced 22q11DS-AssociatedTranslocation | 1 |
| | 47,XX,-22, +der(X)t(X;22)(p22.31;q11.2)mat | Unbalanced Translocation | 1 |
| 10 | 46,XY,dup(22)(q11.2q11.2) | Interstitial 22q11.2 Duplication | 2 |
| 11 | 47,XX,+der(22)t(11;22)(q23;q11.2) | Supernumerary der(22)t(11;22) Syndrome (Emanuel Syndrome) | 3 |
| 12 | 47,XX,+inv dup(22) (p13->q11.2::q11.2->p13) | Cat-Eye Syndrome Chromosome | 1 |
| 13 | 46,XX,t(16;22)(q24;q13.3) | Balanced Translocation Carrier | 1 |
| 14 | 46,XX,t(6;22)(p11.2;q12.2) | De Novo Balanced Translocation Carrier | 1 |

a) See combined MLPA and FISH findings in figure 1.
b) [32]
c) [33]

*Assessment of probe reliability*

The mean Probe SD for each probe set was calculated from the total of 101 control samples that were included in the MLPA experiments described here. A boxplot diagram of signal variability is provided in Figure 2. We utilized the Levene test for homogeneity of variance. Results derived from application of the Levene test indicate that variance in probe signals differed significantly amongst the probes (Levene test, $p < 0.01$). Further, the Pearson correlation coefficient, computed with the mean Probe SD and the total percentage of false results as independent variables (with delta = 0.22) indicated a strong (positive) correlation (Pearson Coefficient 0.80, two tailed p-value: $8.6 \times 10^{-10}$).

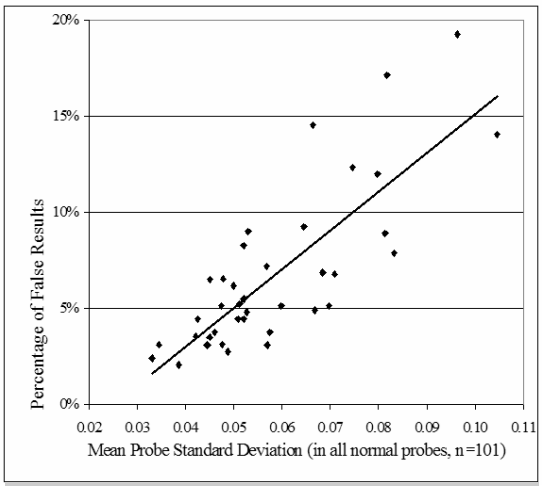
**Figure 2: Signal variability**




Boxplot representation of variability of all probes in the P023 MLPA Kit (MRC-Holland). On the Y-axis are plotted the Dosage Quotients. Since the probe signals in this analysis are exclusively derived from normal samples, the expected Dosage Quotient for each signal is 1.0. The top horizontal line of the box represents the 75$^{th}$ percentile, the bottom line of the box is the 25$^{th}$ percentile. The horizontal line in the middle of the box is the median. Vertical bars outside the box represent the 10$^{th}$ and 90$^{th}$ percentiles.

For example, the probes 22-1229 and 4-1213 had the highest mean Probe SD (0.096 and 0.105 respectively), while the probes 18-0587 and 22-1521 were identified as having the lowest variability in signals, (mean Probe SD: 0.033 and 0.034). Consistent with the strong positive correlation between the mean Probe SD and the fraction of spurious results, 14 – 19% of the total of all signals generated by probes 22-1229 and 4-1213 were spurious results, whereas probes 18-0587 and 22-1521 only produced 2 – 3% spurious results.

**Figure 3: correlation probe variability and false results rate**



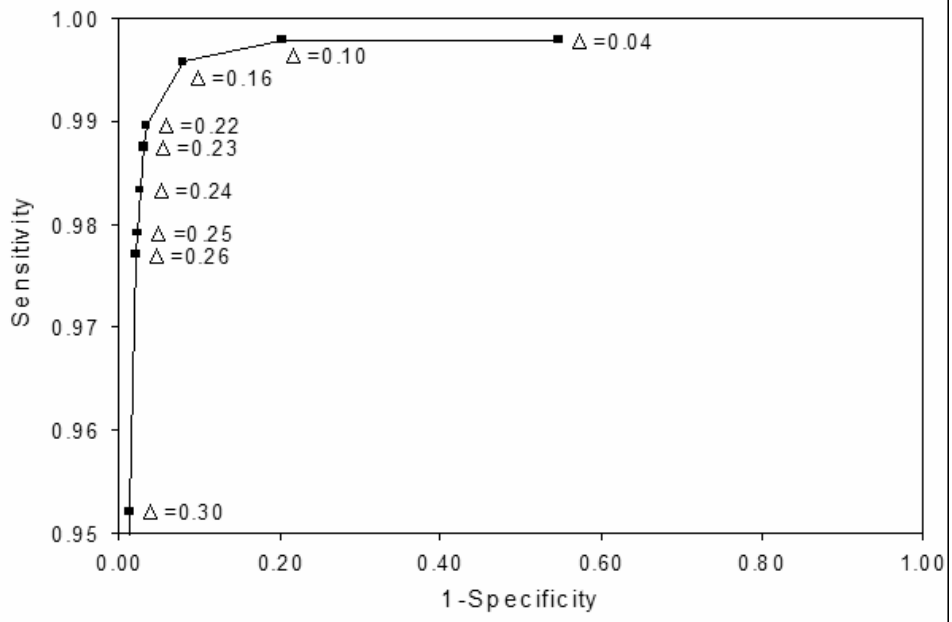

Correlation between the mean variability of individual probes (expressed as a standard deviation, calculated from all normal probes) and the percentage of false results (sum of false positive and false negatives). With Delta = 0.22, the Pearson Coefficient is 0.80, two tailed p-value: $8.6 \times 10^{-10}$.

*Assessment of the optimal Cut-off values*

The empirical assessment of the most optimal cut-off value (expressed as a delta value) consisted of plotting of the percentage of false positive and false negative results from all runs as a function of the delta value. The overall ROC curve indicates a rapid rise in sensitivity (data not shown). The area under the ROC curve is 0.9766, 95% C.I. 0.967-0.986, indicative of an excellent level of accuracy for the test. In order to identify the elbow of the curve, a magnification of the elbow region

is presented in figure 4. The delta value associated with equal weight of a false positive and a false negative (i.e. specificity and sensitivity are equally important), is 0.16. At this point the tangential slope of the ROC curve is 45 degrees. The delta value considered most optimal for the purposes of this specific test is one where the percentage of false negatives is 1% or less (i.e. a sensitivity of 0.99). The outcome of the empirical assessment and the ROC analysis concur at a delta value of 0.22. The corresponding sensitivity for this delta value is 0.99, 95% C.I. 0.977-0.998, and the corresponding specificity is 0.967, 95% C.I. 0.963-0.970.

**Figure 4: Receiver Operator Characteristic Analysis of optimal cut-off value**




Receiver Operating Characteristic (ROC) analysis: the Y-axis represents the fraction of correctly recognized deletions and duplications (i.e. sensitivity), the X-axis represents the fraction of false positives (i.e. 1-specificity). Plotted values are calculated for a range of different cut-off thresholds (delta values).

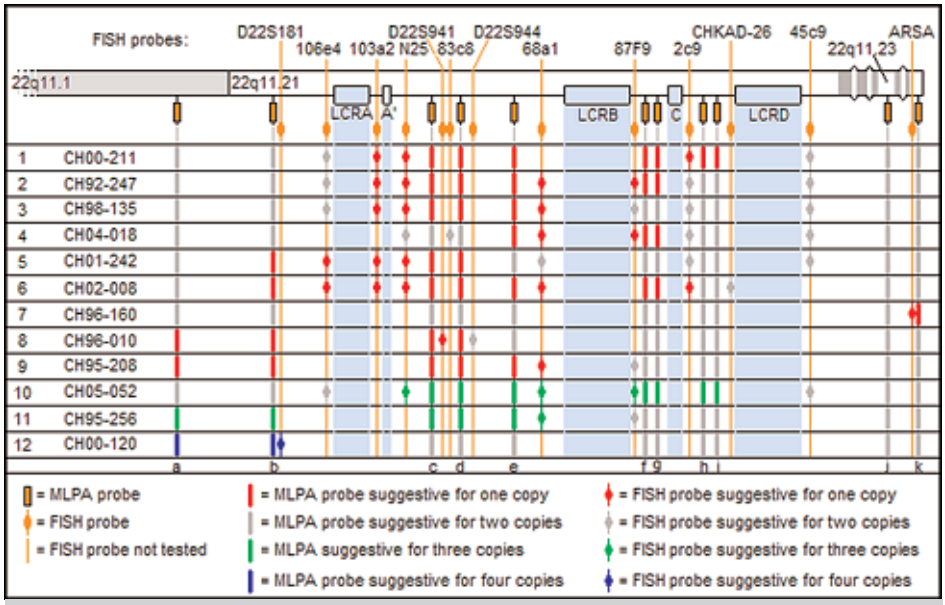
## DISCUSSION

The diagnostic interpretation of MLPA results for a given sample is usually performed by examination of the combination of probe signals. Any copy number change mediated by rearrangements involving LCRs A,B,C, or D will more than likely affect more than one probe in the current P023 MLPA kit and is therefore highly likely to be identified. Indeed, none of the samples in the training set with known copy-number differences went undetected. This included the duplications, which are often difficult to identify using metaphase FISH alone[34, 35]. In addition, there were no instances (Sample SD < 0.20) where a deletion or duplication was spuriously suggested by false positive results for more than 2 adjoining probes. Moreover, in three instances MLPA proved to be more informative than FISH, revealing an extension of deletions centromeric to LCR-A in two cases and an atypical distal endpoint in one case. Additional FISH studies confirmed the findings of MLPA adding to the characterization of these atypical deletions.

Since short regions of copy number change are likely to affect only one probe instead of two or more, the sensitivity and specificity of each individual probe must also be considered for their significance. For example, the 22q13.3 deletion in the training set (CH96-160, table. 1) resulted in only one positive (deletion) signal. Also, a sample from the unbalanced t(4;22)(q35.1;q11.2) generated not only 5 positive (deletion) 22q probes proximal to LCRB, but also a single positive signal (deletion) of a 4q35 probe, indicative of the deletion of 4q at that locus (CH95-208, fig. 1). This result clearly distinguishes the difference in the 4q breakpoints for two similar translocations, CH95-208 and CH95-015, providing molecular evidence for the more distal 4q breakpoint in the latter cell line.

The sensitivity and specificity of diagnostic test results for this MLPA kit using a delta value of 0.3, are a reasonable starting point (sensitivity 0.95, specificity 0.99). However, since we anticipate that this test is likely to be used as an initial screening method, avoiding false negatives is most crucial. Therefore we would advocate the use of a somewhat higher sensitivity, at the cost of a lower specificity. Any uncharacteristic positive result after the initial screening test should be confirmed with a different method (such as FISH). With this approach, the presence of false

**Figure 1: Overview of MLPA results**




Overview of results from MLPA and previous FISH studies on a selection of samples from the training set (Table 1). The number to the left indicates the class of sample as designated in Table 1. Shown above the representation of chromosome 22 are the FISH probes (cosmids) designated either by cosmid address or locus name. The FISH probes (orange ovals) and MLPA probes (orange rectangles) are shown in map order based upon data derived from the UCSC genome browser. All MLPA loci were tested in all samples. FISH studies are indicated by the presence of colored ovals in line with a sample. Copy number for MLPA or FISH probes are indicated by color (grey = normal, 2 copies; red = deleted, 1 copy; green = 3 copies; blue = 4 copies). Locus names of MLPA probes (below, letters a – k): a = 22-1082, b = 22-1767, c = 22-1214, d = 22-1218, e = 22-1223, f = 22-1227, g = 22-1231, h = 22-1235, i = 22-1521, j = 22-1229, k = 22-1093.

results is of lesser consequence. Using the current dataset we were able to compute a delta value (0.22) associated with a high sensitivity (0.99) and a very acceptable specificity (0.97). We suggest that this delta value is more appropriate if the test is used for the aforementioned purposes.

Our findings indicate that the current version of the commercially available MLPA diagnostic kit contains several probes that consistently display greater signal variability when compared with the other probes in the set. This variability, expressed as the mean Probe SD, strongly correlates with the number of spurious results generated by the probe, thus providing a measure of that probe's reliability. This analysis can further offer assistance with the interpretation of results (see boxplot diagram, Figure 2). In addition, our findings have been communicated to the manufacturer so that several of the less reliable probes in the current kit are planned to be replaced in future versions. The higher variability in the performance of some probes may be a result of suboptimal probe characteristics, such as the kinetics of hybridization and ligation of the hemi probe sets, and the amplification of fully ligated probes.

Conceivably, spurious results implying either a deletion or a duplication may in some cases be the consequence of the presence of a Copy Number Polymorphism (CNP), rather than of a cytogenetic abnormality. Recently, it has been shown that CNPs provide a substantial contribution to the (normal) variation of the human genome[36]. Further, the small size of the MLPA hemi probes (20 – 40 nucleotides) may cause a failure of hybridization if a small or single base pair polymorphism is present in the target sequence, leading to an unreliable result.

Nonetheless, the advantage of the small size of the probes is that they allow for a more precise assessment of the boundaries of a rearrangement involving an alteration of copy number. Further, the value of being able to accurately and simultaneously assess multiple short target sequences provides increased sensitivity over similar diagnostic tests performed with individual BAC or cosmid FISH clones. This is due to the fact that these larger clones may hybridize even when the target sequence is only partially present while partial binding and subsequent ligation of MLPA probes is highly unlikely. Further, BAC or cosmid FISH is likely

performed with one or two target sites per hybridization. MLPA expands the number of loci queried in a single experiment.
Finally, MLPA currently costs approximately 15 U.S. dollars per reaction plus the cost of separation on a DNA sequencing machine and multiple samples can be tested simultaneously, requiring only 3 hours hands on time.

In summary, the results of the present study indicate that MLPA is a rapid, relatively inexpensive assay that performs with high sensitivity and specificity. This makes it an appealing alternative to existing methods for the detection of copy number differences of 22q as well as other regions of the genome.

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## CHAPTER 4

# Detailed Analysis of 22q11.2 With a High Density MLPA Probe Set

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**ABSTRACT**

**Introduction:** The presence of chromosome-specific low copy repeats (LCRs) predisposes chromosome 22 to deletions and duplications. The current diagnostic procedure for detecting aberrations at 22q11.2 is chromosomal analysis coupled with fluorescence in situ hybridization (FISH) or PCR-based multiplex ligation dependent probe amplification (MLPA). However, there are copy number variations in 22q11.2 that are only detected by high throughput platforms such as array CGH.

**Method:** We report on the development of a high definition MLPA (MLPA-HD) 22q11 kit which detects copy number changes at 37 loci on the long arm of chromosome 22. These include the 3Mb region commonly deleted in DiGeorge/Velocardiofacial Syndrome (DGS/VCFS), the Cat Eye Syndrome (CES) region and more distal regions in 22q11 recently shown to be deleted. We have used this MLPA-HD probe set to analyze 363 previously well-characterized samples with a variety of different rearrangements at 22q11.

**Results:** The findings of this study demonstrate that it can detect copy number alterations with excellent sensitivity and specificity. In addition to detection of the common recurrent deletions associated with DGS/VCFS, variant chromosome 22 aberrations have been detected. These include duplications within as well as deletions distal to this region. Further, the MLPA-HD detects deletion endpoint differences between patients with the common 3 Mb deletion.

**Conclusion:** The MLPA-HD kit is proposed as a cost effective alternative to the currently available detection methods for individuals with features of the 22q11 aberrations. In patients with the relevant phenotypic characteristics, this MLPA-HD probe set could replace FISH for the clinical diagnosis of 22q11.2 deletions and duplications.

## INTRODUCTION

Genomic copy number alterations at 22q11.2 are associated with several syndromes. They are characterized by a variety of symptoms including cardiac defects, thymic, parathyroid, craniofacial, developmental and behavioral manifestations[1-4]. It is thought that the presence of chromosome-specific Low Copy Repeats (LCRs) or segmental duplications SDs, predisposes this region of chromosome 22 to cytogenetic rearrangements[5, 6].

To date, the majority of rearrangements identified at 22q11.2 have been deletions. It has been demonstrated that the deletion endpoints cluster, and that there is a typically deleted region of ~3 Mb in >85% of patients. It has been shown that there appear to be typical proximal and distal deletion endpoints (DEPs) as well as two recurrent variant distal DEPs occurring in the LCRs[5-9]. In recent years, several deletions with variant atypical DEPs have been identified using an extended set of FISH probes across the region and/or microarray technologies[10-13].

Although both deletions and duplications are expected to occur in equal proportions as a result of reciprocal LCR-mediated events, fewer duplications of 22q11.2 have been described, and the phenotype resulting from these duplications is extremely variable[14-22]. Further, the breakpoints implicated in generating the Cat Eye Chromosome, a supernumerary inverted duplication of proximal 22q, implicated in cat eye syndrome (CES; MIM# 115470), frequently match the proximal LCR or one of the more distal LCRs of the 22q11.2 region[23, 24]. However, detailed analysis of the extent of the duplicated material and its relationship to the ensuing phenotype has been minimal.

The diagnostic procedure most often used for detection of deletions and duplications at 22q11.2 is chromosomal analysis, which is often coupled with fluorescence in situ hybridization (FISH) using commercially available probes located between LCRs A and B. Previously we have shown that DGS/VCFS MLPA kit (MRC-Holland, Amsterdam, the Netherlands) for DiGeorge/velocardiofacial syndrome (DGS; MIM# 188400), is a cost effective, rapid and sensitive method for

the detection of typical recurrent deletions and duplications in proximal 22q11 extending to LCR-D[25].

However, we and others have identified copy number changes at 22q11.2 that would not have been detected by the current commercially available diagnostic FISH probes or the existing MLPA P023 kit[25, 26]. The detection and analysis of these genomic copy number alterations at 22q11.2 is important because to date little information is available with regard to their prevalence and whether there are consistently associated phenotypic differences. Indeed, identification of these variant cases is of particular interest since it may provide insight into which genes or genomic regions are crucial for specific phenotypic manifestations.

Therefore, in collaboration with MRC-Holland, we have developed a high density MLPA (MLPA-HD) probe set that incorporates probes starting proximal to LCR-A and covers the region flanked by the four LCRs distal to LCR-D, which is the distal boundary of the typical 22q11.2 deletions[27]. We have tested the ability of this new high-density (HD) probe set to detect copy number alterations on numerous samples with copy number changes at 22q11.2. Here, we report on the first results using this new probe set, including its reliability, cost effectiveness as well as its capacity to detect rare, difficult to identify copy number changes in 22q11.

## METHODS

Over the past 20 years the Clinical Genetics Center at The Children's Hospital of Philadelphia (CHOP) has enrolled patients with a variety of duplications, deletions and rearrangements of 22q11 under several IRB approved research protocols. Several additional samples used in this study were identified clinically after the parents had consented to genetic testing. Characterization or validation of 22q rearrangements by FISH analysis with cosmid or BAC clones was performed by our research laboratory or by clinical diagnostic laboratories**.** A group of 363 well characterized samples, including typical 22q11.2 deletions, atypical deletions, duplications (trisomy and tetrasomy) and unbalanced translocations, as well as samples without a known rearrangement were selected for the purpose of the validation study for the MLPA-HD kit. The new HD-MLPA22 kit was validated in a research laboratory blinded to the molecular genetic status of the samples. The test includes a group of standard 22q11.2DS deletions (64 cases), several recurrent smaller 22q11.2DS deletions (24 cases), several unique 22q11.2DS associated deletions (seven cases), a group of unbalanced translocations to 22q (four cases), several interstitial duplications of 22q11.2 (11 cases), several supernumerary der(22)t(11;22)(q23;q11) cell lines (three cases), two CES cell lines, and several balanced translocations to 22q (two cases).

*Constitutional 22q11.2 deletions associated with Rhabdoid tumor samples*
DNA samples from tumor tissue, cell lines, and peripheral blood samples were obtained from the four patients for *INI1* analysis according to procedures approved by the Institutional Review Board at The Children's Hospital of Philadelphia. In each case the parents signed consent forms for genetic testing.

*Enhanced MLPA kit generation and validation*
In collaboration with the group at MRC-Holland, an expanded HD-MLPA22 kit (containing 45 probes) was developed to test for copy number variation (CNV) of genomic regions within 22q11. To identify typical and atypical patients, probes are positioned in specified intervals on 22q11.2. There are nine probes between LCR-A and LCR-B (1.103 Mb); three probes between LCR-B and LCR-C (278.8 kb); three probes between LCR-C and LCR-D (272 kb); three probes between LCR-D and

LCR-E (1.05 Mb); one probe between LCR-E and LCR-F (651 kb); four probes between LCR-F and LCR-G (616 kb); one probe between LCR-G and LCR-H (298 kb) and one probe placed distal to LCR H-toward the telomere. There are eight probes in the Type I CEC region proximal to LCR-A (chr22: 16,205,810-17,017,046). The location of the probes is included in Table 1. In addition, the MLPA-HD kit has one probe placed within LCR-A and four placed within LCR-D. The remaining eight loci, which serve as controls, are located at eight different chromosomal positions (chromosomes 2q, 3p, 5q [2 loci], 8p, 10p, 17q, and 19p).

