Genetics of intellectual ability and disability

Beunders, G.

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Gea Beunders

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Genetics of intellectual ability and disability

The importance of common variants and rare syndromes illustrated by studies of SNAP25 and AUTS2

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promotoren: prof.dr. E.J. Meijers-Heijboer
prof.dr. D. Posthuma

copromotor: dr. E.A. Sistermans

Paranimfen	Rian Bierlaagh
	Els Voorhoeve
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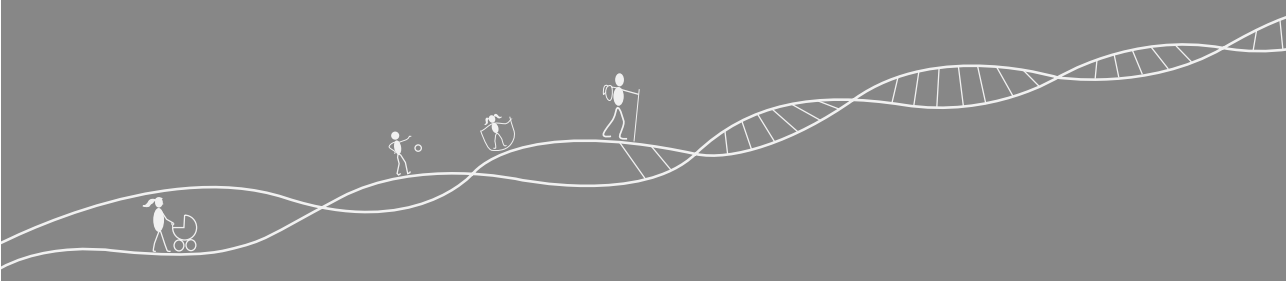
General introduction



GENERAL INTRODUCTION

What is intelligence?

Intelligence describes the psychological quality that allows one to apperceive, reason, orient in space, plan, solve problems, use language, think abstractly, remember and learn from experiences.[1] Intelligence is thus a complex concept, and there is no final consensus on its definition. Wechsler defined intelligence as the capacity to perform goal-oriented actions, to think rationally and to effectively react to one's surroundings.[2]

Models and theories to explain intelligence have changed over the years. The most well-known theory is that of Spearman. He suggested a general factor '*g*', which he described as a form of mental energy that explains the correlation between performances on several different tasks (such as cognitive, creative and problem-solving tasks). [3] Spearman's statistical method of factor analysis hypothesized that the variation between individuals in their performance on cognitive subtests is explained by a common factor 'g' and a test-specific factor 's'. This idea however seems to be too simple because the correlation between tests cannot be explained by just one general factor. [4] Furthermore, although the general correlation ('g') between various cognitive tasks has been reproduced many times, the biological substrate for 'g' is much debated. In the 1970s and 1980s there was criticism of this scheme and broader views were proposed in which at least eight independent forms of intelligence were recognized, including interpersonal ability, creativity and kinaesthetic ability. [5; 6] Today 'g' is still recognized but is less and less regarded as a causal factor by itself; it is instead seen as a measure of the fact that different parts of intelligence partly rely on the same fundamental processes in the brain, such as attention, focus and working memory. [7]

Measuring intelligence is not an easy task, especially when you appreciate the broader definitions or models of intelligence. IQ tests have their limitations, but are generally accepted to be able to measure the variability in intelligence between individuals. Two of the most commonly used tests are the Wechsler Adult Intelligence Scale (WAIS) for adults and the Wechsler Intelligence Scale Children (WISC) for children. [8; 9] Both consist of several subtests such as assort pictures, vocabulary and figure series. For each item, the number of mistakes and the completion time is measured and a raw score is obtained. These raw scores are compared to norm scores deduced from testing a group of people in the general population within the same age group, and a standard score per subtest is computed. Adding these scores leads to a total score for verbal IQ, performance IQ and a total or general IQ score. A score of 100 means that 50% of the people in the general population score higher and 50% score lower, whereas an IQ of 130 means that 97.5% score lower and 2.5% score higher. (see figure 1)

Biology of intelligence: What has caused humans to be intelligent mammals?

Humans evolved from a common ancestor with the chimpanzee. Fifteen phenotypic differences between the humans and the great apes were described by Carroll et al., including brain size,

reduced body hair, language and advanced tool making. [10] Higher level of intelligence and bipedality were not mentioned, but seem to be important differences as well. [11] Chimpanzees and modern humans share >98% of their DNA sequence, but it is likely that there are much larger differences at the protein expression level. [10] Genetic changes like insertions, deletions or point mutations (in protein coding or regulatory DNA sequences) are essential for evolution and explain the differences between species. [12-15] Single nucleotide polymorphisms are responsible for 1.06% of the fixed base pair differences. Copy number variations contribute to 1.5% of unique sequences between the genomes of the chimpanzee and human. [12]

Random DNA mutations that arise in an individual and are passed on to the next generations cause a slow change in a species over time. For more rapid changes to occur, as seems to have occurred in the rise of modern humans, genetic drift and natural selection are necessary. Genetic drift leads to changes in the allele frequency of a genetic variation within a population due to random processes. Examples of random processes include arbitrary allele distribution during gametogenesis but also natural disasters that cause dramatic changes in population size. Natural selection can cause alleles to disappear or to become fixed (meaning that the alternative allele is lost). Positive or negative selection is a non-random driving force for changing allele frequencies. This occurs when a mutation changes the phenotype such that the chance of survival and/or reproduction is affected. [10] Both random and non-random factors seem to have played a role in human evolution. [16] How else can we explain such a rapid increase in intelligence? There are many different theories why positive selection has contributed to the increased intelligence in humans. The 'Intelligence as a disease resistance sign' theory, for example, explains selection by the fact that intelligence provides a survival advantage through, e.g. better avoidance of disease by intelligent choice and preparation of food. [17] Another theory is the 'sexual selection' theory, in which intelligence is seen as a sign of fitness that makes intelligent human individuals more interesting to mate with. [18]

What biological aspects made humans more intelligent? There are several lines of evidence that level of intelligence is correlated to brain size within human evolution, but this correlation does not hold true amongst other species. The correlation overall is modest and does not mean that there is a causal relationship. [19; 20] It thus seems likely that a more effective use of the brain is of greater importance. [21; 22] It has been shown that people with a higher intelligence show lower glucose metabolism during complex tasks yet higher metabolism in areas of the brain that are thought to be important in that specific task. [23; 24] Further, conduction speed is also correlated to intelligence.[25] It therefore seems likely that humans, with their higher intelligence, use their brains more effectively than the great apes.

Variation in human intelligence, heritability and common variants

Intelligence is variable among individuals. Because of the way IQ scores are defined (an individual score is compared to the scores of a reference group of the same age) the mean IQ score is 100. Variation in IQ is normally distributed and 95% scores fall within the range of 70 and 130. Intellectual

disability, as defined by an IQ below 70, can be seen as the lower end of this distribution. There are, however, more patients with severe intellectual disability than would be expected from the Gaussian curve describing the normal distribution [26] (See figure 1). This variation is caused by a complex interplay between genetic and environmental factors.

Intelligence has a high heritability (h^2). Heritability is the portion of the variation of a trait caused by genetic factors and can be calculated in twin studies by doubling the difference in correlation between monozygotic (MZ) twins and dizygotic (DZ) twins (heritability = 2(rMZ - rDZ)). It is important to realize the complexity of the concept of heritability. Heritability is the ratio of the variation of a trait caused by genetic factors against that caused by environmental factors. Heritability, however, does not indicate the part of the variation that is caused by genetic factors in absolute terms and is, to a great extent, dependent on population and environment. [27] The overall heritability for intelligence is around 0.54. [28] Although one would think the genetic influences on intellectual functioning would decrease with age, the opposite is true: heritability increases with age from 0.4 in childhood to 0.6 or even 0.8 in adulthood. [29; 30]

The general consensus is that variation in intelligence is influenced by many genetic variations with a small effect. The effects may be as small as odds ratios of 1.0 to 1.2. [31] Most variants will be common, but there will be rare variants influencing variation in intelligence as well. Until recently only a few genes, including *SNAP25*, showed replicated association with intelligence in the normal range. [32; 33] Recently, a GWAS meta-analysis including 78,308 individuals identified 336 SNPs at 18 loci associated with variation in intelligence. In total, 52 genes were found to be associated with intelligence, 40 of which had not been identified before. In this large study, however, no association with the *SNAP25* gene was found. [34]

The heritability of intelligence is only partly explained by current estimates of SNP-based heritability, a gap that is referred to as the "missing heritability". [35] Gene-gene and gene-environment interaction can be important, and rare variants involved in causing variation in intelligence between individuals are not found by association studies.[29]

High intelligence is not only as familial/heritable as IQ variation in the total range (IQ scores 70-130), it also seems to be influenced by the same genetic variations. High IQ (the top 15% of IQ scores) is likely to be caused by the presence of many positively and few negatively associated alleles that influence the variation in IQ in the normal range as well, rather than by other genes or other gene variants. [36]

Interestingly, the same mechanisms seem to influence IQ in the lower range and even in mild intellectual disability. Siblings of people with mild intellectual disability were found to show mean IQ-scores of 85, while siblings of people with severe to profound intellectual disability have mean IQ-scores of around 100. [37] *De novo* single mutations are known to be the main cause of severe intellectual disability. In mild intellectual disability, monogenetic or chromosomal deleterious mutations are less frequently found. [37; 38] These findings indicate that mild intellectual disability is often just the lower end of the normal distribution and seems to be caused by the additive effect of many negatively (and few positively) IQ-correlated variants. [37]

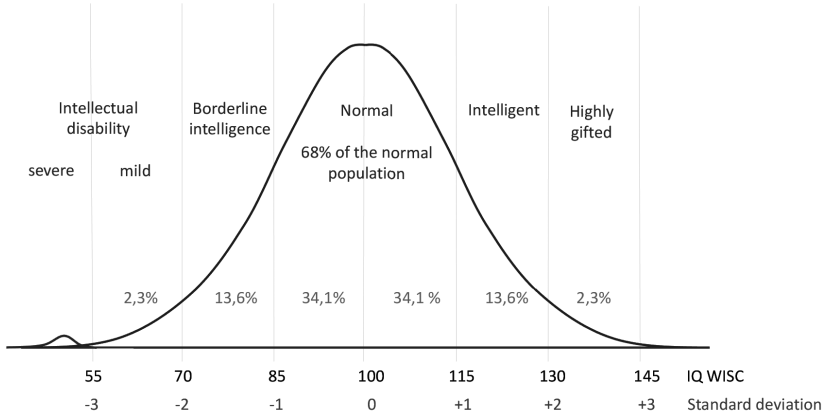


Figure 1. *Variation in IQ scores is normally distributed except for severe intellectual disability.*

Intellectual disability

Intellectual disability is a complex phenotype characterized by suboptimal functioning of the central nervous system. [39] According to DSM-IV, intellectual disability is defined as 'an IQ < 70 with deficits in two or more adaptive skills starting at a childhood age' (American Psychiatric Association, DSM-IV 1994). Alternative classification systems (e.g. those proposed by the American Association for Mental Retardation (AAMR) or following ICD-10) advocate a more multidimensional or multiaxial classification that includes the measurement of intelligence but puts greater emphasis on adaptive functioning and systems of support. Intellectual disability is a complex disorder in which a large number of intellectual skills (e.g. language, motor, social, emotional and visuospatial skills) are suboptimally developed in the patient. The severity of disability ranges from mild to profound disability. The true prevalence of intellectual disability is hard to estimate as most studies report a prevalence based on registration by health or governmental authorities. The true prevalence is, however, a combination of those with intellectual disability who do need social services and those who do not. A meta-analysis of 52 prevalence studies by Maulik et al. showed a prevalence of about 1% for intellectual disability as a whole over the time period 1980-2009. [40] A more recent review suggested that the global prevalence might even be lower than 1% but also found a large variation in the prevalences measured within the different studies (ranging from 0.1% to 1.55%). [41] Roeleveld et al. revealed that the prevalence rate of 0.4% for moderate to profound intellectual disability was rather stable among the different studies, whereas the prevalence rate of mild intellectual disability was much more variable ranging from 0.4% to 8%. This larger variation is due to the fact that different populations where tested in different studies and the fact that there was a large variation in the definition of (mild) intellectual disability. [42]

Intellectual disability can be present in a non-syndromal or a syndromal form. The *non-specific* or *non-syndromal form* presents as an isolated form of intellectual disability with no other distinct

features. In the *syndromal* form, intellectual disability is one feature of a complex of symptoms and can coincide with dysmorphic features or major physical malformations.

Aetiology of intellectual disability

Understanding the causes of intellectual disability is biologically interesting as it teaches us more about normal neurodevelopment processes, but this understanding is also important for individuals with intellectual disability and their parents. A causal diagnosis helps to adjust follow-up, to know if screening for additional developmental disorders or birth defects is important and sometimes to adapt therapy. Causal diagnosis can further help in predicting prognosis and recurrence risk, in enabling prenatal diagnosis in some cases, and in facilitating access to special needs assistance. Aetiology can be genetic, environmental, or a combination of both. However, for many individuals with intellectual disability, we still do not have a causal diagnosis. [38; 43]

Moderate to severe intellectual disability negatively influences reproductive fitness. Most people with intellectual disability will not have children so they will not pass on their genetic defect causing intellectual disability. Still the number of people with moderate to severe intellectual disability has been stable over time. [40] This stability suggests *de novo* mutations of chromosomal aberrations are an important cause of moderate to severe intellectual disability. As described above, in mild intellectual disability a multifactorial model would fit better. Supporting this hypothesis, the chance of finding a genetic cause in patients with moderate to severe intellectual disability is about 60% after extensive genetic investigation, while this chance is lower (around 20%) in patients with mild intellectual disability. [38]

It is not exactly known which proportion of intellectual disability is caused by genetic factors, but it is clear that the genetic causes are very heterogeneous. Trisomy 21 (Down syndrome) was the first cause discovered after karyotyping was introduced, and Down syndrome is still the most common cause of intellectual disability. Down syndrome together with other microscopically visible chromosomal aberrations explain about 10% of cases in selected cohorts of intellectual disability. [44; 45] Smaller chromosomal abnormalities, mostly *de novo,* were recognized as an important cause soon after the technological advances of chromosomal banding techniques and the discovery of fluorescent in situ hybridisation (FISH), Multiplex Ligation-dependent Probe Amplification (MLPA), array comparative genome hybridisation (array CGH) and single nucleotide polymorphism array (SNP array). Clinically relevant sub-microscopic copy number variations (CNVs) are found in about 10 to 15% of intellectual disability patients. [46; 47] Single gene disorders causing intellectual disability could sometimes be recognised because of specific extra features and a specific pattern of inheritance. Linkage analysis, for example, has revealed the causal mutated gene for several x-linked and autosomal recessive diseases. The recent introduction of whole exome sequencing has allowed the identification of many new intellectual disability genes. At present, there is convincing evidence of a causal association with intellectual disability for mutations in more than 700 genes, and this number is still increasing rapidly. [38]

Techniques for finding causal dna or chromosomal variants in individuals with intellectual disability

Over the last decade, technological progress in the field of genetics has been rapid, and I present here a short introduction to the techniques that are now in use.

Karyotyping

Chromosomes are large DNA strands that are wound together and these can be visualized by microscopy during cell division. Special colouring techniques allow the recognition of individual chromosomes by size, form and black and white banding patterns. Chromosomes ordered from large to small makes a karyotype. Our cells contain 46 chromosomes in 23 pairs. We have 22 autosomal chromosome pairs that are the same for males and females and one sex chromosome pair: XX for a female and XY for a male. Karyotyping can reveal extra chromosomes (such as a trisomy 21), structural rearrangements (such as unbalanced or balanced translocations), and copy number variations larger than 10 MB (deletions or duplications within a chromosome).

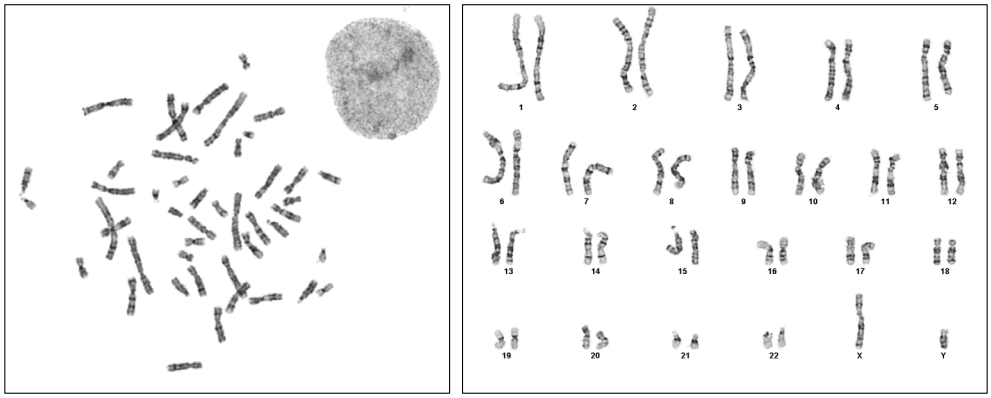


Figure 2. *karyogram of a normal male, 46,XY. Light microscope view of a lymphocyte in its metaphase, showing the chromosomes before and after ordering for interpretation.*

Other techniques to pick up smaller deletions, duplications or chromosomal rearrangements have also been introduced, including FISH (in which fluorescent labels where used to see if a specific locus was present at the right place) and MLPA (a DNA based technique to find specific deletions or duplications under the resolution of karyotyping), but these will not be discussed in detail here.

Array analysis

Array analysis was first introduced in clinical genetic practice around 2006. This enabled the identification of chromosomal abnormalities, deletions and duplications with a much higher resolution on a genome-wide scale. In Array Comparative Genomic Hybridization (Array CGH) the DNA of the patient is labelled with one fluorescent signal (red for example) and DNA of a control is labelled with another fluorescent signal (green for example). Both are then mixed and hybridized

to many locus-specific markers on an array chip. By measuring the colour of the fluorescent signals of the DNA bound to the chip, the relative number of locus-specific copies is deduced. If there is as much DNA from the patient as from the control bound to a specific marker, a yellow signal is picked up. If more or less patient DNA is bound then the signal will be red or green, respectively. These signals are mapped to their chromosomal position and plotted on a graph as shown in figure 3.

In a SNP array, there are locus- and allele-specific markers on the array chip and only the (fluorescent) DNA of a patient is hybridized to the chip. The signal intensity for a specific marker enables the user to deduce not only the copy number but also the alleles of a specific locus. With SNP array or array CGH you can see sub-microscopic gains or losses, but you cannot see the place and orientation of chromosomal material and you are not able to read the DNA sequence of a gene.

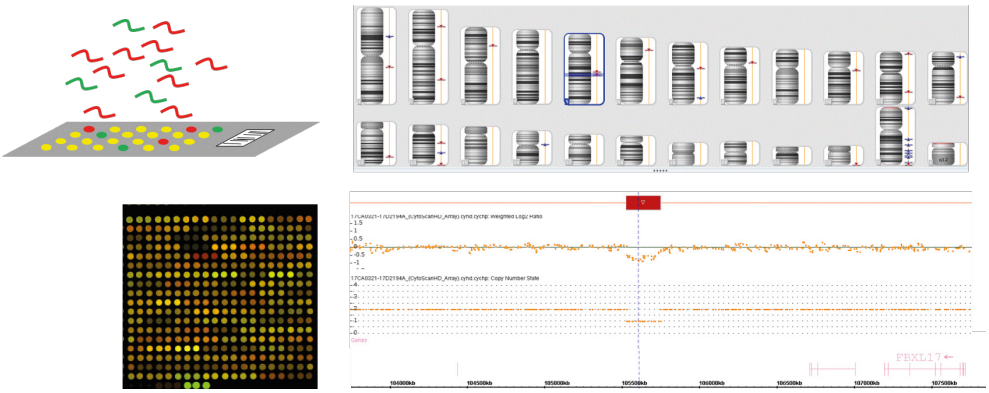


***Figure 3**. array CGH, A. The array chip binding patient DNA fragments (with a red fluorescent label) and control DNA fragments (with a green fluorescent label); B. Part of the array chip showing the fluorescent signal of multiple probes. The colour indicates the proportion of patient DNA. C. This proportion is plotted in a graph (below), and shows a small deletion (indicated by the dotted blue line) on chromosome 5 (as indicated by the blue line in the chromosome overview above). Here several probes are below the average of 0 here because there is less patient DNA bound than control DNA to the array chip.*

Whole Exome Sequencing

DNA is built of four different nucleotides: adenine (A), guanine (G), cytosine (C) and thymine (T) and forms a double helix. The order of these four nucleotides forms the genetic code. A nucleotide sequence that codes for one protein is called a gene. Genes have exons that are protein coding and these are separated by introns (see Figure 4). All the protein coding parts of all the genes in the genome is called 'the exome', and it accounts for about 1-2% of the genome (about 30 million base pairs).

