From the DEPARTMENT OF WOMEN´S AND CHILDREN´S HEALTH

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AUTISM SPECTRUM DISORDERS – GENETIC AND NEURODEVELOPMENTAL ASPECTS IN CHILDREN WITH EARLY DIAGNOSIS

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ABSTRACT

The overall aim of the thesis was to describe the broad variability in neurodevelopmental profiles in preschool children with autism spectrum disorders (ASD) and to relate these findings to co-existing genetic conditions and other medical disorders. Children in the study were assessed with regard to neurodevelopmental characteristics, before and after intervention, and were recommended genetic testing including chromosomal micro-array. Data concerning parental and sibling neuropsychiatric conditions were also collected.

PAPER 1

The objective of the first study was to characterize ASD severity, general cognitive and language level and associated co-existing disorders in a population-based group of 208 preschool children (age 20-54 months at first assessment) with a clinical diagnosis of ASD, before the initiation of early intervention at the Autism Centre for Young Children, in Stockholm County. The study set up base-line data for a 2-year follow-up regarding outcome based on adaptive functions. Intellectual disability and developmental delay were found in a large proportion as well as hyperactivity. A regressive trajectory was found in one fifth and epilepsy in 6 %.

PAPER II

In the second study, certain prenatal risk factors were studied in the group of 208 preschool children with early-diagnosed ASD and the data were contrasted to the general population, using the Swedish Medical Birth register. Compared to the general population, fathers of children with ASD were older and parents more often of non-European origin. Mothers of children with ASD had an increased rate of antidepressant and psychoactive medication use, as well as of scheduled caesarean sections. At parental interview, information was also obtained regarding developmental and psychiatric disorders in the family. Fathers and brothers of children with ASD had high rates of ASD including the broader phenotype. Mothers of children with ASD had high rates of depression and other psychiatric disorders.

PAPER III

The third study reports all available medical information regarding the 208 children with ASD and preliminary results from genetic analyses. All children had received early intervention, intensive or non-intensive. Outcome at the two-year follow up was measured as change in adaptive function, according to Vineland composite score. A significant genetic or other medical condition was found in 18%. Epilepsy prevalence was now 8.6%. Children with a medical/genetic condition, including epilepsy, had been diagnosed with ASD at an earlier age than those without such conditions and the presence of an identified medical disorder correlated negatively with adaptive functioning outcome.

PAPER IV

In the fourth study, 162 of the 208 children with early-diagnosed ASD were analysed with chromosomal micro-array analysis to detect Copy Number Variants (CNVs) associated as risk factors for autism. Pathogenic aberrations were detected in 8.6 % of the patients, and in an additional 8.6 % variants of uncertain significance were present. CNVs were more frequent in children with congenital malformations or dysmorphic features as well as in children with intellectual disability in addition to ASD. Finally, we explored how parentally transmitted CNVs related to neurodevelopmental and psychiatric conditions in the parents. There was a trend towards increased rates of neurodevelopmental and psychiatric conditions in mothers transmitting a potentially pathogenic CNV compared to the mothers of children where no CNV was detected.

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Developmental profiles in preschool children with autism spectrum disorders referred for intervention
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IV. **Eriksson M**, Lieden A, Westerlund J, Bremer A, Sahlin E, Gillberg C, Fernell E, Anderlid B-M
Copy number variants in children with early diagnosed autism spectrum disorders
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I. Turk J, Bax M, Williams C, Amin P, **Eriksson M**, Gillberg C
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Am J Med Genet B Neuropsychiatr Genet, 2013,162:388-403

To Sylvia, Markus and Cecilia

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LIST OF ABBREVIATIONS

ABA	Applied Behavioral Analysis
ABC	Autistic Behaviour Checklist
ACYC	Autism Centre for Young Children
AD	Autistic Disorder
ADHD	Attention Deficit Hyperactivity Disorder
APA	American Psychiatric Association
Array-CGH	Array-based Comparative Genomic Hybridization
ASD	Autism Spectrum Disorder
ASDs	Autism Spectrum Disorders
AST	Autismspektrumtillstånd
Bp	Base pair
CDI	Communicative Development Inventories
CDKL	Cyclin-dependent kinase-like
CHC	Child Health Care center
CI	Confidence Interval
CMA	Chromosomal Microarray
CNS	Central Nervous System
CNTNAP	Contacting Associated Protein-like
CNV	Copy Number Variant/Variation
CNVs	Copy Number Variants
DGV	Database of Genomic Variants
DNA	Deoxyribonucleic acid
DSM	Diagnostic and Statistical Manual of Mental Disorder
DQ	Developmental Quotient
DZ	Dizygotic
EIBI	Early Intensive Behavioral Intervention
FISH	Fluorescent *in situ* hybridization
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X syndrome
FMR	Fragile X Mental Retardation
ICD	International Classification of Diseases
ID	Intellectual Disability
IQ	Intelligence Quotient
Kb	Kilo base pairs
Mb	Mega base pairs
MECP2	Methyl CpG binding protein 2
MLPA	Multiplex Ligation-dependent Probe Amplification
mtDNA	Mitochondrial DNA
mRNA	Messenger RNA
MZ	Monozygotic
NLGN	Neuroligin
NRXN	Neurexin
OR	Odds Ratio
PCR	Polymerase Chain Reaction
PDD-NOS	Pervasive Developmental Disorder – Not Otherwise Specified
POLG	Polymerase (DNA Directed) Gamma
RCT	Randomized Control Trial

RNA	Ribonucleic acid
SHANK	SH3 and multiple ankyrin repeat domains
SMBR	Swedish Medical Birth Register
SNV	Single nucleotide variant
SNVs	Single nucleotide variants
SNP	Single nucleotide polymorphism
SNPs	Single nucleotide polymorphisms
SSRI	Selective Serotonin Reuptake Inhibitor
TEACCH	Treatment and Education of Autistic and related Communication handicapped CHildren
TSC	Tuberous Sclerosis Complex
VABS-II	Vineland Adaptive Behavior Scale
VOUS	Variant of Unclear Clinical Significance

1 INTRODUCTION AND BACKGROUND

Autism Spectrum Disorders (ASDs) represent a clinically and aetiologically highly heterogeneous group of neurodevelopmental conditions characterized by qualitative impairments in reciprocal social interaction and communication and by restricted, repetitive, stereotyped interests and behaviours. There is a considerable variation in clinical symptoms in individuals within the autism spectrum. In many individuals with ASD there are co-existing disorders, such as intellectual disability (ID), ADHD and other psychiatric conditions. The severity of social and communicative impairment varies greatly as well as the cognitive level. Intellectual disability is reported in approximately 70% of children with autistic disorder [1-3]. Within the total autism spectrum, ID is reported in ~50 % in preschool children [3] and in ~15% in school children [4]. Language and communication impairments in ASD include a spectrum varying from severe impairments with extremely low verbal skills to impairments in pragmatic language skills as well as those with more than average language capacity.

Definitions and classification of autism spectrum disorders

In 1943 Leo Kanner published a paper describing 11 cases, "Autistic disturbances of affective contact" [5]. In 1944, Hans Asperger described four male cases with similar but less severe impairments and with higher cognitive function [6]. In the earliest Diagnostic and Statistical Manuals of Mental Disorders (DSM-I 1952 and DSM-II 1968) autism was considered to belong to the childhood onset schizophrenia group. Between the 1950s and the 1970s, autism was assumed to be caused by psychogenic factors and due to poor parenting. The criteria for childhood autism proposed by Michael Rutter in 1978 influenced the DSM III (1980) and infantile autism was no longer included among the childhood schizophrenia disorders.

In the DSM-IV, autism conditions included Autistic disorder (AD), Pervasive developmental Disorder Not Otherwise Specified, (PDD-NOS), Asperger Syndrome and Childhood Disintegrative Disorder [7]. The WHO ASD classification (International Classification of Diseases, ICD-10, 1992), is commonly used in the Swedish Hospital and Medical diagnostic registers [8]. In ICD-10, the term PDD-NOS is replaced by atypical autism.

Autistic disorder is usually considered to be the most severe form with onset before 3 years of age. Pervasive developmental Disorder Not Otherwise Specified or Atypical Autism requires fewer criteria and can be regarded as a less severe form of ASD than AD. In Asperger syndrome the criteria includes significant impairment in social interaction and restrictive and repetitive interest and behaviour.

In the newly launched DSM-5, there is one single umbrella term - Autism Spectrum Disorder, officially recognizing what has been the de-facto term in recent years**.** Asperger syndrome and PDD-NOS are excluded, and are no longer diagnostic entities. The rationale for these major changes from categorical to dimensional criteria was influenced by a US multisite study that demonstrated that "clinical distinctions among

categorical diagnostic subtypes of autism spectrum disorders were not reliable even across sites with well-documented fidelity using standardized diagnostic instruments" [9].

Current DSM-5 criteria now include impairments in two major domains - Persistent deficits in social communication and social interaction and restricted, repetitive patterns of behaviour, interests or activities. The diagnostic practice requires specification of additional information including severity level of ASD, level of adaptive functioning and other important features (such as presence or not of known genetic disorder, epilepsy, and intellectual disability). Symptoms must be present in early childhood (but may not become fully manifest until social demands exceed limited capacities) and limit and impair everyday functioning [10].

Diagnostic Criteria for Autistic Disorder DSM IV

A. A total of six or more items from (1), (2), and (3), with at least two from (1), and one each from (2) and (3):

(1) Qualitative impairment in social interaction, as manifested by at least two of the following:

(a) marked impairment in the use of multiple nonverbal behaviours such as eye-to-eye gaze, facial expression, body postures, and gestures to regulate social interaction

(b) failure to develop peer relationships appropriate to developmental level

(c) lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest)

(d) lack of social or emotional reciprocity

(2) Qualitative impairments in communication as manifested by at least one of the following:

(a) delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)

(b) in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others

(c) stereotyped and repetitive use of language or idiosyncratic language

(d) lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

(3) Restricted repetitive and stereotyped patterns of behaviour, interests, and activities, as manifested by at least one of the following:

(a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

(b) apparently inflexible adherence to specific, non-functional routines or rituals

(c) stereotyped and repetitive motor manners (e.g., hand or finger flapping or twisting, or complex whole-body movements)

(d) persistent preoccupation with parts of objects

B. Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years: (1) social interaction, (2) language as used in social communication, or (3) symbolic or imaginative play.

C. The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder.

Diagnostic Criteria Pervasive Developmental Disorder Not Otherwise Specified (Including Atypical Autism) DSM IV

Severe and pervasive impairment in the development of reciprocal social interaction associated with impairment in either verbal or nonverbal communication skills or with the presence of stereotyped behaviour, interests, and activities, but the criteria are not met for a specific Pervasive Developmental Disorder, Schizophrenia, Schizotypal Personality Disorder or Avoidant Personality Disorder.

Diagnostic Criteria for Asperger's Disorder DSM IV

A. Qualitative impairment in social interaction, as manifested by at least two of the following:

a) marked impairment in the use of multiple nonverbal behaviours such as eye-to eye gaze, facial expression, body postures, and gestures to regulate social interaction

b) failure to develop peer relationships appropriate to developmental level

c) lack of spontaneous seeking to share enjoyment, interests, or achievements with other people (e.g., by a lack of showing, bringing, or pointing out objects of interest to other people)

d) lack of social or emotional reciprocity

B. Restricted repetitive and stereotyped patterns of behaviour, interests and activities, as manifested

a) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity of focus

b) apparently inflexible adherence to specific, non-functional routines or rituals

c) stereotyped and repetitive motor mannerisms (e.g., hand or finger flapping or twisting, or complex whole-body movements)

d) persistent preoccupation with parts of objects

C. The disturbance causes clinically significant impairment in social, occupational, or other important areas of functioning.

D. There is no clinically significant general delay in language (e.g., single words used by age 2 years, communicative phrases used by age 3 years).

E. There is no clinically significant delay in cognitive development or in the development of age-appropriate self-help skills, adaptive behaviour (other than in social interaction), and curiosity about the environment in childhood.

F. Criteria are not met for another specific Pervasive Developmental Disorder or Schizophrenia.

DSM-5 Criteria for Autism Spectrum Disorder

An individual must meet criteria A, B, C and D:

A. Persistent deficits in social communication and social interaction across contexts, not accounted for by general developmental delays, and manifest by all 3 of the following:

1. Deficits in social-emotional reciprocity; ranging from abnormal social approach and failure of normal back and forth conversation through reduced sharing of interests, emotions, and affect and response to total lack of initiation of social interaction.

2. Deficits in nonverbal communicative behaviours used for social interaction; ranging from poorly integrated- verbal and nonverbal communication, through abnormalities in eye contact and body-language, or deficits in understanding and use of nonverbal communication, to total lack of facial expression or gestures.

3. Deficits in developing and maintaining relationships, appropriate to developmental level (beyond those with caregivers); ranging from difficulties adjusting behaviour to suit different social contexts through difficulties in sharing imaginative play and in making friends to an apparent absence of interest in people.

B. Restricted, repetitive patterns of behaviour, interests, or activities as manifested by at least two of the following:

1. Stereotyped or repetitive speech, motor movements, or use of objects; (such as simple motor stereotypies, echolalia, repetitive use of objects, or idiosyncratic phrases).

2. Excessive adherence to routines, ritualized patterns of verbal or nonverbal behaviour, or excessive resistance to change; (such as motoric rituals, insistence on same route or food, repetitive questioning or extreme distress at small changes).

3. Highly restricted, fixated interests that are abnormal in intensity or focus; (such as strong attachment to or preoccupation with unusual objects, excessively circumscribed or perseverative interests).

4. Hyper-or hypo-reactivity to sensory input or unusual interest in sensory aspects of environment; (such as apparent indifference to pain/heat/cold, adverse response to specific sounds or textures, excessive smelling or touching of objects, fascination with lights or spinning objects).

C. Symptoms must be present in early childhood (but may not become fully manifest until social demands exceed limited capacities)

D. Symptoms together limit and impair everyday functioning

Prevalence of ASD

The broad spectrum of ASDs is relatively common, affecting approximately 0.6-1% of the childhood population [1, 11, 12]. However, the prevalence of the most severe Autistic Disorder is often estimated 1/300-1/500 (contributing to 20-40% of all ASD). The prevalence of Asperger syndrome is often estimated to be 0.1-0.5 % [4]. ASDs are much more common than previously believed. In the 1960-80´s ASDs were probably underreported and a dramatic increase of the ASD prevalence has been reported in recent decades – in US from 5 per 10, 000 in 1980 to 1/88 in 2008 as reported in the latest survey from Centers for Disease Control [13]. Alterations and widening of diagnostic criteria have contributed to more children being diagnosed with ASD. The increase of the ASD prevalence may also partially be explained by milder cases with ASD, more often diagnosed as Asperger Syndrome or recognition of PDD-NOS, whereas the estimated prevalence of autistic disorder has not changed to the same extent. In the US study, relatively more children with ASD without ID are currently being diagnosed [13]. Several other, additional factors also contribute to this change of ASD prevalence rates; increased awareness of ASD among professionals and among parents in the general public, rapid expansion of autism diagnostic services, diagnostic substitution or diagnostic transfer (children with intellectual disability/learning disorder or specific language disorder/ADHD, increasingly diagnosed with ASD) and diagnostic accretion (children with ID and children with neurological disorders and syndromes also diagnosed with ASD).
In a review of prevalence studies by Isaksen et al 2013, the authors discussed that reported prevalence rates have been highly variable (4-120 per 10,000 in the period 1966 to 2012) and that various epidemiological methods influence the estimates. During the last decade there has been a stabilization of the ASD prevalence [14]. Three recent studies have been published in Sweden. The prevalence for all 2-year-olds in Gothenburg (2010) diagnosed with ASD was 8 in 1,000 [12]. The ASD prevalence in 6 year-olds born in 2002 in Stockholm was 6.2 per 1,000 [15]. In a large register based study the overall prevalence of ASD in Stockholm 2007 was 11.5 per 1,000 ranging from 6.5 per 1,000 among children aged 4–6years to 14.6 per 1,000 among those aged 13–17 years [16].