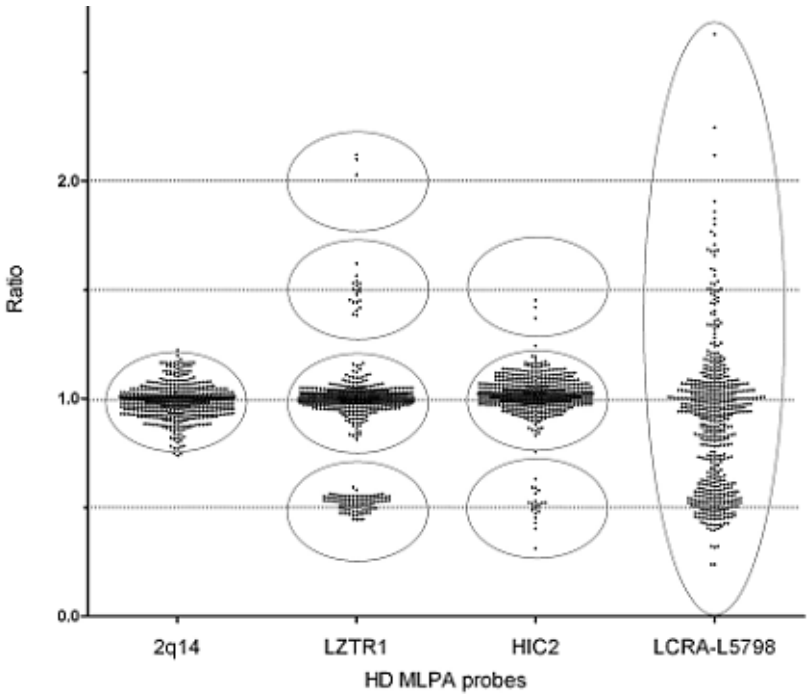
**Table 1: Chromosomal postions of the probes in the MLPA-HD kit (May 2004 assembly)**

| | Probe name | Chr. Position | Chr. Region | | Probe name | Chr. Position | Chr. Region |
|---|---|---|---|---|---|---|---|
| 1 | IL17R | 15,954,293 | | 25 | LCR-Dr1 | 19,773,060 | |
| 2 | CECR1 | 16,062,702 | | 26 | LCR-Dr2 | 19,938,175 | LCR-D |
| 3 | SLC25A18 | 16,417,934 | | 27 | HIC2 | 20,125,240 | |
| 4 | BCL2L13 | 16,546,387 | CES | 28 | LCR-Dr3 | 20,241,722 | |
| 5 | BID | 16,601,309 | | 29 | PPIL2 | 20,241,722 | |
| 6 | MICAL3 | 16,699,273 | | 30 | TOP3B | 20,654,633 | LCR-E |
| 7 | PEX26 | 16,935,706 | | 31 | RAB36 | 21,812,171 | LCR-F |
| 8 | USP18 | 17,007,539 | | 32 | SMARCB1-D01 | 22,453,988 | |
| 9 | LCRA-L57984 | 17,310,399 | LCR-A | 33 | SMARCB1-D02 | 22,500,983 | |
| 10 | GSCL | 17,511,823 | | 34 | MIF-D01 | 22,561,257 | |
| 11 | CLTCL1 | 17,616,215 | | 35 | MIF-D03 | 22,561,884 | LCR-G |
| 12 | HIRA | 17,693,633 | | 36 | UPB1 | 23,215,006 | LCR-H |
| 13 | CDC45(L) | 17,842,094 | | 37 | SEZ6L | 25,013,099 | |
| 14 | CLDN5 | 17,885,934 | | | | | |
| 15 | GP1BB | 18,086,134 | | 38 | C1, NRXN1 | 50,060,856 | 2q14 |
| 16 | TBX1 | 18,121,732 | | 39 | C2, APC | 112,191,603 | 5q31.1 |
| 17 | KIAA 1652 | 18,260,839 | | 40 | C3,SCN5A | 38,593,243 | 3p21 |
| 18 | ARVCF | 18,352,766 | LCR-B | 41 | C4, STK11 | 1,169,489 | 19p13 |
| 19 | ZNF74 | 19,073,049 | | 42 | C5, BRCA1 | 38,505,407 | 17q21 |
| 20 | FLJ14360 | 19,167,921 | | 43 | C6, IL4 | 132,037,671 | 5q22 |
| 21 | PCQAP | 19,261,367 | LCR-C | 43 | C7,CUGBP2 | 11,247,596 | 10p14 |
| 22 | SNAP29 | 19,566,638 | | 45 | C8, MFHAS | 8,786,079 | 8p23.1 |
| 23 | LZTR1 | 19,673,815 | | | | | |
| 24 | P2RXL1 | 19,705,448 | | | | | |

To calculate test characteristics of the MLPA-HD kit, we analyzed individual probe results of 609 runs. Results of 21 runs were excluded because of excessive variance (the standard deviation of the results of the eight control probes exceeded 0.20). We have used receiver operating characteristic (ROC) curve analysis to assess the optimal cutoff values[25]. The usual cause for variance of the results was due to low quality of the DNA preparations or increased variation (>±10%) in DNA concentrations. In addition, in all runs, results of two probes (one in LCR-A, one in

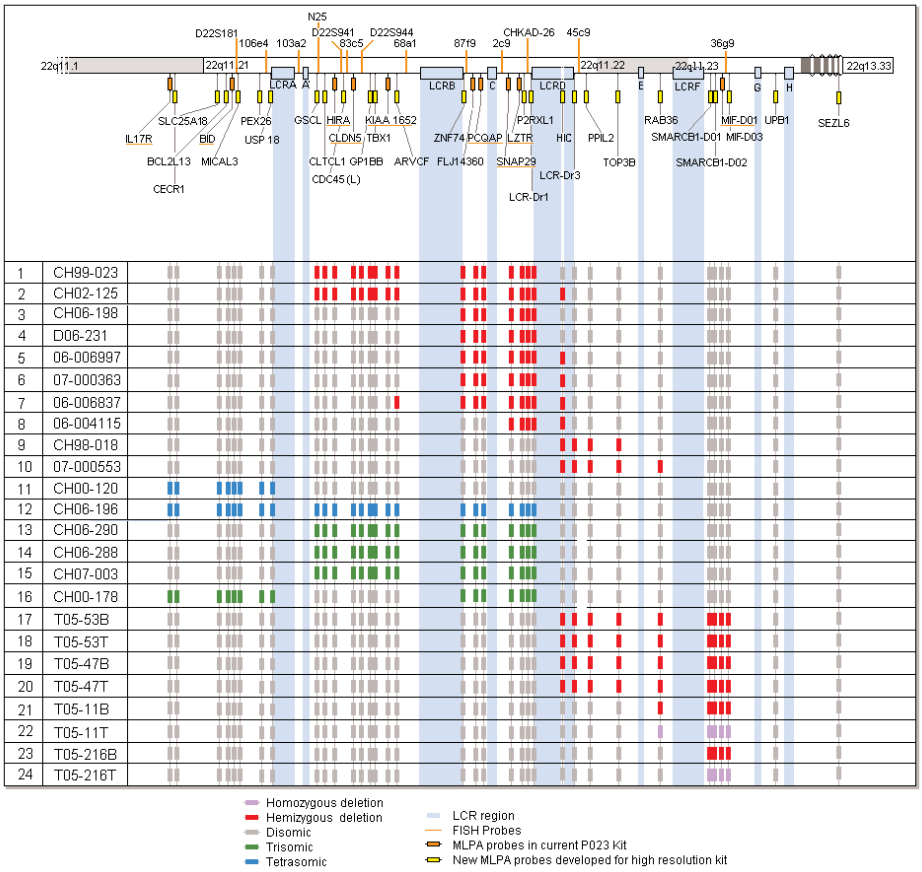
LCR-D) were also excluded from the analysis because of overall high signal variance (Fig. 1). Thus, results of a total of 588 runs, yielding more than 25,000 signals, were analyzed and compared to results obtained from previous studies and/or replication studies. Subsequently, based on these comparisons, each locus signal was individually labeled as false or true negative/positive, respectively.

**Figure 1: A visualization of probe performance**




The ratio for copy number values is expected to cluster around 0.0, 0.5, 1.0, 1.5, or 2.0, representing homozygous deletion, hemizygous deletion, disomic 2 copies; trisomic 3 copies and tetrasomic, 4 copies respectively. The above dot plot shows the performance of a control probe and three test probes based on the analysis of 585 runs. The results of control probe; CNT-2q14 clearly shows a cluster around 1.0 representing 2 copies. Test probes LZTR1 and HIC-2 display clustering closely around 0.5, 1.0, 1.5 and 2.0 representing deletion, disomic, trisomic and tetrasomic representing good performance. In comparison, the results of LCRA-L5798 lack clear clustering around expected values of 0.5, 1 or 1.5 respectively, indicating poor performance.

**Figure 2: Graphical representation of MLPA-HD results**




Shown above the representation of chromosome 22 are the FISH probes (cosmids) used in the laboratory designated either by cosmid address or locus name. The MLPA probes (orange rectangles, existing MLPA PO23 loci; yellow rectangles, new MLPA loci) are shown in map order based upon data derived from the UCSC genome browser. All MLPA loci were tested in all samples. Copy number for MLPA probes is indicated by color (purple=nullisomic, homozygous deletion; gray=disomic, two copies; red=hemizygous deletion, one copy; green=trisomic, three copies; blue=tetrasomic, four copies). All results are confirmed in two independent runs. E.g. sample 4 (D06-231) shows an atypical LCRB to LCRD deletion, sample 13 (CH06-290) shows an LCRA-LCRD duplication. Samples 17 – 24: MLPA-HD results on individuals with a constitutional 22q11.2 deletion and rhabdoid tumor, the notation “B” indicates DNA from whole blood, the notation “T” represent DNA from tumor samples. E.g. samples 21 and 22 (T05-11B and T) show a deletion between LCRE and LCRG, in the tumor (T) sample the deletion is homozygous.

*Laboratory Methods*

The MLPA-HD test was performed on genomic DNA according the manufacturer's method (MRC-Holland; MLPA-HD Kit; www.mrc-holland.com). Briefly, 500 ng of genomic DNA was denatured (5 min at 98 °C) and subsequently hybridized to the new MLPA-HD probe set. PCR amplification was carried out on an ABI 9700 thermocycler (Applied Biosystems, Foster City, CA), and electrophoresis was performed using the ABI 3700 DNA analyzer (Applied Biosystems) with Rox 500 size standards[28, 29].

*Data Analysis*

We have used a commercially available software, Gene Marker from SoftGenetics (State College, PA) to analyze our data. Gene Marker has developed a completely integrated application for MLPA analysis. The Gene Marker program has integrated functions that are specific for the analysis of data derived from the MLPA reactions. The software exploits two selectable normalization methods. The first normalization method is the traditional method based upon the control probes. The second method normalizes peak intensities based upon the statistically most probable median intensities and was the method used for this study (see Supplementary Material, which includes Supplementary Fig. 1; available online at http://www.interscience. wiley.com/jpages/1059-7794/suppmat).

## RESULTS

The MLPA-HD kit was designed to contain a total of 45 loci. Of these, there are 37 loci located on the long arm of chromosome 22. Most of the probe sets are located within genes, of which 15 loci are located between LCR-A and LCR-D. We chose five unique loci located within the LCR regions, one probe positioned within LCRA and four placed within LCR-D for detecting differences in deletion endpoints. Two of these LCR probes, one in LCR-A and one in LCR-D did not pass quality control and were removed from our analysis. The MLPA-HD kit provides more extensive coverage of the typically deleted region (TDR) within 22q11.2, allowing for the identification and characterization of atypical deletions. It also includes 10 probe sets that are located distal to LCR-D in the interval between LCR-D and LCR-H. The remaining eight loci, which serve as controls, are located at eight different chromosomal positions (chromosomes 2q, 3p, 5q [two loci], 8p, 10p, 17q, and 19p). Table 1 lists the probe sets in the MLPA-HD kit and their location in the sequence of chromosome 22 based upon the NCBI reference sequence (May 2004 assembly, hg17). Utilizing a delta value of 0.22, which implicates a deletion at signals below 0.78 and a duplication at signals exceeding 1.22, the MLPA-HD kit demonstrated very robust test characteristics; sensitivity and specificity were 0.997 (95% confidence interval (CI) = 0.994–0.999) and 0.989 (95%CI = 0.987–0.990). These values were not essentially altered when analyzing test results utilizing the more commonly applied delta value of 0.3; the associated sensitivity and specificity values were 0.992 and 0.995, respectively.

*Detecting Atypical 22q11 Deletions*

Figure 2, Patients 1–10, shows a panel of patients with typical proximal, atypical proximal and distal 22q11 deletions that were studied under several different protocols. Patients 1 and 2 are from a cohort of VCFS patients with known typical 3-Mb deletions. Patient 3 was a member of a cohort of patients with conotruncal defects studied in a Specialized Center of Clinical Research funded by the National Heart, Lung, and Blood Institute (NHLBI) (HL74731). In this study the MLPA-HD kit was utilized in an attempt to streamline screening patients with conotruncal defects for the presence of a 22q11.2 deletion. Individual 4 is the parent of patient 3. Patients 5, 6, 7, 8, and 10 were patients with multiple congenital anomalies that

did not fit a specific syndrome who were evaluated by clinical geneticists. Their samples were sent for array comparative genomic hybridization (aCGH) studies (Signature Genomics, Spokane, WA). After a deletion of one or more chromosome 22 BACs was detected by the array, the MLPA-HD kit analysis was able to more precisely assess the extent of the deletion in each of these individuals. Patient 9 has been reported previously[10, 11]. The MLPA-HD kit results agreed with any prior and subsequent confirmatory FISH and molecular analyses. In the patients with known LCR-A to LCR-D deletions (Patients 1 and 2), the MLPA-HD not only confirmed the FISH and existing MLPA kit (P0-23) results but also revealed that the extent of the deletion was different in these two cases. Patient 1 showed a hemizygous loss of one copy of 16 probes covering the region located between LCR-A and LCR-D while Patient 2 showed loss of one copy of an additional probe (HIC2), clearly showing differences in the extent of the deletion between the two patients as a result of different distal deletion endpoints. Patients 3 and 4 (CH06-198 and D06-231) showed loss of one copy of seven probes located between LCR-B and LCR-D, indicating an atypical LCR-B to LCR-D deletion. For patient CH06-198, FISH screening with a probe (N25) in the DGS/VCFS region had been normal, suggesting that there was no 22q11.2 deletion. Patients 5 and 6 (06-006997 and 07-000363) have similar LCR-B to LCR-D deletions. Interestingly, the distal deletion breakpoints are at a different location for these latter two cases when compared with the previous two cases. In these latter cases there is the loss of the HIC2 probe, suggesting a more extensive deleted area. In Patient 7 (06-006837), there are nine loci deleted, expanding the deleted region proximal to the typical LCR located breakpoints. Patient 8 (06-004115) is deleted between LCR-C and LCR-D, such that five probes show a hemizygous loss. Patient 9 (CH98-018) is deleted for one copy of the four probes within the region between LCR-D and LCR-E, while Patient 10 (07-00053) has a deletion that extends farther, such that it also includes the region between LCR-E and LCR-F. It is of interest that none of these atypical cases (Patients 3–10) would have been detected by the N25 or TUPLE/HIRA probes which are the routine FISH probes used for detecting deletions of 22q11. Although patients with deletions between LCR-B and LCR-D (Patients 3–8) would have been detected using the existing MLPA kit; neither of the cases with distal deletions that extend beyond LCR-D (Patients 9 and 10) would have been detected. Differences in deletion endpoints within LCR-D would not be detected utilizing the

existing kit. Further, only the MLPAHD kit is capable of detecting copy number changes within and distal to LCR-D.

*22q11Duplications*

Duplications involving chromosome 22 can be difficult to identify using standard technology. Figure 2 shows a schematic diagram of several cases with a variety of duplications that have been studied with the MLPA-HD kit (Patients 11–16). The bisatellited supernumerary marker chromosomes associated with the cat eye syndrome (CEC) are variable in size and can be asymmetric with regard to the region duplicated[24]. There are no commercially available, unique diagnostic probes for identifying the CEC when identified pre- or postnatally. We have previously determined that the breakpoints of the symmetrical CES chromosome cluster in two intervals such that there are small and large cat eye chromosomes. In Patient 11 (CH00-120) MLPA detected tetrasomy for the eight most proximal probes distributed throughout the cat eye syndrome chromosomal region (CECR). In Patient 12 (CH06-160), all the probes in the CECR as well as the 16 probes between LCR-A and LCR-D are present in four copies, confirming the presence of the Cat Eye chromosome as well as determining the extent of the duplicated material. Thus, Patient 11 has a smaller CES chromosome that is symmetrical, classified as a Type I CES chromosome and has both breakpoints located within LCR-A. Patient 12 has a larger type II CES chromosome that is symmetrical with both breakpoints located in the distal (LCR-D) interval. The MLPA-HD kit provided a rapid assessment of these two supernumerary marker chromosomes. Individuals with 22q11 interstitial duplications have been identified less frequently than anticipated and some seem to share some phenotypic similarities with 22q11.2 deletion syndrome patients[16, 17, 19, 21, 22, 30, 31]. The detection of these individuals has been problematic utilizing FISH because it requires interphase analysis. We have detected a three-generation familial microduplication.

Patient 13 (CH06-290; proband) showed a duplication of all of the probes located between LCR-A and LCR-D. Patients 13 (CH06-288) and 14 (CH07-003) are the mother and maternal grandmother of the patient, who both exhibit the same pattern of duplication. Thus, the MLPA-HD kit provided a rapid screening test for the presence of the duplication.
Patient 16 (CH00-178) carries a complex supernumerary der(22)t(8;22)(q24.1;q11.2)pat chromosome. We previously mapped the breakpoints as well as the translocation junction to the LCRs found on chromosome 22[32]. Trisomic signal was observed in Patient 16 (CH00-178) for the eight most proximal MLPA probes distributed throughout the CECR including the USP18 probe at the proximal edge of LCR-A. All six of the MLPA probes located between LCRs B and D also exhibited trisomic signals, indicating that these sequences are also present on the supernumerary der(22). The derivative chromosome is the result of an inversion of the region between LCR-A and LCR-D prior to the occurrence of the translocation. The nine probes corresponding to loci located between LCR-A and B, however, are disomic. These MLPA-HD results are entirely consistent with FISH and molecular mapping results and confirm the previously reported mechanism presumed responsible for the rearrangement.

*Constitutional 22q11.2 Deletions Associated With RhabdoidTumor*
In the third group of samples we screened four patients with constitutional 22q11.2 deletions associated with rhabdoid tumor. Of these, Patients 17 and 18 had phenotypic findings that were suggestive but not diagnostic for the 22q11.2 deletion (DGS/ VCFS). The initial genetic analysis did not reveal an underlying diagnosis, as the chromosomal analysis and FISH studies using a diagnostic probe (TUPLE1) in the DGS/VCFS region were normal. However, the probands were subsequently diagnosed with malignant rhabdoid tumors.

We were fortunate to have access to blood and tumor samples for these four cases. MLPA-HD confirmed the findings of FISH with probes from the rhabdoid tumor region and microarray analysis[33]. Figure 2, Patients 17–24, represent the findings on these patients. DNA from blood (B) and tumor (T) sample of Patients 17-20 (T05-53 and T05-47) showed the loss of one copy of nine probes, confirming a LCR-D to LCR-G deletion. Genomic DNA from blood from Patient 21 (T05-11 B) revealed

the hemizygous loss of five probes spanning the region between LCR-E to LCR-G. The tumor sample from this patient (Sample 22; T05-11T) not only confirms the same extent of the deletion but also exhibits homozygous deletion for the five probes contained within the deleted region. Patient 23 demonstrated a deletion of the region between LCR-F and LCR-G, revealed by the loss of one copy of four loci covering this region. The DNA from the tumor sample from Sample 24 showed a homozygous deletion for the same four probes. In all of these patients, the MLPA-HD analysis confirmed the deletion of SMARCB1 (INI1), the tumor- suppressor gene inactivated in rhabdoid tumors. Thus, in Patients 21 and 23, MLPA-HD confirmed homozygous deletions of the INI1 gene observed in the majority of rhabdoid tumor samples and also validated the copy number change in one of these cases (Sample 22; T0511T).

**DISCUSSION**

Aberrations of the 22q11 region are amongst the most common constitutional chromosomal abnormalities. The resulting varied and complex phenotypes make these disorders a significant health problem[34-37]. We have previously utilized a DGS/VCFS MLPA kit (P023) to assess copy number changes of 22q11.2[25]. MLPA proved to be a relatively inexpensive assay with high sensitivity and specificity. However, there have been reports from several laboratories about variant deletions and duplications that are neither detected by chromosomal analysis with FISH using the N25 or TUPLE1/HIRA diagnostic probes nor the currently available MLPA kit for DGS/VCFS[11, 38-40].
Therefore, in collaboration with MRC Holland we have developed a MLPA-HD kit for the 22q11 region. The new MLPA probe set detects copy number changes at 37 loci on the long arm of chromosome 22. The MLPA-HD kit not only has a greater density of probe coverage within the typically deleted region LCR-A to LCR-D (18 probes), but also includes probes covering other important regions, including the CES region (8 probes) the region located between LCR-D and LCR-H. (9 probes). There are also eight regions for control probes located on non critical regions associated with DGS/VCFS.

In the current study, results are presented from this new high density MLPA probe set for 22q11. Based on a sizeable number of verified probe signals, we show that this new assay is highly accurate with excellent sensitivity and specificity values. Further, using a unique sample set with a variety of different rearrangements at 22q11, we demonstrate the ability of the new MLPA probe set to correctly detect gain or loss of genomic material, to accurately indicate the number of allelic copies (from n=0 to n=4) and to delineate the extent of the region involved in the rearrangement. Of note, several abnormalities that were detected by the new MLPA probe set would have remained undetected by either FISH using the standard probe at 22q11.2 (N25 or TUPLE1) or the currently available MLPA kit.

The majority of the DGS/VCFS patients have a 3Mb deletion (86.3%) extending from LCR-A to LCR-D. However the presence of copy number variations (CNV) and sequence similarity within the LCRs (>96%) has proved to be an obstacle in

interrogating the extent of the deletion in this group of patients[41-43]. Thus we have addressed this by designing probes for the unique regions interspersed within the LCRs. Five probes were designed within the LCR regions, one within LCR-A and four within the LCR-D (Table 1). Two of the probes, LCR-A and LCR-D2 proved to be problematic; however the performance of the other three, two probes flanking LCR-D and the HIC2 probe inside the unique region of LCR-D gave consistent results.

Investigators have been looking for modifiers of the phenotype in patients with typical LCR-A to LCR-D deletions that reside outside the typical 3 Mb deletion. The variations in DEPs in patient with LCR-A to LCR-D has been reported, using isothermal HR-CGH array[12]. Here, for the first time using a PCR base method, we demonstrate that this group of patients is not a homogeneous group, confirming variation DEPS in patient having TDR. Patients 1 and 2 clearly show a difference in deletion size (Figure 1). We have used samples from 77 patients with typical deletions of LCR-A to LCR-D of whom 5 patients had extended deletions including the HIC2 locus. The signals of the HIC2 probe in normal control samples were found to cluster closely around 1.0 (n=2), which indicates a good performance for this particular probe. It is of interest that although the proximal part of the HIC2 region is within a reported CNV region, the MLPA probe covering HIC2 is outside the CNV region[43] (http://projects.tcag.ca/variation/). This variation in deletion endpoint might eventually be useful in explaining differences in the mechanism of the rearrangement in affected patients as well finding explanations for the extreme variability in the spectrum of the phenotype.