Sequencing genes used to be very time consuming. Using Sanger sequencing, only targeted gene resequencing was achievable. Now, various high-throughput DNA sequencing techniques known as next generation sequencing (NGS) enable the generation of large-scale sequence data. In whole exome sequencing, the DNA code of all protein coding sequences is analysed in a single

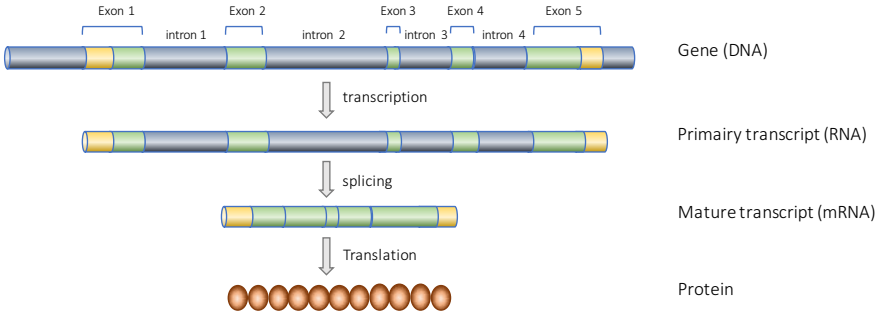


Figure 4. *Gene structure and steps toward protein synthesis*

experiment. The sequence techniques produce sequence reads that need to be mapped to the genome, then the sequence is compared to the reference DNA sequence and variations are called. This results in about 20,000-25,000 variants per individual, most of which are normal variations. Several filtering steps are then necessary to know which variants might be disease-causing. One important filtering step is to filter out variants with high allele frequencies in the general or local population, which are therefore unlikely to cause rare diseases. Subsequently, you can filter for variants with a likely effect on known disease genes (virtual targeted analysis of a gene panel). This is often a first step in the analysis process because greater knowledge of these selected genes helps to interpret the variants and there is less chance of unsolicited findings with this approach. In the majority of cases, targeted analysis is followed by whole exome analysis as no causal diagnosis could be found. The diagnostic yield is much higher when trio analysis is performed, meaning that both parents of the patient are sequenced as well. This enables *de novo* analysis, meaning that variants that are not present in both parents can be identified. These new variants are few in number and more likely to be disease-causing. Finally, by looking for two variants with a possible deleterious effect in one gene (one of both parents) you can find recessive inherited disease-causing variants. [48]

Finding new genes for intellectual disability

In the past, intellectual disability syndromes could only be recognized by a specific pattern of birth defects and/or physical and facial features. Once a new phenotype was recognized, the locus or the gene harbouring the causative genetic defect could be found by linkage studies, by studying patients with chromosomal translocations and by candidate gene sequencing. Examples of syndromes identified this way are Williams Beuren syndrome and Coffin Lowry syndrome, which are a recurrent microdeletion and an X-linked mental retardation syndrome, respectively, and which both have a recognizable facial gestalt and specific pattern of birth defects.[49; 50]

After the introduction of array and WES as genome-wide diagnostic techniques, new syndromes were discovered by comparing the phenotypes of patients with a genetic defect at the same locus or gene. Examples here include Kleefstra syndrome [51] and the AUTS2 syndrome described in this

thesis. [52]

After finding a new candidate gene with these genome-wide techniques, further proof for a causal relation is needed. A *de novo* loss-of-function variant does not prove causality, as generally one or two *de novo* variants are found in the coding sequence of all individuals in the general population and loss-of-function variants are not always disease-causing. Variants predicted to cause a loss of function (even in known disease genes) are found in healthy controls, which indicates that one should be careful with interpretation of variants that seem to be causative based on molecular characteristics only. [53-55] International databases and collaborations between DNA laboratories help identify patients with comparable phenotypes who carry a defect in the same gene, thereby identifying new candidate genes. However, and especially with phenotypes not specific for a certain syndrome, the discovery of 2-3 patients with a mutation in the same gene is not enough. To confirm the pathogenicity of a candidate gene requires statistical evidence (finding a higher percentage of gene disruptions in cases compared to controls), functional studies proving that the variants have a functional effect on mRNA- and or protein- level, or evidence from animal models like zebrafish or mouse knock-out, knock-down and rescue studies.

Informing patients and parents

All these new technological advances and the advancing knowledge about intellectual disability genes have led to great progress in the diagnostic yield in the clinical practice. Compared to just a few years ago, it is now possible to find a causal diagnosis in many more patients with intellectual disability. It is therefore of great value to re-evaluate patients who have visited a geneticist in the past but for whom no causal diagnosis was made. [38] At present patients are told to contact the genetic service themselves when they want a re-evaluation. If families were contacted by the clinical genetics department to update them about the new possibilities, it is likely that more patients would benefit from these advances in genetics. This active contact by the healthcare provider after a consultation in the past is called recontacting. There has been a lot of discussion about the duty to recontact, but recontacting, while preferable, is not common in clinical practice. [56; 57] Patients and physicians in England see practical problems for recontacting programs. Patients think that joint responsibility between patients and caregivers could be helpful and some suggest ICT solutions such as an electronic health record that sends automatic alerts when there are updates. Measures to secure privacy and transparency about who can use these records will be necessary though. [56] It is unclear what patients think about recontacting in the Netherlands. There are few systematic recontact programs at present, although some follow-up policlinics regularly follow patients with intellectual disability with and without a causal diagnosis, which allows clinicians to update patients and their families about new knowledge on known syndromes and additional genetic testing for undiagnosed patients.

Scope of this thesis

In this thesis, our research on genetic aspects of intellectual ability and disability is presented. There is a large variation in intellectual ability. At the lower end of this distribution we find people with intellectual disability (IQ scores below 70). We hypothesize that there is a large overlap in the biological processes causing variation in IQ in the normal range and those causing intellectual disability. It is very important to better understand the processes in the brain and in development that influence the complex trait of intelligence. Understanding the cause of intellectual disability furthermore helps patients and their families to accept the diagnosis and get a better grip on the future.

Our first goal was to contribute to the understanding of the genetic architecture of intellectual disability and show its relation to intelligence in general. Our aims were to:

1. *Find risk factors for mild non-syndromic intellectual disability.* Here we present our work on a variant in the *SNAP25* gene associated with mild non-syndromic intellectual disability. This genetic variation was already associated with the variation in normal intelligence. We have compared allele frequencies of this variant in a high IQ and low IQ (mild intellectual disability) cohort and found the minor allele of this SNP to be a risk factor for mild intellectual disability. Our findings indicate a multifactorial model for mild non-syndromic intellectual disability and support the generalist gene theory. (Chapter 2)
2. *Finding new causes for syndromic/severe intellectual disability.* We describe a new autosomal dominant intellectual disability syndrome: Mental retardation, autosomal dominant 26, also called AUTS2 syndrome. Our work on AUTS2 syndrome illustrates that moderate syndromic forms of intellectual disability are more likely caused by *de novo* autosomal dominant mutations. It also shows the importance of statistical and functional work-up for the proof of causality of variants in new intellectual disability genes. (Chapter 3 and 4)

   The technical possibilities for diagnostic tests have expanded enormously in the last years. One of the important questions in our clinical genetics practice is how to reach patients and their families to inform them about this. Until now we just informed parents about the fact that with time there might be new insights or technological advances and a new referral could be useful. But is this enough? Would active re-contacting when new information or techniques are available be better? If so, how should we inform them? And is it feasible?

   The second goal of this thesis was to enlarge information for patients and their families about the genetics of intellectual disability. Our more specific aims on this subject were:
3. *Improving information for patients, their families and caregivers about the AUTS2 syndrome.* Our explorative and descriptive research produced new information about AUTS2 syndrome by analysing the phenotype and a possible genotype-phenotype correlation in detail. This allows better estimation of the prognosis and that better care can be given to AUTS2 syndrome patients. (Chapter 5)
4. *Exploring how we can better inform families about new techniques with a higher diagnostic*

yield. We recontacted parents of children with intellectual disability to inform them about the possibility for re-evaluation of their child without a causal diagnosis. We here present the evaluations of this pilot and an analysis of the opinions of these parents from a questionnaire study on recontacting to help answering these questions. (Chapter 6)

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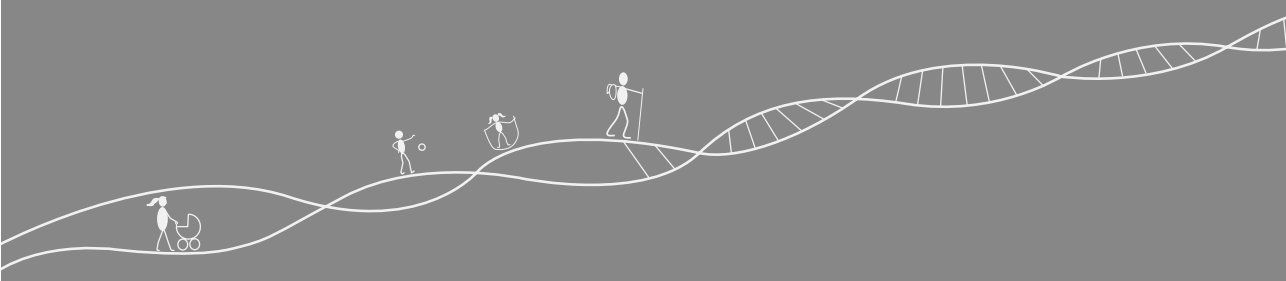
Chapter 2

Common variants in intellectual disability

Supporting the generalist genes hypothesis for intellectual ability/disability: the case of SNAP25.

Rizzi TS, Beunders G, Rizzu P, Sistermans E, Twisk JW, van Mechelen W, Deijen JB, Meijers-Heijboer H, Verhage M, Heutink P, Posthuma D.



Genes Brain Behav. 2012 Oct;11(7):767-71. doi: 10.1111/j.1601-183X.2012.00819.x.

ABSTRACT

Intellectual disability (ID) is an unresolved health care problem with a worldwide prevalence rate of 2–3%. For many years, research into the genetic causes of ID and related disorders has mainly focused on chromosomal abnormalities or X-linked genetic deficits. Only a handful of autosomal genes are known to cause ID. At the same time it has been suggested that at least some cases of ID represent an extreme form of normal intellectual ability and therefore that genes important for intellectual ability in the normal range may also play a role in ID. In this study, we tested whether the autosomal *SNAP25* gene, which was previously associated with variation in intellectual ability in the normal range, is also associated with ID. The gene product of *SNAP25* is an important presynaptic plasma membrane protein, is known to be involved in regulating neurotransmitter release, and has been linked to memory and learning by its effect on long term potentiation in the hippocampus. Allele frequencies of two genetic variants in *SNAP25* previously associated with intellectual ability were compared between a group of 636 ID cases (IQ<70) and a control group of 361 persons of higher than average intellectual ability. We observed a higher frequency of the putative risk allele of rs363050 (P=0.02; OR=1.24) in cases as compared to controls. These results are consistent with a role of *SNAP25* in ID, and also support the notion that ID reflects the lower extreme of the quantitative distribution of intellectual ability.

INTRODUCTION

Intellectual disability (ID) affects 2 – 3% of the (child) population.[1; 2] ID is defined as 'an IQ < 70 with deficits in two or more adaptive skills starting at a childhood age'.[3] ID is a complex disorder in which a large number of intellectual skills (e.g. language, motor, social, emotional, visuospatial) are suboptimally developed in the patient. The severity of disability ranges from profound to mild disability, although the majority of cases (60–85%) are classified as 'mild disability'.[4]

The causes of ID are enormously heterogeneous, and in a significant proportion of patients with ID the cause remains unexplained.[5; 6] Some of the known causes of ID include environmental factors like cerebrovascular incidents associated with premature birth, and genetic factors like chromosomal abnormalities, or rare mutations with major gene effects.[7; 8]

Genetic causes of ID are thought to be present in 25–50% of cases.[9] The Online Mendelian Inheritance in Man (OMIM) database contains 488 identified ID genes (March 2012). Most likely numerous additional ID genes remain to be identified.[10] Current genetic research typically focuses on chromosomal abnormalities and X-linked genetic effects which tend to be related to the more severe forms of ID.[11; 12] From all reported cases of individuals with moderate to severe ID, only about 50% can be traced back to a known cause.[5; 6] In mild ID (IQ between 50 and 70) about 30% has a known cause.[4; 10; 11] It has been proposed that the lack of major gene findings for mild ID suggests that mild ID is influenced by multiple genes, each of relatively small effect.[13] It is also estimated that most remaining ID genes are autosomal.[11]

According to the two related 'Common Disorders are Quantitative Traits' and 'Generalist genes' hypotheses [14; 15], common disorders, such as ID, are the quantitative extreme of a normally distributed trait (intellectual ability), and therefore the same genetic factors are largely responsible for both upper and lower extremes. Genetic polymorphisms associated with variation in normal intellectual ability may thus also be involved in some cases of ID. In this study, we set out to test whether the *SNAP25* gene, which has been associated with variation in intellectual ability in a non-clinical, population-based sample[16; 17], is also important in ID. If that is the case, that would support the notion that at least some cases of ID are indeed at the lower extreme of the normally distributed trait 'intellectual ability'.

MATERIALS AND METHODS

ID cohort (cases)

The ID cohort consisted of 636 Dutch children (518 males, 118 females; mean age of 7.7; SD 3.2) with intellectual delay (IQ < 70), without chromosomal rearrangements and without fragile X syndrome, recruited through the Clinical Genetics department of the VU Medical Centre. Anonymous blood-samples of the ID cohort were collected and genomic DNA was isolated from all samples using Flexigene AGF3000 technology (QIAGEN, Valencia, CA, USA) on an automated AutoGeneFlex 3000

isolator (AutoGene, Holliston, MA, USA) according to the protocols supplied by the provider. DNA was collected for diagnostic purposes and parents consented to anonymous use of the DNA samples for scientific purposes.

Higher than average IQ cohort (controls)

Controls were derived from a 'higher than average IQ' (HTA-IQ) cohort consisting of 361 (170 males and 191 females) Dutch individuals aged between 13 and 14years old at the time of inclusion in the study (mean age = 13.5, SD = 0.5). The HTA-IQ cohort is part of the Amsterdam Growth and Health Study (AGAHLS).[18; 19] This is a longitudinal study that started in 1976 and recruited children who followed the highest level of secondary education in two Dutch secondary schools. Subjects who reported to have been premature at birth were excluded from the study.[19] DNA was isolated from 361 of these participants. A subsample (N = 260) had also performed a Dutch standardized IQ test ('Groninger IQ Test, GIT')[20], which correlates well to the widely used Wechsler Adult Intelligence Scale (WAIS)[21], with correlations of standardized GIT and WAIS-IQ scores ranging from 0.72 to 0.91.[20] In this study, IQ scores in the subsample were only used to confirm the 'higher than average IQ' level of this group, which was used as 'controls' for the ID cases. The mean IQ of this sample was 107.3 (SD = 13.8), which is conform expectation as these individuals were sent to the highest level of secondary school. Both the fact that ID is generally detected at an early age [22] and the fact that all controls entered the highest level of secondary education in the Netherlands, render it highly unlikely that individuals marked as controls would later develop into cases. The study was approved by the medical ethical committee of the VU University Medical Center, and all subjects gave their written informed consent (provided by the parents as the subjects were aged 13–16years).

Genotyping

We selected two SNPs (rs363050 and rs363039) in the SNAP-25 gene that were previously reported to be associated with intellectual ability in two independent Dutch samples.[16; 17] The selected genetic variants are located in the first intron of SNAP25 and are not in strong linkage disequilibrium (LD) ($r^2 < 0.40$).

A TaqMan assay with specific fluorogenic probes in the high throughput 5′ nuclease assay (TaqMan, PE Applied Biosystems, Foster City, CA, USA) was used for genotyping the two SNPs in the SNAP25 gene (rs363039 and rs363050). Deviation from Hardy–Weinberg equilibrium for all genotyped markers was tested using PLINK.[23] Alleles previously reported to be associated with increased intellectual ability by Gosso et al. [16; 17] are G for rs363039 (minor allele: A, major allele: G) and A for rs363050 (minor allele: G, major allele: A). Thus, for both SNPs the minor allele is defined as the 'putative risk allele' for ID.

Statistical analysis

A logistic regression of case–control status on genotype implemented in Plink[23] was performed,

adjusting for the effect of sex. One-sided tests were conducted to test the hypothesis that the putative risk alleles for ID were more frequent in the ID cases as compared to the HTA-IQ controls. The Bonferroni corrected significance level was set at 0.05/2 = 0.025, i.e. correcting for testing two SNPs.

Brain expression analysis

As the selected SNPs (rs363050 and rs363039) are intronic and do not have a known functional role, we investigated whether they were associated with expression of the SNAP25 gene in the brain. To this end, we used the publicly available brain expression dataset[24] that includes genotypes and brain expression data. Brain cortex samples were available from 193 individuals of European descent with age at death greater than or equal to 65 years with no clinical history of stroke, cerebrovascular disease, Lewy bodies, or co-morbidity.[24] All 193 samples were genotyped using Affymetrix GeneChip Human Mapping 500K and the expression analysis was done using Illumina HumanRefseq-8 Expression BeadChip. [24] For all 193 individuals identified from the Myers' database, genomic coverage in the SNAP25 genomic area (±1.5 Mb) was increased by using genomic imputation (MACH).[25] The reference panel used was the HapMap II phased data (NCBI build 36, UCSC hg18). For the brain expression phenotype we made use of the available SNAP25 mRNA intensity information [transcript variant 2 (NM_130811) isoform SNAP25B]. Genetic association of imputed genotypes for all 193 individuals from the Myers' database was carried out using a weighted linear regression analysis implemented in MACH2QTL.[25] This sample size (193) is sufficient to detect SNPs explaining 4% of the variance in expression of SNAP25, given a Bonferroni corrected significance level of 0.025.

In silico binding site analysis

We further investigated if the SNAP25 genetic variants might have functional effects by affecting transcription factor binding sites (TFB). For this we used the UCSC browser, which includes experimental outcomes from published studies.[26-28] In addition, we investigated whether the two variants might alter binding of the transcription factors using the JASPAR binding site prediction program.[29] We selected a 500bp region surrounding the two SNPs and used the web interface for an online sequence analysis of regulatory regions present in the region. The TFB site models for each sequence were selected if the scoring matrices were above 90%. The analysis was done for each allele separately. We ran similar analyses for SNPs in high LD ($r^2 > 0.9$) with the two target SNPs.

RESULTS

Quality control

In total, 997 DNA samples were available for genotyping, 636 in the ID cohort and 361 in the HTA-IQ cohort. The genotyping success rate was 96% average. Missingness occurred due to ambiguous genotype calling in small proportion of the TaqMan assays. Both SNP's where in Hardy – Weinberg

equilibrium in both cohorts separately [ID cohort rs363050: P = 0.47, rs363039: P = 0.10; HTA-IQ cohort rs363050: P = 0.73, rs363039: P = 0.69 as well as for the both cohorts combined rs363050: P = 0.36, rs363039: P = 0.25].

Descriptives

Frequencies of the putative ID-risk alleles (G in rs363050 and A in rs363039) were 0.47 and 0.35, respectively in the ID cohort and 0.41 and 0.30 in the HTA-IQ groups, see Table 1. Figure 1 shows the allele frequencies of the putative risk alleles for cases and controls and for males and females separately.

Table 1: Descriptives of cases of intellectual disability (ID) and controls of higher than average IQ (HTA-IQ)

	ID cohort	HTA-IQ cohort
Age in years (SD)	7.7 (3.2)	13.5 (0.5)
Males/females (%)	81/19	47/53
Total N	636	361
rs363050 Genotypic frequencies (GG/AG/AA)	0.227/0.484/0.289	0.174/0.473/0.353
Risk allele (G) frequency rs363050	0.47	0.41
rs363039 Genotypic frequencies (AA/AG/GG)	0.139/0.427/ 0.434	0.084/0.433/ 0.483
Risk allele (A) frequency rs363039	0.35	0.30

Case–control association analysis

As the cases and controls were not matched for sex (i.e. the cases included relatively more males, which is likely related to the higher prevalence of ID in boys than in girls) all analyses included sex as a covariate, to correct for possible confounding of sex with genotype. The putative risk allele of rs363039 (A) was not significantly associated with ID (OR=1.11, P=0.182), although a relative large difference in allele frequency in females in the hypothesized direction was observed. The putative risk allele of rs363050 (G) was associated with ID (OR=1.24, P=0.020), and showed a higher frequency in cases compared to controls. Analyses for rs363050 were also conducted separately for males and females and showed a significant association in females (OR = 1.44, P = 0.02) but not in males (OR = 1.14, P = 0.17), although the direction of the effect was similar. A separate analysis was run to test for a sex-by-genotype interaction, which showed no significant interaction (P = 0.12). Together with the sex-adjusted analyses, this suggests that although the effect was strongest in females, both males and females showed an association in the same direction.

For rs363039 the test in females and males only did not reach significance, although females showed a trend (OR = 1.34, P = 0.06).

Brain expression and in silico analysis

There was no evidence for an association of either variant with SNAP25 brain expression, using the

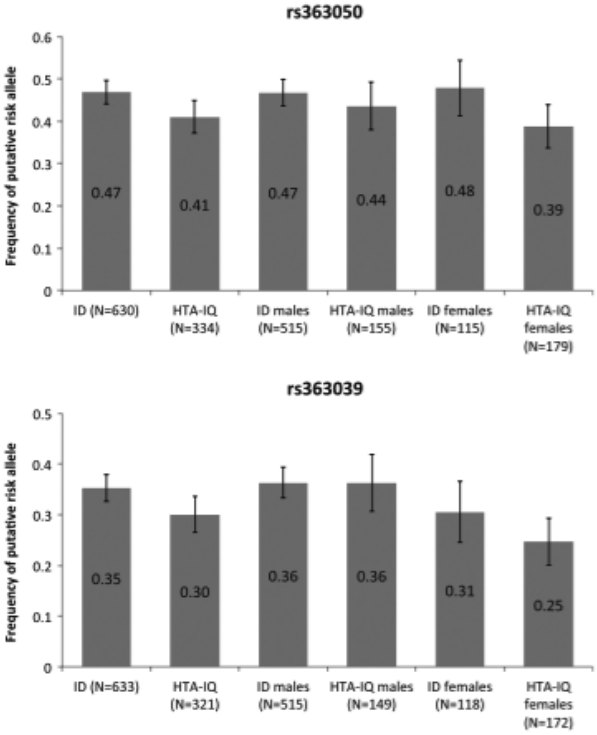


Figure 1: *Putative risk allele frequencies of the two selected SNAP25 SNPs across intellectual disability (ID) cases and higher than average IQ (HTA-IQ) controls, and across males and females. Error bars denote 95% CI.*

Myers database (rs363039: P = 0.89; rs363050: P = 0.60). In addition, neither of the genetic variant was inside reported binding site regions nor in the predicted sites (scoring matrices were below 90%). We also checked SNPs that were in high LD (r2 >0.9) with rs363050 or rs363039 using the CEU hapmap LD structure. There were three SNPs in high LD with rs363050 (rs6104571, rs363016 and rs12626080), which are located in the second intron of SNAP25. None of them were associated with expression of SNAP25, however, two of them were located inside predicted TFB sites: rs6104571 (MEF2A binding site) and rs12626080 (FOXL1 binding site).

Recently Söderqvist et al.[30] conducted an *in silico* analysis using the TESS (transcription element search system) program to search for a functional role of the rs363039 SNP. They reported the predicted presence of a glucocorticoid receptor (NR3C1) binding site in carriers of the A allele (i.e. the putative risk allele) of rs363039, which was lacking in carriers of the G allele. Instead, G allele carriers were predicted to have a zinc finger protein (ZNF589, alias SZF1) binding site. However, these predicted binding sites for rs363039 could not be confirmed by us using data from UCSC or JASPAR.

DISCUSSION

In this study, we tested whether two genetic variants in the *SNAP25* gene, which were previously associated with variation in intellectual ability in the normal range, are associated with ID risk. For one SNP (rs363050) we observed a significantly higher frequency of the putative risk allele in ID cases as compared to the controls of higher than average IQ. For the second SNP (rs363039) the allele frequency difference between cases and controls was not statistically significant although there was a trend in the expected direction.

In silico analysis showed that neither SNP was related to differential brain expression of *SNAP25*. This suggests that if there is a regulatory role, it must be very small (explaining <4% of the variance in *SNAP25* brain expression), and will therefore go undetected with the current sample size of brain expression analysis. Both SNPs were also not predicted to alter TFB sites. However, there were three SNPs in high LD with rs363050 (rs6104571, rs363016 and rs12626080). Two of these were located inside predicted TFB sites: rs6104571 (MEF2A binding site) and rs12626080 (FOXL1 binding site). The MEF2A binding site is of particular interest as the *MEF2A* factor was recently reported to be involved short-term synaptic plasticity in mice.[31] Further research is needed to elucidate the functional role of these SNPs or other SNPs in LD with them.