ASD and gender differences

In previous and recent studies, males are consistently more often diagnosed with ASD. Higher sex ratios are found in studies based on clinically ascertained ASD cases and lower ratios are found in epidemiological population screening studies. A gender ratio of about 3-4:1 is a commonly referred consensus [17-20]. In children with ASD and IQs in the normal range, the male to female ratio is often reported to be even higher (6-15:1) [21], whereas in those with moderate to severe intellectual disability the ratio is lower, in the range of 2:1 [17, 22]. Also, other neurodevelopmental disorders (ID, ADHD, speech- and language impairment) show a male bias [23-26]. In the Stockholm County, a clinical case study estimated the ASD prevalence in 6 year old boys to 10 in 10, 000 and in girls 2 in 10,000 [15]. In the Stockholm County large register study the male: female ratio was overall 2.6:1 and similar for ASD cases with or without ID. However, at age 8 years the ratio was 5.1:1 and 1.9:1 at age 18. A delayed ASD

diagnosis in females may contribute to the decline in sex ratio at older age [16]. Males with ASD, more often than females display externalizing behaviour problems (such as aggression and hyperactivity) while females with ASD more often are affected by internalizing symptoms such as anxiety and depression [27] and show less stereotype and repetitive behaviour [28]. The skewed sex ratio may be explained by under-ascertainment in females. However, a recent large study of dizygotic twins showed that siblings of autistic females had significantly more autistic impairments than siblings of males with ASD. The results suggests that female sex protects girls from autistic impairments and that girls may require greater familial etiologic load to manifest the phenotype [29]. Studies of chromosomal structural variation (CNVs) as well as studies of single nucleotide variants (SNVs) have found that more female ASD cases than males carry *de novo* copy number variants and higher rates of SNVs. The CNVs identified in females are found to affect more genes than those found in males [30-34]. One conclusion is that females are protected from the effects of heritable and de-novo ASD risk variants [35]. Higher penetrance of *de novo* mutations in males than females may contribute to the higher prevalence of ASD in males [36]. An example of male-biased difference in penetrance was identified in a study of inherited *SHANK1* mutation where males had high functioning autism while female carriers of the same rare mutation displayed anxiety but not ASD [37]. Several X-linked genes associated with ID are also found to be ASD risk genes (*FMRP, MECP2, NLGN3, NLGN4*) and sex chromosome aneuploidies are associated with ID and increased ASD risk [35]. In 2002, Baron-Cohen formulated the extreme male brain theory of autism [38]. Subsequent studies have shown correlation between foetal testosterone levels and autistic traits [39] and negative correlation with empathizing [40]. Increased levels of testosterone have been reported in females with Asperger´s syndrome [41]. Poelmans et al. reported that a large number of ASD candidate genes are involved in three major signalling protein networks – one of the networks regulating the production and metabolism of the steroid hormones [42].The skewed sex ratio reflects true differences in ASD liability and also that female ASD phenotypes are not adequately identified and diagnosed [43].

ASD aetiologies and risk factors - overview

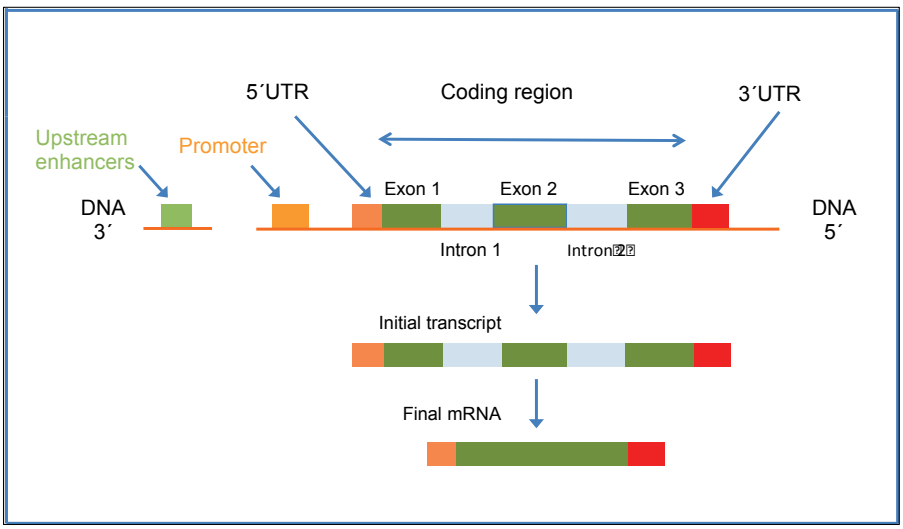
ASDs are the neurodevelopmental disorders with the most clearly documented influence of genetic factors [44]. However, despite the convincing genetic basis for ASDs, a definite cause remains unknown in most individual cases. Single gene disorders and larger chromosomal rearrangements are found in 10-20 % of cases with ASD with the genetic methods used today[4]. However, the majority of ASDs are probably caused by complex interactions between multiple genes (oligo- and polygenic models). Environmental and epigenetic factors modulate gene expression and variation in penetrance and expressivity of influential genes add to the broad diversity of clinical presentations [45]. Several pre- and perinatal factors have been associated with an increased ASD risk, however all these risk factors are also linked to other impairment in neurocognitive outcome [46]. In a recent meta-analysis it was demonstrated that most perinatal and neonatal factors examined, have shown inconsistent results and the preponderance of findings overall have not been statistically significant [47]. Various methodologies, diagnostic practises and case selection explain inconsistent results in some studies. In a recent study comparing maternal conditions and perinatal

characteristics in cases with ID and cases with ASD with or without ID, only weak association was found between perinatal risk factors and ASD without ID [48]. It has been suggested by Dodds that among individuals with low genetic ASD susceptibility, maternal and some obstetric factors may have an independent role in autism aetiology whereas among genetically susceptible children, these factors are of less importance [49].

Genetics: background and human genetic variation

The human haploid genome contains 3 billion base pairs and around 20 500 genes. The protein-coding regions, the genes, consist of exons, introns and regulatory elements. On average a protein is coded by 10 exons, each including approximately 300 base pairs. Transcription is the first stage in the protein synthesis. The gene is transcribed to initial RNA (primary transcript), a continuous RNA copy of the gene with both exons and introns. The initial mRNA is spliced – the intronic sequences are removed and the exonic transcripts are joined together. The mature mRNA is transported to the cytoplasm where it acts as the template for translation – the protein synthesis. Through the process of alternative splicing, joining the exons in multiple combinations a gene may code for multiple proteins. Transcription of DNA is regulated by the degree of DNA methylation and histone modification (histones are proteins around which DNA is wound to form chromatin). Longrange chromatin interactions also play a role in the regulation of the genes [50].

Figure 1



The ENCODE project has shown that ~80 % of the DNA outside the protein-coding regions is linked to biochemical functions. The space between the genes is filled with enhancers (regulatory DNA elements), promoters (the sites at which DNA´s transcription into RNA is initiated) and numerous regions that encode RNA transcripts not translated to proteins but with regulatory roles [50].

It is estimated that more than half of the human genes are expressed in the brain. The transcription of these genes varies over time and within different brain areas. The greatest regional differences occur during the prenatal development.

Human genomes vary as a result of different DNA changes - mutations. These alterations can be classified as sequence or structural variants and usually arise by errors is the gametogenesis – the formation (meiosis) of germ-line cells (sperm/egg). Mutations also occur during somatic cell division (mitosis), either during embryogenesis, in the foetus or later in life.
More than 50 million different single nucleotide variations (SNVs) are catalogued within the human genome (dbSNP database). SNVs that occur in > 1% of the population are referred to as Single Nucleotide Polymorphisms (SNPs) and are part of the normal variation. A point mutation is defined as substitution of a base pair and may occur in coding, regulatory and splicing relevant regions. Indels are insertions and deletions of 1-10 000 base pairs. Mutations also include missing or duplicated chromosomes (aneuploidy) and structural variations such as translocations, deletions, duplications, inversions and complex rearrangements. Other genetic aberrations linked to human disease include DNA triplet repeat expansions, imprinting errors, X-inactivation variants and mosaicisms [51]. More than 120 000 mutations are listed in the Human Gene Mutation Database.

The genetic code defines how one of 64 possible triple nucleotide base combinations (codons) code for one of twenty specific amino acids, start of protein synthesis or stop of translation. A single base substitution within a coding region may lead to an unchanged protein due to the existence of more than one codon coding for a specific amino acid (silent or synonymous mutation). Alternatively it may substitute one amino acid (missense mutation), cause a premature chain termination by introducing a stop signalling codon (non-sense mutation) or a frame-shift mutation where all amino acids after the mutation are substituted and often resulting in a non-functional protein. Frame-shift mutations are often caused by indels that are not multiples of three base pairs in length [51].

Mutations may be classified according to their effects on protein function. Reduced amount or activity of an encoded protein is defined as loss-of-function. Gain- of-function mutations lead to an increased amount or activity of the protein.

Copy Number Variants

A recent and important development in human genetics is the discovery of structural variations, referred to as Copy Number Variants (CNVs), changing the chromosomal architecture and occurring both in phenotypically normal and in diseased subjects. Copy Number Variants (CNVs) are defined as submicroscopic structural variations and with an abnormal number of copies of a DNA-segment (deletions and duplications) and with a size larger than 1000 base pairs. CNVs occur in the normal population and play an important role in the human genetic variation. Human genome variation is explained by single nucleotide polymorphisms (~0.1%) and at a structural level where CNVs contribute to another ~0.1% [52]. CNVs have been found to contribute to a greater percentage of the total number of base pairs differing between normal genomes than

SNPs [53]. On average, each human have > 1000 CNVs, accounting for ~4 million base pairs of difference [52]. CNVs often encompass large regions, affecting more than one single gene. CNVs may have great phenotypic impact by altering gene dosage, disrupting coding sequences or deregulating gene expression. Several CNVs have been found to be associated with diverse neurodevelopmental and psychiatric disorders. CNVs may be inherited from one or both parents (transmitted) or occur *de novo*. A CNV may affect one or several genes on the paternal or maternal alleles (heterozygous) or, rarely, affect both the paternal and maternal alleles in the corresponding region (homozygous). Different CNVs exhibit different penetrance, exemplified by the observation of identical CNVs in healthy controls as in affected individuals. Some CNVs demonstrate variable expression and can result in several disorders or phenotypes (e.g. ASD, ID, schizophrenia and bipolar disorder). In many complex disorders CNVs have identified hot regions with candidate genes and enabled targeted DNA sequencing. Many of these ASD associated genes have been associated as risk factors for other psychiatric and neurodevelopmental disorders, such as deletion 22q13.3 (Phelan-McDermid Syndrome) disrupting the *SHANK3* gene [54-58], deletion 2p16.3, disrupting the *NRXN1* gene [59-62], deletion 7q35-q36, disrupting the *CNTNAP2* gene [63-65] and the postsynaptic neuroligins *NLGN3* and *NLGN4X* located on the X chromosome [66].

Genetic technologies

Standard clinical genetic testing in children with ASD has previously included conventional karyotyping and molecular testing for Fragile X. In children with ASD karyotyping identifies abnormalities in about 2-5% [67, 68] and Fragile X syndrome is identified in about 2% [69]. The resolution of conventional karyotyping is limited compared to new technologies such as chromosomal microarrays. However, karyotyping identifies balanced chromosomal rearrangements, undetectable by micro-array methods and the contribution of balanced translocations or inversions in ASD is approximately 0.4% [70].

Linkage and genome wide association studies

Linkage studies of ASD, using multiplex families, identify shared genetic regions that can be linked to the condition between affected members. Alleles close together on the same chromosome have a high tendency to be transmitted together as an intact unit. Using a large number of genetic markers covering the whole genome, fine mapping of a specific region of interest is possible. Linkage studies have identified ASD risk conferring regions on all chromosomes but only a few regions have been repeatedly replicated. In genome-wide association studies genetic markers are used to compare the rates in unrelated affected with matched controls. The dominant technology that is use of high resolution SNP-arrays. To date, the six Genome Wide Association Studies for ASD that have been published implicate ~200 ASD candidate genes [42].

Comparative genomic hybridization

Chromosomal microarrays or array-CGH , enables a whole genome analysis of gains or losses of DNA segments. Several different platforms exist, based on the type of DNA-probes used. The resolution of the method depends on the number of probes and the distance between them, increasing the resolution 100-fold compared to conventional karyotyping.

Classification and interpretation of Copy Number Variants

To evaluate the clinical significance of rare CNVs, the development of public databases have been essential. The Database of Genomic Variants (DGV (http://projects.tcag-.ca/variation/) catalogues putatively benign CNVs in the general population. Several datasets catalogues putatively pathogenic CNVs (e.g. DECIPHER (http://decipher.-sanger.ac.uk/), SFARI (gene.sfari.org), AutismKB (http://autismkb.cbi.pku.edu.cn).

The interpretation of CNVs should be done according to established guidelines [71]. CNVs can be classified as pathogenic, with uncertain clinical significance or as benign. The size of a CNV must be considered, although even large CNVs can be benign and small CNVs can be pathogenic. *De novo* CNVs and large CNVs > 3-5 Mb are more likely to be pathogenic. In addition, the genomic content of a CNV is important. The interval may contain gene-rich regions and genes previously reported in association with a disorder or encompass a region void of genes. A CNV is categorized as benign if it is reported in multiple publications or in databases and the nature of the CNV is well characterized or represents a common CNV polymorphism (occurring in > 1% of the population). CNVs documented as clinically significant in multiple peer-reviewed publications are classified as pathogenic. Inherited CNVs are more difficult to classify. The CNV may be pathogenic, but not in the unaffected carrier parent due to incomplete penetrance. The carrying parent may have milder subclinical features within the spectrum of the disorder due to variable expressivity. Imprinting effects may add to the complexity. A second mutation not detected by microarray may be present on the corresponding allele or other genetic modifiers (second-hit model) may be present in the probands but not in the unaffected parent. Mosaicism for the CNV in the unaffected parent may be of importance. An unaffected mother may transmit an X-linked CNV to an affected son. In females, phenotypic variation may result due to the possibility of skewed X-inactivation [71].

CNVs of uncertain clinical significance include aberrations, for which at the time being, there is insufficient evidence to determine whether it is pathogenic or benign. A CNV of uncertain clinical significance may be pathogenic if it encompasses a gene, with a function is relevant to the phenotype, even if there are no other reports of similar cases [71].

Microarray platforms

SNP-array

A SNP array platform has a large number of probes with segments of reference DNA (25 Bp oligonucleotide sequences). For each SNP, the array has a matching probe for the two possible alleles. The test DNA is purified, digested and labelled with a fluorochrome and hybridized to the microarray. DNA fragments containing a SNP binds to their allele-specific perfect match probes. After washing the array is scanned for fluorescent signals reflecting the DNA copy number and information on the SNP alleles in the genotype. Due to linkage disequilibrium, the tendency of chromosomal segments to be inherited as blocks (haplotypes), thus single markers (SNPs) can be used to predict the genotype of the surrounding region.

Oligonucleotide-array

Oligonucleotide-arrays use sequences sized 60-80 base pairs as probes. Patient-DNA and reference DNA are labelled with differently coloured fluorochromes and are hybridized to slides with a large number of probes covering the whole genome. The slides are scanned and the ratio between the two fluorescence signals is measured. Loss of a DNA segment in the test DNA will result in an increased signal from the reference DNA and gain will result in an increased signal from the test DNA. By use of 180 000 or 244 000 oligonucleotide probes it is possible to detect changes in copy number of segments sized (30)-50 Kb.