The MLPA-HD kit has eight probes located in the CES region of chromosome 22. We were able to detect variation in the size of the CECs in two individuals carrying a supernumerary chromosome comprised of different duplications of chromosome 22. The availability of the MLPA-HD kit will permit a better understanding of genotype-phenotype correlations in cat eye syndrome patients. Further, the kit will fill a major void in diagnostics for CES as there are no chromosome 22 specific FISH probes currently available for this purpose.

Based on these findings, we suggest that the new MLPA probe set represents an appealing alternative for the currently available detection methods. Naturally, oligonucleotide-based micro-array based methods have a greater resolution than MLPA. In addition, they have the potential of genome-wide screening of copy number variations. However, the high expense associated with array-based techniques discourages the use of these methods for routine screening of patients. This is an important limitation since screening of large numbers of individuals with phenotypic characteristics of the 22q11.2 deletion syndrome is likely to be an important approach to unraveling the enigma of the high level of phenotypic variations observed in patients with 22q11 rearrangements. This MLPA-HD kit will allow for a cost effective and inexpensive screening method for individuals with specific features of the deletion such as the typical associated conotruncal cardiac defects. Further, while several duplications at 22q11 have been reported, to date the total number of identified patients with this condition is too small to accurately describe the associated phenotypic characteristics.

The recent identification of small deletions within or close to the typically deleted portion of 22q11.2 may prove to be relevant with regard to the identification of candidate genes in the region. It is likely that the identification of additional individuals with atypical or small deletions, or duplications will greatly contribute to our understanding of the genes involved in the etiology of different aspects of the 22q11.2 deletion syndrome phenotype. We strongly believe that with the new MLPA probe set screening of high numbers of individuals will be feasible for most medical centers, and therefore the identification of many cases such as those described in this report can be anticipated.

Apart from the implications for research, we propose that the new MLPA probe set could replace FISH with N25 or TUPLE1 for the clinical testing of 22q11.2 deletions in patients with the phenotypic characteristics. In comparison to standard FISH, MLPA is easier to carry out, less expensive and equally reliable in the detection of typical deletions. In addition, with MLPA smaller deletions outside the typically deleted region are identified with precise identification of the extent of the region involved. While duplications can be easily missed using standard FISH on metaphase spreads, with MLPA any copy number change, either a loss or gain of

material, can be accurately assessed. In conclusion, given the many advantages of MLPA over standard FISH methodology for the detection of rearrangements at 22q11, it is likely that it will replace 22q FISH in the very near future.

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## CHAPTER 5

# Prevalence of 22q11.2 deletions in 311 Dutch patients with schizophrenia

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**ABSTRACT**

**Introduction:** The aims of this study were 1) to examine whether the prevalence of 22q11.2 deletion syndrome (22q11DS) in schizophrenia patients with the deficit syndrome is higher than the reported ~2% for the population of schizophrenia patients as a whole, and 2) to estimate the overall prevalence of 22q11DS among schizophrenia patients by combining all available studies.
**Method:** A sample of 311 Dutch patients with schizophrenia, including 146 patients meeting the criteria for the deficit syndrome, was screened for the 22q11.2 deletion utilizing Multiplex Ligation-dependent Probe Amplification (MLPA).
In order to calculate the prevalence of 22q11DS in schizophrenia, all published findings of screening studies for the 22q11.2 deletion in samples of patients with schizophrenia were analyzed.
**Results:** No 22q11.2 deletions were detected in this sample. Our literature research revealed that in eight studies sixteen deletions were identified in 2133 patients with schizophrenia. This corresponds to a prevalence of 0.75% (95%CI: 0.5%-1.2%).
**Conclusion:** The prevalence of 22q11.2 deletions in patients with schizophrenia is less than the 2% which is often quoted in literature, but is still approximately 30-45 times higher than expected.

## INTRODUCTION

The 22q11.2 deletion syndrome (22q11DS) is associated with a range of congenital physical defects, including velopharyngeal anomalies, cardiac anomalies and typical dysmorphic features of the face, hence the name Velo Cardio Facial Syndrome or VCFS. It is also associated with a very high risk (20-30%) of schizophrenia[3, 4]. It is uncertain, however, which proportion of schizophrenia patients carries a 22q11.2 deletion. Results of studies vary widely from 0.3% to 5.3% (see overview in Table 1). Knowledge of the true rate of 22q11.2 deletions in schizophrenia patients is important for several reasons; from a clinical perspective the identification of a 22q11.2 deletion in patients has consequences for genetic counseling. Identification may also lead to the detection of other physical anomalies associated with the clinical syndrome that may need treatment, such as hypocalcaemia. Furthermore, from a research perspective it is important to better define the relationship between 22q11DS and schizophrenia.

We have screened a sample of 311 Dutch patients with schizophrenia, including a relatively large sub-sample of patients meeting the criteria for the deficit syndrome. The deficit syndrome in schizophrenia is characterized by enduring, idiopathic negative symptoms, and is generally associated with a poor prognosis[5]. It is diagnosed in approximately 25%-30% of chronic schizophrenia patients. A number of observations indicate that the deficit syndrome represents a distinctive subgroup within schizophrenia, with an increased heritability[9-11]. Remarkably, the negative symptoms of schizophrenia resemble some behavioral features of 22q11DS patients (with and without schizophrenia). These features are “lack of mental energy”[13], “social withdrawal”, “bland affect with minimal spontaneous facial expression”, “monotonous voice”[14] and “poor social skills”[15].

Consequently, the present study tested the hypothesis of a higher rate of 22q11 deletions in the subpopulation of schizophrenia patients with the deficit syndrome. A second aim of the study was to update the current estimations with regard to the prevalence of 22q11DS among schizophrenia patients. To that end we included the results of this study and the study by Drazinic et al., which is presently in review.

## METHOD

To achieve a maximum number of participants with prominent negative symptoms, the majority of patients were assessed through institutionalized settings for patients with chronic mental illness. Patients underwent diagnostic screening using the Comprehensive Assessment of Symptoms and History (CASH[16]) and additional information from medical records and clinicians. A total of 311 unrelated patients received a primary diagnosis of schizophrenia (DSM-IV). All patients were diagnosed by trained raters. All participating patients provided written informed consent according to the Declaration of Helsinki and the Medical Research Ethics Board of each participating institution approved the project. Of the 311 patients, 284 were further assessed using the Schedule for the Deficit Syndrome (SDS)[5].

Blood was collected from each participant; screening for 22q11DS was done by means of Multiplex Ligation-dependent Probe Amplification (MLPA). MLPA is a Polymerase Chain Reaction (PCR) variant using two oligonucleotide hemiprobes complementary to the target DNA sequence[17]. In a single reaction up to 45 target sequences can be quantified. The two hemi-probes on each target sequence (two PCR primers, one with added variable unique length sequence) are ligated after hybridization, and subsequently amplified, each of the ligated probes producing an amplification product of unique length, which can be detected and quantified. The resulting quantification of each probe product allows for the definition of a normal or abnormal copy number (euploidy versus a deletion or duplication) of that probe. For this screening the MLPA P023 kit was used[18]. The MLPA P023 kit is commercially available (www.mrc-holland.com).

Deletions and other rearrangements at 22q11.2 are thought to be mediated by Low Copy Repeats (LCRs)[19, 20]. In each run DNA from two patients with a known 22q11.2 deletion (proven by fluorescence in situ hybridization (FISH) analysis) was simultaneously analyzed in order to verify reliable detection of the 22q11.2 deletion (one common LCRA-LCRD deletion and one LCRA – LCRB deletion).

In order to calculate the prevalence of 22q11DS in schizophrenia, we synthesized findings that were previously reported on this topic. For this purpose, the key words “22q11”, 22q11DS”, "VCFS" and "schizophrenia" were used in a computerized search of MEDLINE. Bibliographies from identified articles were cross-referenced. Only studies that screened random samples of schizophrenia patients were included in this calculation; studies in which samples of schizophrenia patients were screened for 22q11DS after selection for its phenotypic hallmarks were excluded.

Prevalence and 95% confidence intervals were estimated for patients with schizophrenia in general, as derived from all available studies. Since patients with childhood onset schizophrenia (age of onset<13 years) may represent a homogeneous subgroup with more neuro-developmental and more cytogenetic abnormalities[21], we also estimated the prevalence of 22q11DS in childhood onset patients and in adult onset patients with schizophrenia separately. Prevalences and their 95% confidence intervals were calculated using the Confidence Interval Analysis statistical package (CIA)[22].

**RESULTS**

A total of 146 out of 311 patients met criteria for deficit syndrome as defined by the SDS. Following SDS criteria, 138 patients were classified as non-deficit patients. Among these, 29 patients exhibited negative symptoms that could be secondary to substance abuse or medication. However, in accordance with the SDS criteria, they were classified as non-deficit schizophrenia.
In each run the two positive controls were identified, indicating the reliability of the MLPA kit. In the total sample, including the deficit subgroup, no 22q11 deletions were detected with MLPA.

The results of the literature search on the prevalence of 22q11DS are shown in Table 1. Adding the results of the current study and the report of Drazinic et al. (in review) to the results from the literature search, to date a total of sixteen 22q11 deletions have been identified in 2133 patients with schizophrenia (0.75%, 95% confidence interval: 0.5%-1.2%). In a total of 82 childhood onset schizophrenia patients (combining the results of Ivanov et al.[1] and Sporn et al.[12], four 22q11 deletions were identified (4.9%, 95% confidence interval: 1.9%-11.9%). As a result, twelve 22q11 deletions have been identified in 2051 adult onset schizophrenia patients (0.6%, 95% confidence interval: 0.3%-1%).

**Table 1: Overview of prevalence of 22q11DS in schizophrenia**

| Study | n 22q11DS / n schizophrenia patients | Rate of 22q11DS | Population | Age of onset of psychosis in 22q11DS |
|---|---|---|---|---|
| Karayiorgou et al., 1995 | 2/100 | 2% | U.S., ethnicity not specified | 20 y; 32 y |
| Arinami et al., 2001 | 1/300 | 0.3% | Japan, unrelated patients | 15 y |
| Ivanov et al., 2003 | 1/329 adult onset<br>0/134 early onset[1]<br>0/7 childhood onset[2] | 0.3%<br>0%<br>0% | U.K. (n=415), unrelated patients<br>Bulgaria (n=55)<br>Exclusion IQ<70 | Unclear: between 17 & 21 y |
| Wiehahn et al., 2004[3] | 2/85 | 2.35% | South Africa, Afrikaner | 19 y; 22 y |
| Sporn et al., 2004[4] | 4/75 childhood onset | 5.33% | U.S., ethnicity not specified.<br>Exclusion IQ<70 | Information not provided |
| Horowitz et al., 2005 | 6/634 | 0.95% | Israel, Ashkenazi Jewish | Unknown; anonymous |
| Drazinic et al. (in review) | 0/158 | 0% | U.S., ethnicity not specified | - |
| Hoogendoorn (this study) | 0/311 | 0% | The Netherlands, Dutch | - |

1) Early onset patients with schizophrenia (N=134) with age of onset 13 -17 years[1]
2) Seven childhood onset patients with schizophrenia with age of onset 7-12 years[1]
3) In this study[2] 85 patients with schizophrenia were only subjected to FISH when they met two or more positive screening criteria for 22q11DS. This was the case for six patients; two were found to have a deletion on the 22q11 locus. However, in an independent set of studies[6, 7] the entire sample of 85 patients was genotyped using a panel of SNPs at 22q11.2. Except for the two previously identified patients, the remaining 83 patients were heterozygous for at least one marker; therefore the presence of a 22q11 deletion was excluded in these patients.
4) Usiskin et al. reported a prevalence of 6.4% of 22q11 deletions in childhood onset schizophrenia (COS) patients[8]. However, this sample was not included in our prevalence calculation all participants were already described by Sporn et al[12].

## DISCUSSION

In the present study we tested the hypothesis of a higher rate of 22q11DS in the deficit form of schizophrenia. Furthermore, a literature search was performed to re-estimate the prevalence of 22q11DS among schizophrenia patients. Results that were previously reported on this topic were combined.

Not a single 22q11 deletion was found in a sample of 311 Dutch schizophrenia patients, of whom 146 met criteria for the deficit syndrome. A possible explanation for this negative result is that, contrary to our hypothesis, schizophrenia in 22q11DS patients is not characterized by more prominent negative symptoms as compared to schizophrenia in the general population. Indeed, Bassett et al. reported no significant differences in the core symptoms of schizophrenia, including negative symptoms, between schizophrenia patients with 22q11DS and those without 22q11DS[23]. Also, Murphy et al. reported fewer negative symptoms in schizophrenia patients with 22q11DS than in patients without 22q11DS[3]. However, the sample size of the deficit schizophrenia subgroup in the present study does not allow definite conclusions regarding this issue.

In the entire studied sample of schizophrenia patients, no deletions were detected. This result differs from previous studies reporting up to 2.4% of 22q11.2 deletions in random samples of schizophrenia patients with adult onset (see table 1). Additionally, in concordance with our results, Drazinic et al. (in review) did not find any 22q11 deletions in a sample of 171 patients treated with clozapine, 158 of whom met criteria for schizophrenia or schizoaffective disorder.

The prevalence derived from our literature search is less than the 2% which is often quoted in literature. Nevertheless, given an estimated prevalence of 22q11DS in the general population of 1/4 000- 1/6 000[24-28], the prevalence of 22q11DS in schizophrenia patients is still approximately 30-45 times higher than expected. The results of our literature study also show that the prevalence of 22q11DS in schizophrenia patients with childhood onset (1.9-11.9%) is considerably higher than the prevalence in schizophrenia patients with adult onset (0.3-1%). This finding is in keeping with an onset of psychotic symptoms at an early age in subjects with 22q11DS[29].

In summary, in the current study no 22q11 deletions were detected in a sample of 311 Dutch patients with schizophrenia, in which 146 patients met criteria for the

deficit syndrome. Thus, the present study provided no evidence that this deletion is more common among deficit schizophrenia patients. Contrary to the frequently reported prevalence of 2% of 22q11DS in schizophrenia, a prevalence of 0.5%-1.2% of 22q11DS is calculated when summing op the total of schizophrenia patients that have been tested so far[2, 6, 7, 30-32].

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## CHAPTER 6

# Proline affects brain function in 22q11DS children with the low-activity COMT$^{158}$ allele

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*Submitted*

**ABSTRACT:**

**Introduction:** The association between the 22q11.2 deletion syndrome (22q11DS) and psychiatric disorders, particularly psychosis, suggests a causal relationship between 22q11DS gene(s) and abnormal brain function. The genes catechol-O-methyl-transferase and proline dehydrogenase both reside within the commonly deleted region of 22q11.2. COMT activity and proline levels may therefore be altered in 22q11DS individuals. Associations of both $COMT^{158}$ genotype and elevated serum proline levels with abnormal brain function have been reported. We hypothesized an interaction between the $COMT^{158}$ genotype and proline, predicting the strongest negative effect of high proline on brain function to occur in 22q11DS children who are carriers of the $COMT^{met}$ allele.
**Method:** Fifty-six 22q11DS children and 75 healthy controls were assessed on physiological measures of brain function, including prepulse inhibition of startle (PPI), P50 auditory sensory gating and smooth pursuit eye movements (SPEM). $COMT^{158}$ genotype and plasma proline levels were determined in the 22q11DS children.
**Results:** Of the three physiological measures, only SPEM and PPI were abnormal in the patient sample. With regard to the SPEM performance, there was a significant interaction between the $COMT^{158}$ genotype and proline level with significantly decreased SPEM performance in children with high plasma proline levels and the low-activity $COMT^{met}$ allele. A similar interaction effect was not observed with regard to PPI.
**Conclusions:** These findings are consistent with a model in which elevated proline negatively affects brain function via an increase in dopamine in the prefrontal cortex. 22q11DS patients with low dopamine catabolic capacity are therefore especially vulnerable to this functional disruption.

## INTRODUCTION

The 22q11.2 deletion syndrome (22q11DS) is a congenital multi-system disorder caused by deletion of a small region on the long arm of chromosome 22[1, 2]. Individuals with 22q11DS have specific cognitive deficits and are at increased risk for a variety of psychiatric illnesses. Among children and adolescents, attention deficit hyperactivity disorder, obsessive compulsive disorder, mood disorders and autism spectrum disorders have all been reported[3-8]. In adults, there is an increased prevalence of schizophrenia[9].

In schizophrenia patients without concurrent 22q11DS, physiological measures considered to reflect genetic vulnerability are often abnormal. Among these endophenotypes, prepulse inhibition of startle (PPI), P50 auditory sensory gating (P50) and smooth pursuit eye movements (SPEM) are among the most commonly studied[10]. PPI refers to the reduction in magnitude of the startle response when a startle-inducing stimulus is preceded by a weaker subthreshold stimulus. P50 gating refers to a similar reduction of the P50 auditory evoked potential response to the second of two auditory stimuli presented in rapid succession. SPEM refers to the ability to smoothly follow and maintain a moving target in the central foveal area of the retina without using additional saccadic movements to recapture central fixation. Of these three measures, only PPI has been examined in 22q11DS subjects. Consistent with the increased occurrence of psychotic symptoms in the 22q11DS population, the 22q11DS subjects exhibited PPI deficits similar to those seen in schizophrenia[11].

Given the co-occurrence of a cytogenetic deletion and an increased risk of psychopathology in 22q11DS, a causal relationship between gene(s) in the deleted region of chromosome 22 and abnormal brain function is likely. This paper examines the effects of two specific genes located at 22q11.2, catechol-O-methyl-transferase (COMT) and proline dehydrogenase (PRODH). COMT is an extracellular enzyme involved in the breakdown of catecholamines. A common functional polymorphism of COMT at amino acid position 158 ($COMT^{Met}$) is associated with a significant decrease in enzyme activity relative to the other allelic form ($COMT^{val}$)[12, 13].

In 22q11DS, a critical effect of this polymorphism can be expected since only one copy of the COMT gene is present. Several studies have now reported significant associations between cognition and the COMT$^{158}$ genotype in 22q11DS[14-18], although not consistently[19]. PRODH catalyzes the first step in the degradation of the amino acid proline. Increased plasma levels of proline have been reported in 22q11DS patients[20], caused by haplo-insufficiency and/or functional mutations within the remaining PRODH allele or its promoters[21]. Evidence supporting proline's role in brain function include its modulation of glutamatergic neurotransmission in the murine hippocampus *in vitro*[22, 23] and the presence of high affinity proline transporter molecules in a subset of glutamatergic neurons in the rat brain[24]. Direct evidence that PRODH dysfunction increases proline and leads to altered brain function was provided by a study on PRODH knockout mice, who displayed both elevated levels of proline and reduced PPI[25].

There is evidence to support a functional association between COMT activity and proline. Interference with glutamatergic neurotransmission through antagonism of the NMDA receptor induces dopamine (DA) release in the prefrontal cortex (PFC)[26]. Similarly, the putative modulatory effect of proline on glutamatergic transmission could induce DA release in the PFC. The efficiency with which this increased DA can be catabolyzed is dependent on COMT activity, which in turn can be predicted by the COMT$^{158}$ genotype. Evidence supporting this mechanism comes from a recent study in which brain function was found to be most profoundly disrupted in mice having both increased levels of proline and decreased COMT activity[27]. Given this, we hypothesized a negative effect of elevated proline levels on brain function in children with 22q11DS, with the strongest effect to be expected in the subgroup of children with the low-activity COMT$^{met}$ allele. To test this hypothesis, we measured PPI, P50 and SPEM performance in children with 22q11DS and in typically developing children. 22q11DS subjects were genotyped for the COMT$^{158}$ allele and assessed for plasma proline levels.

## METHOD

*Recruitment*

Children with 22q11DS (n=56) were recruited following presentation at the research child psychiatry clinic at the University Medical Centre Utrecht, Netherlands. Control subjects (n=75) were recruited from high schools in and around Utrecht. Inclusion criteria included age between 12 and 18 years, no known history of closed head injury, neurological illness or endocrine dysfunction, no drug or alcohol abuse over the preceding twelve months, and no use of psychoactive medications. Absence of psychiatric illness was assessed using the Mini International Neuropsychiatric Interview[28, 29]. This study was approved by Dutch Central Committee on Research Involving Human Subjects (C.C.M.O.) and written informed consent was obtained from participants and their parents or guardians.

*Cognitive assessment*

A detailed account of the assessment protocol used for the 22q11DS sample has been published previously[8]. Intelligence level (FSIQ) was assessed using the Dutch version of the Wechsler Intelligence Scales: WISC-III[30]. In three cases the WISC-R and in three cases the adult scale (WAIS-III) was used instead. In one case intelligence assessment was not performed but IQ data from a Wechsler assessment for preschool children, assessed at an earlier age, (WPSSI-R) were available. In the control group, intelligence level was assessed using the WISC-III in children younger than 16 years and the WAIS-III in children 16 years of age or older.

*Proline measurement*

Plasma proline levels were assessed by automated ion-exchange chromatography with post column ninhydrin derivatization. Plasma amino acid analyses were performed on a JEOL AminoTac (JEOL AminoTac JLC-500/V, Tokyo, Japan) following AM blood draw. Blood draw followed a confirmed overnight fast in 25 children; in 27 children overnight fasting status was uncertain. One outlier (proline of 580 μM) was identified in the confirmed fasting group, but not removed since abnormally high proline levels can be seen in 22q11DS. Consistent with the fact that proline is not an essential amino acid, mean proline levels did not differ

between the uncertain fasting (278±70μM) and confirmed fasting (280±110μM) groups (p=0.94). Exclusion of the outlier did not alter these results.

*$COMT^{158}$ genotyping*

COMT genotyping was carried out using allele-specific TaqMan probes. Twenty nanograms of genomic DNA were mixed with the assays and TaqMan® mastermix (Applied Biosystems, Foster City, CA) in a final volume of 5μl. Four replicates were used for each sample. Samples were treated with the following profile: 95°C for 10 minutes pre-treatment to activate the Taq Gold and then 40 cycles of 95°C for 15 seconds and 60°C for one minute. Data were collected during amplification using the Sequence Detection System software (version 2.2) and a post-read run performed for allelic discrimination.