The SNAP25 gene codes for a presynaptic plasma membrane protein that is an integral component of the vesicle docking and fusion machinery that regulates neurotransmitter release.[32-34] It is also implicated in axonal growth and synaptic plasticity [35] and is shown to be involved in hippocampal long-term potentiation (LTP), which is thought to be a form of synaptic plasticity that underlies memory and learning.[36-40] Pavlowsky et al.[41] recently reviewed all known ID-related genes and concluded that gene-products of these genes are enriched in synapses, thereby 'supporting the unifying synapse-based theory for cognitive deficits'.[41] Although there have also been failures to replicate (e.g. see the recent study by Chabris et al.[42] who failed to replicate the rs363050 effect on 'g' in a sample of 6464 individuals), *SNAP25* has been suggested to be associated not only with ID and intellectual ability but also with related traits such as autism[43], ADHD[44] and working memory capacity[30].

Although our results warrant replication in other ID cases and controls to further confirm the role of *SNAP25* in ID, we set out to put the 'Common disorders are quantitative traits' and 'Generalist Genes' hypotheses to a test.[14; 15] We reasoned that if ID is indeed the extreme of the quantitative trait intelligence, than it is likely that genes associated with intelligence are also of importance to ID. Our results support this view by showing that *SNAP25* also plays a role in the lower extreme of the quantitative trait 'intellectual ability'. This also suggests that genes that have already been identified for ID may be of importance in normal intellectual functioning. It should be noted however, that we do not intend to suggest that all cases of ID represent the lower extreme of the normally distributed trait of intellectual ability. ID is a heterogeneous disorder with multiple causes, some of which are environmental, some are monogenic, X-linked, and in addition some may indeed be at the lower

extreme of intellectual ability and share common (genetic) causes.

Intellectual ability is considered to be influenced by hundreds of genetic variants each of small effect.[45] Gene finding for intellectual ability therefore necessitates large sample sizes. If, however some of the genetic variants underlying normal intellectual ability also influence the lower (and upper) extremes of the distribution, then statistical power of genome-wide association analyses for normal intellectual ability can be greatly enhanced by selecting extreme cases of the distribution (i.e. ID vs. high IQ).[14] Gene-finding efforts for both intellectual ability and disability may thus benefit from taking a generalist genes view.

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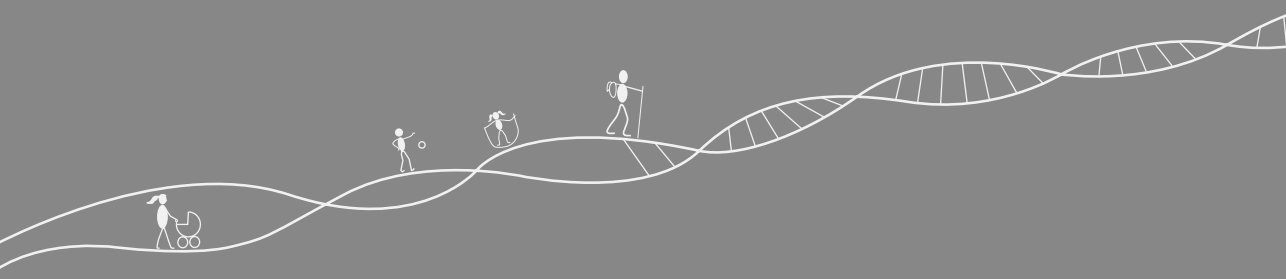
Chapter 3

Rare variants in AUTS2 cause a recognizable intellectual disability syndrome

Exonic deletions in AUTS2 cause a syndromic form of intellectual disability and suggest a critical role for the C-terminus.

Beunders G, Voorhoeve E, Golzio C, Pardo LM, Rosenfeld JA, Talkowski ME, Simonic I, Lionel AC, Vergult S, Pyatt RE, van de Kamp J, Nieuwint A, Weiss MM, Rizzu P, Verwer LE, van Spaendonk RM, Shen Y, Wu BL, Yu T, Yu Y, Chiang C, Gusella JF, Lindgren AM, Morton CC, van Binsbergen E, Bulk S, van Rossem E, Vanakker O, Armstrong R, Park SM, Greenhalgh L, Maye U, Neill NJ, Abbott KM, Sell S, Ladda R, Farber DM, Bader PI, Cushing T, Drautz JM, Konczal L, Nash P, de Los Reyes E, Carter MT, Hopkins E, Marshall CR, Osborne LR, Gripp KW, Thrush DL, Hashimoto S, Gastier-Foster JM, Astbury C, Ylstra B, Meijers-Heijboer H, Posthuma D, Menten B, Mortier G, Scherer SW, Eichler EE, Girirajan S, Katsanis N, Groffen AJ, Sistermans EA



Am J Hum Genet. 2013 Feb 7;92(2):210-20. doi: 10.1016/j.ajhg.2012.12.011.

ABSTRACT

Genomic rearrangements involving *AUTS2* (7q11.22) are associated with autism and intellectual disability (ID), although evidence for causality is limited. By combining the results of diagnostic testing of 49,684 individuals we identified 24 microdeletions that affect at least one exon of *AUTS2*, as well as one translocation and one inversion with one of their breakpoints within the *AUTS2* locus. Comparison of 17 well-characterized individuals enabled identification of a variable syndromic phenotype including ID, autism, short stature, microcephaly, cerebral palsy and facial dysmorphisms. The dysmorphic features were more pronounced in persons with 3' *AUTS2* deletions. This part of the gene is shown to encode a C-terminal isoform (with an alternative transcription start site) expressed in human brain. Consistent with our genetic data, suppression of *auts2* in zebrafish embryos caused microcephaly that could be rescued by either the full-length or the C-terminal isoform of human AUTS2. Our observations demonstrate a causal role of AUTS2 in neurocognitive disorders, establish a hitherto unappreciated syndromic phenotype at this locus and demonstrate how transcriptional complexity can underpin human pathology. The zebrafish model provides a valuable tool to investigate the etiology of AUTS2 syndrome and facilitate gene function analysis in the future.

INTRODUCTION

Neurodevelopmental disorders, including intellectual disability (ID) and autism, have a strong genetic component, but only few of the underlying genes have been identified. Candidate gene discovery has accelerated in recent years by the implementation of high-resolution genomic arrays. However, detected CNVs often encompass either multiple genes or are too rare to provide causal evidence for a particular candidate transcript. Autism susceptibility candidate 2 (*AUTS2*), located on 7q11.22 [MIM 607270], represents such an ID-candidate with inconclusive evidence for causality.
AUTS2 was first identified as a candidate for neurocognitive defects because a translocation breakpoint analysis in twins with autism, developmental delay and epilepsy showed that one of the breakpoints disrupted *AUTS2*.[1] Besides the twins, seven additional cases have now been reported to disrupt the *AUTS2* coding region: four individuals with a translocation breakpoint [2, 3], one with an inversion breakpoint disrupting *AUTS2* [2-4] and two with intragenic deletions.[2, 5] These individuals manifested ID/developmental delay (all nine), dysmorphic features (six), autism (four) and skeletal abnormalities (three). This overview does not include persons with intronic deletions in *AUTS2* because the functional significance of such intronic variation is unclear.[6]

Complicating the candidacy of this locus, some of the genomic rearrangements affecting *AUTS2* disrupt other genes as well. A combination of cytogenetic and sequencing studies suggested that *CNTNAP2* (7q35) might be causal in a individual with a 7q inversion disrupting *AUTS2* and *CNTNAP2* [MIM 604569];[4] likewise, for three larger multi-genic *de novo* deletions in the DECIPHER database that encompass *AUTS2*, it is unclear whether the disruption of *AUTS2* alone drives the phenotype.[7] The data presented by Nagamani et al. on two individuals with intragenic deletions, suggest that deletions in *AUTS2* alone might be pathogenic. However, the number of affected individuals was too small to exclude the role of other genes, or to delineate a phenotype.[5] Here we present direct evidence from both clinical and genetic data and animal studies for the causal relation of *AUTS2* with an ID syndrome and delineate the associated phenotype. Furthermore, we provide evidence that functional elements in the C-terminus of AUTS2 are major contributors to both the neurodevelopmental and craniofacial phenotypes of individuals with C-terminal deletions or rearrangements at this locus.

SUBJECTS AND METHODS

Subjects

Routine diagnostic array CGH was performed for ID and/or multiple congenital anomalies (MCA) for a total of 49,684 individuals across ten diagnostic centers in the Netherlands, Belgium, Great Britain, the USA and Canada (each center using their standard diagnostic platform: in total six analogous platforms were used). In some of these individuals karyotyping was also performed. We selected all individuals from this cohort with a deletion that involved *AUTS2*, as well as one person with a translocation and another person with an inversion in which one of the breakpoints is in *AUTS2*. To

further map the region and to delineate the associated phenotypes peripheral blood samples were obtained and clinical information was collected through either medical letters or a data sheet filled in by the referring physicians with approval of the local medical ethical committee. Results were confirmed using different methods, depending on the laboratory: High density array, MLPA and FISH (see Table S1, S2 and S3). Exact breakpoint delineation of the translocation with one breakpoint in 7q11.22 was performed with FISH and the inversion was characterized using whole-genome sequencing, as previously described. [8-10] Informed consent was obtained from parents or caregivers as appropriate and specific consent for publishing photographs was obtained from all individuals whose photographs are shown here. Institutional approval of the local medical ethical committee was obtained as well. Individuals with a confirmed exonic deletion or a genomic rearrangement involving *AUTS2* and available clinical data were included for phenotypic studies.

Controls

To assess the frequency of *AUTS2* deletions within a large general population, we analyzed CNV data of 16,784 subjects from several control groups. A total of 4,783 DNA samples from the Welcome Trust Case Control Consortium 2 (WTCCC2) were analyzed with SNP array. This control group included individuals from the 1958 Birth cohort and the UK Blood service collection (10-26-2011) that had been nationally ascertained and regarded as healthy.[11] Further control CNV data from 8,329 cell line and blood-derived controls were obtained primarily from genome-wide association studies of non-neurological phenotypes. As these include 2090 controls from the UK Blood service collection this set adds only 6,239 unique controls. Although these data were not ascertained specifically for neurological disorders, they consist of adult individuals providing informed consent as described previously.[12] In addition publicly available data from Hapmap phase 3 (10-26-2011), which consist of 1,056 healthy controls from 11 different populations were checked for deletions involving *AUTS2.*[13] CNV data from four control sets from respectively Canada, Germany, the USA and the Netherlands were available: The Ottawa Heart Institute (OHI) controls (n=1.234); POPGEN controls (n=1.123), SAGE controls (n=1.287) and the Low-Lands-Consortium controls (n=981, a Dutch control cohort).[13-15] See Table S4 for details on all cohorts and the array platforms used. The array platforms used for controls have the same or a comparable resolution as the platforms used for cases. The number of deletions found in cases was compared to that in controls using a Fisher's exact test.

Genotype-phenotype correlations

We received clinical data from 17 individuals and 4 family members carrying an exonic *AUTS2* disruption. These individuals were used to identify features that occurred in at least two unrelated individuals, indicating a minimal frequency of 10%. A recent systematic review of Oeseburg et. al. shows that in a general ID cohort the most frequent additional health conditions (epilepsy and cerebral palsy) are as frequent as 20%, but the remainder of the co-morbid clinical features (including autism and a congenital malformation in general) are seen in less than 10%.[16] Therefore

a frequency of 10% for a specific feature in this AUTS2 cohort will be an enrichment compared to ID cases in general. These recurrent features were scored for all individuals and family members carrying the familial deletion, asymmetrically occurring features were counted as positive. The sum of positive features was counted for each individual and was defined as their individual AUTS2 Syndrome Severity Score.

Since deletions or genomic rearrangements affecting the 3' end of the *AUTS2* coding sequence seem to be associated with a more severe phenotype, persons with exonic deletions were categorized in two groups depending on whether the deletion disrupted the highly conserved *AUTS2* segment (containing exons 9-19) that is also encoded by the alternative 3' transcript (see alternative transcription start sites and the results section). We tested whether the corresponding AUTS2 Syndrome Severity Scores for these two groups differed significantly using a Kolmogorov-Smirnov test.

Alternative transcription start sites

To search for an explanation for the observed genotype-phenotype trend, we first determined the evolutionary conservation of human *AUTS2* exonic sequences. We used the following species for comparison: gorilla (*Gorilla gorilla*; gorGor3), macaque *(Macaca mulatta*; Mmul_1), dog (*Canis familiaris*; Broadd2), cow (*Bos tauris*; Btau_4.0), pig (*Sus scrofa*; Sscrofa9), mouse (*Mus musculus*; NCBIM37), chicken (*Gallus gallus*; Washuc2), clawed frog (*Xenopus tropicalis*; JGI_4.2) and zebrafish (*Danio rerio*; Zf9). Accession numbers of protein sequences are: ENSGGOP00000011519, ENSMMUP00000023254, ENSBTAP00000002697, ENSSSCP00000008253, ENSCAFP00000016549, ENSMUSP00000062515, ENSGALP00000001729, ENSXETP00000007747, ENSDARP00000073379

Two different methods were used. We first aligned the predicted protein of the longest isoform in humans to the predicted amino acid sequences of the orthologous species using the muscle v3.8 software.[17] For that purpose sequences from the latest builds were downloaded from Ensembl. Secondly to detect similarity in non-annotated or non-coding genomic DNA the tblastn algorithm with the human amino acid sequence as query was used.[18] The degree of homology was calculated as the percentage of identical amino acids.

Second we searched for putative alternative transcription initiation sites (TSS) that were associated with a shorter 3' isoform in human brain. We used mRNA from the caudate nucleus and the medial frontal *gyrus* from one donor provided by the Dutch Brain Bank and performed a replication experiment by the same procedure on a mRNA sample from the medial frontal *gyrus* of a second donor. Rapid amplification of 5' cDNA ends (5'RACE) was performed with the Ambion FirstChoice® RLM-RACE kit according to the manufacturer's instructions. Nested PCR amplification was performed with 5'-atgtcttcggctgaaatgct-3' as the outer, and 5'-ggaagagactgtgccggtag-3' as the inner *AUTS2*-specific reverse primer (Figure S1A-B).

Knockdown and rescue experiments in zebrafish embryos

To investigate the role of AUTS2 in the regulation of head size, neuronal development and morphology in general we performed zebrafish knockdown experiments. Zebrafish (*Danio rerio*) embryos were raised and maintained as described.[19] Splice blocker morpholinos (MOs) against the *AUTS2* orthologue *auts2* were designed and obtained from Gene Tools, LLC (sequences available on request). We injected 1 nl of diluted MOs (4.5 ng for 5'MO targeting exon 2 donor splice, and 6 ng for 3'MO targeting exon 10 donor splice) and/or mRNA (100 pg) into wild type zebrafish embryos at the 1- to 2-cell stage (n = 50-100 embryos per injection dose) and performed RT PCR to measure the efficiency of the splice blocking. Injected embryos were scored visually at three days post fertilization (dpf) and classified as normal or microcephalic on the basis of the relative head size compared with age-matched controls from the same clutch. For rescue experiments, the human wild type mRNAs (full-length or short transcript; GenBank# JQ670866 resp. JQ670867) were cloned into the pCS2 vector and transcribed in vitro using the SP6 Message Machine kit (Ambion), 100 pg of the human wild type mRNAs were co-injected with the MOs. All experiments were repeated three times and evaluated statistically with a Student t-test. Alcian blue staining of cartilaginous structures was performed to investigate the morphology of the head. Zebrafish embryos were fixed with 4% PFA and the cartilage structures were visualized by staining with Alcian blue following an established protocol.[20] Further, whole-mount immunostainings with either HuC/D (postmitotic neurons) or phospho-Histone H3 (proliferating cells) were performed to investigate neuronal development and head size regulation at a cellular level. Embryos were fixed in 4% PFA overnight and stored in 100% methanol at -20°C. After rehydration in PBS, PFA-fixed embryos were washed in IF buffer (0.1% Tween-20, 1% BSA in PBS 1X) for 10 minutes at room temperature. The embryos were incubated in the blocking buffer (10% FBS, 1% BSA in PBS1X) for 1hr at room temperature. After two washes in IF Buffer for 10 minutes each, embryos were incubated in the first antibody solution, 1:750 anti-histone H3 (ser10)-R (sc-8656-R, Santa Cruz), or 1:1000 anti-HuC/D (A21271, Invitrogen), in blocking solution, overnight at 4°C. After two washes in IF Buffer for 10 minutes each, embryos were incubated in the secondary antibody solution, 1:1000 Alexa Fluor donkey anti-rabbit IgG and Alexa Fluor goat anti-mouse IgG (A21207, A11001, Invitrogen) in blocking solution, for 1hr at room temperature. Staining was quantified by counting positive cells in defined regions of the head and with ImageJ software.

RESULTS

Genotypes

To assess the candidacy of *AUTS2* in cognitive impairment in humans, we examined the *AUTS2* region in 49,684 individuals with intellectual disability (ID) and/or multiple congenital anomalies (MCA) using array CGH and/or karyotyping. We identified 44 deletions that encompass at least part of *AUTS2* and a maximum of two other genes (*WBSCR17* and *CALN1* [MIM 607176]), while conventional karyotyping revealed one translocation and one inversion with one of the breakpoints in *AUTS2*

(Table S1*)*. Duplications encompassing *AUTS2* that were found in this cohort are not included in this study, since the functional relevance of these lesions is unclear. Twenty-four deletions were found to include at least one *AUTS2* exon, while another 17 did not. For the remaining three deletions it was unclear whether they included an exon due to limited resolution of the array. For these three individuals we had no consent to perform further studies.

Overall, in our cohort of 49,684 affected individuals, we identified 24 persons (0,05%) who harbored deletions disrupting the coding sequence. To assess the significance of this observation, we analyzed 16,784 controls from 12 cohorts using arrays with high-density coverage of the *AUTS2* locus (Table S4). Although nine deletions were found, none of them disrupt an *AUTS2* exon (Table S5). The difference between exonic deletions in the cases (24/49,651) vs controls (0/16,784) was highly significant (p = 0.00092), suggesting that exonic disruptions of *AUTS2* give rise to a highly penetrant phenotype in humans. This is supported by CNV data from the latest version of the Database of Genomic Variance (25th of august 2012), wherein none of the array based studies show CNV's that disrupt an exon and by the fact that none of the 24 probands with an exonic *AUTS2* deletion had a rare *de novo* CNV at another locus (Table S1).

We were able to obtain phenotypic data from 15 out of 24 probands with an exonic *AUTS2* deletion (case 1-15), from the inversion case (16) and the translocation case (17) with one breakpoint in *AUTS2,* as well as from four family members carrying the familial *AUTS2* deletion. In these 17 probands MLPA, FISH, high density array and breakpoint sequencing confirmed the aberrations and further delineated the breakpoints. (Figure 1, Table S1).

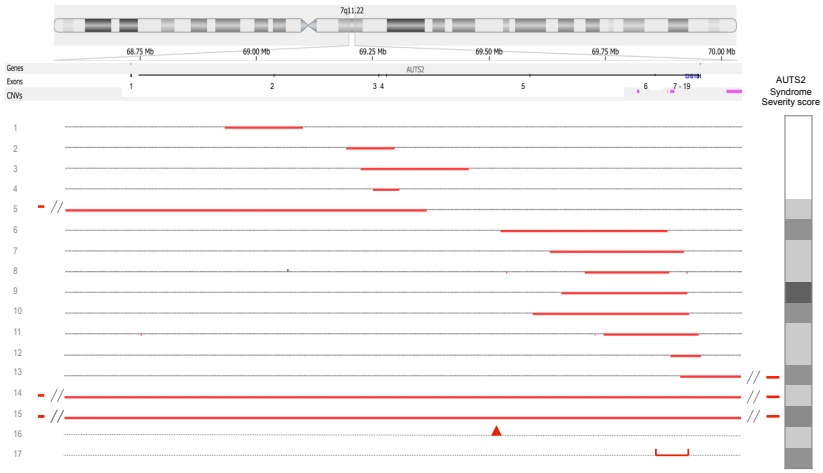


Figure 1. *Overview of AUTS2 aberrations in the probands. The location of the deletions is indicated by the red bars, the inversion breakpoint by an arrowhead, the translocation breakpoint area is indicated by* ⌴ *. CNVs extracted from the Database of Genomic Variance in purple (CNVs found in BAC-studies not included). The AUTS2 Syndrome Severity Score of the probands is shown on the right. Darker shades indicate a more severe and/or more specific phenotype (AUTS2 Syndrome Severity Score: in white: scores <7; in light gray: scores of 7-12; in gray: scores of 13-18; in dark gray: scores >18). See also Figure S2 and Table S6.*

In total, 21 individuals from 17 families were included in our genotype-phenotype study (Table S6). In 8 out of 11 probands in which both parents were available for genetic testing the *AUTS2* aberrations occurred *de novo* (case 5, 8, 10, 12, 14, 15, 16 and 17): the other three probands inherited the *AUTS2* deletion from an unaffected parent (case 1) or an affected parent (case 4 and 6). In six probands the inheritance status of the *AUTS2* deletion could not be fully resolved because one or both parents were unavailable for testing (case 2, 3, 7, 9, 11 and 13). Of the ten individuals with an intragenic deletion (not including the first and last exon), four probands carried a deletion predicted to cause a frameshift (case 6, 7, 8 and 11), whereas in the other six individuals the deletion was in-frame (case 1, 2, 3, 4, 9, 10**)**. Finally in case 14 the deletion also included one downstream gene (*WBSCR17*), while in cases 13 and 15 the deletions also affected two downstream genes (*WBSCR17* and *CALN1*) (Figure 1, Table S6 and Figure S2).