CNV calls can be validated using alternative methods – multiplex ligation-dependent probe amplification (MLPA), FISH or quantitative PCR.

Whole exome and whole genome sequencing technologies

By sequencing it is possible to detect variations in a single base pair. In conventional sequencing single genes are analysed. During the last years, high-throughput DNA sequencing has become faster and much cheaper. Whole exome or whole genome sequencing is now available but the analysis of enormous amounts of data is still time consuming why it is difficult to use the methods in large cohorts. However, by the use of this methodology it is possible to analyse 100-200 selected genes (panels) in larger cohorts.

Genetics and heritability in ASD

Albeit the robust evidence for genetic factors in ASD, a specific genetic aberration can be detected in less than 20-30% of individuals with ASD with the current available technologies. The complex patterns of inheritance display an extreme heterogeneity and various distinctive genetic models contribute to explain the genetic background of subsets of individuals with ASD. Despite the high heritability most genetic findings linked to ASD causality are *de novo* genetic aberrations, i.e. not inherited.

Familial aggregation, twin and adoption studies clearly indicate that genetic factors highly contribute to the risk for ASD. In the last decade a large number of studies on ASD and autistic traits in twins have been published. Heritability (defined as how much of the variance is explained by shared genetic factors) may be calculated comparing the concordance in monozygotic (MZ), identical, and dizygotic (DZ), fraternal twins. Twin studies are also used to estimate the degree of environmental influences and whether the environmental influences are shared or non-shared.

Twin studies

Early twin studies estimated the autism concordance rate in identical twins (monozygotic=MZ) at 36-91% and in fraternal twins (dizygotic=DZ) at 0%. [72-74]. More recent studies have replicated similar high ASD concordance in MZ twins. However, they report substantially higher concordance in DZ twins than in earlier studies. The heritability estimates in the earlier studies ranges between 60-90% [75-77]. The median values for broad ASD concordance were 88% for MZ twins and 31% for DZ twins in three new studies reviewed by Ronald and Hoekstra [78]. Hallmayer et al. (2011) reported conflicting results in their study of 192 twin pairs. The concordance for strict autism in male MZ twins was 0.58 vs. 0.21 in DZ twins and in female MZ twins; the concordance was 0.60 vs. 0.27 for DZ twins. The authors concluded that a) earlier studies have underestimated the concordance in DZ twins b) susceptibility to ASD has a moderate genetic heritability and a substantial shared twin environment component [79]. No twin study has shown complete concordance in MZ twins and prenatal/early postnatal epigenetic, stochastic and environmental factors impact susceptibility to ASDs.

Autistics like traits have been shown to occur in a smooth normal distribution in the general population to the clinical extremes [80, 81]. The concordance rates for autistic traits in twins in the general population were estimated in a UK study. The results showed high heritability; concordance for same-sex MZ males was 0.78 vs. 0.26 in DZ same-sex males. Thus, heritability for autistic traits in males was 72% and 53% in females. The study indicated evidence of similar aetiology across normal variation and the extreme scoring groups [82]. A nationwide twin study, by Lundström et al. demonstrated an aetiological similarity between ASD and autistic like traits in the normal variation and suggested that ASD and autistic like traits are aetiologically linked [83]. In addition, studies have shown increased rates of a broad variety of neurodevelopmental disorders in ASD twins (ADHD, developmental motor disorder and tic disorder [75].

Sibling studies

The recurrence risk for siblings has probably been underestimated in earlier studies with estimates between 3 and 10 %. Constantino et al, 2010, reported a recurrence risk for a traditionally defined ASD to be 10.9 % and that another 20 % of siblings had a history of language delay, one half off whom exhibited autistic qualities of speech [84].

In a baby sibling study, Ozonoff et al. reported an overall recurrence frequency of 18.7 % (26 % for infant sibling boys and 9% for infant sibling girls) [85]. Three recent studies have estimated roughly a 25-fold increased risk for ASD in siblings [77, 84, 85].

Genetic findings in ASD - overview

In about 10 % of individuals with ASD a monogenic disorder can be identified. Most common are Fragile X syndrome, tuberous sclerosis and Rett syndrome. There are numerous other genetic disorders where a subset of affected individuals also present with co-existing ASD; Angelman syndrome, Neurofibromatosis type I, CHARGE syndrome, Moebius syndrome, Cohen syndrome, Smith-Lemli-Opitz syndrome, Timothy syndrome, Soto's syndrome and 22q11 deletion syndrome. ASD is additionally found in individuals with larger chromosomal aberrations such as trisomy 21, Turner syndrome and Klinefelter syndrome).

Fragile X syndrome

Fragile X syndrome (FXS) affects about 1/4000 males and 1/6000 females. FXS is the second most common genetic cause of ID after trisomy 21. About 25% of FXS males and 6% of FXS females have autistic features [4]. In individuals with ASD, FXS is found in about 2% [69]. The molecular basis of FXS is an abnormal number of trinucleotide repeats (CGG sequences) in the *FMR1* gene on the X chromosome (Xq27.3). Normally there is an average of 30 tandem repeats and individuals with Fragile X have more than 200 repeats [4]. The excessive number of repeats produce abnormal methylation of the *FMR1* gene and transcription silencing followed by decreased levels of Fragile X Mental Retardation Protein (FMRP). The decreased FMRP levels affects important signalling pathways in the brain and interferes with dendritic maturation and synaptic plasticity.

Tuberous sclerosis

Tuberous sclerosis complex (TSC) affects about 1/6 000. Inactivating mutation in either the *TSC-1* gene, located on chromosome 9q34 or the *TSC-2* gene, located on chromosome 16p13.3, causes TSC. The neurological manifestations of TSC include infantile spasms, intractable epilepsy, cognitive disabilities, and autism. Half of the children with TSC have ID and 25-40 % have autistic features [86]. In individuals with ASD, TSC is found in 1-3% [4]. Children with TSC and autism display more global cognitive impairment than those with TSC without autistic features. The disorder is characterized by the presence of hamartomas (tumor-like lesions) in multiple organs, including the brain. Epilepsy is common as well as a wide range of behavioural problems. Early onset epilepsy and infantile spasms have been associated with significant risk for ID and ASD [87].

Rett syndrome

Rett syndrome is a genetic disorder affecting about 1/10 000 girls. Among girls with ASD, Rett syndrome has been reported in about 3%. Rett syndrome is caused by mutations in the *MECP2* gene on chromosome region Xq28. The gene encodes for MeCP2 (Methyl-CpG-binding protein) that affects the transcription of a large number of genes. Loss (and gain) of function of MeCP2 leads to a severe clinical condition that present in different variants. Girls with the most typical Rett variant have an apparently normal development during the first 6-18 months. A loss of function/regression occurs with impairment of language and social interaction. There is partial or complete loss of spoken language and loss of purposeful hand skills. Other main criteria include gait abnormalities and stereotypic, often characteristic hand movements. Many have a subnormal head growth resulting in microcephaly. Epilepsy is very common (~60%), with onset about 2-3 years of age and with an improvement of seizures after puberty [88].

ASD and Copy Number Variants

Microarray studies have demonstrated increased rates of CNVs in individuals with ASD (5-15 %) compared to healthy controls (1-2%) [30, 32, 89-96]. These increased rates occur both as sporadic (*de novo*) mutations and as inherited CNVs, often from an apparently healthy parent. An overall increased burden of large CNVs has also been documented in individuals with intellectual disability, schizophrenia and bipolar disorder [52]. CNVs in individuals with ASD are more numerous and contain more genes than compared to controls. Females with ASD, compared to males have more and larger CNVs. In individuals with ASD in combination with ID and/or dysmorphic features more CNVs are detected.

ASD and coding sequence variants

Sequencing of candidate genes has implicated a large number of mutations that appears to be sufficient to cause ASD. In 4-8 % of individuals with ASD, rare highly penetrant mutations (Single Nucleotide Variants) can be identified [33, 34, 97, 98]. The average rate of *de novo* coding-sequence variants, in these studies did not significantly differ between individuals with ASD compared to unaffected siblings and controls. However, a statistically significant difference was found when the analysis was restricted to genes expressed in the brain [99]. Approximately 65 ASD causing mutations were identified in an exome sequencing study by Iossifov et al, and the authors estimate 350-400 autism susceptibility genes. Females with ASD have been found to carry more gene disrupting mutations [100]. *De novo* SNVs as well as *de novo* CNVs often combine with other risk factors [33]. Higher rates of *de novo* mutations in older fathers has recently been documented [101]. Lim et al reported that a minor fraction of ASD cases is explained by double loss of gene function caused by inherited homozygous and compound heterozygous point mutations in autosomes and in the X chromosome. The authors suggested that this enrichment of double loss of function variants contributes to

3% of ASD cases and to another 1.7% in males [102].

ASD and common polymorphisms

In multiplex families, inherited CNVs and SNVs, as well as common risk variants with low effect size contribute to ASD susceptibility. Klei et al, estimated that common genetic polymorphism exerts substantial additive genetic effects on ASD liability and explains at least 60 % of the variance in ASD cases from multiplex families and that a myriad of common variants of very small effect impacts ASD liability [103]. However, common polymorphisms have been difficult to identify and replicate. The relative risk conferred by these loci is small [104, 105]. Devlin and Scherer summarized the latest Genome Wide Association Studies, searching for SNPs association to ASD and concluded that no common variant have substantial impact on risk (Odds Ratio > 1.2) but many common variants may have modest impact [68].

ASD and neurometabolic disorders

Neurometabolic disorders in individuals with ASD are rare and metabolic screening is often negative. Most new-born children in Sweden are enrolled in the neonatal screening for selected metabolic disorders, of which some have been associated to intellectual disability and ASD (e.g. phenylketonuria and [106] homocystinuria [107]). Several studies have reported low diagnostic yield of metabolic testing in children with ASD [107-111]. Selective testing should be performed when there are additional clinical symptoms, such as a history of lethargy, cyclic vomiting, early seizures, dysmorphisms, mental retardation, or regression and if the neonatal screening was not performed [112]. Neurometabolic disorders that have been implied in autistic phenotypes include disorders of purine metabolism, a group of creatine deficiency and creatine transport disorders, biotinidase deficiency, cerebral folate deficiency, succinic semialdehyde dehydrogenase deficiency, Sanfilippo syndrome and Smith-Lemli-Opitz syndrome.

ASD and mitochondrial disorders

Mitochondrial dysfunction measured as different levels of biomarkers compared to healthy controls have been demonstrated in individuals with ASD [113]. Biomarkers that could indicate mitochondrial dysfunction include lactate, pyruvate, ubiquinone and carnitine. In a recent meta-analysis by Rossignol and Frye, the authors conclude that the prevalence of mitochondrial dysfunction was 5% in children with ASD vs. ~0.01% in the general population [114]. Mutations in mitochondrial DNA associated with mitochondrial function have been identified in isolated cases of ASD [115-119]. They include mitochondrial DNA mutations, mtDNA depletions/deletions and a few cases with POLG mutations. Children with ASD and mitochondrial disorder may have a history of regression, sometimes triggered by fever and clinical symptoms could include muscular hypotonia, ptosis, dysarthria and epilepsy. A recent study of a large cohort of individuals with ASD, found no evidence to suggest a major role for mitochondrial DNA variation in ASD susceptibility [120]. The biomarkers indicating

mitochondrial dysfunctions in individuals with ASD appear to be secondary to the underlying pathophysiology in most cases [121]. In summary, truly mitochondrial forms of ASD are rare [122].

ASD and prenatal risk factors

Pre-conception risk factors include advanced maternal and paternal age, autoimmune disorders in the parent, season of conception and birth, birth order (primiparous woman), parental socioeconomic status and fertility treatments. Maternal overweight at birth has also been found to be an ASD risk factor [49]. Prenatal environmental risk factors include multiple births, threatening abortion, bleeding during pregnancy, and intrauterine exposure to maternal psychoactive medication and alcohol. Preeclampsia has been shown to be an ASD risk factor in some studies [123, 124] but did not reach significance in the meta-analysis by Gardener [47], neither in the review by Guinchat [125]. Infectious and immune disturbances during the prenatal period have emerged as potentially important ASD risk factors. Vitamin D deficiency in early life affects neuronal differentiation and axonal connectivity and is increasingly being associated with a number of neurologic (e.g. multiple sclerosis) and psychiatric conditions (schizophrenia). Observation of increased incidence of a disorder in higher latitudes, in individuals born during winter/spring and in the offspring of dark skinned mothers could be indirect indications of risk linked to low vitamin D levels. Some studies have reported such associations with ASD but other studies have not. At present, no study has measured low maternal vitamin D levels during pregnancy and demonstrated an increased risk for ASD in the offspring [126]. Prospective longitudinal studies with larger samples are needed.

Parental age

Advanced paternal [127-129] and maternal age [127, 130] has been associated with an increased risk for ASD in the offspring. Interestingly, a recent study showed an increased risk of autism linked to advanced age in grandparents [131]. Advanced age in fathers [132, 133] and grandparents [134] has also been associated with schizophrenia and advanced paternal age has been associated with increased risk for intellectual disability [135]. In ASD and in ID it has been shown that risk conferring *de novo* mutations more often are of paternal origin [33, 34, 98, 100, 135] and a higher rate of *de novo* mutations in older fathers has recently been found using whole genome sequencing [101]. It has been speculated that in the ASD cases, advanced paternal age at the time of the birth of the first child could reflect a broader ASD phenotype [136]. Advanced maternal age has also been associated as a risk factor for numerical chromosomal aberrations (aneuploidy), structural chromosomal aberrations (increased burden of CNVs) and congenital malformations [137, 138].

Migrational status

Parental migration status and increased risk for ASD has been described in European studies, mostly from Nordic Countries. In UK, children to mothers of Caribbean, African and Asian origin were found to have increased risk for ASD [139]. In the Swedish study by Haglund et al. maternal birth outside the Nordic Countries was associated with autism but not with Asperger syndrome [140]. In a Swedish register study by Magnusson et al., an increased risk for ASD in combination with ID was observed and the highest risk was found when parents migrated from regions with a low human development index, and peaked when migration occurred around pregnancy [141]. An increased prevalence of ASD and ID has been documented in children of Somali origin in Stockholm County [142]. In the review by Guinchat et al. (2012), having a mother born outside Europe, North America or Australia was consistently associated with ASD risk [125].

Autoimmune disorders

Epidemiological studies have shown that the prevalence of autoimmune disorders (e.g. maternal type 1 diabetes, rheumatoid arthritis, ulcerative colitis and celiac disease) is elevated in families of individuals diagnosed with ASD [143-145]. The risk conferring disorders vary between the different studies. In a register study by Keil et al., a 50% increased risk for ASD in the offspring was observed if any parent (maternal OR =1.6 and paternal OR =1.4) had an autoimmune disorder [143]. Recent studies have identified the presence of maternal autoantibodies that react against foetal brain proteins in mothers of ASD children [146, 147]. Maternal autoimmune disorders have been linked to an increased risk for a wide range of adverse neurodevelopmental outcomes [148]. One pathophysiological pathway that has been suggested is that mothers with autoimmune disorders have an increased risk for gestational preeclampsia that may impair the foetal brain development [148].