*P50 data acquisition and processing*

EEG activity was recorded from 12 tin electrodes (Electro-Cap International, Eaton, OH) located at 10-20 system scalp sites (American Electroencephalographic Society, 1991) referenced to left mastoid, using Psylab hardware and software (Contact Precision Instruments, London, UK). Horizontal EOG was recorded from electrodes adjacent to the outer canthus of each eye. Vertical EOG was measured from left infra- and supra-orbital electrodes. Impedances were below 5kOhms. Bandpass filter settings were 0.01-100 Hz. Digital sampling rate was 500 Hz. Auditory clicks, consisting of 86 dB 1.5 ms duration white noise, were presented binaurally through stereo insert earphones (Eartone ABR). Software settings were calibrated by an artificial ear (Brüel and Kjær, type 4152) to ensure that stimulus intensities at the subjects' ears were as intended. Before the actual experiment, 2 click pairs were presented to the subjects, who were instructed to count the click pairs. A block of 36 click pairs, with an inter-stimulus interval of 500 ms and an inter-trial interval of 10 sec, was then presented, after which the subjects were asked how many click pairs they had counted.

Continuous EEG data were digitally filtered with a 10-100 Hz zero phase-shift Butterworth filter (24 dB/octave). An automated artifact rejection algorithm excluded EEG intervals with voltages beyond the range of +/- 75μV. The data were then segmented into artifact-free intervals beginning 50 ms prior to each click and ending 125 ms post-stimulus. Segments were averaged to produce separate ERP

waveforms for the two paired clicks. P50 amplitude was measured as the maximum voltage response at the vertex (Cz), between 50 and 80 ms post-stimulus. The P50 ratio was computed as the amplitude of the response to the second test stimulus divided by the amplitude of the response to the initial conditioning stimulus.

*PPI data acquisition and processing*

The electromyographic activity (EMG) of the right orbicularis oculi muscle was recorded from bipolar electrodes, one placed over the medial aspect of the muscle and one displaced 0.5cm laterally. The EMG was recorded with analog filter settings of 30-200 Hz. Digital sampling rate was 500 Hz.

The prepulse and startle stimuli were bursts of white noise (duration 22.5 and 32.5 ms, intensity 87 dB and 106.5 dB, respectively, rise/fall 0.1 ms), with a fixed inter-stimulus interval of 120 ms. The stimuli were presented binaurally through stereo insert earphones (Eartone ABR) without any background noise. Prior to testing, 4 startle stimuli of rising intensity were presented, with two preceded by a prepulse, to accustom the subjects to the loud noise. The actual experiment consisted of 24 randomized trials: 12 startle stimuli preceded by a prepulse and 12 startle stimuli with no prepulse. The inter-trial interval varied between 12 and 23 sec.

The continuously recorded EMG activity was bandpass filtered between 1 and 1000 Hz (24db/octave slope), using a zero phase-shift Butterworth filter. Data were segmented into intervals beginning 50 ms prior to each startle stimulus and ending 250 post-stimulus. Voltage polarity was rectified to yield all positive amplitudes. Individual startle segments were visually inspected for excessive electrical noise and/or voluntary eye-blinks occurring during stimulus presentation. Acceptable segments were averaged separately for the "startle alone" and "startle with prepulse" conditions. Startle magnitude was defined as the most positive peak occurring 20-100 ms after stimulus presentation. Prepulse inhibition of startle (PPI) was computed as 1 minus the ratio of the "startle with prepulse" divided by the "startle alone" response (expressed as a percentage). Individual PPI results exceeding 100% were relabeled as “100%”, negative PPI results were relabeled as “0%”.

*SPEM data acquisition and processing*

Stimuli were displayed on a 21-inch computer screen (42 by 32 cm) positioned 1 meter in front of the subject. Display resolution was 800 by 600 pixels. Eye movements were recorded using electro-oculography (EOG) with Psylab hardware and software (Contact Precision Instruments, London, UK). Tin electrodes were placed above and below the left eye and adjacent to the outer canthi of both eyes, with a ground electrode at aFz. EOG recordings were filtered online with a bandpass of 0.1 - 100 Hz and digitally sampled at 250 Hz.

The visual target was a white dot of 1 pixel on the 21" monitor, which was clearly visible against a black background. This dot moved horizontally in a harmonic (sinusoidal) motion described by $X(t)=A \sin(2\pi ft)$ (A=300 pixels). The subject was seated at 100 cm from the monitor, so that the amplitude A of 300 pixels (spanning nearly 18cm) was 10 degrees of visual angle. The eyes moved from full left to full right position over a total arc of 20 degrees. Seven trials were presented, each consisting of 10 seconds of sinusoidal motion. Each trial began with an additional 2 seconds, during which the dot began to move slowly in the middle of the screen and speeded up to the desired speed for the trial. The average trial speeds were respectively: 8, 13, 16, 20, 24, 29 and 35 degrees per second. For training purposes, subjects were shown two trials with the slowest velocities and asked to follow the dot carefully. After the experimenter was convinced that the task requirements were well understood, the actual experiment was started.

There was a large amount of data drop-out in the 22q11DS sample for the 3 fastest target frequencies (28.7 % of data). Many 22q11DS children appeared to have trouble following the target at these higher speed levels. In contrast, missing data for the 3 fastest frequencies in the control sample was only 4.5 %. At the 4 slower target frequencies (8, 13, 16 and 20 degrees/second) only 5.6 % of 22q11DS data were missing. Therefore, only the data from these 4 frequencies were included in the analysis. The proportion of smooth pursuit movements relative to the occurrence of saccadic movements, termed "velocity gain", was computed for each target frequency and then averaged across the 4 frequencies (see Supplementary Figure 1).

*Statistical analysis*

All statistical analyses were conducted with SPSS version 11.5 statistical analysis software. The three dependent measures were the auditory P50 gating ratio, the percent prepulse inhibition of startle, and the mean SPEM velocity gain. In order to obtain optimal matching for age and gender distribution with the 22q11DS group, matched subgroups were composed out of the original n=75 controls. Normal distributions of data in the different subgroups were assessed with Kolmogorov-Smirnov tests (significance level 0.05). For each dependent measure, outliers were detected and removed using the boxplot function; (see online Supplementary Figure 2). This resulted in the exclusion of 2 subjects for P50 (1 case, 1 control) and 2 subjects for SPEM (both controls). For 6 cases and 1 control the PPI results were outside the 0 – 100% range, and therefore relabeled to either 0% or 100%.

Comparison of demographic characteristics between the 22q11DS subjects and controls was performed using Student's t-test for FSIQ and age, and the $\chi^2$ test for gender distribution. A two-tailed significance threshold of $p<0.05$ was employed.

Plasma proline levels in the 22q11DS sample were compared to published normative values using a one sample t-test. For the analyses of the effect of proline on the outcome measures, the 22q11DS sample was divided in two subgroups: "high plasma proline" (levels above the group median value of 262 μM) and "low plasma proline" (below 262 μM).

Analysis of the 3 physiological measures proceeded in three stages. Stage 1 examined differences between 22q11DS subjects and controls using analysis of covariance (ANCOVA), while covarying for FSIQ.

Stage 2 examined the effects of proline and the $COMT^{158}$ allele, independently, on those physiological measures that were significantly altered in the 22q11DS group.

Stage 3 addressed the central hypothesis of this study, that the effect of proline would be evident principally in the $COMT^{met}$ subgroup, by examining $COMT^{158}$ genotype * plasma proline level interaction on the outcome measure, while covarying for age, FSIQ and gender using ANCOVA. Prior to these tests, normality of the data distribution in all subgroups was ascertained, and mean age and gender distributions of the $COMT^{158}$ allele and high/low proline subgroups were compared.

Given the previously reported effect of plasma proline levels and the $COMT^{158}$ genotype on FSIQ[14, 17, 31], we examined these associations post-hoc in the current sample utilizing the Student's T-test and ANCOVA.

## RESULTS

*Demographic characteristics*

Table 1 displays the sample characteristics of the 22q11DS and typically developing children with available data for each dependent measure. Between the 22q11DS group and controls mean FSIQ was significantly different in all sub samples (P50, PPI and SPEM), while mean age and gender distribution were not significantly different.

In the 22q11DS sample (proline data available from n=52 subjects), mean plasma proline levels were significantly higher than normal population values ($p<0.01$, see online Supplementary Figure 3).

**Table 1: demographics of the 22q11DS and control samples**

| | **22q11DS** | | | | **Normal controls** | | | | **Statistics[1]** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | n[2] | Mean Age ± SD | Mean FSIQ ± SD | M:F | n[2] | Mean Age±SD | Mean FSIQ ± SD | M:F | Age[3] | IQ[3] | M:F |
| P50 | 49 | 13 yr, 8 m ±2 yr, 7 m | 66±14 | 0.58 | 59 | 14 yr,1 m ±2 yr, 5 m | 108±15 | 0.74 | p= 0.40 | p< 0.01 | p= 0.55 |
| PPI | 40 | 14 yr, 1 m ±2 yr, 6 m | 66±13 | 0.54 | 33 | 14 yr, 6 m ±1 yr, 1 m | 106±13 | 0.57 | p= 0.39 | p< 0.01 | p= 0.90 |
| SPEM | 56 | 13 yr, 7 m ±2 yr, 6 m | 65±14 | 0.56 | 54 | 13 yr, 10m ±2 yr, 11m | 111±15 | 0.74 | p= 0.68 | p< 0.01 | p= 0.46 |

SD = Standard Deviation, yr = year, m = month, M:F = male to female ratio.

1 Comparison of means cases versus controls. For age and FSIQ: Student's T-test, for M:F ratio: X-square test.

2 Sample size after removal of outliers

3 Age and IQ normally distributed in all subgroups of 22q11DS and controls ($p>0.05$,

*Psychophysiological measures*

Data from P50 gating, PPI of startle and SPEM were normally distributed in all subgroups. SPEM and PPI were significantly different (decreased) in 22q11DS compared to controls (see Table 2). Inclusion of outliers did not alter these findings. The results of SPEM were not essentially changed when using velocity gain data from all target speed velocities.

**Table 2: Comparison of psychophysiology results between 22q11DS and controls**

| | **P50[1]** | | **PPI** | | **SPEM[1,2]** | |
|---|---|---|---|---|---|---|
| Mean performance±Standard Deviation: | | | | | | |
| 22q11DS | 0.266±0.236 | | 46,5±30.8 | | 0.326±0.139 | |
| Controls | 0.367±0.246 | | 78.9±13.5 | | 0.481±0.117 | |
| Univariate Analysis of Co-Variance: | | | | | | |
| | F-statistic | P | F-statistic | P | F-statistic | P |
| 22q11DS vs controls | 0.635 | 0.428 | 8.785 | 0.004 | 7.259 | 0.008 |
| FSIQ | 3.458 | 0.067 | 0.002 | 0.968 | 0.595 | 0.442 |

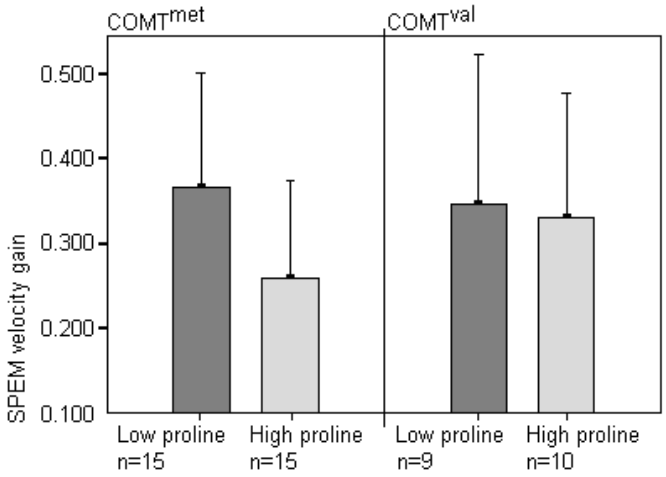
Mean performance of brain function measures (respectively P50, SPEM and PPI) in cases and controls, and results of the Univariate Analysis of Co-Variance with the brain function measure as dependent variable and FSIQ as covariate.

1 Results of SPEM and P50 were not essentially changed when including outliers.

2 Results of SPEM were not essentially changed when using data from all target speed velocities.

**Effects of proline level and COMT$^{158}$ genotype on identified brain measures**
Based upon these findings we proceeded to analyze the effects of proline and COMT$^{158}$ genotype on SPEM and PPI, the measures that differentiated the 22q11DS group from controls. For both SPEM and PPI, there was no main effect of COMT158 allele status or proline level on any of the demographic variables (age, FSIQ, gender).

**Figure 1: The association between proline and SPEM is moderated by the COMT$^{158}$ genotype**




Only in the COMT$^{met}$ subgroup a decreased SPEM performance was associated with high plasma proline levels in 22q11DS individuals ($p = 0.028$), whereas in the COMT$^{val}$ group no significant difference was revealed between the high/low proline subgroups ($p = 0.827$. Proline levels were not available in 7 subjects; therefore the sample size in this analysis is 49.

With regard to PPI performance, there was a trend towards a significant difference between the $COMT^{met}$ (mean PPI±SD: 40.1±32.5) and the $COMT^{val}$ subgroup (59.4±26.4, p = 0.065), while no difference was found between the high/low proline subgroups (p = 0.567). With regard to the mean SPEM performance there was no difference between the $COMT^{158}$ subgroups (p = 0.421), but a difference in SPEM performance was found between those with high proline (0.284±0.130) and those with low proline (0.361±0.164, p = 0.050). However, this finding would not remain significant after applying Bonferroni correction for the number of hypotheses tested in the current study.

Analysis revealed a significant effect of $COMT^{158}$ allele status * proline level on SPEM, while covarying for age, FSIQ and gender (F = 13.825, p = 0.003, see table 3). This effect remained significant after applying Bonferroni correction (p = 0.036). A high proline level was associated with a significantly decreased SPEM performance only in the $COMT^{met}$ group (p = 0.028) but not in the $COMT^{val}$ group (p = 0.827, see figure 1).
With regard to PPI, analyses revealed no significant interactive effect of $COMT^{158}$ allele status * proline level; in both $COMT^{158}$ allele subgroups the mean PPI performance was not significantly different between those with high proline levels and those with low proline levels ($COMT^{met}$: p = 0.748, $COMT^{val}$: p = 0.296).

**Table 3: Effect $COMT^{158}$ * proline on SPEM**

| Variable | F | Sig. |
|---|---|---|
| $COMT^{158}$ allele status | 0.040 | 0.874 |
| Proline high/low subgroup | 6.445 | 0.492 |
| FSIQ | 0.000 | 0.996 |
| Age | 0.092 | 0.764 |
| Gender | 0.649 | 0.524 |
| $COMT^{158}$ * proline | 13.825 | 0.003 |

Univariate Analysis of Co-Variance with SPEM as the dependent measure, $COMT^{158}$ allele status and high/low proline groups as fixed factors and FSIQ, age and gender as covariates.

*Post hoc Analyses*
Mean FSIQ was not significantly different between carriers of the $COMT^{met}$ (64±14) and the $COMT^{val}$ allele (66±14, p = 0.575). Similarly, mean FSIQ was equal in those with low proline (64±13) and those with high proline levels (65±14, p = 0.817). Further analyses did not reveal an interactive effect of $COMT^{158}$ allele status * proline level on FSIQ.

## DISCUSSION

In this study we report decreased SPEM velocity gain and PPI of startle in children with 22q11DS compared to typically developing children of the same age. A decreased SPEM in 22q11DS is a novel finding since to date no studies have reported on the SPEM performance in 22q11DS children. This study replicates the finding of a decreased PPI in 22q11DS subjects, as reported by Sobin et al[11].
We further demonstrate a significant interactive effect of $COMT^{158}$ allele status * proline level on SPEM; proline negatively affects SPEM in 22q11DS subjects who are hemizygous for the $COMT^{met}$ allele. With regard to PPI such interactive effect of proline and $COMT^{158}$ was not found; however a trend towards a main effect of the $COMT^{158}$ genotype was demonstrated; individuals with the $COMT^{met}$ allele showed decreased PPI performance.

Consistent with both the central hypothesis of this study and previous findings in mice[27], we found that the effect of proline on SPEM was contingent upon the $COMT^{158}$ genotype. These findings are also partly consistent with results recently reported by Raux et al.[31], although the main outcome measure in that study was psychiatric diagnosis rather than SPEM. Findings of both studies indicate a negative effect of high proline on brain function in 22q11DS subjects with the $COMT^{met}$ genotype. The consistency of these results is not surprising given the reported association between SPEM abnormalities and psychosis in numerous studies[10]. We did not analyze psychosis as a phenotypic outcome since the young age of the study sample excluded a reliable partition of subjects in this respect. Raux et al. also reported that increased proline levels were significantly associated

with lower FSIQ, independent of the COMT[158] genotype. Our post-hoc analyses did not replicate this correlation.

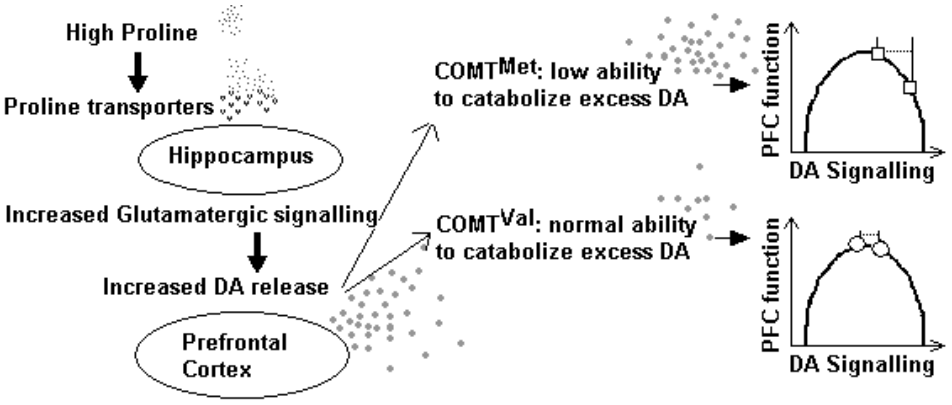
While P50 and PPI are both thought to reflect the brain's capacity to filter information, they tend to be uncorrelated within individuals and, therefore, are likely to be mediated by different neurobiological mechanisms[32, 33]. For PPI, a significant regulatory influence of the striatum is suggested by the findings of impaired PPI in Huntington's Disease patients[34] and in animals with striatal lesions[35]. In contrast, several studies indicate a more critical (though not exclusive role for the prefrontal cortex[36, 37] and the hippocampus[36, 38, 39] in the regulation of P50 gating and SPEM. While the anatomical loci of control for P50 gating and SPEM may overlap, the regulatory neurotransmitter systems are thought to be different. For P50, there are strong indications that cholinergic transmission is an essential part of its regulation[40, 41]. In SPEM, a regulatory role for DA is strongly suggested by the fact that the COMT[158] allele affects SPEM performance in both healthy subjects and schizophrenia patients[42]. Similarly, DA signaling is likely involved in the regulation of PPI, since the administration of DA agonists attenuates PPI[43, 44] and a common functional variant of the Dopamine D3 receptor significantly affects PPI in humans[45].

Given the putative roles of the hippocampus and PFC in regulating SPEM, it is notable that evidence now supports an influence of proline on excitatory neurotransmission in these regions. A high affinity proline transporter has been identified on a subset of glutamatergic neurons[24, 46, 47] and proline-mediated modulation of glutamatergic neuron terminals has been demonstrated[22, 23, 48]. Regions with the highest levels of proline transporter expression include hippocampal (Schaffer collateral commissural and lateral perforant pathway) and cortico-striatal pathways[47]. Importantly, in a PRODH knock-down study, it has been shown that PRODH deficiency not only alters hippocampal glutamatergic transmission, but also significantly potentiates DA release[27].

In summary, the hypothesized model (see figure 2) holds that the action of proline on specific glutamatergic neurons in the hippocampus induces two events: interference with glutamate transmission and secondary potentiation of the DA

response in the PFC. Under this model, one can anticipate that the effect of proline will be accentuated in individuals with the low-activity COMT enzyme, given their decreased capacity to effectively catabolize the augmented DA response in the forebrain. Our finding that proline significantly affects SPEM in the low activity $COMT^{met}$ allele, but not in the high-activity $COMT^{val}$ allele subgroup is both consistent with and supportive of this model.

**Figure 2: Schematic representation of the hypothesized model**




High proline levels induce glutamatergic signaling in the hippocampus. Increased glutamatergic tone causes a release of DA in the PFC. In 22q11DS subjects hemizygous for $COMT^{met}$ (squares in upper panel, right), the inefficiency in catabolizing DA leads to a large shift to the right (dotted line). This, in combination with a starting position somewhat right of the curve's optimum, leads to a decrease in PFC function. In those hemizygous for $COMT^{val}$ (circles in lower panel, right), excess DA can be more adequately catabolized; the resulting shift, if any, on the inverted U-shaped curve is more moderate and does not result in a substantial change in PFC function (inverted U-shape curve adapted from Mattay et al. Proc. Natl. Acad. Sci. U. S. A 100, 6186-6191 (2003))

The effect reported in this study accords with the inverted U-shape relationship between DA signaling and prefrontal function[49]; in this model, optimum prefrontal function occurs within a restricted range of DA signaling, with decreased function in conditions of too high or too low DA availability. Regarding the current study, we would hypothesize that 22q11DS individuals who are hemizygous for the $COMT^{val}$ allele have relatively less DA signaling and are therefore located somewhat to the left of the curve's optimum, such that a moderate increase in DA availability would not have a deleterious effect on prefrontal function. In contrast, 22q11DS carriers of the $COMT^{met}$ allele have relatively more baseline DA availability. This shifts their baseline location towards the downward sloping part of the curve's optimum. A further shift to the right is correlated with a reduction of prefrontal function, which occurs when high proline induces excess DA that cannot be efficiently catabolyzed.