Phenotypes

Next we asked whether there were any recurrent phenotypic features associated with *AUTS2* disruptions. All seventeen probands from whom detailed clinical data were available had intellectual

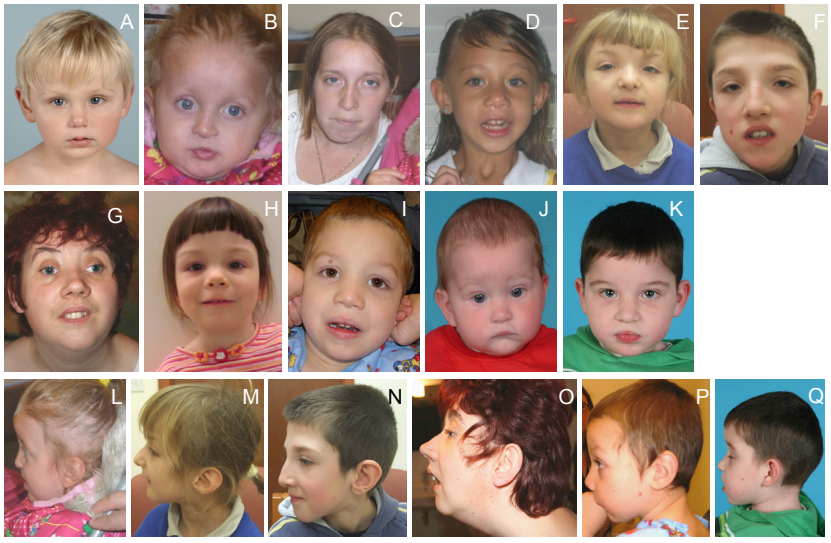


Figure 2. *Facial characteristics of cases with an AUTS2 aberration.* ***A.*** *Case 1 at age 3 years shows no dysmorphic features.* ***B+L.*** *Case 4 at age 2,5 years has a repaired cleft lip, mild proptosis, short and mild upslanting palpebral fissures.* ***C.*** *The mother of case 4 shows a repaired cleft lip, ptosis and retrognathia.* ***D.*** *Case 5 at age 3 years shows highly arched eyebrows, mild downslanting palpebral fissures, epicanthal folds and a short philtrum.* ***E+M.*** *case 6 at age 6 years. She is hyperteloric, has ptosis and downslanting palpebral fissures, a short philtrum and narrow mouth, similar to her brother shown in F+N at ages 10 years.* ***G+O.*** *Case 9 at age 32 years, with hypertelorism, proptosis, upslanting palpebral fissures, a short upturned philtrum and a narrow mouth.* ***H.*** *Case 10 at age 2 years. She shows a prominent nasal tip, anteverted nares and short philtrum.* ***I+P.*** *Case 13 at age 5.5 years. He has hypertelorism, ptosis, a broad nasal bridge, a short and upturned philtrum and a narrow mouth.* ***J+K+Q.*** *Case 15 at age 1 year (J) and 4,8 years (K). He has a broad nasal bridge, short palpebral fissures and a short philtrum and narrow mouth. See also Table 1 and Table S6.*

Table 1. *Clinical features characterizing the AUTS2 syndrome patients.*

Clinical features	Cases	
	This Study n/total (%)	Published n/total (%)
General		
age at examination	11m-32y	3y-16y
sex	13f/8m	5f/4m
De novo occurence	9/13 (69%)	8/9 (89%)
Growth and feeding		
low birth weight	7/17 (41%)	2/8 (25%)
short stature <p10	12/20 (60%)	4/9 (44%)
microcephaly <p2	14/20 (70%)	1/6 (17%)
feeding difficulties	10/21 (48%)	4/5 (80%)
Neurodevelopmental disorders		
intellectual disability/ development delay	20/21 (95%)	9/9 (100%)
autism/autistic behavior	7/21 (33%)	4/6 (67%)
sound sensitivity	2/8 (25%)	2/4 (50%)
hyperactivity/ ADHD	3/21 (14%)	1/4 (25%)
Neurological disorders		
generalized hypotonia	8/21 (38%)	4/7 (57%)
structural brain anomaly	3/11 (27%)	4/9 (44%)
cerebral palsy/spasticity	9/21 (43%)	¼ (25%)
Dysmorphic features		
highly arched eyebrows	8/21 (38%)	1/5 (20%)
hypertelorism	10/21 (48%)	0/5 (0%)
proptosis	6/21 (29%)	2/5 (40%)
short palpebral fissures	8/21 (38%)	2/5 (40%)
upslanting palpebral fissures	4/21 (19%)	1/5 (20%)
ptosis	8/21 (38%)	2/5 (40%)
epicanthal fold	7/21 (33%)	1/5 (20%)
strabismus	5/21 (24%)	3/6 (50%)
prominent nasal tip	5/21 (24%)	2/5 (40%)
anteverted nares	3/21 (14%)	2/5 (40%)
deep/broad nasal bridge	7/21 (33%)	1/5 (20%)
short/upturned philtrum	8/21 (38%)	5/7 (71%)
micro/retrognatia	7/20 (35%)	2/5 (40%)
low set ears	6/20 (30%)	2/5 (40%)
ear pit	2/20 (10%)	0/5 (0%)
narrow mouth	12/21 (57%)	3/5 (60%)
Skeletal abnormalities		
kyphosis/ scoliosis	2/9 (22%)	3/5 (60%)
arthrogryposis/shallow palmar creases	3/20 (15%)	1/3 (33%)
tight heel cords	5/8 (62%)	1/1 (100%)
Congenital malformations		
hernia umbilicalis/inguinalis	2/21 (9%)	1/9 (11%)
patent foramen ovale/ atrial septum defect	3/21 (14%)	1/9 (11%)

This table shows the frequency of clinical features in AUTS2 syndrome as the number of affected individuals with this feature (n) in relation to the total number of individuals for whom information was available for each feature (total). See for a more detailed overview Table S6, which also includes an overview of cases described in literature.[2-5].

disability and/or developmental delay; this had been the reason for diagnostic testing. One of the parents carrying an *AUTS2* deletion had learning difficulties (the mother of case 4), one had mild intellectual disability (mother of case 6) and one had normal intelligence (the father of case 1). Seven probands were diagnosed with autism spectrum disorder (ASD) or showed autistic behavior (case 2, 5, 9, 12, 13, 16 and 17). In addition to the expected neurocognitive defects we also observed a constellation of other recurrent clinical features in individuals with exonic deletions. These included: microcephaly (14 individuals), short stature (12), feeding difficulties (10), hypotonia (8) and cerebral palsy (9). We also found recurrent dysmorphic features: hypertelorism (10), proptosis (6), ptosis (8), short palpebral fissures (8), epicanthal folds (7), a short and/or upturned philtrum (8), micrognathia (7) and a narrow mouth (12). Less frequent features were skeletal abnormalities including (signs of) arthrogryposis (3), umbilical or inguinal hernia (2) and heart defects (3) (Figure 2, Table 1).

The striking phenotypic complexity and variable size and position of the CNVs prompted us to evaluate the clinical information from the 17 probands and 4 family members carrying the familial *AUTS2* deletion included in this study to derive pathology scores based on simple, objective criteria, which we summarized as "the AUTS2 syndrome severity score" (maximum score is 32). Even though this paradigm is a crude approximation of the phenotypic diversity at this locus, we nonetheless observed dichotomization of phenotypes. Cases and family members 1-4, (all with 5' in-frame deletions) scored significantly lower (median AUTS2 Syndrome Severity Score=5) compared

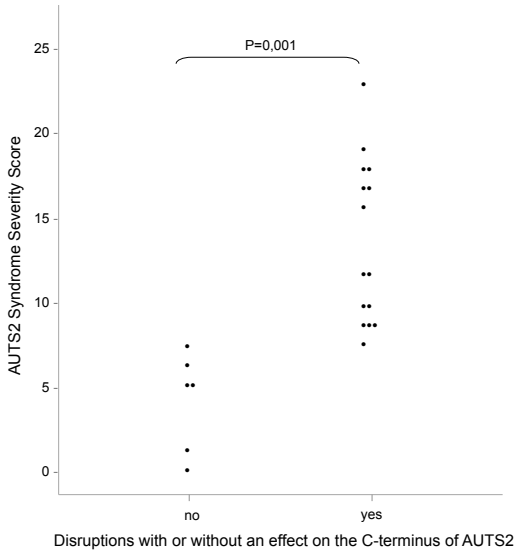


Figure 3. *Scatter plot of the AUTS2 Syndrome Severity Score for disruptions affecting the N- or C-terminus of AUTS2. Scatter plot of the AUTS2 Syndrome Severity Score for the disruptions that have an effect on the highly conserved amino acid sequence block encoded by exon 9 to 19 (yes) and the deletions not affecting this amino acid sequence (no) (see also Table S6 and Figure 4). The numbers refer to case numbers; f= father of patient x, m=mother of patient x, s= sibling of patient x (see Table S6). The AUTS2 Syndrome Severity Score between these groups of cases differ significantly, p=0,001 (Kolmogorov-Smirnov Z-test)*

to cases and family members with deletions of downstream exons, whole gene deletions, or the deletion of exon 1 to 4 that includes the initiation codon (case 5-17) (median AUTS2 Syndrome Severity Score=12) (Figure 1 and 3 and Table S6). This difference was significant regardless of the inclusion or exclusion of affected family members (p=0.001 and p= 0,011 respectively).

Detection of a C-terminal *AUTS2* isoform

The apparent dependence of severity scores on CNV location prompted us to evaluate the evolutionary conservation of each *AUTS2* exon (Figure S3 and S4) which was especially high in the 3' gene region. Given the fact that the ENSEMBL annotation of the *AUTS2* sequences predicts the presence of several splice isoforms, we next looked for the presence of alternative isoforms in human brain mRNA. Using 5'-RACE we identified a short 3' *AUTS2* mRNA variant starting in the middle of exon 9, depicted in Figure 4. All transcripts detected employed the same start site (see also Figure S1C and D). The reading frame of the short transcript is identical to that of the full-length

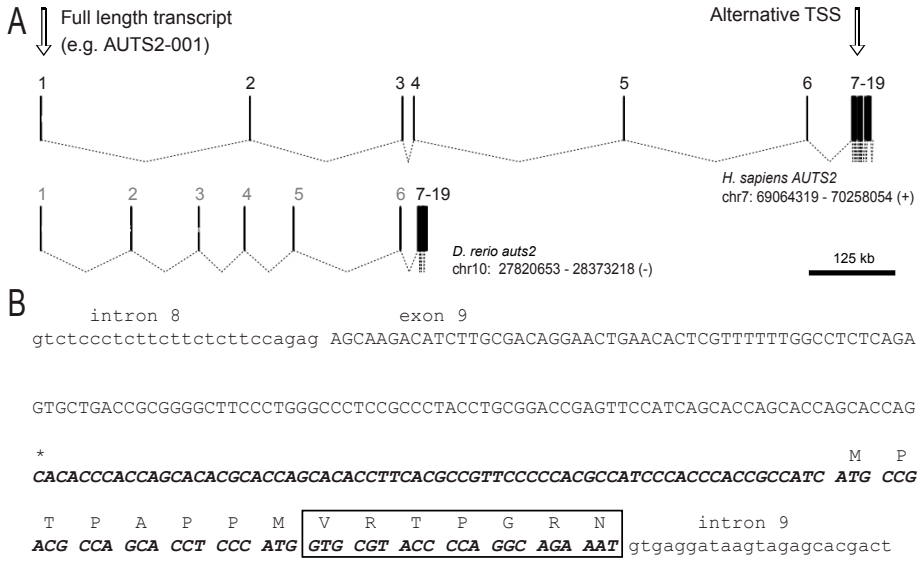


Figure 4. *Exon organization of human AUTS2 and its zebrafish ortholog and identification of a novel transcriptional start site (TSS) in exon 9 of full-length human AUTS2.*

A. *Exon organization of AUTS2 orthologs in humans and zebrafish. Arrows indicate two transcriptional start sites (TSS) used in human brain mRNA. Alternative novel TSS is located 1,17 Mbp downstream of the standard TSS in the cluster containing exons 7-19. Exons shown in grey represent conserved sequences that are not annotated in the current zebrafish genome (for details see Figure S4)*

B. *Identification of an alternative AUTS2 transcript detected in human brain mRNA by 5' RACE (rapid amplification of cDNA ends). The alternative transcript starts in the center of exon 9 (asterisk) and contains the indicated cDNA sequence (italic font). The mRNA was spliced to exon 10 using the second of two known splice donor sites in exon 9, resulting in the incorporation of 7 alternatively spliced amino acids (rectangle). The alternative mRNA uses the same reading frame as the conventional transcript. Conventional exons are in uppercase, introns in lowercase. See also Figures S1 and S3.*

AUTS2 transcript and is predicted to encode a polypeptide of 697 acids instead of the 1,259 amino acids of the full length protein. The evolutionary conservation from humans to zebrafish suggests an important biological function for *AUTS2* and together with the shorter transcript gave us the opportunity to analyze the function of the C-terminus of AUTS2 in a zebrafish model.

***In vivo* analysis of *AUTS2* in zebrafish embryos**

Taken together, our CNV mapping data, our RACE analyses and the strong correlation between phenotypic severity and position of the deletion, suggested that the 3'end of the *AUTS2* locus contains major functional elements that are encoded by both the full-length transcript and the shorter C-terminal isoform. Microcephaly is one of the most consistent clinical features in our cases (14/20; Table 1). We therefore asked whether AUTS2, and the shorter C-terminal isoform in particular, might be involved in the regulation of head size. As we have shown recently how head size evaluations in zebrafish embryos can serve as a surrogate for the evaluation of candidate genes for neurocognitive traits,[21] we decided to create a zebrafish morphant for *auts2*. Using reciprocal BLAST, we identified a single *Danio rerio AUTS2* ortholog (*auts2* on chromosome10; 62% amino acid identity, 72% similarity with the long isoform of human *AUTS2*) (Figure 4A). We were able to detect endogenous *auts2* message by RT-PCR as early as the embryonic 5-somite stage using both 5' and 3' primer sets (data not shown). Next, we designed two splice-blocking morpholinos (sb-MOs): a 5' MO targeting the splice donor site of exon 2, and a 3' MO targeting the splice donor site of exon 10 (targets were chosen to respectively suppressing the full length transcript only or both *auts2* transcripts (if present), see Figure 4A and S5). RT experiments demonstrated that both sb-MOs affected correct splicing of the *auts2* transcript (Figure S5). Masked scoring of embryos at three days post-fertilization (3 dpf) showed a reproducible microcephaly phenotype, 53% and 48% for 5' and 3' sb-MO respectively (Figure 5A-B) that was concomitant with the efficiency of splice blocking of the two sb-MOs, as established by RT-PCR (Figure S5). The phenotype was unlikely to be driven by overall developmental delay; morphants had a normal appearance with regard to their pigment cells, there was no apparent pathology in other internal organs such as the heart or the swim bladder, and their body length was indistinguishable from control embryos from the same clutch (Figure 5C). The phenotype was specific; the observed microcephaly caused by the two sb-MOs could be rescued efficiently with co-injection of wild type human full-length mRNA (GenBank, JQ670866) (Figure 5A-B). Strikingly, microcephalic embryos could also be rescued with the human short *AUTS2* isoform (GenBank, JQ670867) in a manner indistinguishable from full-length, indicating that the observed phenotype is driven by sequences in exons 9-19. We also observed another recurrent dysmorphic feature in knockdown zebrafish morphants: micro/retrognathia. To quantify this defect, we stained embryos injected with either 5' or 3' sb-MO at 5 dpf with Alcian blue and performed quantitative morphometric analysis of the lower jaw. We observed a significant reduction of the distance between the Meckel and ceratohyal cartilages, indicating a reduced lower jaw size comparable to the micro- and retrognathia seen in individuals with an *AUTS2* disruption (Figure 5D-E).

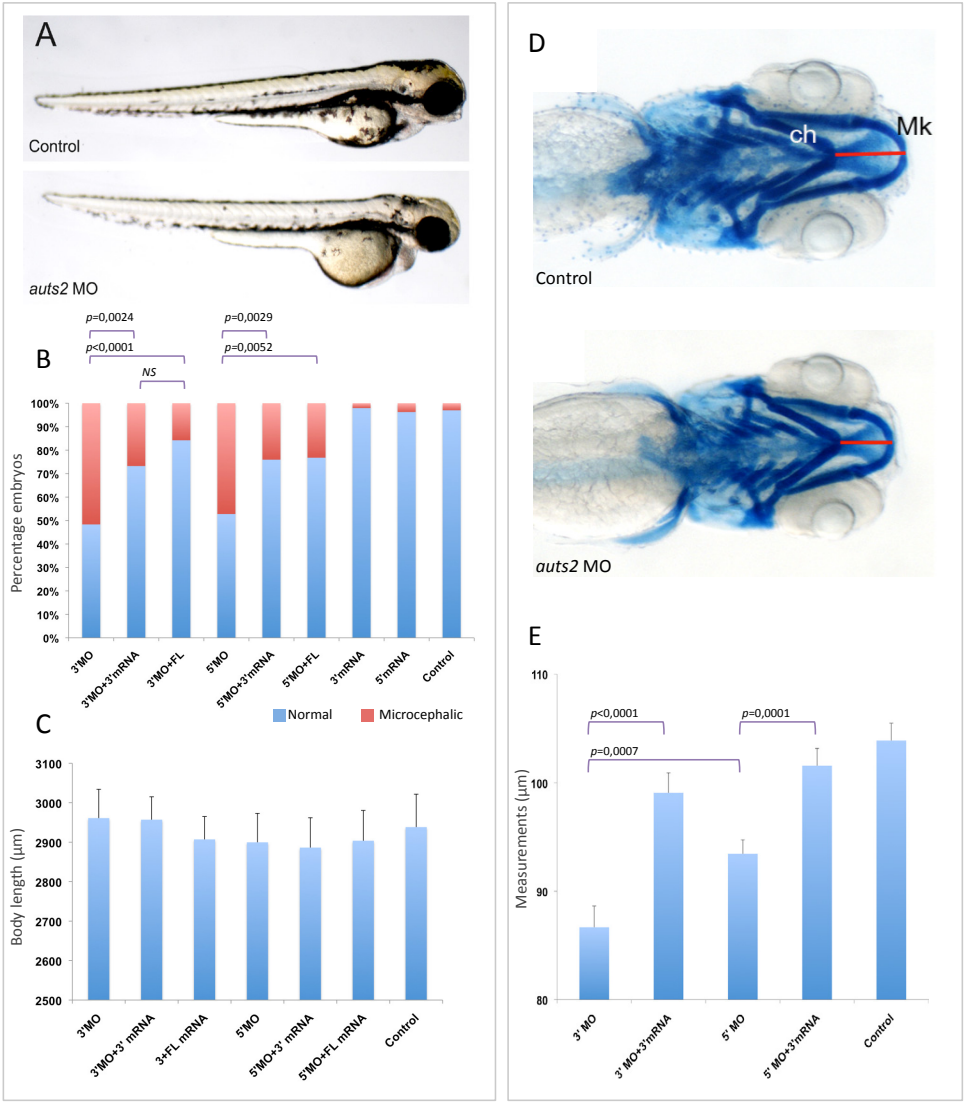


Figure 5. *Suppression of auts2 in zebrafish leads to small head size and craniofacial defects.*
A. *Lateral views of representative control embryos and embryos injected with AUTS2 morpholinos (MO).*
B. *Quantification of microcephaly was performed in embryo batches injected with 4.5 ng 5'MO (targeting exon 2 donor splice) or 6 ng 3'MO (targeting exon 10 donor splice) plus 100 pg wild type human AUTS2 full length (FL) or short isoform (3') mRNAs (n= 56-91 embryos per injection). P-values are denoted on the bar graph. NS, non-significant.* ***C.*** *No significant difference in* ***body length was observed in auts2 morphants and rescued embryos at 2dpf. The bars represent average length of 30 embryos, scored blind to injection cocktail. Data are shown as mean ± SD.*** ***D.*** *Ventral views of representative control embryos and those injected with auts2-MO (either 3' or 5' MOs) at 5 dpf. Cartilage structures were visualized by whole-mount Alcian blue staining at 5 dpf, allowing measurement of the distance between ceratohyal and Meckel's cartilages (red lines).* ***E.*** *Averaged distance measurements presented as mean ± s.e.m. The corresponding p values are denoted on the bar graph; two-tailed t-test comparisons. ch, ceratohyal cartilage; Mk, Meckel's cartilage.*

To probe the underlying cause(s) of the microcephalic phenotype further, we stained embryos at 2dpf with antibodies against phospho-histone H3, an M phase marker, and HuC/D, a marker of postmitotic neurons.[22] This time point was selected because it precedes the development of microcephaly and as such allows us to evaluate the forebrain prior to gross anatomical defects. We observed a striking reduction in phospho-histone H3 and HuC/D positive cells in embryos injected with either the 5′ or 3′ MO, as well as loss of bilateral symmetry in HuC/D protein levels, indicating that the microcephaly phenotype is caused by disturbed neuronal proliferation. Both phenotypes could be rescued with the 3′ human mRNA (Figure 6).

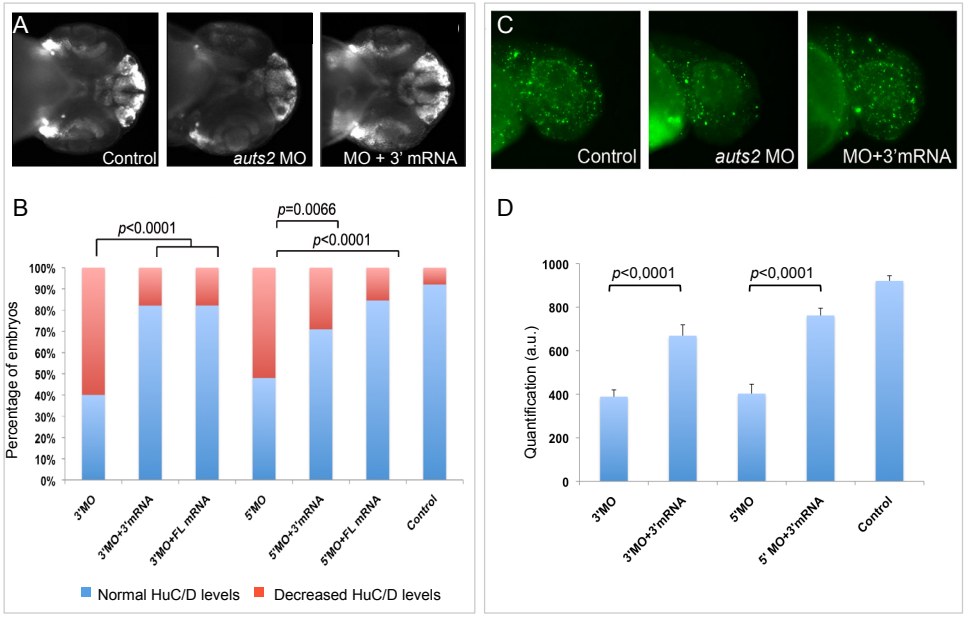


Figure 6. *Suppression of auts2 leads to reduced HuC/D protein levels and less proliferating cells.*
A. *Suppression of auts2 leads to a decrease of HuC/D levels at 2dpf. Representative photographs of ventral views of control, auts2 MO-injected embryo, and rescued embryo injected with auts2 MO plus 3′ human AUTS2 mRNA-injected embryos at 2dpf respectively, stained with HuC/D antibody. HuC/D levels in the anterior forebrain of auts2 MO-injected embryo are reduced considerably compared to control embryos. This defect is rescued significantly by co-injection of full length (FL) or short isoform (3′) human AUTS2 mRNAs.* ***B.*** *Percentage of embryos with normal, bilateral HuC/D protein levels in the anterior forebrain (blue) or decreased and/or unilateral HuC/D protein levels (red) in embryo batches injected with auts2 MOs alone or MOs plus human AUTS2 FL or 3′ mRNAs (MO + 3′ mRNA). P-values are denoted on the bar graph.* ***C.*** *Phospho-histone H3 staining for proliferating cells in zebrafish brain at 2 dpf.* ***D.*** *Quantification of phospho-histone H3 staining intensities from 20 embryos each, injected with either MO, 3′ and 5′ human AUTS2 mRNAs, or control. Data are represented as mean ± s.e.m. The corresponding p values are denoted on the bar graph; two-tailed t-test comparisons between MO-injected and rescued embryos.*

DISCUSSION

Our studies of 49,684 individuals with ID and/or MCA revealed deletions in *AUTS2* in 44 individuals, of which at least 24 involve exons. In contrast, we only found nine *AUTS2* deletions in 16,784 controls, none of which were exonic, strongly indicating that intragenic *AUTS2* deletions that disrupt at least a portion of the coding sequence are a recurrent cause of neurodevelopmental defects in humans. The frequency of exonic deletions that we found is 1 in 2.000 cases, comparable with some of the recurrent deletions described by Cooper et al (2011) such as the 10q23 deletion (*NRG3* [MIM 605533], *GRID1* [MIM 610659]) and deletions causing Sotos- [MIM 117550] or Rubinstein Taybi syndrome [MIM 180849].[12] This observed frequency is likely to be an underestimate because smaller deletions (single exon deletions and small (in)dels within exons) and nonsense mutations are likely to cause AUTS2 syndrome as well and are missed with the techniques used here.