Maternal infections during pregnancy

Several intrauterine infections have been associated with adverse neurological and cognitive outcome and some evidence also suggests that maternal infection and immune dysfunction may be associated with autism. Congenital rubella [149] and cytomegalovirus [150, 151] have been linked to ASD. The meta-analysis by Gardener indicated that intrauterine infections confer a small but significant increased risk for ASD [47]. Maternal influenza infection during pregnancy and prolonged episodes of fever was shown to increase the risk for ASD, however the authors concluded that mild infections, febrile episodes, or use of antibiotics during pregnancy were not strong risk factors for ASD [152]. A Swedish register study found no associations between maternal prenatal infections or infection-related variables and autistic disorders [123] and in the review by Guinchat, maternal infections during pregnancy did not reach significantly increased adjusted odds ratios [125].

Intrauterine exposure

Children exposed in utero to the antiepileptic drug sodium valproate have been found to have an increased prevalence of neurodevelopmental disorders, including ASD [153, 154]. Conflicting results have been published on alcohol exposure and increased risk for ASD. In the meta-analysis by Gardener, maternal alcohol consumption during pregnancy was not associated to ASD and no association was found between average alcohol consumption and ASD in a Danish study [155]. In two Swedish studies, 9-13 % of children whose mothers abused alcohol in pregnancy had ASD [156, 157]. Other teratogen substances that have been linked to ASD are thalidomide and misoprostol, both inducing congenital malformation and neurodevelopmental impairment [158]. In the study by Gardener, a meta-analysis of the two studies that looked specifically at psychiatric medication use during pregnancy, the analyses suggested a significant positive association with the risk of autism [47]. Similar findings were reported by Dodds et al., where maternal use of prescribed psychoactive drugs were more often reported [49]. A 2-fold increased risk of ASD was associated with treatment with selective serotonin reuptake inhibitors by the mother in a Californian study [159]. In a Swedish register study, similar findings were recently reported. A history of maternal depression and antenatal use of antidepressants was associated with an increased risk of ASDs in the offspring, the increased risk confined to ASD without ID [160].

Parental psychiatric history

Parental psychiatric history has been associated with increased risk for ASD in the offspring in clinical [161-163] and epidemiological [164-166] studies. In mothers (but not in fathers) of children with ASD, depression and personality disorders were found to be more common than in mothers of typically developing children [164]. A recent Australian study identified maternal bipolar disorder as a risk factor for ASD. This study showed that maternal schizophrenia/bipolar disorder/unipolar depression also was associated with an increased risk for ID and rare congenital syndromes in the offspring [167]. In a recent Finnish, population-based case-control study by Jokiranta et al., the authors examined associations between parental psychiatric history (based on in-patient records) and risk for ASD in offspring. The study calculated the associations with the three ASD sub-groups (autistic disorder, PDD-NOS and Asperger´s syndrome). Total ASD was associated with maternal schizophrenia, affective and anxiety disorders. Autistic disorder was associated with maternal depression but not with PDD-NOS or Asperger´s syndrome. However, PDD-NOS and Asperger´s was associated with maternal schizophrenia, affective and anxiety disorders. Maternal depression has been identified as a risk factor for ASD in previous studies. In the study by Jokiranta, also paternal affective disorders were associated with a 2-fold risk of ASD in the offspring, suggesting a shared genetic background for ASD and affective disorders. Paternal schizophrenia was also associated with autistic disorder. An adjusted three-fold increase of ASD risk was found if both parents had a psychiatric disorder. Interestingly, strongest association was shown between parental psychiatric disorders and PDD-NOS [168]. As mentioned before, an association between maternal

use of anti-depressants during pregnancy and ASD risk in the offspring has been identified [159, 160].

Peri- and neonatal risk factors

Several perinatal and neonatal risk factors are associated with neurodevelopmental disorders including cerebral palsy. Multiple births, preterm birth, low birth weight, small for gestational age, breech presentation and caesarean section has have all been found to confer ASD risk. Scheduled caesarean section has been identified as an ASD risk factor in several studies [169-172]. Guinchat concludes that during the perinatal period, the predominant risk factors were preterm birth, breech presentation and planned caesarean section. All the documented adjusted odds ratios were between 1.3 and 2.8 [125]. Other neonatal ASD risk factors, that have been reproduced, are low Apgar score, neonatal encephalopathy, congenital malformations and hyperbilirubinaemia.

Low birth weight and preterm birth

Low birth weight and preterm birth contribute to ASD risk. Pinto-Martin et al. estimated the ASD prevalence in children with birth weight less than 2000 g to be five times the population prevalence [173]. Losh et al, comparing autism-discordant twin pairs found that the twins with lower birth weights were three times more likely to meet criteria for ASD than the heavier twins [174]. A foetal growth that was less than two standard deviations below the mean increased the risk of ASD, both with and without ID [175]. Children born extremely preterm have higher prevalence of cognitive and attention deficits. In a recent Swedish study of children born < 27 gestational weeks, 11.3 % had moderate or severe cognitive disability vs. 0.3 % in controls at 2.5 years [176]. Screening children with birth-weight < 1500 g for ASD has indicated that a high proportion (10-25%) have autistic features [177-179]. However, the interpretation of these results must be cautious as motor and sensory impairments lead to high rates of false-positive screens [180]. In a UK study of children born < 26 gestational weeks, 16 % screened positive but only 8 % were diagnosed with ASD [181]. Buchmayer et al. discussed that the risk of autistic disorders in premature children may be mediated by prenatal and neonatal complications [123].

ASD and co-existing conditions

The most common co-existing disorders in children with ASD are intellectual disability, ADHD, epilepsy and psychiatric disorders. Furthermore diverse behavioural problems, sensory abnormalities, delays and deficits in motor functions and birth defects are common co-existing conditions. Sleep- and feeding problems are often present as well as gastrointestinal symptoms.

Intellectual disability and ASD

In children with autistic disorder about 50-70% are also diagnosed with ID, containing the full spectrum of mild to profound ID [1-3]. Including all types of ASD at age 9-12 years, 34% had intellectual disability in a Swedish twin study [75]. Intellectual disability is found in 1-3% of the childhood population and a large proportion has a genetic aetiology. The prevalence of autism increases with the severity of the ID. In adults with ID (18-87 years old) Saemundsen reported a 21% prevalence of autism [182]. Similar to ASD, there is a skewed sex ratio with more males having ID [183]. X-linked mental retardation affecting only males contributes to the skewed ratio. Strong evidence for great overlap between ASD and ID has emerged from genetic studies. In the review by Betancur, 103 gene variants found to be associated with ASD, were all likewise associated with ID [184]. Neale et al. stated that most *de novo* CNVs found in association with autism, often were linked to "a broad range of conditions including ID, epilepsy and schizophrenia" [33]. In individuals with ASD and intellectual functioning within the normal range, it is likely that oligo- and polygenic risk variants play a more dominant role compared to individuals with ASD and ID, where a larger proportion exhibit detectable genetic risk variants. Current genetic findings suggest that many single gene variants and larger structural chromosomal aberrations contribute to multiple phenotypes caused by pleiotropic effects of the genes involved [46].

ADHD and ASD

ADHD as well as ASD, is a highly heritable disorder. Simonoff et al. reported an ADHD prevalence of 28 % in 10-14 year old children with ASD [185]. Hofvander et al. reported an ADHD prevalence of 43% in adults with ASD without ID [186]. A higher than expected rate of autistic symptoms in children with ADHD was shown in a large multicentre study [187]. In the twin study by Lichtenstein et al., 51% of children with ASD (excluding cases with syndromic ASD) also met criteria for ADHD. Concordance for ADHD in MZ twins with ASD was 44% vs. 15% in DZ twins. The result suggests that a large part of the genetic susceptibility for ASD was shared with ADHD. The authors concluded that different neuropsychiatric disorders seem to have a common genetic aetiology [75].

Epilepsy and ASD

Childhood epilepsy, defined as recurrent unprovoked seizures, can be regarded as a spectrum of numerous disorders with extensive clinical and aetiological heterogeneity [188]. The overall prevalence of childhood epilepsy is 0,5-0.7% [189, 190]. The cumulative epilepsy incidence is likely about 1.2-2% at age 25 years [191]. Childhood epilepsies may be caused by several pre- peri - and postnatal conditions, such as genetic disorders, congenital CNS-malformations, intrauterine infections, neonatal encephalopathy, cerebrovascular events, head trauma, brain tumours, hypoxia and numerous neurometabolic disorders. With novel high-resolution SNP-assays, array-CGH and high-throughput sequencing technologies an increasing number of genetic causes are identified.

Among epilepsies with a genetic underpinning most cases are sporadic with no family history and a minor fraction is classified within the familial or inherited epilepsy group. In this group the inheritance exhibits complex patterns and few susceptibility genes have been identified [192]. *De novo* sequence mutations contribute to epilepsy pathogenesis and exome sequencing has revealed high rates of *de novo* mutations in children with Dravet syndrome and other infantile epileptic encephalopathies. A subset of CNVs linked to ID, schizophrenia and ASD have also been associated with epilepsy (e.g. deletion 16p13.11 and deletion 15q13.3) [192]. CNVs may be recurrent (inherited or *de novo*) and confer risk for common epilepsy and non-recurrent large CNVs that arise *de novo* play a role in the epileptic encephalopathies.

The rate of epilepsy in children with ASD is significantly higher than in typically developing children. The epilepsy prevalence in children with ASD has been estimated between 5-46% [193, 194]. The rates of epilepsy in individuals with ASD increase with age. A recent, large epidemiological study by Viscidi et al. established that the prevalence of epilepsy in children with ASD at age 2-17 years was approximately 12%, and by adolescence approximately 26% [195]. Many prior studies have found that epilepsy in children with ASD is strongly associated with intellectual disability. In children with epilepsy, approximately 25% have ID [196]. In the meta-analysis by Amiet et al., a pooled frequency of epilepsy was 21.4% in cases with autism and ID. The rate of epilepsy in subjects with autism without cognitive impairment was 8% [197]. Similar results was reported by Woolfenden et al., in their meta-analysis 1.8% had epilepsy at age under 12 years (majority did not have co-existing ID) and 23.7% in cases > 12 years (majority ASD and ID) [198] . In the Viscidi study, age and cognitive ability was independently associated with epilepsy. For one standard deviation increase in IQ, the odds of having epilepsy decreased by 47% [195]. In children with epilepsy and IQ > 80, the prevalence of ASD was estimated to be 2% [199]. ASD and epilepsy have also been associated with female gender [200] and a history of regression but inconsistent results have been found. When adjusting for IQ, female gender and a history of regression was not associated with epilepsy in the study by Viscidi [195].

Early onset epilepsy has been associated with increased risk of ID and ASD. Saemundsen et al. found that 14 % of children with epilepsy onset in the first year of life developed autism. In children with infantile spasms, the risk for ASD was 46% and in children with more severe structural brain disorder (hypoxic ischemic encephalopathy, cortical dysplasia, tuberous sclerosis) 69% were found to have ASD [87]. In children with active epilepsy, ASD have been found in 37% [201]. Brooks-Kayal have proposed abnormal synaptic plasticity producing an imbalance between excitation and inhibition, a shared finding in Fragile X syndrome, Rett syndrome, tuberous sclerosis, mutations in the *CDKL5* gene and mutations of the neuroligin genes to explain the link between ASD and epilepsy [202]. The association between autism, intellectual disability and epilepsy suggests that these are concurrent symptoms of an underlying brain dysfunction and shared genetic pathways rather than being comorbid associated separate disorders [46].

Sensory abnormalities

Abnormal responses to sensory stimuli are common in children with ASD. These symptoms have been reported to distinguish ASD from other developmental delays [203]. Hyper- or hyporeactivity to sensory input or unusual interest in sensory aspects of the environment have been included in DSM-5 ASD criteria. Klintwall et al. reported that that sensory abnormalities differed among children with autism spectrum disorders according to autistic subgroups and that sensory/perceptual abnormalities were most frequent in the subgroups with nuclear autism without learning difficulties and in the Asperger syndrome group [204].

Motor impairments

In many children with ASD there are co-existing impairments in fine- and gross motor performance. Motor impairments include both developmental delays and deficits. Several studies have documented delays in achieving motor milestones, such as sitting unsupported and walking independently. Children may have clumsiness and dyspraxia (impairments in skilled movements) and gait abnormalities (e.g. toe-walking) [205]. In a meta-analysis by Fournier et al., the authors concluded that substantial motor coordination deficits are present across ASD subtypes, persist over time and are cardinal features of ASD [206].

Birth defects and ASD

A high frequency of minor malformations has been reported in individuals with ASD [207]. Also birth defects are more often found in children with ASD compared to the general population. Schendel et al. reported a 6% rate of birth defects in cases with ASD, compared to 3.2% in controls and that a skewed sex ratio with a male preponderance was found in children with ASD and birth defects. There was an association with more birth defects in children with ASD in combination with ID [208].

Early identification and screening

There is general agreement that ASDs should be identified as early in life as possible, with a view to ensuring that intervention can start as quickly as possible. Because of the extremely heterogeneous aetiology of ASD, with varying degrees of associated brain dysfunction, time of identification will probably have to vary. Identifying atypical behaviours in infant siblings of children with autism has been done in several studies displaying the heterogeneity of onset time as well as the wide variation in presenting symptoms [46].

Screening instruments, (e.g. Modified Checklist for Autism in Toddlers (M-CHAT)) for early identification of ASD have been developed [209, 210]. In Gothenburg, Sweden a general population screening at child health centres has been conducted for children at the age of 2.5 years. The screening instruments used were the M-CHAT

completed with a short observation of the child´s joint attention. The positive predictive value of the screening method was found to be 90%. [211]. Similar results were reported from a Flemish study using a screening instrument at day-care centres [212]. Unspecific symptoms during infancy such as sleeping, crying or feeding difficulties have been reported in children later diagnosed with ASD [213]. Despite behavioural markers being identified within the first year of life, the current average age of clinical diagnosis of ASD remains at approximately 3 years or older [214].

Early intervention programs

There is now a number of early intervention programs available more or less specifically focused on autism. One major intervention model is based on applied behaviour analysis (ABA), founded on basic principles of learning, motivation and positive reinforcement [215]. Another major model is Treatment and Education of Autistic and related Communication handicapped CHildren (TEACCH) emphasizing visual work systems, positive routines and structured teaching [216]. In a meta-analysis by Eldevik et al that involved nine controlled studies measuring the efficacy of Early Intensive Behavioral Intervention (EIBI), a standardized mean difference effect size for two available outcome measures, change in full-scale intelligence and/or adaptive behaviour composite, was demonstrated [217]. Similar results were reported by Virués-Ortega [218]. Most authors agree that EIBI result in some improved outcomes in the short- and intermediate-term perspective [219-224]. However, these studies have demonstrated considerable variability in outcome with low initial IQ repeatedly demonstrated to contribute to a less favourable outcome [225]. It is important to remember that in many of these studies children with co-existing genetic and medical disorders, such as epilepsy and severe ID, have been excluded [222, 225-227]. Recent reviews, conclude that early intensive behavioural intervention is likely to be beneficial [228] but that the evidence regarding efficacy is still limited due to the lack of randomized control studies (RCT). Additional studies using RCT research designs, also considering the broad aetiological panorama are needed to make stronger conclusions about the effects of EIBI for children with ASD [229].

2 AIMS OF THE THESIS

Study I

To characterize and to increase the awareness of the striking variety of co-existing developmental disorders in children with early diagnoses of autism spectrum disorder in a population-based group of 208 preschool children.

Study II

To analyse certain prenatal risk factors in a representative group of preschool children with early diagnoses of autism spectrum disorders and to investigate gender distribution of neurodevelopmental and psychiatric conditions in their first-degree relatives.

Study III

To explore the frequency of genetic and other medical conditions, including epilepsy, in a representative group of preschool children with early diagnosis of autism spectrum disorders and to relate outcome to co-existing medical findings.