Finally, the attenuating effect of the $COMT^{met}$ allele, but not of proline, on PPI in this study is likely the result of haplo-insufficiency, with the low-activity COMT allele increasing DA availability in the striatum. Apparently, this effect occurs despite COMT expression being lower in the striatum than the PFC[50]. The absence of any influence of proline on PPI is consistent with the fact that proline appears to potentiate DA transmission in the murine cortex, but not in the striatum[27]. Findings of this study may contribute to our understanding of the pathophysiological mechanisms that lead to the increased vulnerability for psychosis in 22q11DS subjects. This finding not only is relevant to our understanding of 22q11DS-related psychopathology, but also contributes to our understanding of how factors such as proline influence DA metabolism and transmission in the brain.

**Acknowledgements**

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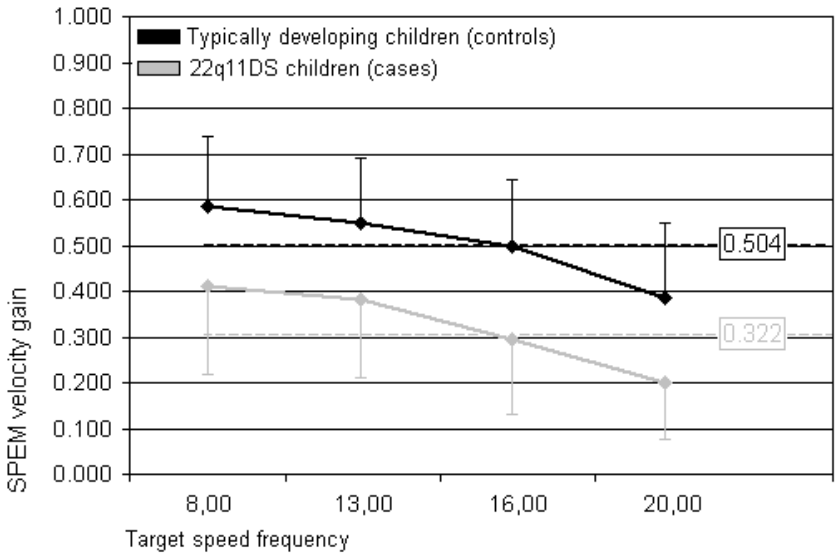
**ONLINE SUPPLEMENTARY MATERIAL**

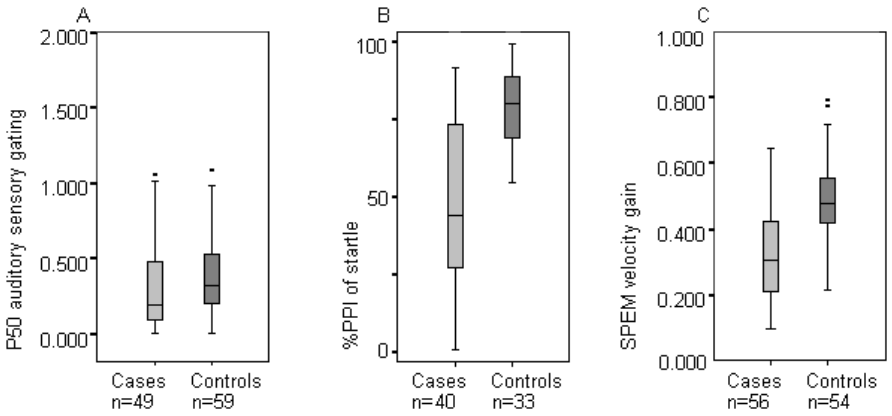
**S_Figure 1: SPEM velocity gain; 22q11DS subjects vs. controls**




Velocity gain of the first 4 target speed frequencies in normal controls (black) and subjects with 22q11DS (gray). For the purpose of testing the hypothesis of this study the averages of the velocity gain (dotted lines) of the first 4 target speed frequencies were calculated for each individual. In this figure group averages are shown (0.504 for controls and 0.322 for the 22q11DS children).

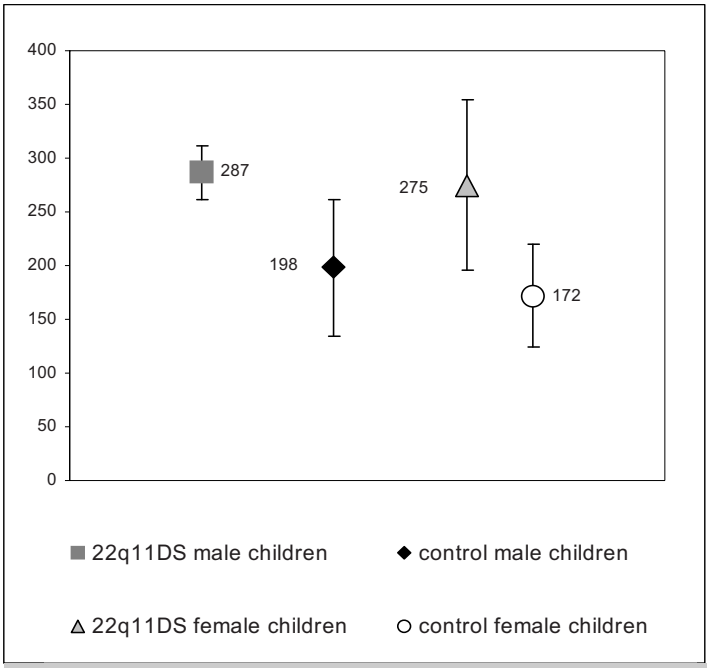
**ONLINE SUPPLEMENTARY MATERIAL**

**S_Figure 2: Data outliers**



**ONLINE SUPPLEMENTARY MATERIAL**

**S_ figure 3: Plasma proline levels in 22q11DS children and normal control children.**




In 52 children with 22q11DS plasma proline levels were assessed. We did not collect data on plasma proline levels in typically developing children. However, proline levels in normal adult subjects (n=62) were available. This adult sample was assessed in the same laboratory facility as where the 22q11DS subjects in the present study were assessed. Average proline values of normal adults tested in our laboratory were equal to the values for adults ($p = 0.99$, t-test), reported by Armstrong et al. (Metabolism, 1973 Apr;22(4):561-9). Thus, we felt sufficiently confident to compare proline levels in our 22q11DS group to proline data on typically developing children reported in the same study by Armstrong et al. (76 boys aged 6 to 18 years and 60 girls aged 6 to 18 years). Plasma proline levels were significantly higher in the 22q11DS sample ($p<0.01$, t-test) in comparison to normal control children.

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## CHAPTER 7

# Association of the PIK4CA schizophrenia-susceptibility gene in adults with the 22q11.2 deletion syndrome

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**ABSTRACT**

**Introduction:** The 22q11.2 deletion syndrome (22q11DS) is associated with an increased prevalence (20-30%) of schizophrenia. Therefore, it is likely that one or more genes within the 22q11.2 region are causally related to schizophrenia. Recently, a significant association with schizophrenia in the general population was reported for three SNPs in Phosphatidyl-inositol-4-kinase-catalytic-α (PIK4CA), a gene located in the 22q11.2 region. In the current study, we tested the hypothesis that the same PIK4CA risk-alleles would be associated with schizophrenia in individuals with 22q11DS.
**Methods:** the distribution of the alleles of the three previously reported SNPs in PIK4CA was tested in a sample of 79 adults with typical 22q11.2 deletions, comparing those with schizophrenia to those without. In addition, sex distribution and mean IQ were compared between cases and controls.
**Results:** Our analysis of the PIK4CA genotypes in this sample of patients with 22q11DS revealed a significant association between the previously reported PIK4CA risk allele and schizophrenia.
**Conclusion:** Our findings represent an independent replication of the previously reported PIK4CA association with schizophrenia in the general population. Second, the results of this study indicate that variation at PIK4A may be a relevant factor influencing the risk of schizophrenia in individuals with 22q11DS.

## INTRODUCTION

In 22q11.2DS patients schizophrenia is far more common than in the general population (20-30% versus 1%)[1]. Conversely, 22q11.2 deletions in schizophrenia are found approximately 30 times more frequently than in the general population[1, 2]. These findings strongly suggest that the 22q11.2 region harbors one or more schizophrenia-susceptibility genes. Nevertheless, approximately 70 – 80% of 22q11.2DS patients do not develop schizophrenia. This suggests the influence of other factors, including genetic variation of the remaining alleles within the 22q11.2 deletion region.

Recently, we reported a significant association of three SNPs within the phosphatidyl-inositol-4-kinase-catalytic-α gene (PIK4CA) with schizophrenia in the general population[3]. PIK4CA is located within the distal half of the region that is typically deleted in 22q11.2DS. We hypothesized that the same PIK4CA SNPs could contribute to the risk of schizophrenia in individuals with 22q11.2DS. We examined the distribution of these PIK4CA SNPs in adults with 22q11.2DS, comparing those with schizophrenia ("cases") to those without ("controls").

**METHOD**

Adults with 22q11.2DS, followed at the Clinical Genetics Research Program, Centre for Addiction and Mental Health (CAMH) at the University of Toronto, Canada, were studied. Participants provided written informed consent, and the study was approved by the Research Ethics Boards of the University of Toronto, CAMH, and University Health Network.

Eighty-four subjects of Caucasian descent were included and assessed by experienced psychiatrists for lifetime DSM-IV psychiatric diagnoses using standard methods, as previously described[4]. In order to exclude a stratification error related to intellectual level, we used IQ results available for 77 participants[4].

Approximately 13% of 22q11.2 deletions differ from the most commonly found deletion with respect to deletion endpoints[5]. In the majority of this subgroup a smaller deletion involves a region that does not contain PIK4CA. Therefore, we used Multiplex Ligation-Dependent Probe Amplification (MLPA)[6] to verify the involvement of PIK4CA in the 22q11.2 deletions. Of the initial 84 subjects, 7 (8.3%) were found to have smaller or atypical 22q11.2 deletions, 5 of which did not include PIK4CA. The latter subjects were therefore excluded, leaving 79 subjects confirmed to be hemizygous at the PIK4CA gene for genotyping studies.
We performed genotyping of previously reported PIK4CA SNPs (rs165793, rs2072513 and rs165862) using an allele specific PCR method (KASPar assay, performed by KBiosciences, Hoddesdon, Hertfordshire, UK). In one sample, genotyping of rs2072513 did not generate reliable results.

Thirty-two patients were diagnosed with schizophrenia or schizoaffective disorder ("cases"; 15 males, 17 females, mean age 40.0 years, SD 8.7) while in 47 participants psychotic illness was excluded ("controls"; 22 males, 25 females, mean age 30.0 years, SD 9.4).
We compared allele and haplotype frequencies between cases and controls using chi-square or Fisher's Exact tests. Sex distribution and mean IQ were compared between cases and controls using chi-square and Student's t-test, respectively (see table 1).

**RESULTS**

Cases and controls did not differ with regard to sex distribution (p=0.995) or mean IQ; (cases: 69.0, SD 10.1, controls: 73.1, SD 10.1, p=0.081). The intronic SNP rs165793 was significantly associated with schizophrenia; the G-allele was found in all 32 cases and in 36/47 controls (77%), p=0.002. The G-allele of rs165862 was found in 22/32 (69%) cases versus 22/47 (47%) controls (p=0.054). The C-allele of rs2072513 was found in 15/32 (47%) cases versus 14/46 (30%) controls (p=0.140). Closer examination showed that the previously reported protective TTA haplotype (rs2072513, rs165862, rs165793)[3] was found in none of the 32 cases and in 10/46 (22%) controls (p=0.004).

**Table 1: Allele and haplotype frequencies**

| | **22q11DS schizophrenia (cases)** | **22q11DS (controls)** | **p** | **Odd's Ratio (95% Confidence Interval)** |
|---|---|---|---|---|
| Sample size (n) | n=32 | n=47 | | |
| Gender (M:F[1]) | 0.88 | 0.88 | 0.995 | |
| Intelligence (mean FSIQ±SD) | 69.0±10.1 | 73.1±10.1 | 0.081 | |
| rs165793[2] (G , A) | 32 , 0 | 36 , 11 | 0.002 | 9.47 (1.16 - 77.56) |
| rs2072513[3] (C , T) | 15 , 17 | 14 , 32 | 0.140 | 2.02 (0.79 - 5.14) |
| rs165862 (G , T) | 22 , 10 | 22 , 25 | 0.054 | 2.50 (0.98 - 6.41) |
| Haplotype[2] (TTA , other) | 0 , 32 | 10 , 36 | 0.004 | 8.61 (1.04 - 71.10) |

1 M:F is male to female ratio.
2 In both instances the Odd's ratios were calculated after artificially attributing one call (respectively rs165793-G and the TTA haplotype) to the cases
3 In one control, genotyping of rs2072513 did not generate reliable results.

Mean IQ of n=66 rs165793 G-allele carriers (71.5, SD 10.8) was not significantly different from that of n=11 A-allele carriers (70.9, SD 6.5, p=0.868).

Calculation of the Odd's Ratio (OR) for the most significantly associated risk allele (rs165793-G) is hampered by the fact that the contingency table of the allele distribution includes an empty cell. When artificially attributing one rs165793-A call in the cases group (cases n=32, rs165793-G: n=31, rs165793-A: n=1), the resulting estimation of the OR is 9.47 (95% Confidence Interval: 1.16 – 77.56). Similarly, for the TTA haplotype, after attributing one TTA call in the cases group (cases n=32, TTA: n=1, other haplotypes: n=31) the resulting OR is 8.61 (95% Confidence Interval: 1.04 – 71.10).

Genotypes of the 5 samples with 22q11.2 deletions that did not include PIK4CA were used as a quality control. Consistent with the shorter deletions identified by MLPA, heterozygosity at any of the three PIK4CA SNPs occurred exclusively in these samples.

**DISCUSSION**

In this study we report a significant association of a common variant at PIK4CA with schizophrenia in adults with 22q11.2DS. We identified the same protective haplotype (TTA) as in our previous study[3] to be significantly more prevalent in controls. The findings represent an independent replication of our previous study with similar frequencies of PIK4CA alleles in this special sample of 22q11.2DS patients as in general population samples[3]. Further, the absence of effect of the rs165793 risk allele on IQ in the current study suggests an influence on brain function that is related to risk of schizophrenia in 22q11.2DS rather than to intellectual level. PIK4CA is a catalytic enzyme in the phosphatidylinositol (PI) pathway, involved in the regulation of signal transduction, synaptic transmission and possibly of cell shape of neurons or oligodendrocytes[3]. Interestingly, PIK4CA is expressed in the gray matter, with higher expression in fetal than adult brain [7], consistent with a neurodevelopmental pathogenesis of schizophrenia[8].

In conclusion, 22q11.2DS patients who carry the rs165793-G-allele in their single copy of the PIK4CA gene are at increased risk of developing schizophrenia. Findings of the current study suggest that variation in PIK4CA may affect the likelihood of expression of schizophrenia in 22q11.2DS and support the potential power of this population to examine risk factors for associated phenotypes[1]. The results provide additional evidence that variation at PIK4A may be involved as one of the multiple genetic factors that contribute to the risk of schizophrenia.

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## CHAPTER 8

# Identification of DIAPH3 as a new autism susceptibility gene suggests role of inherited copy number variations in disease susceptibility through double-hit mechanism

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**ABSTRACT**

**Introduction:** Recent studies have demonstrated that more than 10% of autism cases are caused by *de novo* structural genomic rearrangements, including cytogenetic abnormalities and copy number variants (CNVs). Given that some heritable CNVs have been observed in patients as well as healthy controls, to date little attention has been paid toward the potential role of these non-*de novo* deletions or duplications in the causation of autism.
**Method:** A normally intelligent patient with autism spectrum disorder (ASD) with non-affected parents was identified with a maternally inherited 10Mb deletion at chromosome 13q21.2.
**Results:** Sequence analysis of the genes within the deletion resulted in the identification of a paternally inherited non-synonymous amino acid substitution at position 614 of DIAPH3 (proline to threonine; Pro614Thr). This variant, present in a highly conserved domain, was not observed in 328 healthy control subjects. Our results further demonstrated transient expression of *Diaph3* in the developing murine cerebral cortex. DIAPH3 is involved in cell migration, axon guidance and neuritogenesis, and is suggested to function downstream of SHANK3, another recently reported autism susceptibility gene.
**Conclusion:** Our findings strongly suggest DIAPH3 as a novel autism susceptibility gene. Moreover, this case report illustrates that not only *de novo* genomic variants in patients should be taken seriously for further study but that inherited CNVs may also provide valuable information. We suggest that targeted sequencing of genes in suspected haploid regions is an important tool for the identification of autism susceptibility genes in these highly informative “double hit” cases such as presented in the current report.

## INTRODUCTION

Autism spectrum disorder (ASD) is a group of developmental disorders characterized by impairments in social interaction, communication and the presence of restrictive or repetitive patterns of behavior. The autistic spectrum (MIM 209850) ranges from severe cases (the core syndrome called autism or autistic disorder) to milder forms which include pervasive developmental disorder, not otherwise specified (PDD-NOS) and Asperger syndrome. Despite the high heritability estimate of autism at 90%[1], the identification of causative common genetic variants has proven to be challenging. Until recently, disease-causing mutations for autism have been reported in a limited number of genes, which together may account for only a small percentage (~5-10%) of autism cases[2, 3]. Recently, there is a growing interest in the role of structural genomic rearrangements including cytogenetic abnormalities and copy number variants (CNVs) associated with autism[4-6]. Indeed, findings of these and other recent studies[7, 8] suggest that an even larger proportion of autism cases may be caused by genomic rearrangements[2].

When a chromosomal or genomic abnormality coincides with a disease phenotype in some patients, but unaffected carriers are also identified, a causal relation between the genetic defect and the phenotype is often considered unlikely. However, such inconsistent concurrence of a structural genomic abnormality and a disease phenotype is frequently encountered in neuropsychiatric disorders. For certain genetic defects a causal relationship with psychiatric disease is widely accepted, even though the concurrence of the defect and the phenotype is variable. For example, several studies have reported a high rate of schizophrenia or psychotic symptoms in subjects with the 22q11.2 deletion syndrome (22q11.2DS)[9-11]. Although it is highly probable that the deletion at 22q11.2 is causally related to psychosis, the majority of subjects with 22q11.2DS do not have psychotic symptoms. Similarly, a causal genotype-phenotype association in individuals with autism spectrum disorders and with genomic duplications at chromosome 15q11-15q13 is generally accepted, even though the co-segregation is far from complete[12].

Hence, such scenarios necessitate further research into other factors that could explain the variable expression of the phenotype. For instance, "modifier" genes located elsewhere in the genome are likely to influence the phenotype related to the genomic variation. Alternatively, it is possible that a structural genomic alteration, such as a deletion, unmasks a mutation in an otherwise recessive gene on the non-involved homolog.

Here, we present a male proband with an autistic disorder with a maternally inherited deletion at the long arm of chromosome 13. Closer examination of the paternally inherited homolog revealed a non-synonymous point mutation in DIAPH3, one of the genes located within the deleted region at 13q. These findings strongly suggest that the concurrence of a deletion and a point mutation in the remaining allele has led to the homozygous inactivation of DIAPH3 in the proband. Further studies demonstrated that *Diaph3* is transiently expressed in the developing murine cerebral cortex. Our results suggest that DIAPH3 may be a novel susceptibility gene for autism.

## METHOD

The proband was born from Caucasian parents, the third child in a family of 4 children (see figure 1).His eldest brother deceased at the age of 13 years; his family currently consisted of one elder brother (+3 years) and one younger sister (-8 years). At the age of twelve, the proband was referred to the child psychiatry outpatient clinic at the University Medical Center Utrecht, the Netherlands.

The assessment protocol including the psychiatric, cognitive and genetic studies was approved by the Dutch Central Committee on Research Involving Human Subjects. Additional genetic studies involving DNA from the proband and his parents and siblings was carried out at the Division of Human Genetics at the Children's Hospital in Philadelphia, U.S.A. with the approval of the Institutional Review Board and the Committee for Protection of Human Subjects. Written informed consent was obtained from all participants in this study.

*Medical, Psychiatric and cognitive assessments*

Phenotyping was performed at the Child and Adolescent Psychiatry Department of the University Medical Centre Utrecht. Psychiatric assessment included structured and semi-structured interviews of the parents and proband, including a DSM-IV semi-structured interview, the sections mood disorder and psychosis of the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL)[13], the Autism Diagnostic Observation Schedule-Generic (ADOS-G)[14], and the Autism Diagnostic Interview-Revised (ADI-R)[15]. In addition, in the proband intelligence level was assessed using the Dutch version of the Wechsler Intelligence Scales: WISC-III[16].

Parents, brother and sister of the proband were screened for psychiatric disorders with a DSM-IV semi-structured interview while the possibility of ASD was examined using the ADOS and ADI-R (siblings), the ADI-R (the deceased brother; parental interview items were scored relating to the presence of behaviors “ever”), and the DSM-IV semi-structured interview (parents).

All psychiatric examinations were carried out by an independent clinician without prior knowledge of the genetic status of the subjects. Regarding the deceased sibling, all available medical information was obtained from the hospital in which he had received care during his life.

*Genetic studies of the subjects*

In the proband and his mother, fluorescent In Situ Hybridization (FISH) studies were carried out with peripheral blood samples, using a series of bacterial artificial chromosomes (BACs) along the predicted breakpoints based on the karyotype results. The BACs were obtained from the clone library at the Children's Hospital Oakland Research Institute. In the proband, the results from the FISH studies were confirmed by Comparative Genomic Hybridization (CGH; Illumina 550K Bead Studio). In all family members the absence or presence of the deletion was confirmed by genotyping 17 polymorphic SNPs of which 8 were located within and 9 outside the deleted region.

Subsequently the involved genomic region was searched for gene content using the University of California Santa Cruz (UCSC) human genome browser, April 2004 assembly. This way, four genes were identified with a total of 48 exons.
All exons were amplified using conventional PCR techniques and adequate primer pairs. Sequencing was performed with BigDye terminator cycle sequencing kit v3.1 and the ABI 3730 DNA Analyzer, per the manufacturer's protocol (Applied Biosystems Group). Sequences were compared to the consensus human genome sequence (UCSC genome browser).

*Mutation screening in controls*

After identification of the non-synonymous mutation in DIAPH3 we screened 128 DNA control samples of mixed ethnic descent for the presence of this polymorphism. Since this sample was not formally screened for the presence of psychiatric symptoms and this reported mutation was observed in a family from the Netherlands, we screened an additional sample of 200 individuals with Dutch ancestry in whom a life time psychiatric diagnosis was ruled out by the Comprehensive Assessment of Symptoms and History (CASH)[17].