The individuals with an *AUTS2* aberration affecting the coding sequence studied here (cases 1 to 17), together with the cases reported previously, allowed us to delineate recurrent phenotypic features of the AUTS2 syndrome: ID, autism, microcephaly, mild short stature, feeding difficulties, hypotonia, cerebral palsy and dysmorphic features. Only 1 of the 21 persons studied in detail did not have any features of the AUTS2 syndrome (the father of case 1) indicating a penetrance of around 95%. Although the phenotype of the AUTS2 syndrome is variable and the features are sometimes subtle, there are other examples where reversed genomics have shown variable phenotypes associated with the same locus.[23, 24] Several lines of evidence support the causality of *AUTS2* deletions for this broad phenotypic spectrum, namely (a) the significant enrichment of exonic deletions in cases, (b) *auts2* zebrafish morphants show microcephaly and smaller lower jaw size comparable to the human phenotype, that can be fully rescued by both full length and a short 3' human *AUTS2* transcript, (c) none of the individuals with an exonic deletion carried a second rare *de novo* CNV and (d) all exonic deletions are *de novo* or inherited from an affected parent except for the in-frame exon 2 deletion of case 1.

Individuals with in-frame exonic deletions in the 5' part of the gene (exon 1-5) show a milder phenotype, mainly restricted to neurocognitive defects with no or limited dysmorphology, or can even be normal, like the father of case 1. In contrast, deletions of the C-terminal part, encoded by both the short and full-length transcript, cause a more severe phenotype including dysmorphology. This could potentially be related to the gene structure: because exons 7-19 are closely packed, deletions in this part of the gene often result in larger disruptions of the coding sequence. However we also observed severe phenotypes in cases with small in frame 3' deletions and in 3' MO zebrafish, where the shorter 3' transcript was sufficient to rescue the dysmorphology (microcephaly and smaller jaw size). This might suggest that the C-terminal part of the protein contains the crucial region for the observed dysmorphology. It is uncertain if the shorter 3' transcript is expressed at sufficiently high levels to explain the milder phenotype in humans with in-frame 5' deletions. The milder phenotype might well be explained by the fact that *AUTS2* alleles with these deletions can still be transcribed,

resulting in a protein that contains the important C-terminal sequences.

In aggregate, our data indicate that *AUTS2* deletions, in particular when they involve the C-terminus, give rise to a highly penetrant syndrome that includes neurocognitive defects. Our data highlight transcriptional complexity at the *AUTS2* locus and show that careful genomic, genetic and functional dissection of such complexity can offer both clinical and mechanistic insights. Although little is known about the function(s) of AUTS2 or its isoforms, a role in neurodevelopment is suggested by the reduction of postmitotic neurons and loss of bilateral symmetry that might be driven by neurogenesis and/or migration defects in the zebrafish *auts2* morphants. The zebrafish model can be of great value for further studies of *AUTS2* function and can be helpful to define the pathogenicity of specific genomic disruptions.

In conclusion, detailed analysis of the *AUTS2* locus allowed us to delineate a hitherto unrecognized microdeletion syndrome, occurring with a frequency that approximates the frequency of deletions causing Sotos syndrome or Rubinstein Taby syndrome.[12] This AUTS2 syndrome presumably remained undetected because (a) the specific characteristics of the resulting phenotype are subtle (b) the severity of the syndrome is highly variable and (c) the penetrance is dependent on location and type of deletion.

Aknowledgments

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JAR and NJN are employees of Signature Genomic Laboratories, a subsidiary of PerkinElmer, Inc. EEE is on the scientific advisory boards for Pacific Biosciences, Inc., SynapDx Corp, and DNAnexus, Inc

Accession numbers

Microarray data have been deposited in the Gene Expression Omnibus under the accession number GSE37657. The nucleotide sequence of the human_*AUTS2*_full length transcript is submitted to GenBank under the accession number JQ670866 and the nucleotide sequence of the shorter 3' alternative human_*AUTS2*_transcript under the accession number JQ670867.

Web resources

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

Welcome Trust Case Control Consortium 2: https://www.wtccc.org.uk/ccc2/

HapMap: http://hapmap.ncbi.nlm.nih.gov/downloads/raw_data/hapmap3_affy6.0/

Database of Genomic Variance: http://projects.tcag.ca/cgi-bin/variation/gbrowse/hg18/#search

Online Mendelian Inheritance in Man: http://www.omim.org/

Gene Expression Omnibus: http://www.ncbi.nlm.nih.gov/geo/

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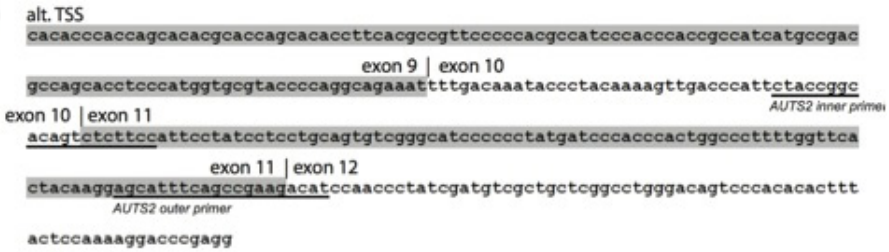
APPENDIX CHAPTER 3

SUPPLEMENTAL DATA

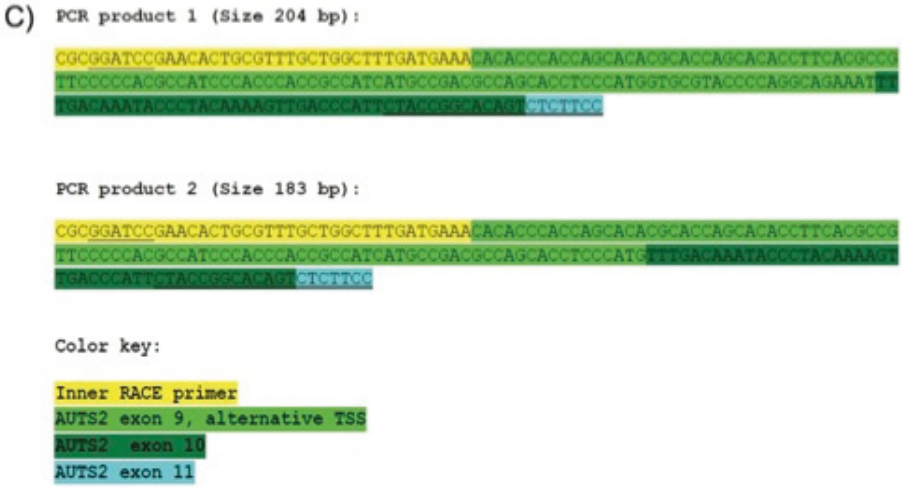
A)



B)

Name	Oligo Sequence
RACE adapter	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA
AUTS2_RACE_outer	ATGTCTTCGGCTGAAATGCT
AUTS2_RACE_inner	GGAAGAGACTGTGCCGGTAG
Universal RACE outer primer	GCTGATGGCGATGAATGAACACTG
Universal RACE inner primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG

C)



D)

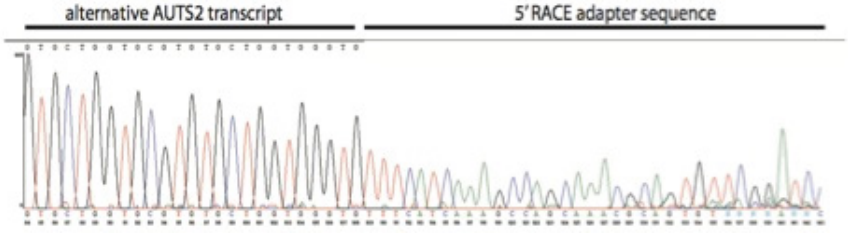


Figure S1. *Strategy used for 5' RACE amplification and obtained sequence results.*
A. *Position of AUTS2-specific reverse primers in AUTS2 mRNA. The primers were designed to recognize the splice boundaries between exon 10 and 11 (inner primer) or between exon 11 and 12 (outer primer).* ***B.*** *Sequences of 5'RACE adapter RNA, universal and AUTS2-specific primers.* ***C.*** *After subcloning and sequence analysis, two types of PCR products were observed: product 1 containing the long variant of exon 9; and product 2 containing a shorter variant of exon 9. Both transcripts offer a start codon in the same reading frame as full-length AUTS2, differing 7 amino acids in length.* ***D.*** *Chromatogram from the antisense strand of the 5'RACE product, showing the transition between the 5' transcriptional start site and the RACE adapter. All transcripts detected had the same start site as confirmed by direct sequencing of the RACE products and of more than 10 independent clones from two independent experiments.*