Study IV

To investigate the frequency of copy number variants using chromosomal microarrays in a representative group of preschool children with early diagnosed autism spectrum disorders.

3 PARTICIPANTS

Study I

The group in study I included 208 children, (176 boys and 32 girls, sex ratio 5.5:1) 20–54-month-old when referred to a specialized habilitation centre with a diagnosis of ASD. They were drawn from a population-based group of 313 children (born 2002–2006) with ASD diagnoses in Stockholm County, a region with approximately 28,000 births per year. Of these 313 children, 288 had been referred for early intervention to a specialized habilitation centre for preschool children with ASD.
Twenty-five children of the 313 children with more severe medical conditions and syndromes in combination with ASD had been referred to general habilitation centres due to more complex medical needs. At these centres, no specific ASD interventions were provided. This group was not included in any of the studies. Of the 288 children, 24 had been referred to the centre prior to study start and could not be included. Of the remaining 264 children, 37 parents declined participation, 15 parents could not communicate in either Swedish or English, two families moved abroad and another two children had been referred back to general habilitation centres due to their complex medical needs. No exclusion of children with ASD and identified medical conditions/or significant ID was done. Of the total group of 208 children, 64 (31%) had been referred before the age of 36 months, 83 (40 %) between 37-48 months and 61 (29%) between the ages of 49–54 months.

Study II

In study II, 206 of the 208 children were included in a) the register study and b) the parental interviews. Two children were adopted why pre- and perinatal data were unavailable.

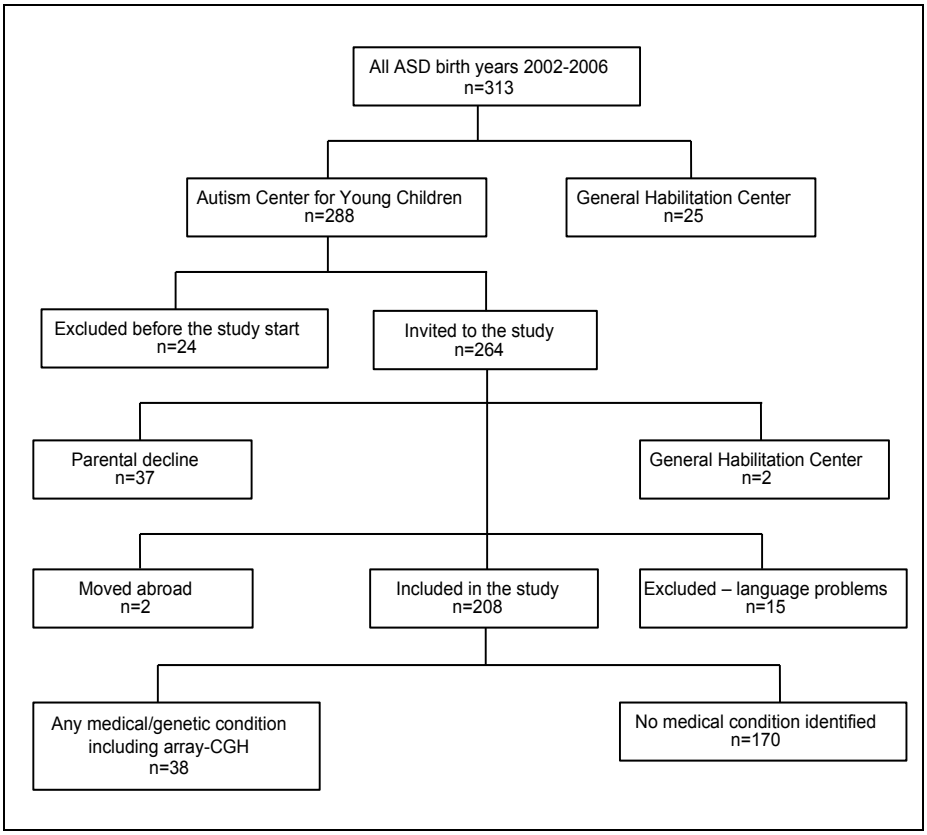
Study III

Of the 208 children, 198 participated in the intervention outcome follow-up study after two years. Complete cognitive data was obtained in 196, and data regarding type of ASD in 197 children. Vineland Adaptive Behavior Scale-II data (VABS-II) were available in 192 children.

Study IV

Parents of the 208 children with early diagnosis of autism spectrum disorder were recommended genetic testing with chromosomal micro-array (CMA) and testing for Fragile X. CMA was performed in 162 children.

Figure 2
Flow-sheet participants in the study



4 METHODS

Study I

The clinical research team consisted of four physicians (two neuropaediatricians, one paediatrician, and one child psychiatrist), two clinical psychologists and two speech and language pathologists.

Paediatric clinical examination

All children were examined by one of the physicians who also interviewed at least one parent, evaluated all available medical reports and collected data from the maternity and obstetric units. Data regarding developmental milestones according to the Swedish Child Health Care Centers protocol were collected.

PARIS schedule

The parental interviews followed a structured protocol, the PARIS schedule (Paris Autism Research International Sib pair Study), including the DSM-IV diagnostic checklist, detailed family history interview, a variety of clinical developmental data, symptoms and signs and a schedule for brief neuromotor examination of the child [230].

Vineland Adaptive Behavior Scales-II (VABS-II)

An interview was performed according to the Vineland Adaptive Behavior Scales-II [231]. VABS-II is a valid and often used outcome measure of adaptive skills in children with ASDs [223, 226].

Autistic Behavior Checklist (ABC)

The Autistic Behavior Checklist, a questionnaire pertaining to core symptoms of autism, was used as parent interview while observing the child during the research paediatric examination [232].

Cognitive test data

The research psychologists scrutinized psychological records from the first referring assessment team, with a view to establishing the level of overall cognitive function of the child at referral. For some children a complete cognitive test had not been possible to perform according to the referring psychologist and in some children no formal testing had been done. However, the research psychologists could make inferences based upon analyses of all available data, including reports pertaining to developmental data, recorded observations during assessments, test data reported and

from preschool visits and make a conclusion about the child's general cognitive ability and then classify the child into an appropriate cognitive group:

(a) Clear indication of or highly suspected learning disability/mental retardation according to DSM-IV (Learning disability/mental retardation group)

(b) Indications of learning problems and developmental delay, but a definite classification into a specific cognitive level group was not possible (Developmental delay group).

(c) Several indications of general cognitive ability within the normal range (Normal intelligence group).

Speech and language assessment

The speech and language pathologist in the research team performed a structured multi-item telephone interview or sent a questionnaire containing the same items to the parents [233].

Study II

The study includes two parts: a) a register study and b) a parental interview.

a) In the register study, the ASD group was contrasted with the general population regarding pre- and perinatal data, obtained from The Swedish Medical Birth Register (SMBR) and Statistical Central Bureau of Sweden (for information on paternal age and country of birth). To perform the study, an ethical approval and an approval from The Swedish Board of Health and Welfare were required. Consequently, all personal data were coded.

The comparison group consisted of all children born 2002-2006 in Stockholm County, excluding children with diagnosis of ASD.

The variables that could be analyzed using register data were: parental country of birth, parental age at child's birth, maternal pre – and intrapregnancy medication and mode of delivery. For 21 children register data was not available in the SMBR.

b) In the parental interviews the following information was investigated: the occurrence of first-degree relatives with ASDs and other neurodevelopmental problems (intellectual disability, ADHD, speech- and language problems and dyslexia – clinically diagnosed or qualifying for educational support) and/or a history of psychiatric disorder. First-degree relatives were classified as ‘‘broader phenotype’’ when there was a report of ‘‘mild autism’’ – (social aloofness/social phobia/extreme shyness that had not been registered as a clinical diagnosis of ASD).

Study III

Medical work-up

Comprehensive medical information on each child was collected from maternity and obstetric care units, the Child Health Care Centre (CHC) and all hospital and outpatient clinics attended by the child. In addition to data from records, parents were interviewed regarding the child's pre- and perinatal history, early development, and any genetic, neurologic or other clinically significant medical condition, including epilepsy.

Parents were interviewed regarding a history of regression in the child and this information was compared to available data in CHC and medical records. Consistency was required between parental information and the notes in the records from CHC. Regression was defined as loss of more than five spoken words used communicatively in children more than 15 months of age. In children younger than 15 months, regression was determined when there was a clear indication of loss of social interest and contact.

Participating families were recommended genetic testing using array-based Comparative Genomic Hybridization (array-CGH) and testing for Fragile X. Conventional chromosomal karyotyping had previously been performed in many of the children. In girls with a clinical suspicion of Rett syndrome analysis of mutation *MECP2* was performed.

Medical and genetic conditions were defined as: (1) a significant intrauterine harmful exposure, (2) substantial prematurity (gestational age less than 29 weeks), (3) an identified genetic disorder including significant genomic imbalances identified with array CGH, (4) abnormal brain MRI findings and (5) a clinical diagnosis of epilepsy.

Intervention

All 208 children received intervention based on principles of Applied Behaviour Analysis (ABA). The study design was naturalistic. There was no randomization to type of treatment. Parental preference was the most decisive factor. The presence of medical/genetic condition did not affect the choice of intervention type.

One group received ‘‘intensive’’ and the remainder ‘‘non-intensive’’ targeted intervention. The different levels of interventions were defined as: Intensive intervention based on ABA, i.e., early intensive behavioral intervention (EIBI), given at the preschool and by the parents at home with assistance from the centre, with the intention to treat (a) 15 h or (b) 25–30 h per week or 30–40 h per week. Non-intensive targeted intervention based on ABA, consisting of different targeted types of training (toilet training, speech and language training, training of compliance or other specific training that the child was deemed to need).

Outcome measure

The primary outcome measure was change in Vineland Adaptive Behavior Scale (VABS-II) composite scores between Time 1 (prior to intervention) and Time 2 (after two years of intervention). Numbers of children in the outcome analyses reflect the number of individuals that could be assessed according to the studied variable and to the Vineland change score. Raters blind to the type of intervention given measured the outcome.

Study IV

Collection of clinical data

All available information regarding the child's neurodevelopmental profile (subtype of ASD diagnosis, cognitive level and medical data (presence of congenital anomaly/malformation, any medical/genetic condition including epilepsy) were collected. Interviews examined the occurrence of parental conditions (occurrence of ASD, other neurodevelopmental problems, intellectual disability, attention-deficit/hyperactivity disorder (ADHD), speech and language problems and dyslexia – clinically diagnosed or qualifying for educational support) and/or a history of psychiatric disorder. Parents were classified as having "broader phenotype ASD" when there was a clear report of "mild autism" – (social aloofness/social phobia/extreme shyness that had not been registered as a clinical diagnosis of ASD). These data were all collected prior to the genetic testing.

Genetic testing

Conventional karyotyping had been performed in some children prior to the study start. Testing with chromosomal micro-array was performed with different array-CGH platforms (244 and180 K Agilent Technologies and 180 K Oxford Gene Technology). All had whole genome coverage. Analysis of microarray data was performed using DNA analytics (Agilent technologies) or Cytosure Interpret (Oxford Gene Technology) software.

Where samples were available, parental testing was carried out to investigate mode of inheritance. Different methods were used (array-CGH, FISH, MLPA).

Classification and interpretation of identified CNVs was based on available information in medical literature and different databases (DGV, Decipher) and done according to current guidelines [71] and recent reviews [90]. CNVs were classified in three categories: pathogenic, variants of unclear clinical significance (VOUS) and benign. Mode of inheritance (*de novo* or transmitted), type of CNV (deletion/duplication) and gene content were taking into consideration. CNVs previously known to be associated with a genomic disorder, *de novo* CNVs and large (> 500 Kb), rare CNVs and CNVs

that affected known candidate genes (e.g. *NRXN1, CNTNAP2*) were classified as pathogenic.

Molecular analyses of *MECP2* mutation was done when there was a clinical suspicion of Rett syndrome and testing for Fragile X was performed in a large proportion of the children when the chromosomal microarray was normal.

5 STATISTICAL ANALYSES

In all studies, comparisons between two groups (e.g. ASD vs. non-ASD) of the distribution of a dichotomous variable (e.g. gender) were performed. In some studies we focused on odds ratios with corresponding confidence intervals and in some studies we only reported p-values. In all cases when we reported a significant difference we confirmed that the difference was significant by using chi-square analysis using SPSS (version 19) exact test.

Group means were compared using a) Students t-test, or b) ANOVA followed by pair-wise post hoc analyses (Fischer's LSD). For heavily skewed data and/or unequal group sizes, a Kruskal-Wallis test, followed by separate Mann-Whitney *U*-tests were used instead.

An alpha level of .05 was used for all statistical analyses in all studies.

6 RESULTS

Study I

ASD diagnoses

The referral diagnosis of the 208 children were; autistic disorder (n=133), PDD-NOS (atypical autism) (n=62), Asperger's syndrome (n=6) and Childhood Disintegrative disorder (n=2) and in 5 cases an unspecified diagnosis of autism spectrum disorder had been given.

Cognitive levels

Of the 208 children, 80 belonged to the learning disability/mental retardation group, 81 to the developmental delay group, and 47 belonged to the normal intelligence group.

Table 1
Cognitive groups

	Normal intelligence	Developmental delay	Learning disability	Total
Boys	40	70	66	176
Girls	7	11	14	32
Total	47(23%)	81(39%)	80(38%)	208

Adaptive behavior – Vineland Adaptive Behavior Scales (VABS-II)

The three cognitive level groups were compared with respect to results on total score and on the four domains of the Vineland Adaptive scales II, Communication, Daily activities, Social and Motor function. Means (95% confidence intervals) and effect sizes are presented in Table 3.

The results on the four domain scores of the VABS-II were related to cognitive levels. The developmental delay group and the learning disability/mental retardation group exhibited profiles with very low results on the Communication and Socialization scales and higher scores on Daily Living Skills and Motor Skills. The group with normal intelligence showed similar results albeit not as prominent.

Table 2
Cognitive groups and Vineland Adaptive Behavior Scales-II

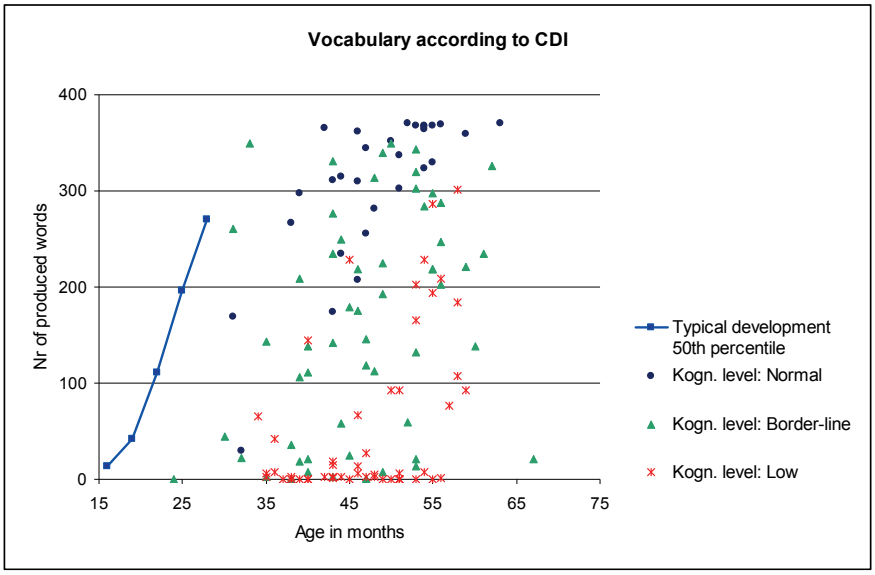
Vineland domain	Normal intelligence	Developmental delay	Learning disability	Effect size (η^2)
Composite Score	78.89 (76.46-81.32)	70.37 (68.54-72.20)	61.18 (59.30-63.07)	0.40
Communication	87.85 (84.58-91.12)	69.76 (67.28-72.26)	56.03 (53.46-58.60)	0.53
Daily Living skills	84.00 (80.91-87.09)	76.74 (74.39-79.10)	66.00 (64.15-69.01)	0.29
Socialization	74.85 (72.23-77.47)	69.78 (67.78-71.77)	63.57 (61.51-65.62)	0.19
Motor skills	81.33 (78.07-84.58)	78.26 (75.80-80.72)	70.24 (67.70-72.77)	0.14

Speech- and language level

At the time of the first assessment 27 of the 208 children (13%) of the children had no words at all, 68 (33%) had a few single words, 48 (23%) had a few communicative sentences, and 65 (31%) had phrase speech with or without echolalia.