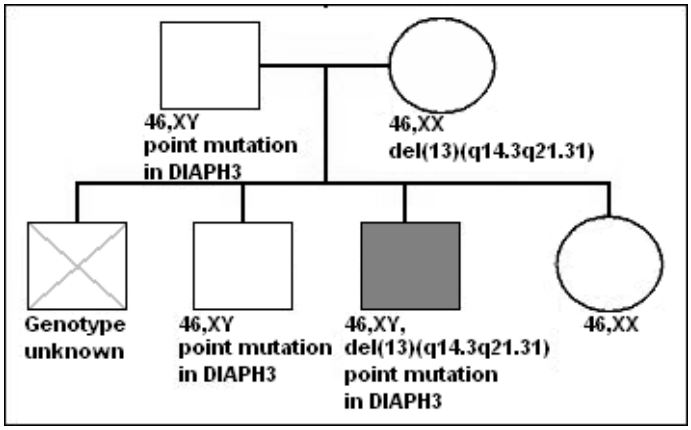
*Review of gene function and predicted effect of the identified mutation.*
Available scientific literature on DIAPH3 function was identified through Pubmed and reviewed. Conservation of the DNA sequence containing the mutation was examined using the PhastCons Conserved Elements, 28-way Multiz Alignment track in the UCSC human genome browser. In this track the prediction of conserved elements is calculated based on a whole-genome alignment of vertebrates, and for the placental mammalian subset of species in the alignment. Each predicted element is assigned a log-odds "conservation score". For more details of this method, see Siepel et al.[18].

*RNA in situ hybridization of Diaph3* during *mouse development.*
A detailed description of this method was reported previously[19]. In short, a 688 basepair fragment of the mouse homolog of DIAPH3 (*mDia2, Diaph3*, NM_019670) was amplified from 14.5 days post coitum (dpc) mouse brain total RNA using gene specific primer sequences (mDia2for: TTCTCCATGAGCTGAAAATGG; mDia2rev:TCAAGGCGATGGAAAAACTC) and the OneStep RT-PCR kit (Qiagen). The RT-PCR product was subsequently cloned into the pGEMteasy vector (Invitrogen), and DIG–labeled cRNA probes were made using T7 or Sp6 dependent RNA polymerase (Roche). Sense and antisense probes were hybridized to 16 µm sagital cryosections of various mouse embryonic stages (12.5 to 18.5 dpc) and adult mouse brain, and expression was visualized using NBT-BCIP (Roche). Images were recorded on a Zeiss Axioskop2 Plus microscope with a Sony Power HAD DXC-950P 3CCD colour video camera. Expression was considered genuine only when the sections hybridized with the sense probe showed no significant staining.

**RESULTS**

The proband was a male subject of 13 years of age. At physical examination he was found to be of small stature (length < 2.5 standard deviation (SD) below the norm average), of normal weight (0 SD) and head circumference (occipital frontal; 0 SD). He displayed mild dysmorphic features consisting of slightly low implanted protruding ears, long eye lashes, hypotelorism (outer canthal distance -2 SD), a high palate, large and irregular front teeth and a mildly triangular shaped face. He was diagnosed with hypermetropic vision at both eyes (S+4.0). In addition, a mild lumbar lordosis and a small leg length difference was found, causing a slightly askew pelvis. Standard laboratory screening including thyroid function and additional clinical studies including an echo of the heart and kidneys, an electrocardiogram (ECG), an electroencephalogram (EEG) and magnetic resonance imaging (MRI) of the brain revealed no structural abnormalities.

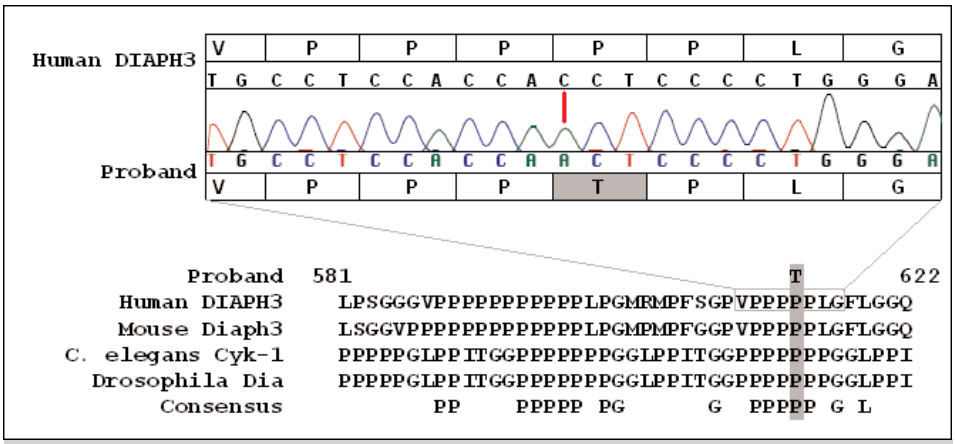
**Figure 1: Genogram.**




He fulfilled DSM-IV diagnostic criteria for autistic disorder according to clinical consensus and the results of the ADI-R and ADOS-G. He also showed problems with regard to his attention span and increased impulsivity. However these symptoms did not meet sufficient criteria of attention deficit and hyperactivity disorder. His cognitive abilities were in the normal range (Full IQ: 98, Verbal IQ: 93, Performance IQ: 105). Both parents and the two siblings did not display dysmorphic features and had a normal psychomotor development. None of the family members displayed autistic features.

The eldest brother was born with a congenital heart defect (single ventricle defect due to mitral valve atresia). Cardiac surgery was post-operatively complicated by renal insufficiency necessitating chronic treatment with diuretics and cardiotonic medications, and growth hormone later in life. At the age of 13, he died of acute heart failure, and autopsy was not performed. Available pictures of him did not suggest facial dysmorphic features. Information from the parents indicated that he had acquired psychomotor developmental milestones in time and had not displayed autistic behavior. A karyotype performed on the proband indicated a deletion of chromosome 13q.

**Figure 2: Proband and consensus DNA and amino acid sequence**




Upper panel: consensus DNA and amino acid sequence versus sequence results in the proband. Lower panel: Formin homology 2 (FH2) domain in human DIAPH3 carrying a C>A point mutation that results in substitution of a conserved Pro by Thr at position 614. This amino acid motif is conserved amongst species as diverse as drosophila, C. elegans, and mammalian species including man.

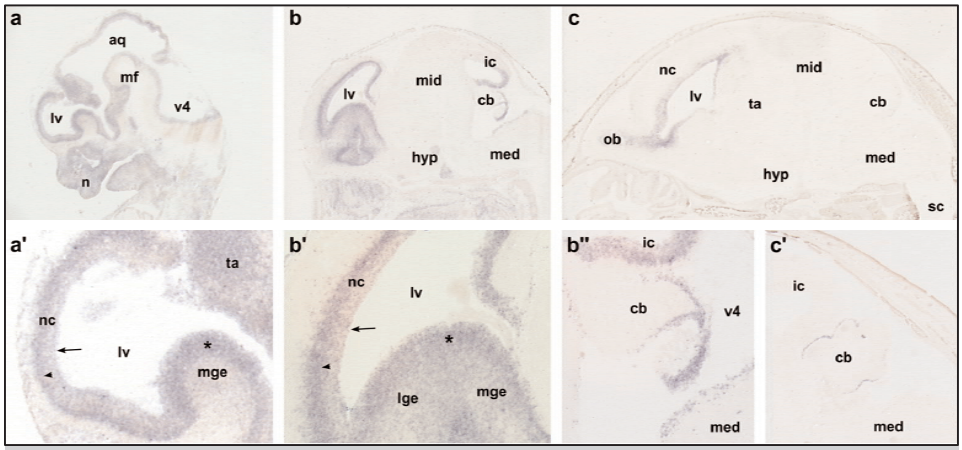
Results of the additional serial FISH experiments (RP11-209J19: 2n, RP11-77O2: n, RP11-67L17: n, RP11-520F9: 2n) narrowed the breakpoints of the deletion to 52,524,189 - 52,955,445 (proximal) and 63,071,144 - 63,447,585 (distal). These findings were confirmed and further specified by the results of the 550K Illumina SNP array, showing that the deletion spanned almost 10Mb, starting at 52,784,996 - 52,805,428 (proximal), and ending at 63,062,087 - 63,082,454 (distal). The same deletion was found in the mother. Further, heterozygosity of the 17 SNPs was consistent with the deleted genomic region in the proband and his mother, and the absence of the deletion in his father and his two siblings (data not shown). There were neither DNA for molecular analysis nor cells for karyotyping available from the deceased brother.

The deleted region contained only 4 genes: protocadherin 17 precursor (PCDH17), diaphanous homolog 3 (DIAPH3), tudor containing 3 (TDRD3) and protocadherin 20 precursor (PCDH20). Sequencing of all exons of these 4 genes revealed a non-synonymous single nucleotide variant in exon 15 of DIAPH3 on the remaining allele. Subsequently, we found the father and brother of the proband to be heterozygous for the same variant in DIAPH3 (see figure 1). No other exonic variants were detected, except for one known untranslated exonic SNP, also in DIAPH3 (rs339531).

The identified point mutation (C>A) results in the substitution of proline by threonine at amino acid position 614 (Pro614Thr) located in the formin homology 1 (FH1) domain of DIAPH3 (see figure 2). This particular polymorphism was not reported in the genome databases. In addition, we did not identify this mutation in our screening of 128 control individuals of mixed ethnic descent and an additional sample of 200 individuals with Dutch ancestry without a life time psychiatric diagnosis. The DIAPH3 Pro614Thr was not observed in these 328 control subjects (656 chromosomes), suggesting a low allele frequency.

In order to establish whether DIAPH3 may have a role in the developing and adult brain, RNA *in situ* hybridization of *Diaph3* specific probes was performed on cryosections of various mouse embryonic stages and adult mouse brain. During embryonic development expression was mainly observed in the subventricular zone (SVZ) of the brain (see figure 3a-c). Initially the signal was present in the ventricular zone (VZ) of the entire brain up to the spinal cord (10.5 (not shown) and 12.5 days post coitum (dpc), figure 3a), at later stages expression became progressively restricted to the SVZ of the forebrain and roof of the midbrain (14.5 and 16.5 dpc, figure 3b, c). At 18.5 dpc only a low level of expression was maintained in the SVZ of the forebrain (figure 3d). Additionally, from 14.5 dpc onwards staining was observed in the granular cell layer of the cerebellum (3e). In the adult mouse brain no signal was detected. Outside the brain *Diaph3* expression was detected in the neural layer of the retina, the liver, the cortical region of the kidneys, and in the developing teeth (not shown). Sense probed sections did not show significant staining at any of the stages analyzed.

**Figure 3: Developmental expression of *Diaph3* in the murine brain**




All sections shown were hybridized with antisense probe, sense probed sections did not show significant staining. a). At 12.5 and 10.5 days post coitum (dpc) a signal was detected in the ventricular zone of the entire central nervous system (10.5 dpc not shown). b). At 14.5 dpc expression was detected in the VZ and SVZ of the forebrain and region of the inferior colliculus. a', b'). In the future cerebral cortex of respectively 12.5 and 14.5 dpc embryos staining was intenser in the subventricular zone (SVZ), compared to the ventricular zone (VZ) immediately lining the ventricles (arrows indicate the border between the lateral ventricle and the neocortex, arrowheads indicate the SVZ with more intense staining. In more posterior brain regions expression was uniformly distributed over the ventricular zones, e.g. the medial ganglionic eminence (asterix)). c). At 18.5 dpc only the ventricular zone of the forebrain shows staining. b'', c'). From 14.5 dpc onwards, additional staining in the brain was detected in the outer cell layer of the cerebellar primordium. Magnification: a, b, c: 12.5X, a',b', b'': 50X, c': 25X. Abbreviations: aq: aqueduct; cb: cerebellar primordium; hyp: hypothalamus; ic: inferior colliculus; lge: lateral ganglionic eminence; lv: lateral ventricle; med: medulla oblongata; mf: mesencephalic flexure; mge: medial ganglionic eminence; mid: midbrain; nc: neocortex; n: nose; ob: olfactory bulb; sc: spinal cord; ta: thalamus; v4: fourth ventricle.

**DISCUSSION**

In this case report a normally intelligent patient with an autistic disorder is presented in whom a rare genetic double hit consisting of a maternally inherited deletion and a paternally inherited functional single nucleotide mutation strongly suggests a homozygous loss-of-function in the DIAPH3-gene. Consequently, DIAPH3 could be assigned a causative role in autistic disorder. Parents and one sibling of the proband were found to be clinically unaffected carriers of either the deletion or the mutation but not both, showing the recessive nature of the alterations. In view of the recessive nature, DIAPH3 mutations resulting in ASD are likely to be rare. However the implication of DIAPH3 in the proband provides insight into the pathological processes leading to autism.

To our knowledge, there is only one case report of an autistic individual with a chromosomal rearrangement involving DIAPH3; (XY,del(13)(q14q22)[20]. In addition, in the database of genomic variants (HG build 36) a large genomic deletion at 13q such as described in the current paper is not reported. However, small CNVs within DIAPH3 have been identified in normal controls[21, 22]. Findings of the current case report and the additional studies reported here provide a reasonable level of plausibility that DIAPH3 is a candidate gene for autism. Apart from suggesting a plausible candidate gene for autism, the findings of this study are also relevant for the understanding how structural genomic abnormalities may be involved in the etiology of genetic disease.

*DIAPH3, a candidate gene for autism*

Protein diaphanous homolog 3 is a formin homology (FH) protein which plays a central role in the assembly of actin filaments through the combined action of its functional domains FH1 (actin recruitment) and FH2 (construction of the actin filament)[23]. It is related to the paralogs DIAPH1 and DIAPH2. The Pro614Thr substitution occurs in the middle of a proline-rich amino-acid sequence which is highly identical with the same sequence in FH1 of DIAPH3 and DIAPH2[24]. The C>A mutation is situated in a short nucleotide sequence element which is highly conserved across vertebrates (chr13:59,443,088-59,443,116, Lod=56) and across a subset of mammalian species (Chr13:59,443,091-59,443,116, Lod=17).

During brain development neurons migrate from the ventricular zones to specific cortical layers where they form synaptic connections with other neurons[25]. This migratory process is dependent on the tightly orchestrated and highly dynamic process of cytoskeletal actin remodeling. As a first step in neuronal migration the leading edge of a cell is extended by formation of membrane protrusions (filopodia). This process is mediated by two distinct actin networks; the lamellipodium and the lamella. Lamellipodia at the leading edge are involved in a repeating process of extension and retraction movements, but persistent leading edge advance and cell migration rely on the expansion of the lamella actin network[26]. Recently, it was shown that the DIAPH3 regulates the actin network dynamics within the lamella and that inhibition of DIAPH3 significantly slows cell migration[27]. However, DIAPH3 also appears to have a significant role in the expansion process of lamellipodia[28].

In addition, recent evidence indicates that lamellipodia and filopodia are not only important for efficient axonal guidance and neuronal migration, but also play an essential role in neurite formation[29, 30]. Fusion of lammelipodia results in the formation of neurite extensions which are the precursors to dendrites, dendritic spines and axons. DIAPH3 has been shown to induce filopodia formation in neurons, suggesting that DIAPH3 can also induce neurite formation[29]. Interestingly, SHANK3, a gene which was recently reported as a novel candidate gene for autism spectrum disorders[31, 32], plays in important regulatory role in the process of the initiation and maturation of filopodia resulting in the formation of functional dendritic spines[33]. In this process, SHANK3 interacts with cortactin, a protein implicated in connecting the actin network sites with receptor signaling complexes[34]. Based on these findings one could speculate that DIAPH3 acts downstream of SHANK3 regulation.

Spatiotemporal expression of DIAPH3 suggests a dual role in cortical development. During early corticogenesis DIAPH3 is expressed in the VZ of the whole brain, while in later stages expression shifts to the SVZ of the cortex only. This suggests that DIAPH3 may participate initially in proliferation of neural progenitors. The restricted expression in the cortical SVZ points to post-mitotic functions such as migration, axon outgrowth and neuritogenesis. The role of DIAPH3 in the

development of the cortex appears transient, since in the adult cortex DIAPH3 is no longer expressed (data not shown). This also provides a neurodevelopmental window in which the pathogenesis of autism may occur. Particularly the second phase of DIAPH3 expression in the SVZ would fit with observations in patients showing subtle cortical defects, but no gross abnormalities due to proliferation defects.

In summary, the expression profile together with the available functional data suggests that DIAPH3 may have a pivotal role in cell migration, axon guidance and neuritogenesis. In addition, DIAPH3 may exert these functions in a biological pathway in which also SHANK3 acts.

A causal relationship between DIAPH3 and autistic disorder in the presented proband is plausible based on the concurrent findings of this study.
First, while the deletion and the point mutation are both identified in unaffected first degree family members, the proband is the only one to be a compound heterozygote. Autism is a relatively rare disorder, and both the point mutation and deletion appear to be very rare events at the population level which makes the concurrence in one person an extremely rare event. According to the principle of parsimony, one all-inclusive explanatory hypothesis is the most plausible.

Second, our findings indicate that it is very likely that the function of DIAPH3 is disrupted in the proband; the point mutation in the remaining copy leads to an amino-acid substitution in a protein domain that is essential to its function, within a sequence highly conserved between the other DIAPH genes and across mammalian species.

Third, the function and expression profile of *Diaph3* is indicative of an important role in the developing cortex during embryogenesis. This is consistent with the current etiologic models of autism which imply a disruption of normal development leading to abnormal organization and connectivity of cortical neurons, e.g.[35].

*The interpretation of inherited structural abnormalities in studies on ASD*

When a structural genomic abnormality is identified in a patient, establishing evidence for a causal relationship between the disease phenotype and the observed genomic abnormality is not straightforward. Naturally, causality is more likely to exist if more than one of such concurrences of the phenotype and the genomic abnormality are identified. In contrast, when other cases are observed (within or outside the index patient's family) with the same genomic defect but without the phenotype, it is often concluded that a causal relation is unlikely.

The interpretation of incomplete co-segregation patterns is not only clinically relevant for genetic counseling, but is also important for the interpretation of results from screens of cytogenetic abnormalities and CNVs in disease populations. CNVs and cytogenetically detectable chromosomal abnormalities are generally regarded as not causally related to a disease if the event is not *de novo*, i.e. when one of the unaffected parents (or siblings) is found to be a carrier.

This case illustrates how a causal relation between a structural genomic abnormality and a disease phenotype may be very plausible, even if unaffected individuals with the same genomic abnormality are identified. This is a relevant issue given that in all hallmark publications so far all non-*de novo* CNVs or CNVs that were also found in unrelated normal controls were excluded from the analyses[4, 5]. While this approach is understandable as an effort to reduce false positive findings, one may also reason that inherited CNVs do in fact contribute to ASD in some cases. For instance, it has been argued that penetrance of genetic defects may be low in females[36]. Alternatively, a gene within the implicated region may be affected by a common variant or a functional mutation, such as is demonstrated in the current case report. Such a “double hit” scenario has been described for several genetic diseases, for instance the situation of compound heterozygosity involving two different point mutations in retinal dystrophy[37], or involving two different genomic rearrangements in a person with Cystic Fibrosis[38], or involving a deletion and a mutation in an individual with the 22q11.2 deletion syndrome and the Bernard-Soulier syndrome[39].

Our findings suggest that assuming that only *de novo* genomic rearrangements are causally related to ASD is incorrect and too restrictive, potentially leading to loss of valuable information and insight. Moreover, one could argue that a "double hit" scenario, i.e. the disruption of a gene by two mutational events affecting both alleles is probably not a frequent cause of ASD but has the great advantage of pinpointing the genetic contribution to a particular gene. We therefore suggest that an effort should be made for a re-sequencing project of genes involved in *non-de novo* and *non*-unique large genomic deletions in individuals with ASD. Provided the results of our study we anticipate that such an effort will efficiently lead to the identification of new autism genes that can further be studied. Given the current difficulty of identifying autism genes despite the evidence of a strong genetic contribution to this disorder, this effort is certainly worthwhile.

**Web resources**

Online Mendelian Inheritance in Man (OMIM),
http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM
Clone library at the Children's Hospital Oakland Research Institute,
http://bacpac.chori.org/
UCSC Human Genome Browser Gateway (http://www.genome.ucsc.edu).
Pubmed, http://www.ncbi.nlm.nih.gov
The Database of Genomic Variants, http://projects.tcag.ca/variation/

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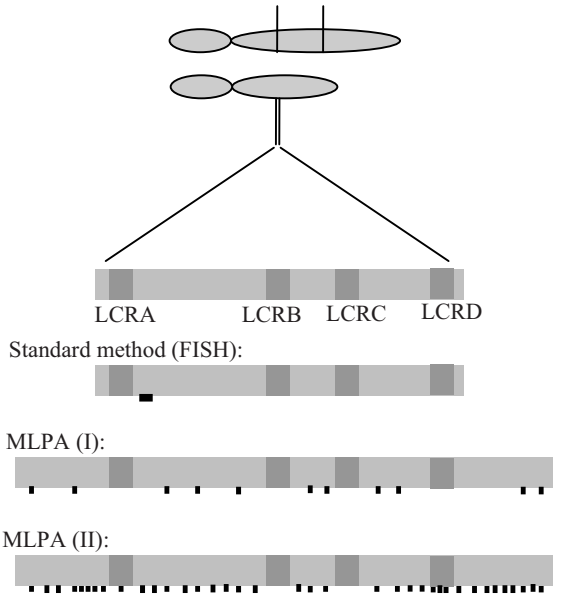
# CONCLUSION

**In the preceding chapters** hypotheses, methods and results are presented of 8 studies that are all related to the central theme of this thesis; the idea that relevant risk genes for autism and schizophrenia can be identified through careful analysis of clinical and genetic aspects of individuals in whom these disorders are present in conjunction with a structural genomic abnormality.