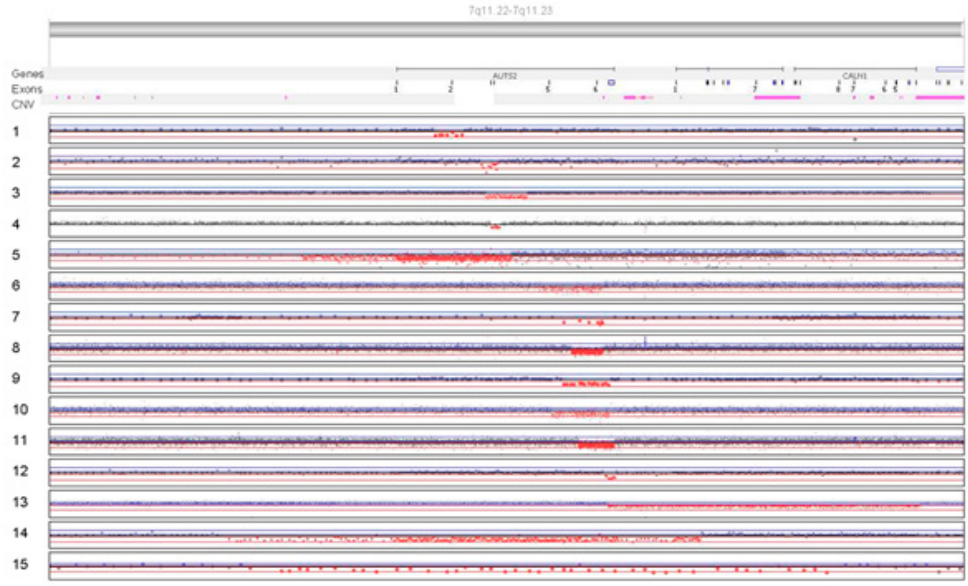


Figure S2. *Probe view of deletions found in AUTS2.*
The deletions of 15 probands on whom detailed genotypic and phenotypic data were available are shown here. The picture was created using NEXUS software and the deleted and undeleted probes of the different array platforms are plotted. CNV's extracted from the Database of Genomic Variance in purple (CNV's found in BAC-studies not included). This figure shows the data on which figure 1 is based.

```
                |exon 1
H.sapiens       MDGPTRGHGLrkkrrsrsqrdrerrsrgglgagaaggggagrtralslaSSSGSDKEDNG 60
G.gorilla       MDGPTRGHGLRKKRRSRSQRDRERRSRGGLGAGAAGGGGAGRTRALSLASSSGSDKEDNG 60
M.mulatta       MDGPTRGHGLRKKRRSRSQRDRERRSRGGLGAGAAGGGGAGRTRALSLASSSGSDKEDNG 60
C.familiaris    MDGPTRGHGLRKKRRSRSQRDRERRSRGGLGAGAAGGGGAGRTRAPSLASSSGSDKEDNG 60
B.taurus        MDGPTRGHGLRKKRRSRSQRDRERRSRGGLGAGAAGGGGAGRTRAPSLASSSGSDKEDNG 60
S.scrofa        MDGPTRGHGLRKKRRSRSQRDRERRSRGGLGAGAAGGGGAGRTRAPSLASSSGSDKEDNG 60
M.musculus      MDGPTRGHGLRKKRRSRSQRDRERRSRAGLGTGAAGGIGAGRTRAPSLASSSGSDKEDNG 60
G.gallus        MDGPARCNGLRKKRRSRSQRDRERRAQGGR-RGAAAGGAPGPGAAPALSSS-GSEKEDNG 58
X.tropicalis    MDGP-RCNGFRKKRRSKSQRDRDMRSKSGLGVARTG----------SLLSSSGSEKEDNE 49
D.rerio         MDGP-RCSGIRKKRKSRSVRNRERISNGIRNNHVRG---------SVLRFSSDSEKED-G 49
                **** *  *:****:*:* *:*:  :..       .          *  * .*:***

                                                          |   exon 2
H.sapiens       KppssapsrprpprrkrrestsAEEDIIDGFAMTSFVTFEALEKDVALKPQERVEKRQTP 120
G.gorilla       KPPSSAPSRPRPPRRKRRESTSAEEDIIDGFAMTSFVTFEALEKDVALKPQERVEKRQTP 120
M.mulatta       KPPSSAPSRPRPPRRKRRESTSAEEDIIDGFAMTSFVTFEALEKDVALKPQERVEKRQTP 120
C.familiaris    KPPSSAPSRPRPPRRKRRESTSAEEDIIDGFAMTSFVTFEALEKDVALKPQERVERRQTP 120
B.taurus        KPPSSAPSRPRPPRRKRRESTSAEEDIIDGFAMTSFVTFEALEKDVALKPQERVEKRQTP 120
S.scrofa        KPPSSAPSRPRPPRRKRRESTSAEEDIIDGFAMTSFVTFEALEKDVALKPQERVEKRQTP 120
M.musculus      KPPSSAPSRPRPPRRKRRESTSAEEDIIDGFAMTSFVTFEALEKDVAVKPQERAEKRQTP 120
G.gallus        PPP---PSRPRPPRRKRRESSSAEEDIIDGFAMSSFVTFEALEKDVALKPQERVEKRQNP 115
X.tropicalis    NPTGSSLPRPKPPRRKRKESSSAEEDIIDGFAMTSFVTFEALEKEGALMPEETDEKQQTP 109
D.rerio         STNPSSSSRPRPPRRKRKESSSAEEDIIDGFSIAGFMTLEALEKDMTLKPHERRQNQAGP 109
                 .    .**:******:**:**********:::.*:*:*****: :: *.*  :.:  *

                                                                      | exon 3
H.sapiens       LTKKKREALTNGLSFHSKKSRLSHPHHYSSDRENDRNLCQHLGKRKKMPKALRQLKPGQN 180
G.gorilla       LTKKKREALTNGLSFHSKKSRLSHPHHYSSDRENDRNLCQHLGKRKKMPKALRQLKPGQN 180
M.mulatta       LTKKKREALTNGLSFHSKKSRLSHPHHYSSDRENDRNLCQHLGKRKKMPKALRQLKPGQN 180
C.familiaris    LIKKKREALTNGLSFHSKKSRLSHPHHYSSDRENDRNLCQHLGKRKKTPKGLRQLKPGQN 180
B.taurus        LTKKKREALTNGLSFHSKKSRLSHPHHYSSDRENDRNLCQHLGKRKKMPKGLRQLKPGQN 180
S.scrofa        LTKKKREALTNGLSFHSKKSRLSHPHHYSSDRENDRNLCQHLGKRKKMPKGLRQLKPGQN 180
M.musculus      LTKKKREALTNGLSFHSKKSRLSHSHHYSSDRENDRNLCQHLGKRKKMPKGLRQLKPGQN 180
G.gallus        LAKKKREALTNGLSYLPKKNRLHH-HQYSSDRENDRNLCQHLGKRKKLLKGLRQLKPGQN 174
X.tropicalis    LTKKKREILSNGLPYKSQK-NNKLSPNYGSDRENDRSLCQQFG-KKTFQKRYKQLKARQN 167
D.rerio         LRKKKPGRVANGLSLDLHKDRLNHSNHQHSDQENNPRLARTHS-KKKKKKHLQKLKPGQN 168
                * ***   ::***.   :* .     :  **:**:  *.:  . :*.  *  ::**. **

                                          | exon 4    | exon 5  | exon 6
H.sapiens       SCRDSDSESASGESKGFHrsssrerlsdssapssLGTGYFCDSDSDQEEKASDASSEKLF 240
G.gorilla       SCRDSDSESASGESKGFHRSSSRERLSDSSAPSSLGTGYFCDSDSDQEEKASDASSEKLF 240
M.mulatta       SCRDSDSESASGESKGFHRSSSRERLSDSSAPSSLGTGYFCDSDSDQEEKASDASSEKLF 240
C.familiaris    SCRDSDSDSASGESKGFHRSSSRERLSDSSAPSSLGTGYFCDSDSDQEEKASDASSEKLF 240
B.taurus        SCRDSDSESASGESKGFHRSSSRERLSDSSAPSSLGTGYFCDSDSDQEEKASDASSEKLF 240
S.scrofa        SCRDSDSESASGESKGFHRSSSRERLSDSSAPSSLGTGYFCDSDSDQEEKASDASSEKLF 240
M.musculus      SCRDSDSESASGESKGFQRSSSRERLSDSSAPSSLGTGYFCDSDSDQEEKASDASSEKLF 240
G.gallus        SCRDSDSESASGESKGFHRSSSRERLSDSSAPSSLGTGYFCDSDSDQEEKASDASSEKLF 234
X.tropicalis    NCRDSDIESPMKETKPSKRNASRERLSDSSAASSLGTGYFCDSDSDQEEKVSDASSEKLF 227
D.rerio         NCKDSDSESVSGESKPSIRSSSRDRLTDPSAPSKTGLGHYCDSESDQEDKGSDASSEKLF 228
                .*:*** :*   *:*   *.:**:**:*.**.*. * *::***:****:* *********

                        | exon 7
H.sapiens       NTVIVNKDPELGVGTLPEHDS-QDAGPIVPKISGLERSQEKSQDCC-------------- 285
G.gorilla       NTVIVNKDPELGVGTLPEHDS-QDAGPIVPKISGLERSQEKSQDCC-------------- 285
M.mulatta       NTVIVNKDPELGVGTLPEHDS-QDAGPIVPKISGLERSQEKSQDCC-------------- 285
C.familiaris    NTVIVNKDPELGVGTLPEHDS-QDAGPIVPKISGLERSQEKSQDCG-------------- 285
B.taurus        NTVIVNKDPELGVGALPHHDG-HDAGPIVPKVSGLERSQEKSQDCG-------------- 285
S.scrofa        NTVIVNKDPELGVGALPEHDG-HDAGPIVPKISGLERSQEKSQDCG-------------- 285
M.musculus      NTVLVNKDPELGVGALPEHN--QDAGPIVPKISGLERSQEKSQDCC-------------- 284
G.gallus        N-AVTNKGKELGVSALAENADPGARGPAVPKISGLERSQEKSQDSS-------------- 279
X.tropicalis    HTISNNGDTELGVRLLTTRGSKNATGQVVPKVSGLERSQEKSQDSD-------------- 273
D.rerio         STAAVKVP-DFSVDTLSTNASQELRGLGIPKVSGLERSQEKSQETSREISSATPPLVPTS 287
                     :   ::.*  *. .      *  :**:***********:

H.sapiens       --KEPIFEPVVLKDPCPQVAQPIP-------QPQTEPQLRAPSPDPDLVQRTEAp-pqpp 335
G.gorilla       --KEPIFEPVVLKDPCPQVAQPIP-------QPQTEPQLRAPSPDPDLVQRTEAP-PQPP 335
M.mulatta       --KEPIFEPVVLKDPCPQVAQPIP-------QPQTEPQLRAPSPDPDLVQRTEAP-PQPP 335
C.familiaris    --KEPIFEPVVLKDPCPQAPQPLP-------QPQAEPQPRAPSPDPDLVPRTEAP-PQPP 335
B.taurus        --KEPLFEPVVLKDARPQVPPPPP-------QPQAEPPPRAPSPDPASGPRAEPP-PPPP 335
S.scrofa        --KEPIFEPVVLKDPRPQVPPPPP-------QPQAEPPPRAPSPDPASGPRAEPP-PPPP 335
M.musculus      --KEPVFEPVVLKDPHPQLPQLPS-------QAQAEPQLQIPSPGPDLVPRTEAP-PQFP 334
G.gallus        --KDPILEPVVPKDPCPQVAQPLA-------QPQLEPQPEVPCPSPALAPRPEAPAPPAP 330
X.tropicalis    --KELQLDVLVSKDP-ILPSLPVV-------HADLESRPILVSPGPTLDSCTEIP----L 319
D.rerio         HSKPPLPAPLHLQPPPSSRGLPLPSRPAQIQNPCPERTLRPLSPPIALPQSQGQELSQAP 347
                  *      :  : .                :.  *      .*
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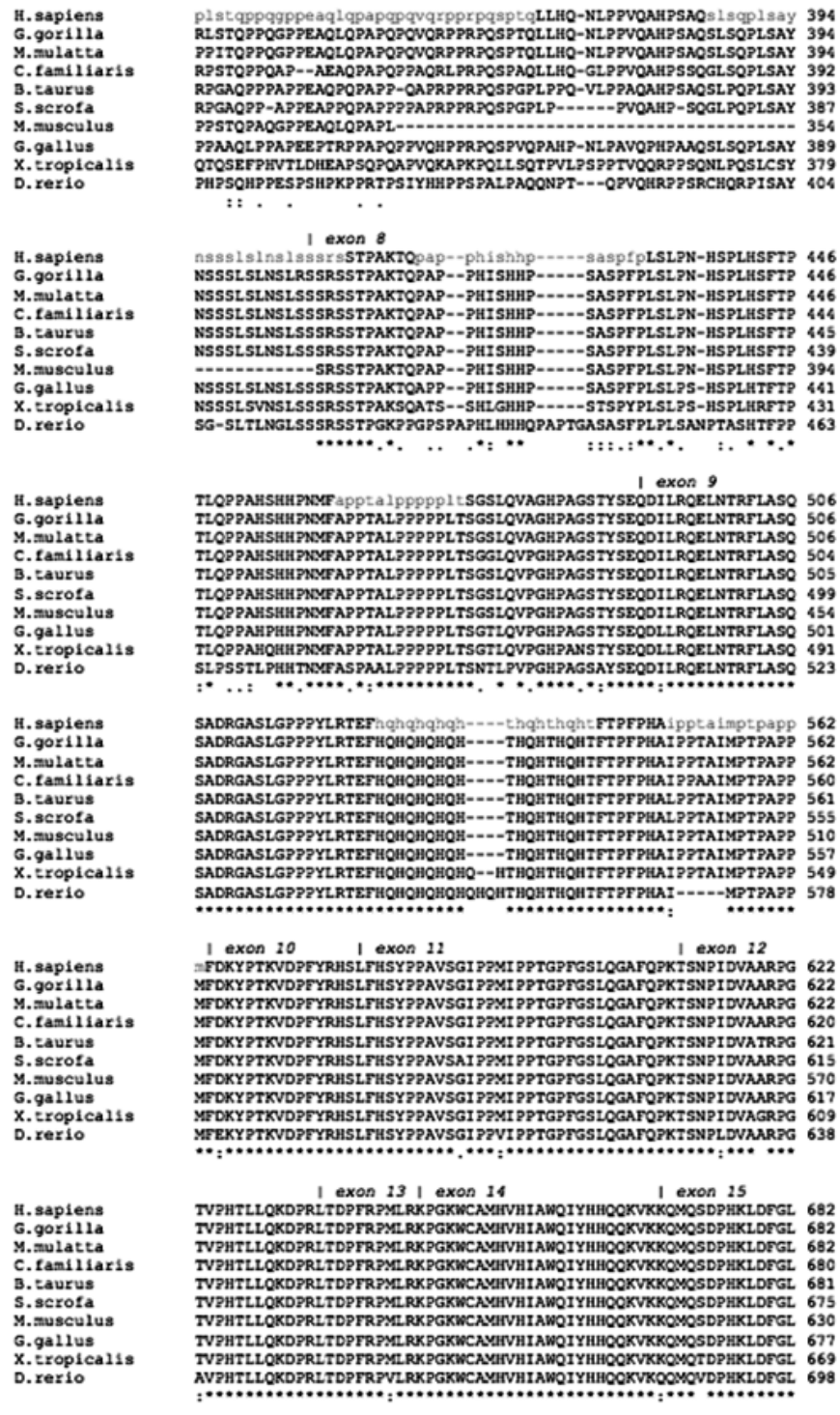
H.sapiens plstqppqgppeaqlqpapqpqvqrpprpqsptqLLHQ-NLPPVQAHPSAQslsqplsay 394
G.gorilla RLSTQPPQGPPEAQLQPAPQPQVQRPPRPQSPTQLLHQ-NLPPVQAHPSAQSLSQPLSAY 394
M.mulatta PPITQPPQGPPEAQLQPAPQPQVQRPPRPQSPTQLLHQ-NLPPVQAHPSAQSLSQPLSAY 394
C.familiaris RPSTQPPQAP--AEAQPAPQPPAQRLPRPQSPAQLLHQ-GLPPVQAHPSSQGLSQPLSAY 392
B.taurus RPGAQPPPAPPEAQPQPAPP-QAPRPPRPQSPGPLPPQ-VLPPAQAHPSAQSLPQPLSAY 393
S.scrofa RPGAQPP-APPEAPPQPAPPPPAPRPPRPQSPGPLP------PVQAHP-SQGLPQPLSAY 387
M.musculus PPSTQPAQGPPEAQLQPAPL---------- 354
G.gallus PPAAQLPPAPEEPTRPPAPQPPVQHPPRPQSPVQPAHP-NLPAVQPHPAAQSLSQPLSAY 389
X.tropicalis QTQSEFPHVTLDHEAPSQPQAPVQKAPKPQLLSQTPVLPSPPTVQQRPPSQNLPQSLCSY 379
D.rerio PHPSQHPPESPSHPKPPRTPSIYHHPPSPALPAQQNPT---QPVQHRPPSRCHQRPISAY 404
exon 8
H.sapiens nssslslnslsssrsSTPAKTQpap--phishhp-----saspfpLSLPN-HSPLHSFTP 446
G.gorilla NSSSLSLNSLRSSRSSTPAKTQPAP--PHISHHP-----SASPFPLSLPN-HSPLHSFTP 446
M.mulatta NSSSLSLNSLSSSRSSTPAKTQPAP--PHISHHP-----SASPFPLSLPN-HSPLHSFTP 446
C.familiaris NSSSLSLNSLSSSRSSTPAKTQPAP--PHISHHP-----SASPFPLSLPN-HSPLHSFTP 444
B.taurus NSSSLSLNSLSSSRSSTPAKTQPAP--PHISHHP-----SASPFPLSLPN-HSPLHSFTP 445
S.scrofa NSSSLSLNSLSSSRSSTPAKTQPAP--PHISHHP-----SASPFPLSLPN-HSPLHSFTP 439
M.musculus ----------SRSSTPAKTQPAP--PHISHHP-----SASPFPLSLPN-HSPLHSFTP 394
G.gallus NSSSLSLNSLSSSRSSTPAKTQAPP--PHISHHP-----SASPFPLSLPS-HSPLHTFTP 441
X.tropicalis NSSSLSVNSLSSSRSSTPAKSQATS--SHLGHHP-----STSPYPLSLPS-HSPLHRFTP 431
D.rerio SG-SLTLNGLSSSRSSTPGKPPGPSPAPHLHHHQPAPTGASASFPLPLSANPTASHTFPP 463
exon 9
H.sapiens TLQPPAHSHHPNMFapptalpppppltSGSLQVAGHPAGSTYSEQDILRQELNTRFLASQ 506
G.gorilla TLQPPAHSHHPNMFAPPTALPPPPPLTSGSLQVAGHPAGSTYSEQDILRQELNTRFLASQ 506
M.mulatta TLQPPAHSHHPNMFAPPTALPPPPPLTSGSLQVAGHPAGSTYSEQDILRQELNTRFLASQ 506
C.familiaris TLQPPAHSHHPNMFAPPTALPPPPPLTSGGLQVPGHPAGSTYSEQDILRQELNTRFLASQ 504
B.taurus TLQPPAHSHHPNMFAPPTALPPPPPLTSGSLQVPGHPAGSTYSEQDILRQELNTRFLASQ 505
S.scrofa TLQPPAHSHHPNMFAPPTALPPPPPLTSGSLQVPGHPAGSTYSEQDILRQELNTRFLASQ 499
M.musculus TLQPPAHSHHPNMFAPPTALPPPPPLTSGSLQVPGHPAGSTYSEQDILRQELNTRFLASQ 454
G.gallus TLQPPAHPHHPNMFAPPTALPPPPPLTSGTLQVPGHPAGSTYSEQDLLRQELNTRFLASQ 501
X.tropicalis TLQPPAHQHHPNMFAPPTALPPPPPLTSGTLQVPGHPANSTYSEQDLLRQELNTRFLASQ 491
D.rerio SLPSSTLPHHTNMFASPAALPPPPPLTSNTLPVPGHPAGSAYSEQDILRQELNTRFLASQ 523
SADRGASLGPPPYLRTEFhqhqhqhqh----thqhthqhtFTPFPHAipptaimptpapp 562
G.gorilla SADRGASLGPPPYLRTEFHQHQHQHQH----THQHTHQHTFTPFPHAIPPTAIMPTPAPP 562
M.mulatta SADRGASLGPPPYLRTEFHQHQHQHQH----THQHTHQHTFTPFPHAIPPTAIMPTPAPP 562
C.familiaris SADRGASLGPPPYLRTEFHQHQHQHQH----THQHTHQHTFTPFPHAIPPAAIMPTPAPP 560
B.taurus SADRGASLGPPPYLRTEFHQHQHQHQH----THQHTHQHTFTPFPHALPPTAIMPTPAPP 561
S.scrofa SADRGASLGPPPYLRTEFHQHQHQHQH----THQHTHQHTFTPFPHALPPTAIMPTPAPP 555
M.musculus SADRGASLGPPPYLRTEFHQHQHQHQH----THQHTHQHTFTPFPHAIPPTAIMPTPAPP 510
G.gallus SADRGASLGPPPYLRTEFHQHQHQHQH----THQHTHQHTFTPFPHAIPPTAIMPTPAPP 557
X.tropicalis SADRGASLGPPPYLRTEFHQHQHQHQHQ--HTHQHTHQHTFTPFPHAIPPTAIMPTPAPP 549
D.rerio SADRGASLGPPPYLRTEFHQHQHQHQHQHQHTHQHTHQHTFTPFPHAI-----MPTPAPP 578
exon 10
exon 11
exon 12
H.sapiens mFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVAARPG 622
G.gorilla MFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVAARPG 622
M.mulatta MFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVAARPG 622
C.familiaris MFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVAARPG 620
B.taurus MFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVATRPG 621
S.scrofa MFDKYPTKVDPFYRHSLFHSYPPAVSAIPPMIPPTGPFGSLQGAFQPKTSNPIDVAARPG 615
M.musculus MFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVAARPG 570
G.gallus MFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVAARPG 617
X.tropicalis MFDKYPTKVDPFYRHSLFHSYPPAVSGIPPMIPPTGPFGSLQGAFQPKTSNPIDVAGRPG 609
D.rerio MFEKYPTKVDPFYRHSLFHSYPPAVSGIPPVIPPTGPFGSLQGAFQPKTSNPLDVAARPG 638
exon 13
exon 14
exon 15
H.sapiens TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 682
G.gorilla TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 682
M.mulatta TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 682
C.familiaris TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 680
B.taurus TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 681
S.scrofa TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 675
M.musculus TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 630
G.gallus TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQSDPHKLDFGL 677
X.tropicalis TVPHTLLQKDPRLTDPFRPMLRKPGKWCAMHVHIAWQIYHHQQKVKKQMQTDPHKLDFGL 669
D.rerio AVPHTLLQKDPRLTDPFRPVLRKPGKWCAMHVHIAWQIYHHQQKVKQQMQVDPHKLDFGL 698

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                                                                  | exon 16
H.sapiens           KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSaagaahptgtpfgpppphh-SNFLNPA 738
G.gorilla           KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSAAGAAHPTGTPFGPPPHH-SNFLNPA 738
M.mulatta           KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSAAGAAHPTGTPFGPPPHH-SNFLNPA 738
C.familiaris        KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSAAGAAHPTGTPFGPPPHH-SNFLNPA 736
B.taurus            KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSAAGAAHPTGTPFGPPPHH-SNFLTPA 737
S.scrofa            KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSAAGAAHPTGTPFGPPPHH-SNFLNPA 731
M.musculus          KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSAAGAAHPTGTPFGPPPHH-SNFLNPA 686
G.gallus            KPEFLSRPPGPSLFGAIH---HPHDLARPSTLFSTASGAHPAGTPFGPPPHH-SNFLNPA 733
X.tropicalis        KPEFLSRPPGPSLFGAIHPHPHPHDLARPSTLFSAAGGTHPAAAPFGPPPHH-SNFLNPS 728
D.rerio             KPEFLSRPPGPTLFGAIH---HPHDLARPASLFSAAGSTHPAAAPFGHPPHHPSNFLTPA 755
                    **********:******   ********::***:*..:**:.:*** **** ****.*:

                        | exon 17                   | exon 18
H.sapiens           AHLEPFNRPSTFTGLAAVGGNAFGGLGNPSVTPNSMFGHKDGPSVQN-FSNPHEPWNRLH 797
G.gorilla           AHLEPFNRPSTFTGLAAVGGNAFGGLGNPSVTPNSMFGHKDGPSVQN-FSNPHEPWNRLH 797
M.mulatta           AHLEPFNRPSTFTGLAAVGGNAFGGLGNPSVTPNSMFGHKDGPSVQN-FSNPHEPWNRLH 797
C.familiaris        AHLEPFNRPSTFTGLAAVGGNAFGGLGNPSVTPNSMFGHKDGPSVQN-FSNPHEPWNRLH 795
B.taurus            AHLEPFNRPSTFTGLAAVGGNAFGGLGNPSVTPNSVFGHKDGPSVQS-FSNPHEPWNRLH 796
S.scrofa            AHLEPFNRPSTFTGLAAVGGNAFGGLGNPSVTPNSMFGHKDGPSVQN-FSNPHEPWNRLH 790
M.musculus          AHLEPFNRPSTFTGLAAVGGNAFGGLGNPSVTPNSVFGHKDSPSVQN-FSNPHEPWNRLH 745
G.gallus            AHLEPFNRPASFSGLTAVGSNAFGGLGNPTVTPNSMFGHKDGPSMQS-FSNPHEPWNRLH 792
X.tropicalis        AHLEQFNRPSTFSGLAAVGGSAFGGLGNPSVSSNSMFSHKEGPNMQS-FNNPHEPWNRLH 787
D.rerio             PHLEPFSRPPSFGGLASLSTAAFGGLGNPALASNSVFGHKDSPSAQQHFSGPHEPWNRLH 815
                    .*** *.**.:* **:::.  ********:::.**:*.**:.*. *. *..*********

                                                                                | exon 19
H.sapiens           RTppsfptpppwlkpGELersasaaahdrdr--------dvdkrdssvskddkeresVEK 849
G.gorilla           RTPPSFPTPPPWLKPGELERSASAAAHDRDR--------DVDKRDSSVSKDDKERESVEK 849
M.mulatta           RTPPSFPTPPPWLKPGELERSASAAAHDRDR--------DVDKRDSSVSKDDKERESVEK 849
C.familiaris        RTPPSFPTPPPWLKPGELERSASAAAHDRDR--------DVDKRDSSVSKDDKERESVEK 847
B.taurus            RTPPSFPTPPPWLKPGELERSASAAAHDRDR--------DVDKRDSSVSKDDKERESVEK 848
S.scrofa            RTPPSFPTPPPWLKPGELERSAPAAALDRDR--------DVDKRDSSVSKDDKERESVEK 842
M.musculus          RTPPSFPTPPPWLKPGELERSASAAAHDRDR--------DVDKRDSSVSKDDKERESVEK 797
G.gallus            RTPPSFPTPPPWLKPGELERSSSAAAHDRDR--------DVDKRDSSVSKDDKERESIEK 844
X.tropicalis        RTPPSFPTPPPWLKPGESERSSSVASHER----------DADKRE-TATKDDKERENVEK 836
D.rerio             RTPPSFPTPPPWLKPGESERSASVSSHERERERDRERERDLDKRDSSVNKDDKEREPVEK 875
                    ***************** ***:..:: :*          * ***: :..******* :**

H.sapiens           RHSSHPSPAPVLPVNALGHTRSSTE-QIRAHLN-TEAREKDK--PKERERDHSES-RKDL 904
G.gorilla           RHSSHPSPAPILPVNALGHTRSSTE-QIRAHLN-TEAREKDK--PKERERDHSES-RKDL 904
M.mulatta           RHSSHPSPAPILPVNALGHTRSSTD-QIRAHLN-TEAREKDK--PKERERDHSES-RKDL 904
C.familiaris        RHSSHPSPAPILPVSTLGHNRSSAE-QIRGHLN-TEAREKDK--SKERERDHSES-RKDL 902
B.taurus            RHSSHPSPAPVLPVSTLGHGRSSSE-QIRGHLS-TEAREKDK--CKDREREHSES-RKDL 903
S.scrofa            RHPSHPSPAPVLPVSTLGHSRSSTE-QIRGHLN-TEAREKDK--SKDRERDHSES-RKDL 897
M.musculus          RHPSHPSPAPPVPVSALGHNRSSTDPTTRGHLN-TEAREKDK--PKEKERDHSGS-RKDL 853
G.gallus            RHSSHPSPAPIHPVNSLGHNRSSNE-QIRSHLN-PEVREKEK--PKERERDHSES-RKEI 899
X.tropicalis        RHSSHPSPAPINQVNSTGHNRSLSE-QNRNNLN-NDVREKEK--PKERERENSDS-WKEA 891
D.rerio             RHQNHQSPLPVNPLTLLGHTRPSEP--SRNHISSSEPRDKDKDKPKDRERDREHSDWKDS 933
                    ** .* ** *   :.  ** *.      * ::.  : *:*:*   *::**::. *  *:

H.sapiens           AADEHKAKEGHLPEKD--ghghegraageeaKQLARVPSPYVRTPVVESARPNSTSSREA 962
G.gorilla           AADEHKAKEGHLPEKD--GHGHEGRAAGEEAKQLARVPSPYVRTPVVESARPNSTSSREA 962
M.mulatta           AADEHKAKEGHLPEKD--GHGHEGRAAGEEAKQLARVPSPYVRTPVVESARPNSTSSREA 962
C.familiaris        TADEHKAKEGHLPEKD--GHSHEGRAVGEEAKQLARVPSPYVRTPVVESARPGSTSSREV 960
B.taurus            GTDEHKAKEGPLAEKD--GHGHEGRTAGEEAKQLARVPSPYARAPAADSARPGSAASRDA 961
S.scrofa            GADEHKAKEGPLPEKDGHGHGHEGRSAGEEAKQLARVPSPYARAPVADSARPGSAASREA 957
M.musculus          TTEEHKAKESHLPERD--GHSHEGRAAGEEPKQLSRVPSPYVRTPGVDSTRPNSTSSREA 911
G.gallus            NVEEHKVKDNYISEKE--SLSHESR-VVEESKQMSRVPSPYARPPVVESNRPNSTSNREA 956
X.tropicalis        SIEEHKTKDNHILEKD--NLVHESR-TIDEAKQLNRVPSPYVRPPTMENTRPNSNSNRET 948
D.rerio             NTDEHKLKENHHSDKD-TPVIHDGR-VSEDKPANRITASPYMRPGGMD--RVNGGLTRDI 989
                      :*** *:.   :::     *:.* . ::      ..*** *.   :  * ..  .*:

H.sapiens           EPRKGEPAYENPkkssevkvkeerkeDHDLPP-EAPQTHRAseppppns----sssvhpg 1017
G.gorilla           EPRKGEPAYENPKKSSEVKVKEERKEDHDLPP-EAPQTHRASEPPPPNS----SSSVHPG 1017
M.mulatta           EPRKGEPAYENPKKNSEVKVKEERKEDHDLPP-EAPQTHRASEPPPPNS----SSSVHPG 1017
C.familiaris        EQRKSEPAYENPKKSSEVKVKEERKEDHDLPP-EAPQTHRSSEAPPPSS----SSSVHPG 1015
B.taurus            EPRKGEPAYENPKKSAEVKVKEERKEDHDLPP-EAPPTHRSSEPPPPPPPSSSAGVHPG 1020
S.scrofa            EARKGEPAYENPKKSAEVKVKEERKEDHDLPP-EAPQTHRSSEPPPPPP--SSSASVHPG 1014
M.musculus          EPRKGEPAYENPKKNAEVKVKEERKEDHDLPT-EAPQAHRTSEAPPPSS--SASASVHPG 968
G.gallus            E-RKNEPAYDNPKKSNEVKVKEERKEDHDAQH-EGPQTHRSSDQPPPIS----TSNVHPS 1010
X.tropicalis        ERKTET--YDNQKKSNDVKVKEERKEEHDIVVPDGSQIHRTSEQPPPIS----TSNVHPS 1002
D.rerio             LEKKAEITYE--KKNSEVKVKEERKEEQDGPTERSPEQRSTPQAPPPPP----PAALHP- 1042
                      :.    *:  **. :*********::*     ..  : :.: *** .    .: :**
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H.sapiens          pLASMPMTVGVTGIHPMNSISSLDRTRMMTPFMGISPLPGGERFPYPSFHWDPIRDPLRD 1077
G.gorilla          PLASMPMTVGVTGIHPMNSISSLDRTRMMTPFMGISPLPGGERFPYPSFHWDPIRDPLRD 1077
M.mulatta          PLASMPMTVGVTGIHPMNSISSLDRTRMMTPFMGISPLPGGERFPYPSFHWDPIRDPLRD 1077
C.familiaris       PLASMPMTVGVTGIHPMNSISSLDRTRMMTPFMGISPIPGGERFPYPSFHWDPIRDPLRD 1075
B.taurus           PLASMPVAVGXPGIHPMNSISGLDRTRMMTPFMGLSPIPGGERFPYPSFHWDPIRDPLRD 1080
S.scrofa           PLASMPMTVGVTGIHPMNSISSLDRTRMMTPFMGISPIPGGERFPYPSFHWDPIRDPLRD 1074
M.musculus         PLASMPMTVGVTGIHAMNSIGSLDRTRMVTPFMGLSPIPGGERFPYPSFHWDPMRDPLRD 1028
G.gallus           PLVSMPMTVGVTGIHHINSISGLDRTRMMTPFMGISPIPGGERFPYPSFHWDPMRDPLRD 1070
X.tropicalis       HLASLPMAMGVAGMHPMNSIGGLDRTRMMTPFMGISPIPGGERFPYPSFHWDPMRDPLRD 1062
D.rerio            -PSSMPVPMGMASMHPINSISSLERTRMVAPFMGISPIPGAERFPYPAFHWDPMR----D 1097
                      *:*:.:*:..:* :***..*:****::****:**:**.******:*****:*    *

H.sapiens          PYRELDIHRRDPLGRDFLLRNDPLHRLSTPRLYEADRSFRDREPHDYS-hhhhhhhhPLS 1136