Children (n=156) demonstrated a marked delay in the development of expressive vocabulary according to CDI/Mac Arthur. Statistical analysis comparing expressive vocabulary across the three cognitive subgroups confirmed that the variability in word production was significantly related to the cognitive level.

Figure 3
Expressive vocabulary in ASD cases and typically developing children



The scatter plot displays the expressive vocabulary from CDI in number of words by the children's age in months. The 50th percentile for typically developing children aged 16–28 months is indicated by the ascending solid line.

Independent walking and toe-walking

Of the 208 children 138 (66%) started to walk unsupported before age 15 months, 48 (23%) between 15 and 18 months, and 21 (10%) after 18 months of age. One child did not walk at first examination (age 40 months). Median age for walking was 13 months. Toe walking from the start of walking was present in 70 of the 208 children (34%), 39 of these (19%) had very clear and persistent toe walking.

Activity level

Hyperactivity was reported in a large proportion of the children, 87/208 (42%) unanimously by the parent and the examining physician. 98/208 (46%) had an activity level within the normal variation. Only seven children (3%) were classified as hypoactive.

Co-existing epilepsy

Twelve children (6%) had a history of epilepsy at the referral. Three of the twelve had their epilepsy onset classified as infantile spasms.

Regression

Regression was defined as loss of expressive language skills (loss of > 5 words used communicatively). In 46/208 (22%) a regressive trajectory was described and medical records from CHC supported this information. In 20 (10%) regression occurred after an apparently normal development and 26 children had a history of regression after a delayed early development.

Study II

Register data revealed that parents born outside Europe had a two-fold increased risk of having a child with ASD compared to parents born in Europe.

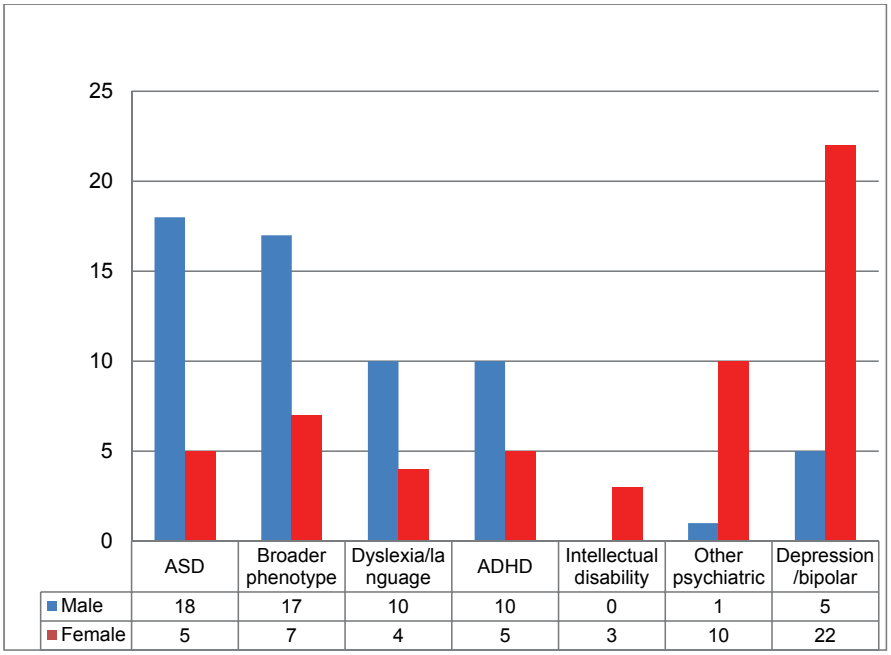
Advanced paternal age (> 40 years) at the child's birth was associated with an increased risk for ASD in the offspring (95 % CI= 1.0-2.1). No increased risk was found in association with advanced maternal age.

The use of psychoactive medication during pregnancy was significantly higher in the ASD mother group compared to the register controls. Antidepressants (SSRI) were used in 4.3 % of ASD mothers vs. 1% in the controls (95 % CI=2.19-9.05). Similar differences were revealed with regard to the use of neuroleptics, sedatives and sleep-inducing medication (95 % CI=2.5-8.0).

The frequency of caesarean section was significantly higher in mothers of children with ASD (27.8% vs. 19.8% (95%, CI = 1.12–2.13, p=0.009). The difference was accounted for by more scheduled sections (95% CI = 1.2–2.7, p=0.003). The frequency of breech presentation and other obstetric rationales were not increased in the ASD-group.

The interviews showed significant gender differences as to types of neurodevelopmental/psychiatric disorders in first-degree relatives. Female first-degree relatives displayed higher rates of depression, including bipolar disorder, and other psychiatric disorders (anxiety disorder, recurrent psychosis, anorexia nervosa, and obsessive–compulsive disorder) than male relatives, who had comparatively higher rates of ASDs, broader phenotype, dyslexia, speech- and language impairment and ADHD.

Figure 4
Gender distribution of conditions in first-degree relatives



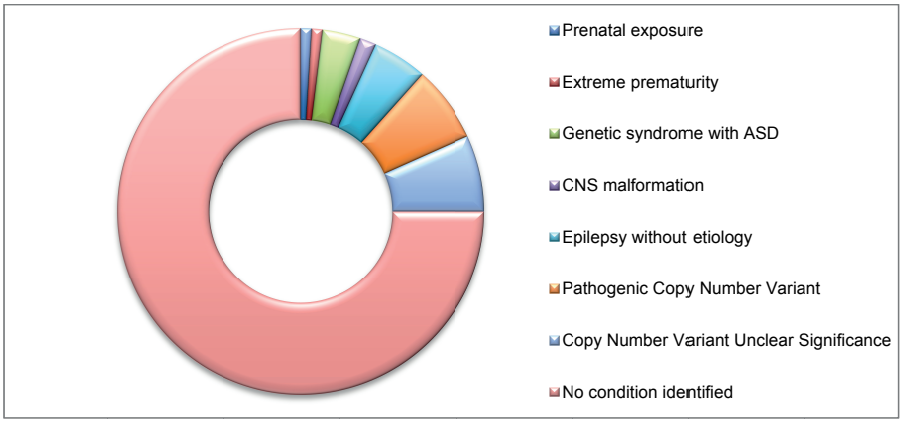
	ASD	Broader phenotype	Dyslexia/language	ADHD	Intellectual disability	Other psychiatric	Depression /bipolar
Male	18	17	10	10	0	1	5
Female	5	7	4	5	3	10	22

Study III

A medical/genetic condition, including epilepsy, was identified in 38 of the 208 children (18%). A single gene disorder was found in seven of the children (tuberous sclerosis complex (3), Fragile X (1), Rett syndrome (2) and Pyridoxine (B6) dependency (1). A clinical diagnosis of Pierre Robin syndrome was present in one child. In one child a deletion 22q11.2 was detected by conventional chromosomal analysis. In 11 of 162 children that could be analyzed with array-CGH a clinically

significant aberration was identified. Two children had been born prematurely (gestational week 24 and 28). One child had been exposed to valproic acid during pregnancy and in one adopted child a there was clinical presentation consistent with a foetal alcohol spectrum disorder. Three had abnormal MRI findings and one child had a congenital hydrocephalus. Of these 27 children 7 also had epilepsy. Epilepsy was present in a total of 18 children. In ten of these no specific aetiology had been identified (see fig 5)

Figure 5
Overview ASD aetiologies in 208 children



Any medical/genetic condition including epilepsy was more often, but not significantly, found in children with the combination of ASD and ID, as compared with those who had ASD without ID (CI 95% = 0.98–4.17, p = 0.057).

Of the 32 girls, 10 had an identified medical/genetic condition. The corresponding rate in boys was 28/176 (95 % CI = 1.03–5.62, p = 0.043).

In children with the combination of ASD and ID, 13/102 had epilepsy, whereas in children with ASD and borderline or average cognitive function, 5/106 had epilepsy (95 % CI = 1.01–8.60; p = 0.047).

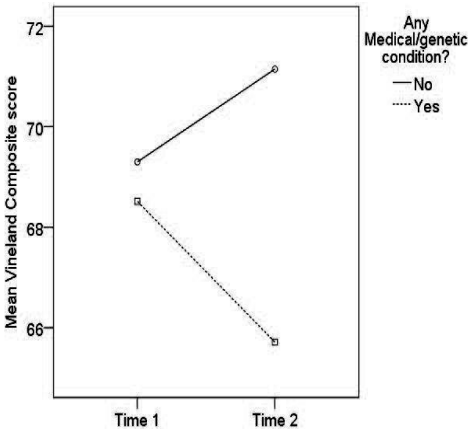
A history of regression was described in 46/208 children (22 %). A history of regression was more common in children with a clinical diagnosis of autistic disorder as compared with children with atypical autism/PDD-NOS (95% CI = 1.37–8.14, p = 0.008). In children with the combination of ASD and ID, regression was reported in (35/102) vs. (11/106) in children with ASD without ID (95 % CI = 2.14–9.51, p=0.001).

Epilepsy was not more prevalent in children with regression than in those without (95 % CI = 0.19–2.47, n.s.).

Outcome in relation to medical/genetic conditions, including epilepsy

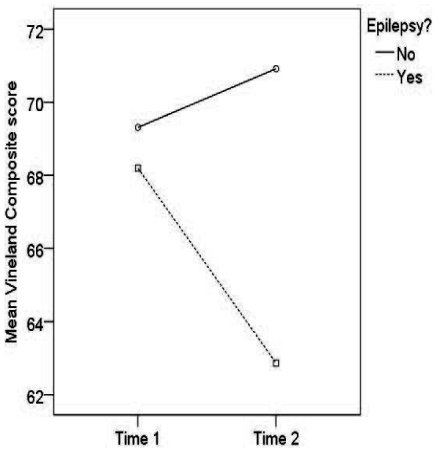
Children without medical/genetic condition had a positive VABS-II change score at Time 2 (compared to Time 1) whereas children with a medical/genetic condition (including epilepsy) had a negative VABS-II change score. The difference was not significant. Type of intervention, intensive vs. non-intensive, did not significantly affect outcome in any of the four groups.

Figure 6 Mean VABS Composite Score at Time 1 and Time 2 in children with (*n*=34) and without (*n*=158) any medical/genetic condition including epilepsy.



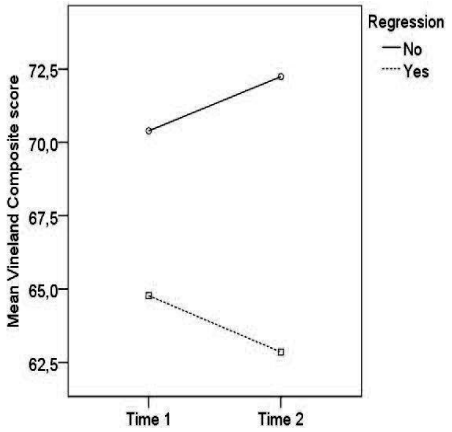
Children without epilepsy had a positive VABS-II change score at Time 2 (compared to Time 1) whereas children with epilepsy had a negative VABS-II change score. The difference was significant.

Figure 7 Mean VABS Composite Score at Time 1 and Time 2 in children with (*n* = 15) and without (*n* = 177) epilepsy.



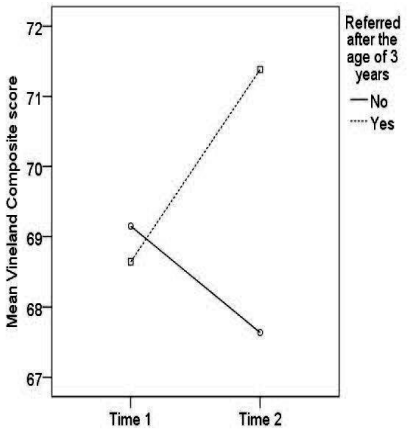
Children without a history of regression had a positive VABS-II change score at Time 2 (compared to Time 1) whereas children with a regression had a negative VABS-II change score. The difference was significant.

Figure 8
Mean VABS Composite Score at Time 1 and Time 2 in children with (*n*=40) and without (*n*=150) regression.



Children who had been referred after the age of three years had increased their VABS-II score at Time 2 (compared to Time 1) whereas children who had been referred before the age of three had decreased their VABS-II score. The difference was significant.

Figure 9
Mean VABS Composite Score at Time 1 and Time 2 in children who had been referred before (*n*=65) or after (*n*=127) age three years.



Study IV

Genetic testing

Genetic testing with array-CGH was performed 136 boys and 26 girls. Seven children had a previously identified genetic disorders and two were not assigned to testing since their ASD affected siblings had normal chromosomal microarray. Testing for Fragile X was performed in 142 of the children and a mutation was found in one boy. Conventional karyotyping had been performed in 80 children. Targeted analysis with FISH, revealed a deletion in chromosome region 22q11.2in one girl. This case has been included as a pathogenic CNV in the following analyses since it would have been detected by array-CGH. Of the 26 girls, three were clinically suspected of having Rett syndrome and two of these were found to carry a *MECP2* mutation.

Array-CGH results

A pathogenic CNV was identified in 13/162 (8%) children and an additional *de novo* deletion 22q11.2 was previously identified. In another 14 children, CNVs classified as VOUS were found – thus, in 28 of the 162 of the children (17%) a putative risk conferring genomic imbalance was identified. In 24/28 children the inheritance status could be determined. Seven of the 24 children had a *de novo* chromosomal aberration (4.3 % of the total ASD sample). Ten of the CNVs were paternally inherited and nine CNVs maternally inherited. In one case, both parents transmitted a heterozygous deletion in the same gene. Two families declined parental testing and two children were adopted.

In some cases more than one CNV or other chromosomal rearrangements were identified. In the group of children with pathogenic CNVs, one boy had a paternally transmitted duplication in region 16p11.2 and an additional mosaic trisomy chromosome 9. One girl had a *de novo* deletion in region 22q13.3, affecting the *SHANK3* gene. In addition two more CNVs were found - a maternally transmitted duplication in region 9p24.2 and a paternally transmitted deletion in region 9q33.1. In the group of children with CNVs classified as VOUS one boy had two deletions – one detected in region 4q22.1 and the second in region 13q21.2, both paternally inherited. One boy had two paternally transmitted duplications, one in region 18p11.23 and a second in region 18p11.22. One boy had a paternally inherited duplication in region 7q36.1 and a maternally inherited duplication in region 11p15.1.

Six of 26 girls (23%) were found to have a CNV. Of these, four were classified as pathogenic and two as VOUS. Three girls had a *de novo* CNV, one was paternally, one maternally inherited and in one case both maternally and paternally transmitted. Twenty-two boys of 136 (16%) were found to have a CNV. Ten boys had a pathogenic CNV (7%) and 12 boys had VOUS. Five boys had a *de novo* CNV, in eight the CNVs were paternally inherited and in seven boys it was maternally inherited.