In **chapter 1** the results are presented of an extensive literature review of all published case-reports on individuals with autism and a concurrent structural genetic abnormality. Consistent with the hypothesis, combining the information from these records resulted in various regions on the human genome where several independently reported cases overlapped. A certain degree of validation of this approach was provided by the finding of several regions (chromosome 7 (several loci), 15q11-q13 and 5p14) in which the overlap occurred at loci previously identified by linkage and / or association studies. Furthermore, utilizing the arbitrary threshold of a minimal overlap of 4 independent cases at the same locus, the results revealed 10 previously unidentified loci: 2q37, 5p15, 11q25, 16q22.3, 17p11.2, 18q21.1, 18q23, 22q11.2, 22q13.3 and Xp22.2-p22.3.
Interestingly, two independent recent studies have identified a gene (SHANK3) within one of these regions, 22q13.3, as an autism susceptibly gene[2, 3].

In **chapter 2** findings are presented of a clinical study on the psychiatric phenotype of 22q11DS. In a sample of 60 Dutch children aged 9 through 18 years with a confirmed deletion at 22q11.2, 50% were diagnosed with an autism spectrum disorder, 12% were diagnosed with a psychotic disorder and 8% were found to have a mood disorder. On a symptom level, an even larger proportion (27%) of children was found to have psychotic symptoms (hallucinations and or delusions). The mean age at which these symptoms were diagnosed was approximately 14 years. With regard to autistic behaviors; of the 50% of children who did *not* meet criteria for a formal diagnosis of one of the ASDs, a large proportion displayed nevertheless behaviors consistent with deficits in one or two core domains of autism (i.e. deficits in social interaction, communication and the presence of stereotyped behaviors). There was no correlation between intelligence level and autistic or psychotic symptoms within the study sample, suggesting that these behavioral features have some specificity for 22q11DS and may not be explained by a non-specific effect of intelligence. However, a definitive clarification of this matter can only be provided by results of studies in which the behavioral features of 22q11DS children are compared to an IQ-matched control group. Recently a study of this type was published which showed that several behavioral domains, including social problems and withdrawn behaviors, were observed significantly more in children with 22q11DS in comparison to age and IQ-matched controls[4].
One implication of the findings in the study reported in **chapter 2** is that the neurodevelopmental consequences of a 22q11.2 deletion may predispose a child with this syndrome to behaviors similar as those seen in autistic children in the

general population. Interestingly, the results of the preceding study presented in **chapter 1** already indicated that the 22q11.2 region is a relevant region of interest for autism. In addition, in an ensuing study by a different group the finding of a high prevalence of ASD amongst children with 22q11DS was replicated[5].
These particular results have generated a debate as to whether 22q11DS-related ASD can be considered as comparable to ASD in the general population[6, 7]. The question was raised whether the ASD symptoms observed in children with 22q11DS should be considered as prodromal signs of schizophrenia, rather than as clinical markers of autism. This is a relevant issue that can only be solved with information from longitudinal studies in which children with 22q11DS are followed up until adulthood.




**Detection of copy number changes:** with the standard FISH method, one region between LCRA and LCRB is queried for copy number changes (black box). With the first MLPA set multiple regions between and outside LCRA and LCRD are reliably examined. Improvement of the first MLPA (MLPA I) probe set resulted in a higher density of probes across the entire region (MLPA II). In this figure the representation of genomic locations of the probes is approximate. *(Figure adapted from Shaikh et al.[1])*

In **chapter 3** the test characteristics of a novel method for the detection of copy number changes, MLPA, are reported. The results demonstrate that MLPA reliably identifies typical and atypical 22q11.2 deletions of varying size, as well as duplications and unbalanced translocations involving this genomic region. Utilizing a threshold for the comparative signal strength which was determined with a receiver operator characteristic (ROC) curve analysis, the sensitivity and specificity of MLPA were 0.99 and 0.97, respectively, indicative of excellent test characteristics. The fact that MLPA is potentially more informative than the standard test method, FISH, was illustrated by the discovery of variant deletion endpoints in three cases which had remained unnoticed upon analysis with FISH before the MLPA study.
However, even though the MLPA method was shown to provide highly accurate information on changes in copy number for several loci at 22q11.2, there were still

rare structural genomic abnormalities that would not have been detected by the available MLPA kit, since none of the MLPA probes queried those regions. Therefore, an effort was made to develop a new MLPA set with an increased density of probes across the 22q11.2 region. The results of this effort are reported in **chapter 4**. While the first MLPA kit contained 7 probes that queried the 22q region between LCRA and LCRD, the revised MLPA kit contained 18 probes between LCRA and LCRD and an additional 17 probes distributed proximally and distally from this region. Results of this study showed that the new kit reliably identifies copy number changes in this region. In addition, utilizing several probes located in the LCR regions it was demonstrated that typical LCRA to LCRD deletions differ from each other with regard to the exact endpoints within the LCR regions. This variation in deletion endpoints may be relevant with regard to the molecular genetic mechanisms that cause 22q11.2 deletions in the population.

In **chapter 5** the MLPA method was used to screen a sample of 311 Dutch patients with schizophrenia for the presence of 22q11.2 deletions. The sample included 146 patients with deficit schizophrenia; a subgroup characterized by the presence of prominent negative symptoms. The hypothesis of an increased prevalence of 22q11.2 deletions in this specific group of patients was not confirmed; indeed none of the 311 patients was found to have a deletion at 22q11.2. When adding this finding to all the available screening studies for 22q11DS in adult patients with schizophrenia, to date twelve 22q11.2 deletions have been identified in a total of 2051 patients with adult-onset schizophrenia. This indicates a prevalence of 0.6%. Even though this is less than the often quoted 2% prevalence, it is still 30–45 times higher than expected given the estimated prevalence of 22q11DS in the general population[8-12].

In **chapters 6 and 7** results of two studies are presented in which associations between genes within the 22q11.2 region and psychiatric phenotypes are examined. In **chapter 6** three physiological measures of brain function were assessed in 56 children with a confirmed deletion at 22q11.2, and 76 typically developing children. The three measures, prepulse inhibition of startle (PPI), P50 auditory sensory gating and smooth pursuit eye movements (SPEM), were selected because on average, individuals with schizophrenia show a decreased performance on these measures[13]. After matching the two study groups for age and gender, the test results were compared. Only performance on PPI and SPEM was found to be significantly decreased in the 22q11DS group, an effect that occurred independently of the IQ difference between the two groups. Then, PPI and SPEM performances were used to test the hypothesis that a significant negative effect of high plasma proline levels on brain function would occur only in 22q11DS children with the $COMT^{met}$ allele. High plasma proline levels can be expected in some 22q11DS patients as a result of variation at the remaining allele of the gene PRODH.
The results of the study demonstrated the anticipated interaction between high plasma proline and the $COMT^{met}$ allele with regard to SPEM. A plausible

mechanism to explain this finding would be that elevated proline negatively affects brain function via a neuromodulatory action on glutamatergic signaling, leading to an increase in dopamine in the prefrontal cortex. The resulting relative redundancy of dopamine may be particularly problematic for 22q11DS patients whose remaining COMT allele is the (low activity) $COMT^{met}$ variant.
The relevance of these findings may be the potential contribution to our understanding of how brain function is impaired in subjects with the 22q11.2 deletion syndrome. Interestingly, the results are consistent with the findings reported by Raux et al[14]: in their study involving adults with 22q11DS, significantly more psychotic disorders were found in the subgroup with high plasma proline levels in conjunction with the low activity $COMT^{met}$ allele.
The effect between increased plasma proline and prefrontal dopaminergic signaling demonstrates in an elegant way how other variables may modulate the effect of the $COMT^{158}$ genotype on brain processes. The latter aspect is particularly interesting in the context of the numerous conflicting results of genotype-phenotype studies with regard to this common polymorphism.

In **chapter 7** a significant interaction is presented between a risk allele of the gene PIK4CA and schizophrenia. This allele was previously reported to confer an increased risk of schizophrenia in the general population[15]. Given its location at 22q11.2 it was hypothesized that individuals with 22q11DS who carried the PIK4CA risk allele would also be at increased risk of schizophrenia. The results of this study were in accordance with this expectation; the PIK4CA risk allele was significantly more present in a group of adult 22q11DS patients with schizophrenia in comparison to adult 22q11DS patients without schizophrenia. The effect size of this association was high (an estimated Odd's Ratio of ~8), suggesting that variation at PIK4CA may explain to some extent the high prevalence of schizophrenia in patients with 22q11DS.

In **chapter 8** another study aimed at the identification of an association between a gene and a psychiatric disease is reported. However, there are several differences in comparison to **chapters 6 and 7**. This study involves chromosome 13 instead of 22q11.2, the psychiatric phenotype is autism instead of (related to) schizophrenia, and the identified genotype-phenotype association is not based on a statistical correlation. Nevertheless, like the two preceding chapters, the central idea behind this study is that relevant risk genes can be identified in patients in whom a psychiatric phenotype is present in conjunction with a structural genomic abnormality.
Clinical and molecular genetic findings are reported concerning a normally intelligent proband with autism who was found to carry a maternally inherited large deletion (~10Mb) of chromosome 13q. Because his mother was not affected with autism, it was hypothesized that the proband could have a functional mutation in a remaining allele of one of the genes located in the deleted region. The results from the sequencing studies confirmed this "double hit" hypothesis; the proband carried a

paternally inherited non-synonymous point mutation in DIAPH3, one of the genes in the region affected by the maternally inherited deletion. This mutation was not found in a large sample of controls, suggesting that it is not a common allele in the population. In addition, results of expression experiments with DIAPH3 in the mouse demonstrated expression of DIAPH3 in specific areas of the developing cortex of the murine brain. This, together with available literature data on the functions of DIAPH3, suggests that DIAPH3 may have a pivotal role in cell migration, axon guidance and neuritogenesis, and may exert these functions in a biological pathway in which also SHANK3 acts. SHANK3 was recently reported as an autism susceptibility gene[2, 3].

Taken together, these findings provide a reasonable amount of evidence that put DIAPH3 forward as a novel autism risk gene. In addition, these results illustrate that the widely accepted assumption that only *de novo* genomic rearrangements are causally related to autism may in fact lead to the loss of valuable information.

**Conclusion**

The research presented in this thesis is centered around one question: What can we learn from the study of psychiatric phenotypes related to structural genomic abnormalities? In this thesis this subject is examined, with most studies focused on the clinical and genetic aspects of the 22q11.2 deletion syndrome.

First, the results of the different studies may help improve the quality of clinical care provided to individuals with 22q11DS. For instance, results presented in **chapter 2** may increase the awareness of parents, medical professionals and educators that many children with 22q11DS present problems similar to those seen in children with autism spectrum disorders.

Second, and on a different level, some results may prove relevant to the understanding of the biological mechanisms that lead to the psychiatric phenotypes associated with 22q11DS. For instance, the findings presented in **chapter 7** indicate that variation in the gene PIK4CA plays an important role in the causation of schizophrenia in individuals with 22q11DS. Following up on this finding, further studies can be undertaken to elucidate the biological role of PIK4CA in the brain. Such information may improve our understanding of schizophrenia in 22q11DS and generate opportunities for the development of better treatments.

Third, in addition to the potential relevance for people with 22q11DS, the results in this thesis may also be relevant for our understanding of schizophrenia and autism in the general population. For instance, the interaction between high proline levels and the COMT[158] genotype presented in **chapter 6** suggests that dopamine metabolism in the frontal cortex is modulated by the action of proline on glutamatergic neurons. This finding connects two prominent hypotheses about the pathophysiological mechanisms that underlie schizophrenia; the “dopamine hypothesis” and the “glutamate hypothesis”[16, 17]. Similarly, findings described in **chapter 8** suggest that DIAPH3 is an autism susceptibility gene. Subsequent studies on the function of this gene may generate relevant information with regard to the developmental pathways that are disturbed in individuals with autism.

In conclusion, results of the presented research favor the view that studying psychiatric phenotypes associated with structural genomic abnormalities may generate valuable information about the genetic, and thus the biological factors that lead to psychiatric disorders, such as autism and schizophrenia.

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# NEDERLANSE SAMENVATTING

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*Autisme en schizofrenie*

Autisme is een aandoening die gekenmerkt wordt door stoornissen in de sociale interactie en de communicatie, en de aanwezigheid van stereotype gedragspatronen. Schizofrenie is een ziekte die gekarakteriseerd wordt door het voorkomen van psychose; een toestand van verminderd contact met de realiteit waarbij de waarneming en de gedachteninhoud verstoord kunnen zijn. Kenmerkend voor schizofrenie is ook de aantasting van cognitieve vermogens zoals aandacht en planning, een verminderde initiatiefname en een afgenomen sociale interactie. Zowel autisme als schizofrenie gaat gepaard met een verminderd algemeen functioneren. Bij autisme is dit per definitie waarneembaar vroeg in de ontwikkeling, vóór het 3e levensjaar. In het geval van schizofrenie wordt een verminderd functioneren doorgaans eerst zichtbaar in de late adolescentie / vroege volwassenheid, zodat wel gesproken wordt van “een knik in de ontwikkeling”.

Voor beide stoornissen geldt dat er op dit moment geen genezende behandeling voor handen is. De gangbare behandeling bestaat uit een combinatie van medicatie, psycho-educatie en rehabilitatie. In het geval van schizofrenie resulteert de medicamenteuze behandeling vaak tot een vermindering van psychotische symptomen. Bij autisme is medicatie enkel effectief in het verminderen van co-morbide gedragsproblemen zoals agressie.

Een belangrijke reden dat er nog geen curatieve behandeling bestaat voor deze ernstige aandoeningen is dat er onvoldoende inzicht is in de onderliggende biologische mechanismen. Een beter begrip van de aard van de verstoring van de neurobiologische functies bij mensen met autisme en schizofrenie kan nieuwe mogelijkheden bieden voor de ontwikkeling van betere behandelingen.

*De relevantie van het ontdekken van ziekte-gerelateerde genen*
Een manier om het begrip van de biologische basis van een ziekte te vergroten is het identificeren van variatie in genen met een causale relatie met de ziekte. Studies met tweelingen laten zien dat zowel bij schizofrenie als bij autisme genetische variatie een belangrijke rol speelt in het ontstaan van de ziekte. Een gen is een code voor een eiwit; variatie in die code kan in sommige gevallen leiden tot een aantasting van de structuur van het eiwit en daarmee in potentie een verstoring van het biologische mechanisme waarin dat eiwit een functie uitoefent. Andersom geredeneerd kan door de identificatie van een ziekte-geassocieerde variatie in een gen, via het bestuderen van de functie van het eiwit, dus een beter inzicht worden verworven in de biologie van de ziekte.

In dit proefschrift wordt een specifieke benadering beschreven die gericht is op de identificatie van ziekte-geassocieerde genen. De essentie van deze benadering bestaat uit de studie van personen met de ziekte én structurele genomische afwijkingen. Voorbeelden daarvan zijn het ontbreken (deletie) of het dubbel aanwezig zijn (duplicatie) van een deel van een chromosoom. Bij dergelijke afwijkingen in de chromosomale structuur zijn vaak verscheidene genen betrokken. De redenering is dan dat het aannemelijk is dat, als een structurele genomische afwijking en een ziekte tegelijkertijd voorkomen, één of meerdere genen in het genomische gebied van de afwijking te maken heeft met de ziekte.

Het 22q11.2 deletie syndroom (22q11DS) is hiervan een voorbeeld; mensen met dit syndroom missen een deel van chromosoom 22 waarbij ongeveer 30 – 50 genen zijn betrokken. Verschillende onderzoeken hebben aangetoond dat ongeveer 20 tot 30% van de volwassenen met 22q11DS schizofrenie ontwikkelt. Dit is vele malen meer dan het voorkomen van schizofrenie in de algemene bevolking (ongeveer 1%). Volgens de redenering die in de voorafgaande alinea is uiteengezet, is het waarschijnlijk dat één of meerdere genen in het gebied van de deletie (het 22q11.2 gebied) een rol speelt bij het ontstaan van schizofrenie. Een studie naar de genetische factoren van schizofrenie in een groep van mensen met 22q11DS kan zich dus beperken tot 30 tot 50 potentiële risicogenen. Ter vergelijking: in een vergelijkbare studie in de algemene bevolking moet rekening gehouden worden met ~25.000 potentiële risicogenen.

*De studies in dit proefschrift*

De inhoud van dit proefschrift is de weerslag van een poging om genetische oorzaken van autisme en schizofrenie te identificeren door het bestuderen van mensen bij wie deze ziektebeelden samengaan met een structurele genomische afwijking. In het bijzonder, maar niet uitsluitend, richten de studies zich op mensen met het 22q11.2 deletie syndroom.

**Hoofdstuk 1** is een review met alle studies in de medische literatuur die het samengaan van autisme en een structureel genomische afwijking in patiënt(en) rapporteren. De hypothese van deze review was dat door het samenvoegen van alle gerapporteerde casus op bepaalde genomische gebieden een clustering van verschillende genomische afwijkingen zichtbaar zou worden. De aanwezigheid van verschillende afwijkingen in samenhang met autisme op eenzelfde genomische gebied suggereert dat in het betreffende gebied één of meerdere risicogenen voor autisme te vinden zijn.

De resultaten van de studie waren consistent met deze hypothese. Ten eerste bleek clustering op te treden in gebieden die door klassieke genetische studies in autisme al waren aangewezen als regios waar risicogenen van autisme kunnen liggen. Dit was onder andere het geval voor verschillende gebieden op chromosoom 7, voor 15q11-13 en 5p14. In zekere zin vormde deze bevinding een validatie van het principe.

Ten tweede werd een clustering zichtbaar op 10 genomische gebieden die niet eerder door klassieke genetische studies naar voren waren gebracht: 2q37, 5p15, 11q25, 16q22.3, 17p11.2, 18q21.1, 18q23, 22q11.2, 22q13.3 en Xp22.2-p22.3. Een interessante ontdekking in twee verschillende studies[1, 2] die volgden op deze studie, was het gen SHANK3, gelegen op één van gerapporteerde gebieden (22q13.3), als autisme risicogen.

In **hoofdstuk 2** worden de bevindingen gerapporteerd van een klinische studie naar de psychiatrische symptomen en stoornissen in een groep van 60 kinderen in de leeftijd van 9 tot 18 jaar met 22q11DS. Vijftig procent van deze groep werd gediagnosticeerd met een autisme spectrum stoornis (ASS), 12% met een psychotische stoornis en 8% met een stemmingsstoornis. Wanneer niet zozeer de

formele diagnostische classificatie maar ook (sub)klinische symptomen als eindmaat werden gebruikt, bleek 27% van de kinderen last te hebben van frequente psychotische symptomen zoals hallucinaties en / of wanen. De gemiddelde leeftijd waarop deze psychotische symptomen begonnen was ongeveer 14 jaar. Wat betreft autistische symptomen viel op dat de meeste kinderen in de groep bij wie géén diagnose ASS werd gesteld, tóch relevante defecten vertoonden in de sociale interactie en / of communicatie, dan wel een neiging tot stereotype gedrag hadden. Er bleek geen correlatie te bestaan tussen intelligentie niveau en de autistische en psychotische symptomatologie binnen deze groep, dat suggereert dat het verhoogde voorkomen van ASS en psychose bij 22q11DS een specifiek kenmerk is van dit syndroom en niet uitgelegd kan worden door de gemiddeld lagere intelligentie van deze populatie. Een definitieve bevestiging van deze interpretatie kan echter pas worden geleverd door het vergelijken van de psychopathologie van kinderen met 22q11DS met een groep kinderen zonder 22q11DS, maar met een vergelijkbaar intelligentieniveau. Dit is gebeurd in een studie van recentere datum, niet in dit proefschrift, waarin de gedragspatronen zijn vergeleken van 22q11DS kinderen met een IQ-gematchde controle groep. De bevindingen tonen aan dat de 22q11DS groep een significant hogere probleemscore laat zien op verschillende gedragsdomeinen, onder andere wat betreft sociale problemen en teruggetrokken gedrag[3].
Een belangrijke implicatie van de bevindingen die worden gerapporteerd in **hoofdstuk 2** is dat het effect van een deletie van 22q11.2 op de ontwikkeling van het centrale zenuwstelsel dusdanig is dat het kind met 22q11DS een hoog risico heeft op een gedragsontwikkeling zoals die gezien worden bij kinderen met ASS. In een recente studie van een andere onderzoeksgroep werd de bevinding van een verhoogd voorkomen van ASS bij kinderen met 22q11DS gerepliceerd[4]. Relevant in dit verband is dat in **hoofdstuk 1** autisme al in verband was gebracht met structurele genomische afwijkingen van het 22q11.2 gebied, onafhankelijk van deze studies. Vanuit het perspectief van de research betekent dit dat het aannemelijk is dat er één of meerdere risicogenen voor autisme in het 22q11.2 gebied liggen. Tegen deze achtergrond is een debat ontstaan over de vraag of de autistische symptomen bij kinderen met 22q11DS gezien moet worden als passend bij ASS, dan wel gezien moeten worden als vroege tekenen van een schizofrene ontwikkeling[5, 6]. Dit is een relevante vraag waar enkel door longitudinaal (follow-up) onderzoek antwoord op zal kunnen worden gegeven.

In **hoofdstuk 3 en 4** worden de resultaten van twee moleculair genetische studies gepresenteerd waarin een nieuwe methode, Multiplex Ligation-dependent Probe Amplification (MLPA), voor het detecteren en karakteriseren van de 22q11.2 deletie wordt onderzocht. MLPA zou verschillende voordelen hebben ten opzichte van de tot op heden meest gebruikte detectiemethode (Fluorescent In Situ Hybridization, FISH):

1. Met MLPA kunnen tegelijkertijd verschillende genomische regios worden onderzocht in in plaats van één gebied met FISH.
2. MLPA is goedkoper, zowel wat betreft materiaalkosten als wat betreft arbeidsinvestering.
3. Met behulp van MLPA kunnen niet alleen deleties maar ook duplicaties worden gedecteerd. Het detecteren van duplicaties met FISH is weliswaar mogelijk, maar vereist een speciale techniek.