G.gorilla          PYRELDIHRRDPLGRDFLLRNDPLHRLSTPRLYEADRSFRDREPHDYS-HHHHHHHHPLS 1136
M.mulatta          PYRELDIHRRDPLGRDFLLRNDPLHRLSTPRLYEADRSFRDREPHDYS-HHHHHHHHPLS 1136
C.familiaris       PYRELDIHRRDPLGRDFLLRNDPLHRLSTPRLYEADRSFRDREPHDYN-HHHHHHHHPLS 1134
B.taurus           PYRELDIHRRDPLGRDFLLKNDPLHRLSTPRLYEADRSFRDREPHDYNHHHHHHHHHPLA 1140
S.scrofa           PYRELDIHRRDPLGRDFLLRNDPLHRLSTPRLYEADRSFRDREPHDYN-HHHHHHHHPLS 1133
M.musculus         PYRDLDMHRRDPLGRDFLLRNDPLHRLSTPRLYEADRSFRDREPHDYS-HHHHHHHHPLA 1087
G.gallus           PYRELDIHRRDPLGRDFLLRNDPLHRLSTPRLYEADRSFRDREPHDYN--HHHHHHHPLS 1128
X.tropicalis       PYRGLDIHRRDPLGRDFLLRNDPLHRLTAPRLYEAERSFRDREPHDYN-----HHHHHLA 1117
D.rerio            PYRGLDIHRRDPLARELLLRNDPLHRLAAPRLYEAERSYRDREPHDFN----RDHPHGLT 1153
                   *** **:******.*::**:*******::******:**:*******:.     .* * *:

H.sapiens          VDPRREHERGGHLDERERLHMLREDYEHTRLHSVHPASLDGHLPHPSLITPGLPSMHYPR 1196
G.gorilla          VDPRREHERGGHLDERERLHMLREDYEHTRLHSVHPASLDGHLPHPSLITPGLPSMHYPR 1196
M.mulatta          VDPRREHERGGHLDERERLHMLREDYEHTRLHSVHPASLDGHLPHPSLITPGLPSMHYPR 1196
C.familiaris       VDPRREHERGAHLDERERLHMLREDYEHTRLHPVHPASLDGHLPHPSLIAPGLPSMHYPR 1194
B.taurus           VDPRREHERGAHLDERERLHVLREDYEHPRLHPVHPASLDGHLPHPGLLTPGLPSMHYPR 1200
S.scrofa           VDPRREHERGAHLEERERLHMLREDYEHPRLHPVHPASLDGHLPHPGLITPGLPSMHYPR 1193
M.musculus         VDPRREHERGGHLDERERLHVLREDYEHPRLHPVHPASLDGHLPHPSLLTPGLPSMHYPR 1147
G.gallus           VDPRREHERGNHLDDRERLHMLREDYEHARLHSVHPAALDGHLPHPSLITPGLPSMHYPR 1188
X.tropicalis       VDPRREHERVSHLEERERLHMLREDYEHARLHAVHPAALDGHLPHPSLITPGLPSMHYAR 1177
D.rerio            LEQRREQER-AHLEERERLHLLREDYEQGRLHPMHHPVLDGHLPHPGLMAPGLPSMHYPR 1212
                   :: ***:**  **::*****:******: ***.:* . ********.*::********.*

H.sapiens          ISP----TAGNQNGLLNKtpptaalsappplistlGGRPVSPRRTTPLSAEIRERPPSHT 1252
G.gorilla          ISP----TAGNQNGLLNKTPPTAALSAPPPLISTLGGRPVSPRRTTPLSAEIRERPPSHT 1252
M.mulatta          ISP----TAGHQNGLLNKTPPTAALSAPPPLISTLGGRPVSPRRTTPLSAEIRERPPSHT 1252
C.familiaris       ISP----TAGHQNGLLNKTPPTAALSAPPPLISTLGGRPVSPRRTTPLSAEMRERPPSHT 1250
B.taurus           ISP----TAGHQNGLLNKTPPTAALSAPPPLISTLGGRPVSPRRTTPLSAEIRERPPSHT 1256
S.scrofa           ISP----TAGHQNGLLNKTPPTAALSAPPPLISTLGGRPVSPRRTTPLSAEIRERPPSHT 1249
M.musculus         ISP----TAGHQNGLLNKTPPTAALSAPPPLISTLGGRPGSPRRTTPLSAEIRERPPSHT 1203
G.gallus           VSP----PTGHQNGILNKTPPTAALSAPPPLISTLGPRPGSPRRTTPLSAEMRERPPSHT 1244
X.tropicalis       VSP----TTGHQNGILNKTPPTAALSAPPPLISTLGGRPGSPRRTTPLSTEIRDRPPSHT 1233
D.rerio            VSPSSAVAAAAQNGILNKTPPTASLSAPPPLIPTLGARSSSPRRTTPLGPDIRDRPPSHT 1272
                   :**    .:. ***:********:********.*** *. ********..::*:******

H.sapiens          LKDIEAR 1259
G.gorilla          LKDIEAR 1259
M.mulatta          LKDIEAR 1259
C.familiaris       LKDIEAR 1257
B.taurus           LKDIEAR 1263
S.scrofa           LKDIEAR 1256
M.musculus         LKDIEAR 1210
G.gallus           LKDIEAR 1251
X.tropicalis       LKDIEAR 1240
D.rerio            HKDIEAR 1279
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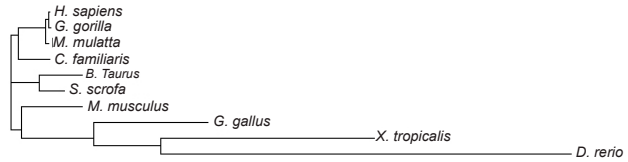
Figure S3. *Amino acid alignment of AUTS2 orthologs.*

Amino acid alignment (determined by ClustalW2) of human AUTS2 orthologs in gorilla, macaque, dog, cow, pig, mouse, chicken, clawed frog and zebrafish. Exon boundaries are indicated above the alignment. Fully conserved or similar residues are marked by symbols below the alignment. Grey lowercase letters indicate low-complexity or repetitive segments of the human amino acid sequence according to the XNU + SEG algorithms.[1-3]

a



b

exon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	all
size (aa)	*103*	*71*	*34*	*12*	*10*	*18*	*157*	*85*	*73*	*15*	*32*	*24*	*10*	*24*	*48*	*26*	*28*	*74*	*415*	*1259*
G. gorilla	100	100	100	100	100	100	98	100	100	100	100	100	100	100	100	100	100	100	99	99
M. mulatta	100	100	100	100	100	100	98	100	100	100	100	100	100	100	100	100	100	100	99	99
B. taurus	99	98	100	100	100	100	75	98	98	100	100	95	100	100	100	96	100	97	88	92
S. scrofa	99	98	100	100	100	100	78	98	98	100	96	100	100	100	100	100	100	97	92	94
C. familiaris	99	94	97	100	100	100	88	97	98	100	100	100	100	100	100	100	100	100	94	95
M. musculus	96	94	97	100	100	94	83	98	100	100	100	100	100	100	100	100	100	97	89	93
G. gallus	78	84	100	100	100	70	64	91	98	100	100	100	100	100	95	92	78	95	83	85
X. tropicalis	69	53	61	91	100	66	40	82	98	100	100	95	100	100	97	76	85	73	76	73
D. rerio	52	41	76	37	80	64	31	50	94	93	96	91	90	100	89	69	57	81	57	60

Figure S4. *conservation amongst species per exon.*

a. *Phylogenetic tree based on clustalW2 alignment of the full-length amino acid sequences.[2]* ***b.*** *To assess the conservation of individual exons, the percentage amino acid identity was calculated and is depicted here. The orange colour shade is an indication of the degree of conservation, darkest colours are most conserved. Conservation of the full-length protein is given in the last column. Italic numbers indicate the number of amino acids encoded by each human exon. Grey numbers indicate conserved sequences not included in the longest annotated protein of that species.*

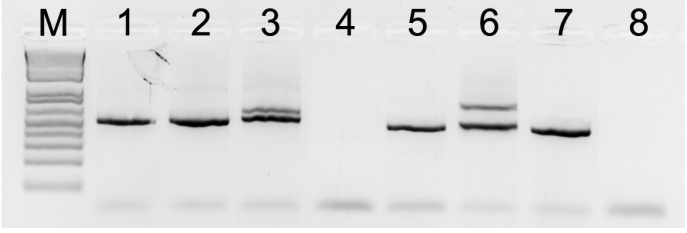


Figure S5. *Efficiency of splice blocking.*

RT-PCR using zebrafish 5' (1-4) and 3' (5-8) auts2 primer sets on RNA extracted from control (1, 5), auts2-5' MO-injected embryos (3,7), auts2-3' MO-injected embryos (2,6) at 3 dpf. RT-PCR using water is shown in (4,8). M: marker (1kb plus ladder).

Table S1. *Overview of the genotypic data of all the cases with an AUTS2 aberration.*

nr	case nr	Sex	Inheritance	Initial array platform	Minimal breakpoint(s)	Maximal breakpoints	Type of abberation	(Min) exon cont.	nr of amino-acids missing	Confirmation	Other CNV's
D_1	1	M	Pat	Agilent, 105k	chr7:68,906,975-69,053,679	chr7:68,890,372-69,070,952	In-Frame	2	71	MLPA	no
D_2	2	F	?	Agilent, 244k	chr7:69,158,443-69,251,631	chr7:69,147,715-69,257,044	In-Frame	3-4	46	MLPA	no
D_3	3	M	?	custom Roche Nimblegen, 135k	chr7:69,186,254-69,407,211	chr7:69,180,268-69,413,687	In-Frame	3-4	46	-	no
D_4	4	F	Mat	Affy, 2,7M	chr7:69,211,398-69,263,338	chr7:69,209,046-69,264,563	In-Frame	3-4	46	FISH	no
D_5	5	F	DN	SignatureChipOS® v2.0 12-plex	chr7:67,063,120-69,321,364	chr7:67,001,077-69,322,899	5' CDS Truncation	1-4	220	FISH, HD-array	no
D_6	6	F	Mat	Affy, 6.0	chr7:69,478,104-69,827,159	chr7:69,475,784-69,827,248	Frameshift	5-6	NA	MLPA	no
D_7	7	M	?	custom Agilent, 105k	chr7:69,612,584-69,827,895	chr7:69,548,807-69,895,670	Frame-shift	6	NA	FISH	no
D_8	8	M	DN	SignatureChipOS® v2.0 12-plex	chr7:69,653,708-69,830,227	chr7:69,653,553-69,830,562	Frame-shift	6	NA	FISH, HD-array	no
D_9	9	F	? (not pat)	Agilent, 105k	chr7:69,612,584-69,858,682	chr7:69,596,512-69,878,246	In-Frame	6-9	333	MLPA	no
D_10	10	F	DN	Affy, 6.0	chr7:69,544,961-69,869,585	chr7:69,544,254-69,874,855	In-Frame	6-11	380	MLPA	no
D_11	11	F	?	SignatureChipOS® v1.0	chr7:69,692,978-69,892,380	chr7:69,692,923-69,892,480	Frame-shift	6-18	NA	HD-array	no
D_12	12	F	DN	Agilent, 244k	chr7:69,842,425-69,895,730	chr7:69,827,895-69,902,739	3' CDS Truncation	7-19	?	MLPA	no
D_13	13	M	? (not mat)	Illumina, Omni 2.5M	chr7:69,855,702-70,057,754	chr7:69,855,618-70,059,580	3' CDS Truncation	7-19	?	MLPA	no
D_14	14	F	DN	Agilent, 244k	chr7:67,771,230-70,370,488	chr7:67,703,093-70,376,527	whole gene	all	1259	MLPA	inherited (mat) chr2:38531520-38948843 dup
D_15	15	M	DN	Bluegnome Cytochip, ISCA 60k	chr7:67,885,578-71,060,555	chr7:67,832,322-71,167,545	whole gene	all	1259	array	no
D_16	-	M	?	SignatureChipOS™ v2.0 12-plex	chr7:66,442,679-68,929,350	chr7:66,070,718-68,936,261	5' CDS Truncation	1	103	FISH	chr7:72,382,850-75,860,222 del
D_17	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:68,707,130-69,744,098	chr7:68,701,577-69,748,756	In-Frame	2-5	127-230	FISH	chr16:28,740,937-28,953,785 dup
D_18	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:68,786,965-69,633,048	chr7:68,781,789-69,634,584	In-Frame	2-5	127	FISH	no
D_19	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:69,609,388-69,811,449	chr7:69,604,279-69,817,541	Frame-shift	6	NA	FISH	no
D_20	-	M	DN	SignatureChipOS® v2.0 12-plex	chr7:69,648,328-69,828,289	chr7:69,645,699-69,838,508	Frame-shift	6	NA	FISH	no
D_21	-	M	Mat	Agilent 180K	chr7:69,783,660 -69,842,625	chr7:69,768,151-69,858,652	Frame-shift	6	NA	-	inherited (pat) chr5:58,208,850-58,317,663 dup
D_22	-	M	Pat	SignatureChipOS® v2.0 12-plex	chr7:69,705,411-69,859,679	chr7:69701217-69868537	Frame-shift	6	NA	FISH	no
D_23	-	M	?	SignatureChipOS® v2.0 12-plex	chr7:69,828,229-69,868,537	chr7:69820620-69870901	Frame-shift	7-8	242-315.67	FISH	no

D_24	-	M	DN	Signature Select (bac-based)	chr7:69,795,761-70,015,770	chr7:67835761-70762480	3' CDS Truncation	6-19	?	-	no
D_25	-	M	?	custom Roche Nimblegen, 135k	chr7:68,820,750-68,912,599	chr7:68,809,325-68,917,095	Intronic	-	0	FISH	no
D_26	-	M	?	SignatureChipOS® v2.0 12-plex	chr7:68,775,850-68,809,385	chr7:68,768,454-68,820,810	Intronic	-	0	-	no
D_27	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:68,834,947-68,877,657	chr7:68,828,461-68,887,471	Intronic	-	0	FISH	no
D_28	-	M	Mat	SignatureChipOS® v2.0 12-plex	chr7:68,877,597-68,908,695	chr7:68,869,791-68,912,659	Intronic	-	0	-	no
D_29	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:68,936,201-68,949,312	chr7:68,929,290-68,958,988	Intronic	-	0	FISH	no
D_30	-	M	Mat	Agilent 180K	chr7:69,244,541-69,393,802	chr7:69,227,818-69,411,912	Intronic	-	0	-	inherited (mat) chr6:42,162,186-42,249,855 dup
D_31	-	M	DN	Agilent, 180k	chr7:69,288,622-69,475,409	chr7:69,266,221-69,507,743	Intronic	-	0	MLPA	no
D_32	-	M	?	SignatureChipOS® v2.0 12-plex	chr7:69,005,252-69,082,988	chr7:69,002,786-69,087,674	Intronic	-	0	FISH	no
D_33		F	?	SignatureChipOS® v2.0 12-plex	chr7:69,040,232-69,191,396	chr7:69,040,022-69,191,461	Intronic	-	0	FISH	no
D_34	-	M	?	SignatureChipOS® v2.0 12-plex	chr7:69,044,108-69,167,230	chr7:69,041,764-69,174,667	Intronic	-	0	FISH	no
D_35		M	Mat	SignatureChipOS® v2.0 12-plex	chr7:69,134,520-69,203,300	chr7:69,134,315-69,203,553	Intronic	-	0	FISH	inherited (mat) chrX:57,356,516-57,658,457 dup
D_36	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:69,145,807-69,216,828	chr7:69,139,154-69,230,835	Intronic	-	0	-	no
D_37	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:69,313,697-69,359,559	chr7:69,309,002-69,364,318	Intronic	-	0	-	no
D_38	-	F	?	custom Roche Nimblegen, 135k	chr7:69,359,499-69,458,435	chr7:69,342,009-69,463,755	Intronic	-	0	-	no
D_39	-	M	Pat	SignatureChipOS® v2.0 12-plex	chr7:69,551,796-69,642,184	chr7:69,547,337-69,645,759	Intronic	-	0	FISH	no
D_40	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:69,551,796-69,642,184	chr7:69,547,337-69,645,759	Intronic	-	0	FISH	chr17:31,462,510-33,281,801 dup
D_41	-	F	?	custom Roche Nimblegen, 135k	chr7:69,828,229-69,859,679	chr7:69,820,679-69,868,479	Intronic	-	0	FISH	no
D_42	-	F	Mat	SignatureChipOS® v2.0 12-plex	chr7:69,828,229-69,859,679	chr7:69,820,620-69,868,537	Unclear	? (max 7-8)	0-242	-	no
D_43	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:69,828,229-69,859,679	chr7:69,820,620-69,868,537	Unclear	? (max 7-8)	0-242	-	no
D_44	-	F	?	SignatureChipOS® v2.0 12-plex	chr7:69,828,229-69,859,679	chr7:69,820,620-69,868,537	Unclear	? (max 7-8)	0-242	FISH	chr6:105,798,153-112,890,365 del
I_1	16	F	DN	46,XX, inv(7)(q11.22,q36.3)	chr7q11.22: 69,685,967-69,685,977	chr7q36.3: 157,577,848-157,577,842	Inv	Intron 4	NA	Break point seq.	NA
T_1	17	F	DN	t(7;22)(q11.22;q12.1)	chr7q11.22: 70,178,753-70,185,711	chr22q12.1: 27,932,064-28,119,112	Trl	Intron 6	NA	FISH	NA

Table S1. *This table summarizes the array and karyotyping data from 44 cases with a deletion encompassing (part of) AUTS2 (and a maximum of two neighboring genes) and the inversion and translocation case found in our international cohort of ~50000 individuals with intellectual disability and/or multiple congenital malformations. M: male, F: female, Pat: paternal, Mat: maternal, DN: de novo, NA: not applicable, Inv: inversion, Trl: translocation. Karyotyping was performed on standard synchronized cultures of peripheral blood lymphocytes. In general GTG-banded chromosomes were analyzed at the 550-band level. Sequencing of the breakpoints of case 16 (inv(7)) is described.[4,5] For the initial CNV analysis ten different designs were used on six analogous platforms. All signal intensities were analyzed in hg18 (build 36). In a subset of the cases validation and high resolution breakpoint delineation was performed using two types of custom Roche NimbleGen 135K microarrays with probes tiled across 7q11.22 (hg18; chr7:65,992,311-72,003,221) at a median density of 1000 bp (case 5) and 75 bp (cases 8 and 11).[6]*

Labelling and hybridization of Agilent, Affymetrix and Roche NimbleGen arrays were performed as described.[4, 7-10] With the Infinium HumanOmni2.5-Quad v1.0 BeadChip (Illumina Inc., San Diego, CA, USA) 2,443,177 markers were genotyped according to the manufacturer's protocol and scanned with default settings using the Illumina iScan.

Data analysis for the Agilent platforms was performed with CGH Analytics or Genomic Workbench Standard Edition 5.0.14 (Agilent Technologies) or an in-house developed program (http://medgen.ugent.be/arrayCGHbase/). The relative DNA copy numbers at the SNP/CNV loci from the Affymetrix platforms were determined by comparison of the normalized array signal intensity data for the proband's DNA sample against the HapMap270 reference file provided by Affymetrix, using Genotyping Console or ChAS software (default settings). All rare CNV's were checked using Nexus.[7] Analysis and intra-chip normalization of the Illumina image files was performed using Illumina's GenomeStudio Genotyping Module software v.2010.3 with default parameters. Genotype calls were generated using the Illumina-provided genotype cluster definitions file (HumanOmni2.5-4v1_B.egt, generated using HapMap project DNA samples). CNV analysis was performed using a multi-algorithm approach.[9] Data analysis of the NimbleGen arrays was as described.[11]

Table S2.

All probes are sorted for the start position with numbering according to genome build HG18. The exon content of intragenic deletions was verified by MLPA in all cases that were not confirmed otherwise. Probes were designed for exons 1 to 19 of the full length AUTS2. Oligonucleotides were synthesized by Integrated DNA Technologies (Leuven, Belgium); all other reagents were from MRC-Holland (Amsterdam, The Netherlands). The samples were separated on a 3730 automated sequencer (ABI Systems) and analyzed using Gene marker v1.95 software (Soft genetics).

Table S2: *Overview of the location of the probes used for MLPA analysis.*

		Hybridizing region (hg18)	
Exon	**MLPA probe**	**Start**	**End**
exon 1		68702256	68702885
	MLPA 102	68702795	68702847
exon 2		69002209	69002421
	MLPA 110	69002227	69002281
exon 3		69221055	69221156
	MLPA 118	69221075	69221136
exon 4		69237459	69237494
	MLPA 126	69237710	69237775
	MLPA 130	69538370	69538429
exon 5		69538675	69538704
	MLPA 096	69542823	69542872
		69596454	69596513
	MLPA 136	69598011	69598086
	MLPA 140	69604531	69604615
	MLPA 144	69608563	69608660
		69612586	69612645
	MLPA 100	69801299	69801351
exon 6		69801492	69801543
		69858624	69858683
	MLPA 104	69865791	69865850
exon 7		69865793	69866264
exon 8		69867675	69867928
	MLPA 108	69867966	69868027
exon 9		69869037	69869257
	MLPA 112	69869343	69869403
exon 10		69870947	69870991
	MLPA 116	69870967	69871035
	MLPA 120	69874308	69874382
exon11		69874472	69874567
exon12		69876951	69877022
	MLPA 124	69876959	69877036
	MLPA 128	69877860	69877941
		69878248	69878307
exon13		69878280	69878309
	MLPA 132	69879948	69880038
exon14		69880026	69880097
	MLPA 134	69884478	69884565
exon15		69884538	69884679
exon16		69887865	69887942
	MLPA 105	69888063	69888120
	MLPA 138	69888864	69888956
exon17		69888878	69888961
exon18		69890132	69890354
	MLPA 107	69890571	69890635
exon19		69892671	69895991
	MLPA 136	69895018	69895108

Table S3. *FISH probes used for breakpoint mapping on chromosome 7 and chromosome 22 in case 17.*

Name	Chr	Start (bp)	End (bp)
RP4-736H5	chr7q11.22	67029247	67175902
RP11-358M3	chr7q11.22	67400859	67525501
RP11-156A14	chr7q11.22	67909795	68062117
RP11-3P22	chr7q11.22	68613015	68778577
RP4-715F13	chr7q11.22	69466935	69610812
RP11-290M1	chr7q11.22	69819029	69993539
RP11-689B18	chr7q11.22	69996264	70178753
G248P87196G1 (WI2-2151M1)	chr7q11.22	70259728	70298275
G248P82441H1 (WI2-1017P1)	chr7q11.22	70206291	70247727
G248P84061D2 (WI2-1371H3)	chr7q11.22	70239978	70279409
RP11-575M4	chr7q11.22	70185711	70372702
RP11-26L10	chr7q11.22	70525506	70703364
RP11-409J21	chr7q11.22	71049273	71204976
RP4-562A11	chr7q11.23	77133285	77277250
RP4-560O14	chr7q21.11	81518832	81663290
CTA-115F6	chr22q11.1	17779327	17965779
CTA-433F6	chr22q11.21	20729567	20874531
CTA-322B1	chr22q11.23	24315264	24392055
CTA-125H2	chr22q12.1	26230801	26404213
RP11-259P1	chr22q12.1	26612062	26660045
RP11-322L06	chr22q12.1	26964530	27132594
CTA-992D9	chr22q12.1	27411607	27566652
RP11-263G19	chr22q12.1	27629570	27813359
RP11-699H18	chr22q12.1	27816054	27979854
RP11-1056M20	chr22q12.1	27932064	28119112
RP11-772E17	chr22q12.1	28105947	28295835
RP3-353E16	chr22q12.1	28167384	28357044
RP11-329J7	chr22q12.1	28914764	28987692
RP11-664C16	chr22q12.1	29341003	29489465
CTA-57G9	chr22q12.1-q12.2	29512541	29626406
RP1-76B20	chr22q12.2	30049384	30221065
RP4-539M6	chr22q12.2	30787513	30946905
CTA-221H1	chr22q12.3	34571571	34574493
CTA-150C2	chr22q13.1	39280299	39481341