A pathogenic CNV was more often identified in girls although the difference was not statistically significant (*OR*=2.29, 95% *CI* = 0.66-7.96).

No association was found between paternal or maternal age and the rate of *de novo* CNVs.

A pathogenic CNV was identified in 9% of children with autistic disorder and in 6% of children with PDD-NOS. Including CNVs characterized as VOUS, any CNV was found in one in five children with autistic disorder vs. one in ten in children with atypical autism. No pathogenic CNV was found in the nine children with Asperger's syndrome. However, two cases with Asperger´s syndrome had CNVs characterized as VOUS. Furthermore, a pathogenic CNV or a VOUS was found in 3/14 children who did not meet full criteria for ASD but had autistic traits in combination with other neurodevelopmental disorders.

Figure 10
Pathogenic CNVs

Sex	Phenotype	Cytogenetic location	Gain/Loss	Size	Inheritance	Gene identifiers
m	AD/ID/Non-syndromic	2p16.3	loss	110kb	maternal	*NRXN1*
f	AD/ID/Non-syndromic	2p16.3	loss	419kb	maternal	*NRXN1*
		2p16.3	loss	169kb	paternal	*NRXN1*
m	AA/N-low/ Syndromic	2q21.1	loss	434kb	maternal	*ARHGEF4,GPR148*
m	Aut traits/ID/Non-syndromic	7q35	loss	151kb	paternal	*CNTNAP2*
m	AA/N/ Syndromic	16p11.2	loss	545kb	*de novo*	*KCTD13*
m	AD/N-low/Non-syndromic	16p11.2	loss	523kb	paternal	*KCTD13*
m	AD/ID/ Syndromic	16p11.2	loss	220kb	*de novo*	*SH2B1*
m	AA/N-low/Non-syndromic	16p11.2	gain	547kb	paternal	*KCTD13*
		chr9	trisomy(mos)	NA	NA	NA
m	AD/ID/Syndromic	17p11.2	gain	3,47Mb	*de novo*	*RAI1*
m	AD/ID/ Syndromic	17p13.3	gain	1.02Mb	*de novo*	*YWHAE, CRK*
m	AA/ID/Non-syndromic	20p13.3	gain	549kb	*de novo*	*SCRT2*
f	Aut traits/syndromic	22q11.2[1]	loss		*de novo*	
f	AD/ID/ Syndromic	22q13.31q13.33	loss	3,04Mb	unknown	*SHANK3*
f	AD/ID/Syndromic	22q13.33	loss	25kb	*de novo*	*SHANK3*
		9p24.2	gain	420kb	maternal	*GLIS3*
		9q33.1	loss	22kb	paternal	*ASTN2*

NA=Non-applicable, m=male, f =female, AD=Autistic Disorder, AA=Atypical Autism, Asp=Asperger, Aut traits= autistic traits, ID=intellectual disability IQ<70, N-low=border-line IQ 70-80 N=IQ>70.[1]Deletion detected by karyotyping, array-CGH not done

Pathogenic CNVs were more often identified in children with ASD and co-occurring ID or borderline intellectual functioning – 10.7% compared to 2.4% in children with ASD and normal cognitive level (OR=2.15, 95% *CI*=0.46-10.0 *n.s*). Including CNVs categorized as VOUS, any CNV was more often found in the ASD+ID group compared to ASD without ID (*OR*=2.4, 95% *CI*=1.00-5.64, *p*=. 061).

Among children with a pathogenic CNVs or a VOUS (n=28), ten had a congenital malformation or major dysmorphology vs. twelve of 134 in children with no CNV detected. A significantly increased rate of CNVs was found in children with congenital malformations or dysmorphology compared to those without (*OR*=5.65, 95% *CI*=2.1-15.0, $p < .001$).

CNVs were not more prevalent in children with ASD and co-occurring epilepsy.

A non-significant trend indicated that in children with a history of autistic regression pathogenic CNVs and VOUS were more often identified compared to children with no history of autistic regression (*OR* = 2.12; 95% *CI*= 0.82-5.48,*n.s*.).

Figure 11
Copy Number variants of unclear significance

Sex	Phenotype	Cytogenetic location	Gain/Loss	Size	Inheritance	Gene identifiers
m	AD/ID/Syndromic	4q22.2	loss	75kb	paternal	*GRID*
		13q21.2	loss	60kb	paternal	*DIAPH3*
m	AD/ID/Non-syndromic	5p15.1p14.3	gain	1.46Mb	unknown	*BASP1*
m	AD/N-low/Non-syndromic	5p15	gain	769kb	maternal	*FBXL7*
m	AD/ID/Non-syndromic	8p23.1	gain	184kb	paternal	*MCPH1*
f	AA/N/Non-Syndromic	9q22.1	gain	845kb	paternal	*CENPP,ECM2,IPPK,BICD*
m	Asp/N-low/Non-syndromic	9q34.3	gain	89kb	maternal	*GRIN1,MAN1B1*
m	AD/ID/Non-syndromic	10q23.1	loss	62kb	maternal	*NRG3*
m	Aut traits/N/Non-syndromic	14q23.3	loss	75kb	unknown	*GPHN*
m	AD/ID/Non-syndromic	15q13.1	gain	301kb	paternal	*OCA2*
		15q13.1	gain	270kb	paternal	*APBA2*
m	AD/ID/Non-syndromic	16p12.3	gain	332kb	paternal	*ITPRIPL2, SYT17, TCM5*
f	Asp/N/Non-Syndromic	18p11.21	loss	297kb	maternal	*IMPA2*
m	AD/ID/Non-syndromic	18p11.23	gain	433kb	paternal	*PIPRM*
		18p11.22	gain	116kb	paternal	*ANKRD12*
m	AD/ID/Non-syndromic	7q36.1	gain	310kb	paternal	*XRCC2, ACTR3B*
		11p15.1	gain	186kb	maternal	*NELL1,SLC6A5*
m	AA/N-low/Syndromic	Xp11.4	gain	144kb	maternal[3]	*TSPAN7*

NA=Non-applicable, m=male, f =female, AD=Autistic Disorder, AA=Atypical Autism, Asp=Asperger, Aut traits= autistic traits, ID=intellectual disability IQ<70, N-low=border-line IQ 70-80 N=IQ>70

Array-CGH results and neurodevelopmental/psychiatric conditions in parents

Of the 28 identified CNVs, eight were *de novo* and 18 were inherited. In two cases parental samples were not obtainable. A parental history of any neurodevelopmental condition, psychiatric disorder or being classified as being within the broader phenotype autism spectrum was reported in 59 of the 320 parents (18%). The frequency

of these conditions was compared between those parents who transmitted a CNV to those who did not transmit any CNV.

Nine of the children had a maternally transmitted CNV. Maternal depression, prior to the birth of the child was more often reported in mothers who transmitted a CNV (classified as pathogenic or VOUS) compared to those mothers where no CNVs were identified in the offspring (*OR*= 3.22, 95% *CI*= 0.74-14.0 *n.s*.). Two mothers transmitted deletions affecting the *NRXN1* gene. One mother diagnosed with ADHD and mild epilepsy transmitted a deletion in 2q21.1. Moreover, one mother, transmitted a deletion in 18p11.21 had symptoms within the broader phenotype autism spectrum. Any neurodevelopmental or psychiatric condition was significantly more often reported in mothers who transmitted a pathogenic CNV/VOUS (5/9) compared to those mothers where no such CNV was identified in the offspring (*OR*=4.7, 95% *CI*=1.2-18.8, *p* =. 031).

The study could not demonstrate any association between paternal transmission of CNVs and paternal neurodevelopmental or psychiatric conditions (*OR*=1.4, 95 % *CI* = 0.28-7.08 *n.s*.).

When combining mothers and fathers who transmitted CNVs, an increased frequency of neurodevelopmental and psychiatric conditions was found in parents who transmitted a CNV to the offspring. Any parental condition was found in 7/19 CNV transmitting parents compared to 48/268 in cases where no transmitted CNV was identified. According to using Fisher´s exact test the difference was however not significant (*OR*= 2.67, 95% *CI*= 1.0-7.1, *p*= .061).

7 DISCUSSION

General findings

The study group of preschool children with ASD that had been referred for early intervention differed considerably with regard to severity of the core symptoms of ASD and exhibited a wide variety of co-existing disorders and conditions. Approximately 50% of the children had a general cognitive level within the intellectual disability spectrum. In a substantial subgroup genetic and other medical conditions were found. The epilepsy prevalence was significantly higher than in the general child population.

The results regarding pre- and perinatal ASD risk factor characteristics were consistent with previous and more recent epidemiological studies; e.g. advanced paternal age, parents born outside Europe, maternal psychoactive medication and scheduled caesarean section were all associated with increased ASD risk in the off-spring.

Psychiatric and neurodevelopmental conditions in 1st degree relatives differed according to gender. Male 1st degree relatives often had ASD or broader phenotype symptoms, speech-and language impairments and ADHD. Female 1st degree relatives more often had a history of depression, bipolar disorder or other psychiatric disorders.

The presence of an identified medical/genetic condition, including epilepsy resulted in a poorer adaptive outcome at the 2-year follow up. Children with such conditions had been referred at an earlier age. A poorer outcome was also found in children with a history of regression.

The genetic work-up including array-CGH yielded a significant increase of identifiable causes. Children with ASD and ID/congenital malformation more often had a causative CNV.

The overall findings implicate that comprehensive and collaborative neuro-developmental, cognitive and medical work-up is warranted in children with ASD.

Limitations and strengths

The children in this population based study group had been diagnosed at Child and Adolescence Mental Health Services and at Neuropaediatric units in Stockholm County. Diagnostic assessment policies varied between referring units. Children were young, the youngest around two years of age, weakening the diagnostic precision. In this group many children had severe cognitive and behavioural symptoms prompting an early identification. A small group, not included in the study, had been referred to their

local habilitation centre, due to ASD in combination with more complex medical conditions and needs.

Thus, our study group was population-based and considered to be representative of preschool children with early-diagnosed ASD, except for the most severely impaired children. The high proportion of children with ID can probably be attributed to that most children with high functioning autism/Asperger syndrome will be diagnosed later in life.

Our sample size is small, which affects the power of the statistical analysis and the results indicating significant findings must be interpreted with caution. Furthermore, associations not detected might have been present with a larger sample size.

Strengths of the study, was the very low attrition rate, (> 95% participated in the two-year follow-up assessment) and that the developmental profiles included evaluation at two time points. Comprehensive clinical and medical data could be obtained for each child and genetic analyses were performed in a large group. Outcome was measured by observers blind to the type of intervention given.

8 Discussion of results in each of the four studies

Study I

Highly heterogeneous clinical profiles were found in the study group. Major subgroups at referral 65%, had been diagnosed with autistic disorder, PDD-NOS had been found in 30% and in 3% Asperger syndrome. In addition, 2% had been diagnosed with unspecified ASD. Intellectual disability and borderline intellectual functioning was found in approximately 75% and correlated to adaptive behaviour (VABS-II). In some children the autistic symptoms were presenting and dominating, whereas in other children a general cognitive delay was more prominent than the autistic features. In this group, many children also had a delayed gross motor function, some had epilepsy and some exhibited dysmorphology. The highly variable speech- and language function also add to the vast heterogeneity in the study group. One subgroup (42%) exhibited significant hyperactivity. The conclusion of the study was that preschool children diagnosed or with suspected ASD require comprehensive and broad clinical assessments, including a medical evaluation. Children diagnosed with ASD at early age also need cognitive and medical re-evaluation.

Study II

Pre- and perinatal risk factors; e.g. paternal age, parents born outside Europe, maternal use of SSRI, other psycho-active medication during pregnancy and scheduled caesarean section were all found to confer a significant increased risk for ASD in the offspring.

Advanced parental age has been associated with ASD in several studies. High paternal age is also a risk factor for ID and schizophrenia. *De novo* mutations in spermatogenesis increase with age and it has been shown that ASD risk conferring sequence mutations more often have a paternal origin. We were not able to detect maternal age as a risk factor, probably due to the limited size of our study.

The risk associated with ASD in children with parents born outside Europe has been confined to ASD combined with ID. Parental migrational status has also been shown to be a risk factor in severe ID [234]. Several mechanisms have been studied and discussed; consanguinity, vitamin D deficiency, maternal stress and exposure to a different panorama of viral infections during pregnancy.

The increased risk for ASD associated with maternal use of SSRI was confined to ASD without ID in a recent Swedish study [160].

The increased rate of scheduled caesarean section has likewise been shown in other studies. However, no conclusions with regard to underlying factors have been drawn. Our interpretation, based on maternity records, of the observed difference is that scheduled caesarean sections were more common among mothers of ASD children, due to emotional vulnerability and fears of natural delivery.

Psychiatric and neurodevelopmental conditions in 1st degree relatives differed according to gender; male 1st degree relatives often had ASD or broader phenotype symptoms, speech-and language impairments and ADHD. Female 1st degree relatives more often had a history of depression, bipolar disorder or other psychiatric disorders. ASD susceptibility genes are probably not specific to ASD but to a wider spectrum of neuropsychiatric developmental disorders. This could well reflect that shared genetic susceptibility factors present in gender specific patterns.

Study III

All 208 children had received intensive or non-intensive/targeted early intervention based on Applied Behavioural Analysis (ABA) principles. The study was naturalistic and there was no randomization to treatment type. Parental preference was the most decisive factor and the presence of a medical/genetic condition did not affect choice of intervention type.

A relatively large proportion (18%) of the preschool children with ASD had an identifiable medical/genetic disorder including epilepsy - reaching 24 % in the group of children with ASD and ID. About one in five had a history of autistic regression and about one third had been referred before three years of age. All these factors were associated with a more negative outcome measured as change in VABS-II score during the 2-year follow-up. Children identified at a low age will have an increased risk of having co-occurring intellectual disability and genetic and medical co-existing disorders, reflecting an apparent and more severe underlying brain dysfunction

compared to children diagnosed at higher ages. The results underscore the importance of considering medical/genetic aspects in young children with ASD and the requirement to individualize and tailor intervention according to their specific needs.

Study IV

The overall result of the study was coherent with previous studies using chromosomal microarray in children with ASD. A clinically significant CNV could be identified in 8.6 % of children with early-diagnosed ASD. *De novo* CNVs were identified in 4.3 % and inherited CNVs in 4.3%. It is unlikely that CNVs classified as pathogenic are the single ASD genetic risk factor in an affected individual – rather a detected genomic imbalance is acting in concert with multiple modifiers with lower effect size resulting in a distinctive phenotype.

Albeit the robust evidence for genetic factors in ASDs, a specific genetic cause can be detected in about 25 % of individuals with ASD with current available technologies. In individuals with ASD, Copy Number Variants, conferring ASD susceptibility are found in 5-15%, mostly identified as *de novo* CNVs in sporadic cases. These CNVs are often rare, highly penetrant and the parental origin of *de novo* CNV events points to a trend with excess of maternal origin [32, 89]. Furthermore recurrent and inherited CNVs contribute to ASD susceptibility. Recurrent CNVs include those found in established microdeletion and microduplication syndromes, such as deletions and duplications on chromosome region 16p11.2, duplications 15q11.2, deletions and duplications 15q13.2, duplications 17q11.23 and deletion on chromosome region 22q11.2.

In this study, CNVs were 2-fold more often identified in children with autism spectrum disorder and co-existing intellectual disability and significantly more often in children with ASD and congenital malformations or dysmorphology. Several studies have reported high proportion (10-20%) of pathogenic CNVs in individuals with intellectual disability without ASD [94, 135] and in this study a large proportion of the children had co-existing ID. Some authors emphasize the genetic overlap between ASD and ID [184] while others report that the detection of a *de novo* CNV is not a useful predictor of low IQ in ASD [32]. It has been shown that lower IQ in individuals with ASD is associated with the number of genes affected by CNVs [32, 235]. The presence of dysmorphology/congenital anomalies increases the chances of detecting a de novo copy number variant in individuals with ASD [99].