In **hoofdstuk 3** worden deze aspecten van MLPA voor de detectie van structurele dosisveranderingen (deleties, duplicaties, triplicaties, etc) van 22q11.2 onderzocht. De resultaten laten zien dat MLPA een excellente sensitiviteit van 0.99 (dat wil zeggen dat er vrijwel geen afwijkingen worden gemist) en specificiteit van 0.97 (dat wil zeggen dat indien MLPA géén afwijking detecteert, er in werkelijkheid vrijwel altijd daadwerkelijk géén afwijking aanwezig is). Deze conclusies konden worden gemaakt omdat in de studie de MLPA blind uitgevoerd was op een grote set DNA-samples van patiënten met een verscheidenheid aan structurele genomische afwijkingen (met dosisverandering) die in de voorgaande jaren door middel van verschillende moleculair genetische technieken al waren gekarakteriseerd.
In dit verband is het belangrijk te realiseren dat het gebied op 22q11 dat aangedaan is door de 22q11 deletie variatie vertoont in exacte locatie en lengte. Daarnaast bestaan er ook patiënten met een dosisvermeerdering van 22q11.2 (duplicatie, triplicatie). De samenhang van de variatie in deze genomische afwijkingen met de variatie in de klinische kenmerken (het zogenaamde “fenotype”) kan belangrijk zijn voor een beter begrip van de rol van 22q11.2 genen in het ontstaan van het (psychiatrische) fenotype.
Met de bestaande MLPA kit werden 7 genomische gebiedjes binnen het 22q11 deletie gebied getest. Het bleek echter dat er nog steeds atypische deleties op 22q11.2 bestonden die door deze bestaande MLPA kit niet werden gedetecteerd.

Daarom is in samenwerking met de fabrikant, MRC-Holland, een poging ondernomen om een gereviseerde MLPA kit te ontwikkelen met een hogere dichtheid van probes in het 22q11.2 gebied. De resultaten van deze poging worden beschreven in **hoofdstuk 4**.
De gereviseerde MLPA kit bevat 18 probes in de 22q11.2 deletie regio, en 17 probes in de directe omgeving van dit gebied. De resultaten van de studie met de vernieuwde MLPA kit laten opnieuw een hoge sensitiviteit en specificiteit zien. Dosisveranderingen variërend van n=0 tot n=4 worden betrouwbaar gedetecteerd. Daarbij leidde de hogere resolutie van de gereviseerde kit tot een preciezere analyse van de eindpunten van de afwijkingen. Een bijkomende bevinding was dat sommige patiënten bij wie voorheen eenzelfde lengte van de deletie was verondersteld, (daarom vaak “typische 22q11.2 deletie genoemd), toch kleine verschillen bleken te vertonen in de precieze lengte. Deze subtiele variatie draagt mogelijk bij aan de variatie van het fenotype, maar ook aan een beter begrip van de moleculaire mechanismen die leiden tot het ontstaan van 22q11.2 deleties en duplicaties in de bevolking.

In **hoofdstuk 5** is de prevalentie van 22q11.2 deleties met behulp van MLPA in een groep van ruim 300 Nederlandse patiënten met schizofrenie onderzocht. Aangezien de 22q11.2 deletie leidt tot een sterk verhoogd risico op het ontwikkelen van schizofrenie kan andersom verwacht worden dat in de populatie van mensen met schizofrenie méér 22q11.2 deleties voorkomen dan in de algemene bevolking. In eerdere studies werd gerapporteerd dat inderdaad ongeveer 2% van de mensen met schizofrenie de 22q11.2 deletie heeft. Omdat bij veel mensen met 22q11DS symptomen van teruggetrokken gedrag, een tekort aan energie en beperkte sociale vaardigheden worden beschreven was de hypothese van deze studie dat in een subgroep van mensen met schizofrenie én een prominente aanwezigheid van dezelfde klachten (in de context van schizofrenie “negatieve symptomen” genoemd), de 22q11.2 deletie nog vaker dan in 2% aantoonbaar zou zijn. Prominente negatieve symptomen waren aantoonbaar in 146 van de in totaal 311 geïncludeerde patiënten met schizofrenie. Echter, in géén enkel geval van het totale sample werd de 22q11.2 deletie gedetecteerd, zodat deze hypothese niet bevestigd kon worden.

Een meta-analyse van deze, en eerdere prevalentie studies wijst op een prevalentie van 22q11.2 deleties in schizofrenie patiënten van 0.6%. Hoewel lager dan de eerder gerapporteerde 2%, is dit nog steeds 30 tot 45x hoger dan verwacht kan worden op grond van de geschatte prevalentie van 22q11DS in de algemene bevolking.

In **hoofdstuk 6** zijn drie electrofysiologische maten van hersenfunctie getest in 56 22q11DS kinderen en 76 gezonde controles. Deze drie maten, prepulse inhibition of startle (PPI), P50 auditory sensory gating en smooth pursuit eye movements (SPEM) waren geselecteerd vanwege hun associatie met schizofrenie; gemiddeld presteren mensen met schizofrenie op deze drie testen minder goed in vergelijking met gezonde controles. 22q11DS kinderen en controles waren gematched voor wat betreft de geslachtsverdeling en leeftijd, maar verschilden van elkaar voor wat betreft intelligentie niveau. Voor dit verschil is statistische correctie toegepast. Uit de analyse bleek dat alleen de prestaties van PPI en SPEM significant verminderd waren in de 22q11DS groep. Vervolgens werden deze twee maten gebruikt om een gecombineerd (interactief) effect van twee genen in het 22q11.2 gebied, PRODH en COMT, op de functie van de hersenen te onderzoeken.

Een hoog proline gehalte is beschreven in een 22q11DS patiënten en hangt samen met het feit dat het individuen met 22q11DS vanwege de deletie maar één kopie hebben van het gen PRODH. PRODH codeert voor een enzym dat een belangrijke rol speelt in de afbraak van proline. Ook het COMT gen ligt in het 22q11.2 gebied; van dit gen is een veel voorkomende variant bekend ($COMT^{met}$) dat geassocieerd is met een lagere activiteit van het gecodeerde enzym in vergelijking met het meest voorkomende variant ($COMT^{val}$). Dit betekent dat 22q11DS patiënten ingedeeld kunnen worden in twee (ongeveer even grote) groepen; de groep met de $COMT^{met}$ variant en de groep met het $COMT^{val}$ variant. Het enzym COMT speelt in de hersenen een belangrijke rol in de afbraak van de neurotransmitter Dopamine. Fundamentele (dier)onderzoeken naar de rol van proline suggereren dat een verhoogde concentratie van dit aminozuur mogelijk leidt tot een verhoogde beschikbaarheid van Dopamine. Op grond van deze twee observaties is een interactie tussen proline en COMT goed mogelijk; als proline de beschikbaarheid

van Dopamine in de hersenen verhoogt dan vormt dat vooral een probleem voor diegenen bij wij de afbraak van Dopamine niet optimaal is.
Dus luidde de hypothese van deze studie dat de hersenfunctie (uitgedrukt in de prestatie op SPEM en PPI) in 22q11DS kinderen met de $COMT^{met}$ variant negatief beïnvloed wordt door een verhoogd proline gehalte in het bloed, terwijl voorspeld werd dat een hoog proline gehalte in kinderen met de $COMT^{val}$ variant géén of weinig effect op de prestatie heeft.
De resultaten van deze studie kwamen overeen met deze hypothese; voor wat betreft SPEM bleek er een significante interactie tussen de $COMT^{158}$ variant en de proline concentratie in 22q11DS kinderen; alléén in de $COMT^{met}$ groep was een negatief effect van verhoogde proline meetbaar. De implicatie van deze bevinding zou kunnen zijn dat in 22q11DS, personen met de $COMT^{met}$ variant én een verhoogde proline concentratie relatief het grootste risico op psychose hebben. Deze interactie werd in een voorafgaande studie door Raux et al. al aangetoond; in de subgroep van 22q11DS volwassenen met zowel de $COMT^{met}$ variant als een verhoogd proline kwamen significant meer psychotische stoornissen voor[7]. Deze bevindingen zijn relevant met betrekking tot het inzicht in het ontstaan van psychose in het kader van het 22q11.2 deletie syndroom. Er is ook een bredere relevantie; het interactie effect tussen proline en COMT demonstreert dat het effect van de veel bestudeerde $COMT^{158}$ variant op hersenfunctie gemoduleerd wordt door andere variabelen.

In **hoofdstuk 7** werd de hypothese getest dat variatie in het PIK4CA gen geassocieerd is met schizofrenie. De studie betrof een groep van ongeveer 80 volwassenen met 22q11DS, waarvan een deel lijdt aan schizofrenie. De hypothese was gebaseerd op een recent gerapporteerde significante associatie van PIK4CA met schizofrenie in de algemene populatie[8] en op het feit dat het PIK4CA gelegen is in het 22q11.2 gebied. De meeste 22q11DS patiënten hebben dus, net zoals het geval is voor COMT en PRODH, maar één kopie (allel) van het PIK4CA gen.
De resultaten van de studie bevestigden de associatie van PIK4CA met schizofrenie; de risico-variant kwam significant meer voor in 22q11DS patiënten met schizofrenie in vergelijking met 22q11DS patiënten zonder schizofrenie. Deze bevinding repliceert de eerder gevonden associatie in de algemene bevolking én suggereert dat juist in de context van een 22q11.2 deletie, de variatie van PIK4CA

een belangrijke factor van invloed op het uiteindelijke schizofrenie risico in deze populatie is. Toekomstige studies naar de rol en functie van het eiwit dat gecodeerd wordt door PIK4CA kunnen meer inzicht verschaffen in de aard van de verstoring van biologische mechanismen die leiden tot schizofrenie.

In **hoofdstuk 8** wordt het uitgangspunt van dit proefschrift toegepast op een geheel andere structurele genomische afwijking. In dit hoofdstuk staat niet een gebied op chromosoom 22, maar een gebied op chromosoom 13 centraal. Het betreft een klinische en moleculair genetische studie van een normaal intelligente patiënt met autisme, en zijn familie. Uit nader onderzoek bleek zijn moeder, die geen autistische stoornis had, drager te zijn van dezelfde deletie op chromosoom 13. Een voor de hand liggende conclusie kan dan zijn dat de genomische afwijking in kwestie géén causaal verband houdt met het ziektebeeld, in casu autisme. Echter, een alternatieve verklaring is dat de patiënt behalve de deletie óók een mutatie heeft in één van de genen in het gebied van de deletie. Van die genen heeft de drager van deletie immers maar één kopie in plaats van de normale situatie van twee kopieën voor ieder gen. Indien in het enige kopie van een gen een mutatie optreedt kan daarvan het effect groter zijn dan indien dezelfde mutatie optreedt terwijl er nog een normale kopie van het gen aanwezig is.
In het gebied van de deletie op chromosoom 13 bleken 4 genen te liggen. Bij nader onderzoek van deze genen bleek dat de patiënt in één gen, DIAPH3, een mutatie te hebben. Deze mutatie was overigens ook aantoonbaar bij zijn (niet autistische) vader. In het artikel wordt de term “double hit” gebruikt om deze situatie te beschrijven; het is waarschijnlijk (maar niet bewezen) dat het autisme in de patiënt ten dele samenhangt met de gevolgen van de combinatie van de van zijn moeder overgeërfde deletie en de van zijn vader overgeërfde mutatie in DIAPH3.
Aanvullende onderzoeken toonden aan dat dit gen tot expressie komt in het muizenbrein tijdens de embryonale ontwikkeling. Daarnaast bleek het waarschijnlijk dat het door DIAPH3 gecodeerde eiwit in hetzelfde biologische mechanisme een rol speelt als het gen SHANK3 dat onlangs in verschillende studies naar voren werd gebracht als kwetsbaarheidsgen voor autisme[1, 2].
Deze aanvullende bevindingen verlenen verdere steun aan de conclusie dat er een causaal verband bestaat tussen de “double hit” in het gen DIAPH3 en de aanwezigheid van autisme in de patiënt.

*Conclusie*

De onderzoeken in dit proefschrift zijn uitgevoerd vanuit het uitgangspunt dat er belangrijke informatie kan worden vergaard door de studie naar de rol van structurele genomische afwijkingen en autisme en schizofrenie. De meeste, maar niet alle studies in deze dissertatie richten zich in het bijzonder op de genetische en klinische aspecten van het 22q11.2 deletie syndroom. De bevindingen van de studies bevestigen het voornoemde uitgangspunt en suggereren dat verdere studies volgens dit principe zeker de moeite waard zijn en een relevante bijdrage kunnen leveren aan ons begrip van de oorsprong van autisme en schizofrenie.

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Ik ben ontzettend dankbaar dat ik de mogelijkheden heb ontvangen en ook de gelegenheid krijg om me bezig te houden met wetenschappelijk onderzoek, iets dat ik beleef als een passie. In één baan deze passie mogen combineren met de medische zorg voor mensen met een psychiatrische stoornis is voor mij een droom die werkelijkheid is geworden.

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[1] Geen valse bescheidenheid maar een feit. Overigens ook géén reden tot zorg; als u dit boekje in handen heeft dan is er gerede kans dat de leescommissie van mening is dat mijn aandeel voldoende is geweest dat ik dit werk in het openbaar mag verdedigen.
[2] M.i. is dit een pleonasme.
3 Eigenlijk: *hep* zijn voordeel, maar het getrouwd zijn met een taalkundige schept bepaalde verplichtingen.

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[4] Dit dankwoord is géén goed voorbeeld van bondig schrijven, maar wordt dan ook niet in een wetenschappelijk tijdschrift gepubliceerd (gelukkig maar).
[5] "*In mijn artikelen geen Libelle-verdriet*" schreef je eens in de kantlijn van een alinea die je met twee grote halen had doorgestreept.

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[6] Prof. Dr. Emanuel from the Children's Hospital of Philadelphia told me that knowing that I was your protégé was what had convinced her to agree that I could come to her lab, "*what comes from Frits must be good*".
[7] Die waren er namelijk ook.
[8] Op een recent landelijk congres legde een onderzoeker / clinicus uit het Noorden des lands zijn werkzaamheden als volgt aan het publiek uit: "*ik ben een soort Wiepke Cahn van het Noorden*".

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[9] Tijdens deze vlucht overkwam ons ook de nachtmerrie van elke medicus: "*is there a doctor on board?"*. Het viel gelukkig mee; samen een huilende baby gekalmeerd en een fles champagne van de stewardess als dank. Waar is die fles trouwen gebleven, Wouter?

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[10] Het mag gerust een wonder genoemd worden dat ik in de afgelopen periode nog geen één keer onder de nood douche heb moeten staan.

Prof. Dr. Emanuel, dear Beverly. Thank you for giving me the opportunity to work with you in your excellent lab at the Children's Hospital Of Philadelphia. The two years in Philadelphia have been extraordinarily rich in positive experiences, both on a professional as on a personal level.
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A part from working in the lab, I was fortunate to spend a considerable amount of time doing clinical work in the department of Child and Adolescent Psychiatry of the Children's Hospital of Philadelphia. This would never have been possible if it weren't for the dedicated supervision of Dr. Tami Benton and Dr. Peter Meyer. Dear Peter, thank you for letting me have a good look in your kitchen; I learned much from your impressive clinical expertise, dedication and communicative skills with children. And besides that, it was just plain fun working with you. Kirstin Thode, thank you for your help with setting up the questionnaire studies; we will analyze those data when they become available, trust me.

Prof. Dr. Turetsky, dear Bruce, it was a real pleasure working with you and your transatlantic contributions have been very important to this dissertation. On a different level, I sincerely wish I will one day be able to have some of your ever so cheerful ways of dealing with excessively negative reviewer's comments[12].

[11] One important finding we did make, though not reported in this thesis, is that singing at the start of a PCR significantly improves the results (and lab atmosphere).
[12] Here is an example: "*Leaving aside reviewer #2's obsessive personality and the bee in his bonnet, it seems like much of this is just a matter of careful rewriting and editing*".

The PIK4CA study has only been possible because the psychiatry genetics research group, in particular Prof. Dr. Bassett en Dr. Chow, at the University of Toronto was willing to go along with our hypothesis and to provide the necessary patient material to perform the tests. Dear Anne, thank you for your trust and friendship. I truly look forward to more collaborative projects in the future.

Terugkomend uit de Verenigde Staten maakte ik in Nederland kennis met verschillende wetenschappers die vooral met betrekking tot de laatste hoofdstukken in dit proefschrift van groot belang zijn geweest. Prof. Dr. Burbach, beste Peter, veel dank voor je input en hulp bij het schrijven van het DIAPH3 artikel. Het is onder jouw leiding buitengewoon prettig werken in het onderzoeksteam Autisme & Genetica, bedankt voor je vertrouwen. Bert van de Zwaag ben ik veel dank verschuldigd voor het uitvoeren van de DIAPH3 expressie experimenten in de muis en voor zijn inhoudelijke bijdrage aan het artikel. Eric Strengman en Esther Janson voerden de sequencing experimenten uit, dank! Roel Ophoff wil ik graag bedanken voor zijn hulp bij het schrijven van verschillende artikelen en bij het submitten! Beste Roel, onze samenwerking is nog jong maar ik stel jouw gemakkelijke toegankelijkheid en bereidheid om mee te denken met onderzoeksproblemen of nieuwe ideeën nu al ontzettend op prijs. Ik verheug me enorm op het vervolg van onze samenwerking.

Albert van der Krabben en Rogier van Gelder, wat is er nou mooier dan je gesteund te weten door zulke goede vrienden? We hebben gezamenlijk al zoveel prachtige dingen meegemaakt[13], en er ligt nog zoveel moois in het verschiet. Dank voor jullie vriendschap.

Dear Reinhild and Max Muenke. There are no words that can express my gratitude for everything that you have done for me, us, during and after our stay in Bala Cynwyd. Your friendship is a real gift to us.

[13] Mijn eerste auto bezat ik samen met jullie; een zo! goed! als! nieuw! Ford Thunderbird (V8) uit 1980. De ratio [tijd zittend in de auto]:[tijd liggend onder de auto] was ongeveer 1, wat voor een eerste auto helemaal niet zo een gek resultaat is hoor.

Mijn lieve ouders, broers en zussen. Wat een voorrecht om op te mogen groeien in een warm en veilig nest. Om een goede kinderpsychiater te worden verwierf ik de benodigde vakkennis en ervaring tijdens mijn opleiding. Maar het meest noodzakelijke kreeg ik van jullie: een visie van de mens als uniek en geliefd wezen.

Très chers beaux-parents; j'ai une telle chance d'avoir rencontré Emmanuelle, et par elle, de vous avoir rencontré. Vôtre façon de vivre continue à être une source d'inspiration pour moi.

En dan nu de uitsmijter. Mijn lieve Emmanuelle, leven met jou is een groots en prachtig avontuur. Ik geloof niet er veel over zou blijven van wat in de bladzijden hiervoor gescheven staat als jij niet in mijn leven was geweest. Wat heb ik toch eindeloos gezwijnd[14] met jou. En dat je steeds weer, dag in dag uit, opnieuw kiest om met mij door te gaan…verbazingwekkend. Daar moet haast wel een bijzondere genetische aanleg aan ten grondslag liggen[15].
En met jou, door jou, wil ik nog maar eens uitspreken hoe intens trots ik ben op onze vier kinderen: Isaure, Alexis, Thijs en Fleur. Wat een rijkdom.

[14] Voor de linguisten onder ons: van het Duitse *"er hat Schwein gehabt"*. Niet gemakkelijk naar het Frans te vertalen, of eigenlijk wel gemakkelijk (*"Il a eu du sanglier")* maar dan al snel leidend tot misverstanden.
[15] De aanwezigheid van twee X chromosomen biedt in dit verband onvoldoende verklaring, weet ik uit ervaring.

# PUBLICATION LIST & CURRICULUM VITAE

**LIST OF PUBLICATIONS**

Jalali GR, **Vorstman** JAS, Errami A, Vijzelaar R, Biegel J, Shaikh T, Emanuel BS.
Detailed analysis of 22q11.2 with a high density MLPA probe set.
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Executive dysfunction in relation to behavioural problems in children and adolescents with 22q11.2 Deletion Syndrome.

Le Pichon-Vorstman EMM, **Vorstman** JAS**,** de Swart HE, van den Bergh H.
Communication Awareness in Young Multilingual Children: the Relevance of a Conscious Language Learning Experience

**CURRICULUM VITAE**

Jacob Vorstman werd op 17 mei 1972 geboren te Oss. Na zijn eindexamen gymnasium β aan het Jeroen Bosch College te 's Hertogenbosch (1990) woonde en werkte hij gedurende een jaar in l'Arche in Trosly-Breuil, Frankrijk. l'Arche is een internationale associatie van huizen waar vrijwilligers samen wonen en leven met mensen met een verstandelijke handicap. In dit jaar behaalde hij tevens het colloquium doctum scheikunde. Tijdens zijn studie en co-schappen geneeskunde aan de Vrije Universiteit te Amsterdam (1991 – 1998) deed hij in 1994 gedurende 8 maanden kinderoncologisch onderzoek aan het Alberta Children's Hospital in Calgary, Canada (onder leiding van Prof. Dr. M.J. Coppes). In 1996 volgde hij gedurende 3 maanden een deels klinische (co-schap) en deels onderzoeksstage aan de afdeling neuro-oncologie van het ziekenhuis La Pitié Salpêtrière in Parijs, Frankrijk (onder leiding van Prof. Dr. J.Y. Delattre).
Na zijn artsexamen (december 1998) werkte hij als assistent geneeskunde, niet in opleiding op de afdelingen neurologie (2 maanden) en spoed eisende geneeskunde (6 maanden) in het Flevoziekenhuis Almere, en op de afdeling kindergeneeskunde van het Waterlandziekenhuis Purmerend (8 maanden).
In september 2000 startte hij de opleiding psychiatrie en kinder en jeugdpsychiatrie aan het Universitair Medisch Centrum Utrecht, bij Prof. Dr. R.S. Kahn (psychiatrie) en Prof. Dr. H. van Engeland (kinder en jeugdpsychiatrie). In 2001 begon hij als Assistent Geneeskundige in Opleiding tot Klinisch Onderzoeker (AGIKO) met het onderzoek van dit proefschrift.
In het kader van dit onderzoek was hij van november 2004 tot juli 2006 werkzaam aan het Children's Hospital of Philadelphia, USA. Hij verrichtte moleculair genetische studies in het laboratorium van de divisie Human Genetics and Molecular Biology (onder leiding van Prof. Dr. B.S. Emanuel). Tevens deed hij klinisch werk aan de afdeling Child and Adolescent Psychiatry (onder supervisie van Dr. T. Benton en Dr. Peter Meyer).
Sinds 1 maart 2008 is hij als psychiater werkzaam bij de zorglijn psychotische stoornissen van het UMC Utrecht.

# APPENDIX

**Appendix A: genes in the 22q11.2 region**