Also in children with significant neurodevelopmental impairments in combination with autistic traits, but not meeting ASD criteria, a pathogenic CNV was present in as many as 14%. Pathogenic CNVs were slightly more often found in girls. Previous studies have documented that a higher proportion of females with ASD carry detectable de novo copy-number events than do males and that the events are larger [31] and that a trend toward more gene-rich CNVs has been observed in females [32].

Eight of the 162 children in the study were found to have a well-established microdeletion or microduplication syndrome. CNVs involving 16p11.2 (deletions and duplications) were found in 2.4 % of the children. This region has repeatedly been reported in a substantial fraction (~0.8%) of individuals with ASD [31, 32, 236]. Deletions and duplications in this region have been associated with ID, non-ASD psychiatric disorders and have also been detected in unaffected individuals.

A double-hit model where CNVs or other chromosomal structural rearrangements combine and cause the ASD phenotype has been described in previous studies [70, 90]. In this study two cases had a pathogenic CNV and a minor second CNV. Three cases had CNVs classified as VOUS and additional minor aberrations.

There are a several specific genes, where mutations have repeatedly been found to be risk factors in ASD, such as *NLGN3* [66], *NLGN4* [66, 237], *SHANK2* [91, 238] , *SHANK3* [54, 239]*, NRXN1 [59, 60, 92], NRXN3 [240], SHANK 1* [37]*, ASTN2* [96, 241], *CNTN4* [242] and *CNTNAP2 [243]* [52]. In this study, seven cases had a CNV encompassing one of these genes (*NRXN1, SHANK3, ASTN2* or *CNTNAP2*).

Advanced paternal age has been shown to be a risk factor for ASD and ID [129, 135, 136]. In a study of cases with ID (without ASD) by Hehir-Kwa et al, rare *de novo* CNVs was associated with a paternal origin and with higher paternal age[135]. In this study we could not document that parental age affected the rate of de novo CNVs in the offspring.

There was a trend indicating higher rates of maternal psychiatric or neuro-developmental conditions prior to the child´s birth in mothers who transmitted CNVs (classified as pathogenic or VOUS) compared with mothers who did not transmit such CNVs. In fathers transmitting such CNVs, no such trend could be documented.

Recent guidelines suggest the use of chromosomal micro-array for children with ASD or ID. Chromosomal micro-array increases the chance of giving an aetiological explanation to the parents and to provide better data when discussing recurrence risk for future siblings.

9 CONCLUDING REMARKS

Autism spectrum disorders are not uncommon and have a major impact on the affected individual, the family and on the requirements for educational and societal support. Our study has demonstrated that preschool children with ASD differ significantly in cognitive and language abilities and in many other developmental aspects (hyperactivity, epilepsy). Prenatal risk factors play a role in ASD aetiology, although these risk factors are non-specific and the relative risk increase is mostly modest. Nearly one fifth of the children in our study had a medical or genetic condition identified. An important observation is that the presence of such a condition may influence the outcome of early intervention. The compartmentalization of services for young children with neurodevelopmental disorders may result in suboptimal recognition and treatment of co-existing medical and neuropsychiatric conditions. Thus, preschool children with ASD should have access to comprehensive neurodevelopmental, medical and genetic assessment, according to the "ESSENCE" concept (Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations) [244].

Our study could identify pathogenic Copy Number Variants in a subset of children with ASD. Interestingly, the CNVs observed were in many cases affecting genes involved in synaptic development and function. Another group of CNVs occurred in regions in recently defined microdeletion or microduplication ASD-associated syndromes. Recent guidelines suggest the use of chromosomal micro-array for children with ASD or ID, the rationale for this being the superior diagnostic yield compared to conventional karyotyping. Chromosomal micro-array increases the chance of giving an aetiological explanation to the parents and to provide better data when discussing recurrence risk for future siblings. Hopefully, better understanding of specific phenotypic characteristics in cases with a defined aetiology will improve the medical and educational support.

10 FUTURE DIRECTIONS

Rapidly evolving genomic technologies, such as whole genome sequencing, combined with increasingly large study cohorts will establish the extreme aetiological heterogeneity in ASD and other complex disorders and display the highly pleiotropic effects of ASD associated mutations [104]. An increasing number of ASD conferring specific genes will be identified and the role of common genetic polymorphisms will be clarified. There is increasing evidence that in addition to protein-coding genes microRNAs may be involved in ASD aetiology [245]. The hypothesis that a large number of ASD risk gene variants affect proteins in specific functional networks will be better understood. It has been suggested that neurodevelopmental and psychiatric disorders are pathway disorders, i.e., that genes involved in ASD seem to converge on common pathways altering synaptic homeostasis. Recently it has been proposed that "synaptic clinical trials" should be designed and carried out to investigate the possibility of reversing phenotypes [246]. It is likely that the aetiological and clinical overlaps between childhood developmental disorders and adult psychiatric disorders will be better elucidated. The future diagnostic procedures will increasingly include not only more specific phenotypic symptom criteria but also far more genotypic data. Identifying specific functional networks involved in ASD and other complex disorders will speed up the development of targeted medications.

11 SVENSK SAMMANFATTNING

Under de senaste 10 åren har det skett en mycket snabb utveckling av genetiska analysmetoder och möjlighet till databearbetning av extremt stora informationsmängder. Det mänskliga genomet har kartlagts i detalj. Många höggradigt komplexa interagerande mekanismer är involverade i regleringen av våra gener, liksom mellan gener och faktorer i miljön.

Vi har fått nya metoder för att kartlägga orsaksfaktorer till utvecklingsneurologiska och psykiatriska tillstånd. Överlappande identiska genetiska förändringar återfinns hos barn med utvecklingsstörning, autismspektrumtillstånd (AST), ADHD och hos vuxna individer med psykiatriska diagnoser och pekar mot att gränserna mellan dessa tillstånd förefaller vara oskarpa. Begreppet ESSENCE(Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examination), som lanserades av Gillberg 2010 beskriver hur tidigt debuterande utvecklingsneurologiska symtom och funktionsnedsättningar i mycket hög utsträckning samexisterar och överlappar.

Vid både utvecklingsstörning och autismspektrumtillstånd har man idag identifierat många hundra underliggande genetiska förändringar. Många av de gener som kopplas till dessa tillstånd är involverade i hjärnans utveckling. Särskilt verkar gener som styr synapsens mognad och funktioner ha en central roll.

Autismspektrumtillstånd definieras i s.k. diagnosmanualer (DSM-IV, DSM-5 och ICD-10). De grundläggande svårigheterna hos individer med AST innefattar svårigheter med socialt samspel, med verbal och icke-verbal kommunikation samt förekomst av begränsningar i beteenden, rutiner och intressen. AST definierat i DSM IV innefattar autistiskt syndrom, atypisk autism (något lindrigare form) samt Aspergers syndrom, som kännetecknas av svårigheter med socialt samspel, förmåga till kommunikation och med begåvning inom normalvariationen men med begränsningar i intressen/beteenden. I den aktuella DSM-5 finns en övergripande diagnosterm; autismspektrumtillstånd. Hos barn och vuxna med AST förekommer i hög frekvens andra samtidiga svårigheter. En stor andel har varierande grad av utvecklingsstörning eller kognitiv förmåga i nedre normalområdet. Många individer med AST uppfyller även kriterier för samtidig ADHD med eller utan överaktivitet/impulsivitet. Epilepsi är inte ovanligt och hos unga vuxna med autism har närmare en tredjedel epilepsi.

Kunskapen om autismspektrumtillstånd har ökat och det har under de senaste trettio åren skett en kraftig ökning av antalet individer som får en diagnos inom autismspektrat. Ett sådant tillstånd diagnosticeras numer hos ~1% av befolkningen. Fler pojkar än flickor får AST diagnos; relationen anges ofta till 3-4:1, ännu mer förskjuten hos personer med AST och normal begåvning.

Epidemiologiska studier bl.a. av enäggs- och tvåäggstvillingar med AST, studier av syskon till barn med AST samt förekomsten av mildare symtom på autism hos släktingar – pekar på att genetiska faktorer spelar en avgörande, central och dominerande roll.

Studier har också identifierat ett flertal riskfaktorer som är relaterade till miljön – i huvudsak under graviditeten då fostrets hjärna utvecklas men även vissa bakgrundsfaktorer hos föräldrarna har kopplats till ökad risk för AST.

Vår forskningsgrupp har undersökt barn som fått diagnos AST före 4.5 års ålder och som remitterats till ett specialinriktat habiliteringscenter: Autism Center för Små barn i Stockholm. Barnen har diagnostiserats vid Barn- och ungdomspsykiatrisk mottagning (BUP) eller på någon av länets tre Universitetssjukhus med enheter för Neuropediatrik. Vi startade våra studier 2006 med syftet att följa upp och utvärdera effekter av tidig intervention. Totalt 208 barn var med i studien, 176 pojkar och 32 flickor.

Vår första studie är en kartläggning av 208 barn som remitterats i åldrar 20-54 månader med avseende på typ av AST, förekomst av samtidig utvecklingsstörning, tal – och språknivå och medicinska tillstånd. Kartläggningen utgjorde en bas inför den planerade 2-års uppföljningen. En stor andel av de tidigt remitterade barnen hade utöver AST också en samtidig utvecklingsstörning eller tecken på försenad generell kognitiv utveckling. Många uppvisade hyperaktivitet. Epilepsi fanns hos 6 %. Ungefär en femtedel av barnen hade haft en tillbakagång i sin utveckling, ofta omkring 18 månaders ålder.

I den andra studien jämfördes barnen med AST med en stor jämförelsegrupp med hjälp av Medicinska Födelseregistret med avseende på några riskfaktorer. Vi fann att barn med AST oftare hade en äldre fader och föräldrar födda utanför Europa. Mödrar till barnen med AST hade en signifikant högre användning av antidepressiva läkemedel, liksom annan psykofarmaka under graviditeten. Planerade kejsarsnitt var vanligare i gruppen med AST. I samband med föräldraintervjuer efterfrågades förekomst av utvecklingsneurologiska och psykiatriska tillstånd hos nära släktingar. Fäder och bröder hade en hög förekomst av AST eller hade mildare autismsymtom (broader phenotype). Mödrar och systrar hade en hög förekomst av depression och andra psykiatriska tillstånd.

I den tredje studien redovisas medicinska och preliminära resultat från genetisk undersökning med s.k. array-CGH. Ett medicinskt eller genetiskt tillstånd förelåg hos 18 % av de 208 barnen. Andelen barn med epilepsi när barnen är < 6.5 år är 9 %.
Som mått på hur barnets adaptiva förmågor utvecklats, jämfördes skillnader i poäng på Vineland Adaptive Behavior Scales (åldersrelaterat instrument som mäter barnets fungerande i det dagliga livet). En mätning gjordes före start av tidig intervention och en ny efter två år. Resultaten visade att barn som remitterats före 3 års ålder/eller hade ett identifierat medicinskt eller genetiskt tillstånd inklusive epilepsi hade en sämre adaptiv utveckling.

I den fjärde studien redovisades resultaten där vi använt s.k. array-CGH för att genetiskt undersöka 162 barn. Vid utredning av barn med AST, har man tidigare ofta rekommenderat traditionell kromosomanalys och analys av Fragile X. Med dessa metoder påvisas orsak till AST hos drygt några procent av de undersökta. Den nya metoden - array-CGH - har ca 100 gånger bättre ”upplösningsförmåga” än den traditionella kromosomanalysen.

Normalt har en individ en DNA uppsättning från modern och en från fadern, vilket innebär att alla sekvenser finns i två upplagor. Med array-CGH, kan man se om ett segment saknas (deletion) eller om ett segment förekommer i fler än två kopior (duplikation). Sådana variationer i antalet kopior (eng. Copy Number Variants = CNVs) förekommer hos friska och utgör en del av variationen mellan olika individer. Dock kan i synnerhet stora förändringar, där det saknas DNA segment kopplas till risk för sjukdom. Med hjälp av array-CGH kan man hos individer med AST identifiera olika ovanliga CNVs hos 5-15%, jämfört med hos friska kontroller (1 %). En CNV kan vara nedärvd från en förälder eller uppkommit *de novo,* i bildningen av spermier/ägg och då återfinnas i barnet men inte hos någon av föräldrarna.

Av de 162 undersökta barnen hade 8.6 % en påvisbar CNV, som med största sannolikhet har relevans för barnets tillstånd. Hos ytterligare 8.6 % påvisades en CNV där det finns en osäker relevans. Hos barn med AST i kombination med utvecklingsstörning påvisades i något högre andel en CNV, liksom hos barn med AST i kombination med medfödd missbildning eller yttre avvikelser. Åtta av barnen hade en s.k. *de novo* CNV och sexton barn hade en nedärvd CNV från en förälder (i ett fall från båda). En möjlig men osäker association påvisades där en högre andel mödrar med egen CNV hade depression eller annan psykiatrisk diagnos före barnets födelse, jämfört med mödrar där ingen CNV identifierades.

Sammanfattningsvis uppvisar barn med en tidig AST diagnos en stor variabilitet avseende hur svår deras autismproblematik är och avseende hur stor andel som har en samtidig lindrig till svår utvecklingsstörning. Det fanns i vår studerade grupp barn som helt saknade talat språk till barn med nära normal språkfunktion. En stor andel barn hade betydande samtidig hyperaktivitet. Epilepsi förekom hos nästan var tionde barn vid ålder 4.5 - 6.5 år.
Inom gruppen barn som fått diagnos AST tidigt återfanns flera barn med neurologiska sjukdomar som tuberös skleros. Några hade andra väldefinierade syndrom som Fragile X och två flickor visade sig ha Rett syndrom. Extrem underburenhet och prenatal exponering för alkohol och epilepsiläkemedel bedömdes vara bidragande prenatala riskfaktorer hos några barn.

Hög ålder hos fadern, utomeuropeisk förälder och om modern medicinerat med antidepressiv medicinering och/eller annan psykofarmaka ökade risken för AST hos barnet. Vi påvisade en markant skillnad där fäder och bröder till barn med AST hade högre förekomst av AST/broader phenotype medan särskilt mödrarna hade depression

och andra psykiska tillstånd. Underliggande förklaring kan vara att genetisk sårbarhet visar sig olika hos män och kvinnor samt att kvinnor oftare diagnosticeras med psykiatriska tillstånd utan att en AST problematik uppmärksammas.

I uppföljningsstudien fann vi att de barn som diagnosticerats i låg ålder oftare hade ett samtidigt medicinskt eller genetisk tillstånd. Dessa barn hade en sämre adaptiv utveckling.

I den avslutande genetiska studien med array-CGH identifierades avvikelser hos 8.6%, hälften nedärvda och hälften s.k. *de novo*.

Avhandlingen belyser att barn med AST har mycket heterogena utvecklingsprofiler och en hög förekomst av utvecklingsneurologiska/neuropsykiatriska och medicinska tillstånd, inklusive epilepsi. Det går idag att identifiera genetiska orsaker till AST hos 10-15% med hjälp av array-CGH, analys av Fragile X och andra mer riktade genetiska analyser. Vår rekommendation är att array-CGH bör ingå i utredning av barn med AST, särskilt vid samtidig utvecklingsstörning. Barn med AST bör ha tillgång till tidig intervention men även till medicinsk och genetisk utredning. Om diagnosen satts vid låg ålder behövs ofta en förnyad utredning efter ett par år, särskilt för att göra en noggrannare bedömning av den generella kognitiva förmågan.

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