Showing probe name, chromosome band, start position and end position according to the GRCh37 genome assembly. Locus specific FISH probes for chromosome 7q11.22 and 22q12.1 were selected using the Ensembl and UCSC genome browser database (http://www.ensembl.org, http://genome.ucsc.edu, 6 December 2010) (genome build GRCh37). Using nick translation the probes were either labelled with SpectrumGreen or SpectrumOrange (Abbott Molecular, Belgium). Metaphase FISH with probes from the region shown to be deleted by array CGH was also used to visualize the deletions in cases 4, 5, 7 and 8 and their parents (if available), according to previously described methods.[12]

Table S4. *Control cohorts. The control cohorts tested for CNV's in AUTS2 with the number of controls per cohort and the references that give more information on these cohorts.*

nr	Control cohort	# Control individuals	Array	deletions in AUTS2	Reference (with pubmed ID)
1	Ottawa Heart Institute (OHI) controls from Canada	1.234	Affy 6.0	2	Dataset and analysis described in Lionel et al. 2011 (PMID: 21832240)
2	POPGEN controls from Germany	1.123	Affy 6.0	0	Dataset and analysis described in Lionel et al. 2011 (PMID: 21832240)
3	SAGE controls from USA	1.287	Illumina 1M	3	Dataset and analysis described in Pinto et al. 2010 (PMID: 20531469)
4	Welcome Trust (WTCCC) controls	4.783	Affy 6.0	4	https://www.wtccc.org.uk/ccc2/
5	HapMap phase 3 controls	1.056	Affy 6.0	0	Dataset and analysis described in International HapMap 3 Consortium et al. 2010 (PMID: 20811451) raw data available at http://hapmap.ncbi.nlm.nih.gov/downloads/raw_data/hapmap3_affy6.0/
6	combined adult controls set (HGDP, NINDS, PARC/PARC2, London, FHCRC, In CHIANTI) (excluding the WTCCC2 cohort because of overlap with control dataset 4)	6.239	Illumina 240K-650K	0	Dataset and analysis described in Cooper et al. 2011 (PMID: 21841781)
7	Lowlands Consortium controls	981	Agilent 105K/180K	0	personal communication with Kok K. UMCG, Department of Genetics, Groningen, the Netherlands

Table S5. Splice-Blocker Morpholinos against the *AUTS2* Ortholog *auts2*

Morpholino	Sequence
auts2-5′	5-ACTTTAGGTGGTGACTGGTACCTGA-3
auts2-3′	5-TCTCTGGATAGTCCACTCACCTGCT-3

Table S6. *Deletions in AUTS2 found in control cohorts. Nine deletions were found in the control cohorts, the breakpoints and localization of the deletions in AUTS2 are depicted here. idel: intragenic deletion, - (in x): no exons involved, but intron x deletion.*

Nr	Control cohort	sub cohort	id number	array platform used	Aber-ration	breakpoints	*AUTS2* exons involved	# base pairs	# pro-bes
4	WTCCC2	birth cohort	BC1088	Affy 6.0	idel	chr7:69,665,498-69,777,260	- (in 5)	~111763	111
4	WTCCC2	birth cohort	BC0081	Affy 6.0	idel	chr7:68,857,867-68,892,176	- (in 1)	~34310	19
4	WTCCC2	blood service collection	NBS1906	Affy 6.0	idel	chr7:69,328,576-69,469,301	- (in 4)/ alternative exon 5	~140726	70
4	WTCCC2	blood service collection	NBS1607	Affy 6.0	idel	chr7:69,067,672-69,194,966	- (in 2)	~127295	72
3	SAGE	-	B556806	Illumina 1M	idel	chr7:69,040,697-69,120,581	- (in 2)	~79885	20
3	SAGE	-	B847353	Illumina 1M	idel	chr7:69,658,096-69,693,762	- (in 5)	~35667	17
3	SAGE	-	B415965	Illumina 1M	idel	chr7:69,822,721-69,861,129	- (in 6)	~38409	21
1	OHI	-	CONT-1960	Affy SNP6.0	idel	chr7:69,826,272-69,862,679	- (in 6)	~36407	40
1	OHI	-	NCA06139	Affy SNP6.0	idel	chr7:69,286,147-69,390,077	- (in 4)/ alternative exon 5	~103931	48

Table S7 (next page). *This table provides an overview of genotypic and phenotypic features of the 17 probands and their (affected) family members. Nonrecurring features are not scored. Cases: f=father, m=mother, s=sibling. Type of AUTS2 aberration: d=deletion, i=insertion, t=translocation. 4*=breakpoint in intron 4, n.a.=not applicable, tr=truncation. Other genes involved: W=WBSCR17, C=CALN1, P=PTPRN2. Inheritance: P=paternal, M=maternal, D=de novo, nd=not determined. Age at examination: x y x m= x years and x months old, sex: m= male, f= female. Intellectual disability / developmental delay: mi=mild, mo=moderate, s=severe, b=borderline. Additional clinical features: a) maternal duplication chr2:38531520-38948843, b) almost no speech, c) white matter abnormalities, d) hyperintense signal periventricular white matter, e) ataxic gait, f) plachiocephaly, brachycephaly, facial asymmetry and prominent forehead, g) low columella, h) downslant, i) large hand and feet, j) short forehead, k) prominent lips, l) large tongue, m) prominent cheeks, n) suppination limitation left arm, o) left, p) mild pectus carinatum, q) metopic synostosis, r) clinodactily digiti V, s) camptodactily digiti V, t) unilateral cleft lip, u) cleft lip, v) sensorineural deafness, w) sacral dimple, x) premature birth; intraventricular hemorrhage, y) hypospadias, eventration of diafragm (right). #: published cases.* [13-16]

Table S7. *Genotypic and phenotypic features characterizing the AUTS2 syndrome.*

Cases	**1**	**f1**	**2**	**3**	**4**	**m4**	**5**	**6**	**s6**	**m6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**Total**	**#**
type of *AUTS2* aberration	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	i	t		
exons deleted	2	2	3-4	3-4	3-4	3-4	1-4	5-6	5-6	5-6	6	6	6-9	6-11	6-18	7-19	7-19	all	all	4*	6*		
frame shift	-	-	-	-	-	-	n.a.	+	+	+	+	+	-	-	+	tr	tr	tr	tr	tr	tr		
translocation/inversion partner																				7q36.3	22q12.1		
other genes involved	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	W, C	W	W, C	P	-		
other CNV's/second hits	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	a)	-	-	-	-		
inheritance	P	D	nd	nd	M	nd	D	M	M	nd	nd	D	not P	D	nd	D	not M	D	D	D	D	9/13 D	8/9 D
General																							
age at examination	3y	nd	3y	2y6m	2y4m	22y	1y7m	2y3m	6y	31y	5y6m	11m	32y	2y1m	1y11m	7y2m	5y7m	9y1m	4y9m	6y8m	1y8m	11m-32y	3y-16y
Sex	m	m	f	m	f	f	f	f	m	f	m	m	f	f	f	f	m	f	m	f	f	13f/8m	5f/4m
Growth and feeding																							
low birth weight	-	-	nd	+	-	nd	-	+	+	nd	nd	-	-	+	-	+	-	+	-	-	+	7/17	2/8
short stature <p10	-	-	nd	+	-	-	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+	12/20	4/9
microcephaly <p2	-	-	-	+	+	nd	+	+	+	-	+	+	+	+	+	-	+	-	+	+	+	14/20	1/6
feeding difficulties	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	+	+	-	+	+	+	10/21	4/5
Neurodevelopmental disorders																							
intellectual disability/ DD	mi	-	+	+	+	b/mi	+	mi	mi/mo	mi	+	+	mo	mi/mo	mi	+	s	+	mi	mi/mo	mo	20/21	9/9
autism/autistic behavior	-	-	-	+	-	-	+	-	-	-	-	-?	+	-	-	+	+	-	-	+	+	7/21	4/6
sound sensitivity	-	-	nd	nd	nd	-	nd	nd	nd	nd	nd	-	-	nd	nd	nd	nd	+	+	nd	-	2/8	2/4
hyperactivity/ ADHD	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-	-	-	3/21	1/4
Other																	b)						
Neurological disorders																							
generalized hypotonia	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	+	-	+	+	+	8/21	4/7
structural brain anomaly	nd	nd	+	-	nd	nd	-	nd	nd	nd	nd	-	nd	+	-	+	-	-	-	nd	-	3/11	4/9
cerebral palsy/spasticity	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	-	-	9/21	1/4
Other			c)											d)			e)						
Dysmorphic features																							
highly arched eyebrows	-	-	-	-	-	-	+	+	+	+	-	-	+	-	+	-	+	-	-	-	+	8/21	1/5

hypertelorism	-	-	-	-	-	-	-	+	+	+	-	+	+	-	-	+	+	-	+	+	+	10/21	0/5
Proptosis	-	-	-	-	+	-	-	+	-	+	-	-	+	-	+	-	+	-	-	-	-	6/21	2/5
short palpebral fissures	-	-	-	-	+	-	+	+	+	-	-	-	+	-	-	-	-	-	+	+	+	8/21	2/5
up slanting palpebral fissures	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	4/21	1/5
Ptosis	-	-	+	-	-	+	-	+	+	+	-	-	+	-	-	-	+	-	-	-	+	8/21	2/5
epicanthal fold	-	-	-	-	-	-	+	-	-	-	+	+	-	+	+	-	+	-	-	-	+	7/21	1/5
strabismus	-	-	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	5/21	3/6
prominent nasal tip	-	-	-	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	-	-	+	5/21	2/5
anteverted nares	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	3/21	2/5
deep/broad nasal bridge	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	-	+	+	+	-	+	7/21	1/5
short/upturned philtrum	-	-	-	-	-	+	+	+	+	-	nd	-	+	-	-	-	+	-	+	+	-	8/21	5/7
micro/retrognatia	-	-	-	-	-	+	nd	+	+	-	-	+	+	-	-	-	+	-	+	-	-	7/20	2/5
low set ears	-	-	-	-	-	nd	-	+	+	+	-	+	+	-	-	-	-	-	-	-	+	6/20	2/5
ear pit	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	2/20	0/5
narrow mouth	-	-	-	-	+	+	+	+	+	-	+	-	+	+	-	-	+	-	+	+	+	12/21	3/5
Other					f)			g)			h)	i)	j)				k)	l)	m)				
Skeletal disorders																							
Kyphosis/ scoliosis	-	-	nd	-	-	-	-	-	-	-	-	-	+	mild	-	-	-	-	nd	-	-	2/9	3/5
arthrogryposis/shallow palmar creases	-	-	nd	-	-	-	-	-	+,n)	-	-	-	+	-	-	-	-	-	-	+	-	3/20	1/3
tight heel cords	-	-	nd	-	nd	nd	nd	nd	nd	nd	+	nd	nd	+	nd	+	nd	+	+, o)	nd	nd	5/8	1/1
Other							p)								q)			r)		s)			
Congenital malformations																							
hernia umbilicalis inguinalis	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	2/21	1/9
patent foramen ovale/ atrial septum defect	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	3/21	1/9
Other					t)	u)				v)				w)		x)			y)				
AUTS2 syndrome severity score / Total number of items	1/31	0/31	5/26	6/31	6/30	5/27	11/29	16/29	17/29	8/28	9/27	8/31	22/31	15/31	8/30	9/31	18/30	7/32	16/32	11/29	17/31		

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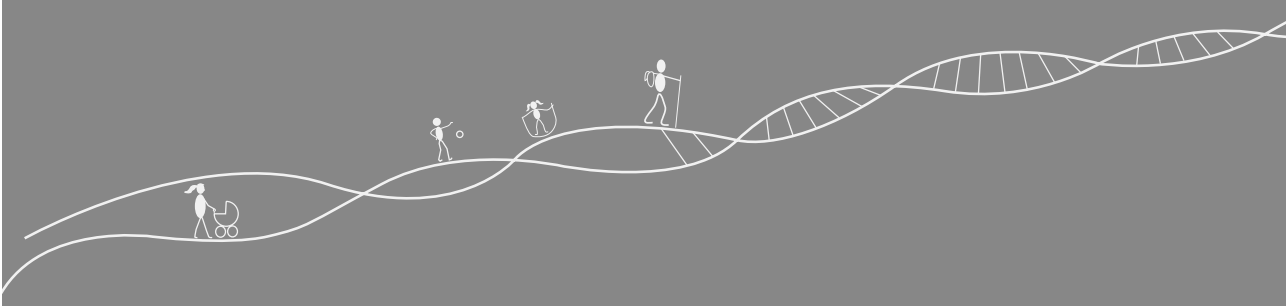
Chapter 4

Loss of function mutation (at the nucleotide-level) in AUTS2 cause AUTS2 syndrome

Two male adults with pathogenic AUTS2 variants, including a two-base pair deletion, further delineate the AUTS2 syndrome.

Beunders G, de Munnik SA, Van der Aa N, Ceulemans B, Voorhoeve E, Groffen AJ, Nillesen WM, Meijers-Heijboer EJ, Kooy FR, Yntema HG, Sistermans EA.



Eur J Hum Genet. 2015 Jun;23(6):803-7. doi: 10.1038/ejhg.2014.173.

ABSTRACT

AUTS2 syndrome is characterised by low birth weight, feeding difficulties, intellectual disability, microcephaly and mild dysmorphic features. All cases thus far were caused by chromosomal rearrangements, mutations at the base pair level disrupting *AUTS2* have not yet been described.
Here we present the full clinical description of two new adult cases with AUTS2 syndrome found by diagnostic exome sequencing and array CGH.
The phenotypic features of both male cases include: intellectual disability, microcephaly, feeding difficulties, dysmorphic features and joint contractures. Both cases have intragenic *AUTS2* mutations (one two-basepair deletion in exon 7 and one deletion of exon 6) that are predicted to cause a frame shift of the full length transcript but are unlikely to affect the shorter 3' transcript starting in exon 9 that is expressed in human brain.
The similarities between the phenotypes of both cases are striking and further confirm that AUTS2 syndrome is a single gene disorder with a recognisable phenotype.

INTRODUCTION

Disruptions of *AUTS2* by translocations, inversions or deletions cause a syndromic form of intellectual disability (ID).[1] Forty-three pathogenic disruptions of *AUTS2* have been described: 6 translocations and 2 inversions with one breakpoint in *AUTS2* and 35 deletions containing at least one exon. Most of these aberrations occurred *de novo*.[1-10] A detailed study of 17 patients with a disruption of *AUTS2* revealed a distinct AUTS2 syndrome characterized by intellectual disability, microcephaly, mild short stature, feeding problems, cerebral palsy or hypotonia and facial features including ptosis, highly arched eyebrows, narrow mouth and micro/retrognatia. [1] The AUTS2 syndrome severity score, expressed as the sum of all features seen more than once in independent cases, is a measure of the severity and specificity of the phenotype. The median AUTS2 syndrome severity score of cases with a genomic rearrangement/deletion involving the 3' region of *AUTS2* was significantly higher than that of cases with 5' deletions. The 3' end of the gene harbours an alternative transcript expressed in human brain and starting in exon 9 which is able to rescue the phenotype of *auts2* zebrafish morphants. These two observations indicate that the 3' region of *AUTS2* contains important functional domains. [1]

Here we report the first small mutation in *AUTS2*, a deletion of two nucleotides, in a young adult with a syndromic form of ID. His phenotype is compared to another new AUTS2 syndrome case of similar age and gender with an intragenic deletion of exon 6. Both mutations result in a frame shift and only disrupt the full length transcript. Both adult cases have a very similar phenotype and fit the AUTS2 syndrome, confirming it to be a single gene disorder and re-emphasizing the importance of AUTS2 in neurodevelopment.

PATIENTS AND METHODS

Clinical reports

Case 1 is the second child of healthy non-consanguineous parents. He has a healthy older sister and younger brother. He was born after 40 weeks of gestation. The pregnancy was complicated by growth restriction from 6 months of gestation. Apgar scores were 9 and 10 after 1 and 5 minutes respectively. Birth weight was 2745 grams (<2th percentile) and length was 49 cm (10th percentile). In the first year, feeding difficulties and mild delay in development and eye contact were observed. At the age of one year he got a tympanostomy after which his social interaction improved. Intellectual development remained delayed. He started walking at 20 months of age with a tendency to walk on his toes and started talking at 2.5 years of age. He has been operated for a misalignment of his feet and for cryptorchidism.

Physical examination at the age of 3 showed a height of 92 cm (p10), a weight of 12.2 kg (<p10) and a head circumference (OFC) of 46 cm (<p10), mild ptosis, narrow os frontale and down slanting palpebral fissures. Neurological examination at that time revealed an immature motor function with normal muscle tone, no paresis, no extrapyramidal movement disorder, no ataxia and normal

to high deep tendon reflexes and a normal sensibility. Psychological evaluation revealed a global developmental delay with a severe delay in speech and language development and a pervasive developmental disorder not further classified (PDD-NOS). Hyperactive behaviour and sound sensitivity were also observed. CT of the brain was performed and showed no anomalies. A nerve conduction study was normal. The Brainstem Auditory Evoked Potential (BAEP) was normal and Electroencephalogram (EEG) was diffusely abnormal with too many slow waves and diffuse beta-waves but no signs of epilepsy. Chromosomal analysis showed a normal male karyotype (46,XY). At the age of 13 years he was evaluated by a clinical geneticist. Fragile X testing was negative and no other causal diagnoses could be made. Re-evaluation at the age of 24 years showed a length of 177 cm (-1SD) and a head circumference of 52.5 cm (-3 SD) and mild dysmorphisms, thick eyebrows, a mild down slant of the palpebral fissures, a deep nasal bridge and a prominent nasal tip with a broad nasal base, a wide mouth with thick lips and a short mildly upturned philtrum. He has a slender build, a long neck, long fingers and contractures of the PIP joints of digit V of both hands (see Figure 1 and Table 1). He has a moderate intellectual disability with a severe language delay and an autism spectrum disorder. His IQ was tested to be 45. Array CGH, metabolic screening and mutation analysis of the PQBP1 gene to exclude Renpenning syndrome were normal (data not shown).

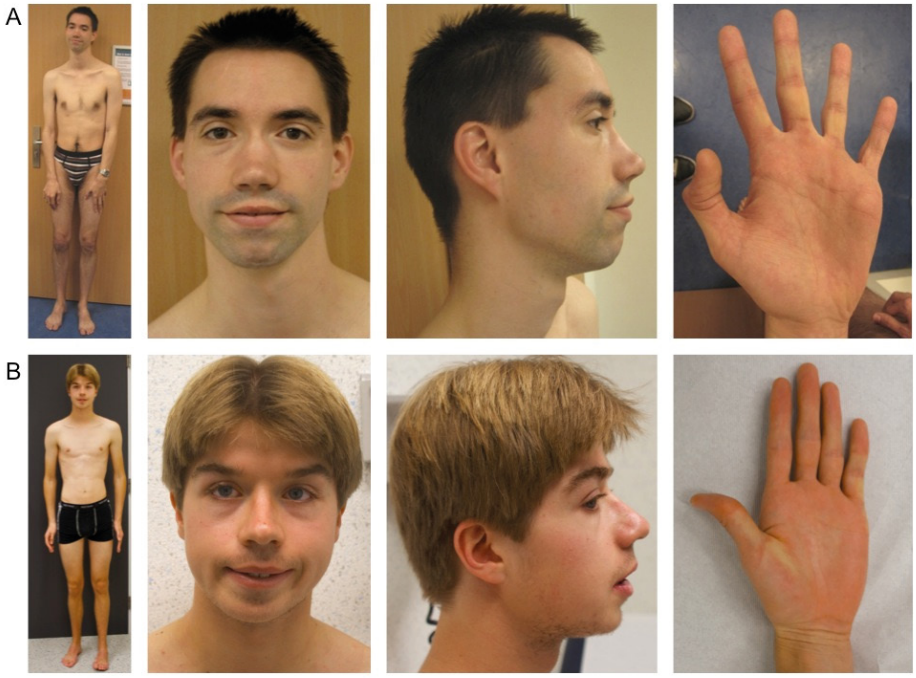


Figure 1: A. *Case 1 at the age of 24 years.* ***B.*** *Case 2 at the age of 20 years. These photographs show the slender build, the mild ptosis, small ears, deep nasal bridge, prominent nasal tip and broad nasal base, the thick lips and short philtrum in both cases. The mild down slanting palpebral fissures and the camptodactyly of the thumbs is evident in case 1 and shallow palmar creases of the PIP joints on digit V in case 2. Parental consent was obtained.*

The second case is a 20 year old male. He is the second child of healthy non-consanguineous parents and has a healthy older brother. The pregnancy and delivery were uneventful. His birth weight was 3200 gram (15th percentile), length was 49 cm (10th percentile) head circumference was 34 cm (3rd percentile) at a gestational age of 40 weeks. Apgar scores were 2/5/10 after 1, 5 and 10 minutes respectively. He had feeding difficulties and a poor weight gain. Evaluation for failure to thrive that occurred in the first months of his live did not reveal an underlying cause. His social and motor development was delayed. He started walking at the age of 2 years. He was hypertonic, had joint contractures and frequent infections as an infant. His joint contractures improved significantly with physiotherapy as did his hypertonia. Extensive evaluation by a paediatric neurologist at the age of two years and 10 months did not reveal a cause for the motor delay. Hypertonia was not objectified anymore, but he still had a camptodactyly of his thumbs and immature, stiff movements. EEG, BAEP, Electromyography (EMG), Visual evoked potential (VEP), CT and MRI brain, metabolic screening, karyotyping and DNA analysis for fragile X were all normal (data not shown). Eye examination revealed strabismus. He visited a special school where he was tested to have an IQ of 74 at the age of 5 years with a disharmonic profile, verbal IQ of 87 and performance IQ of 64. He had signs of autism, namely poor interaction with peers, obsessions, stereotypic behaviour and movements. His personality was described as kind and calm.

Now at the age of 20 years, he still has stiff and immature movements. He has some degree of independence (he can dress and feed himself and is able to use public transport), but needs too much assistance in his daily care to live independently. A recent IQ test is not available, but based on his educational and independence level his IQ is estimated to be between 60 and 70. Physical examination reveals a slender build and a height of 175 cm (-1 SD), a weight of 52.5 kg (-2 SD) and a head circumference of 52 cm (-3.2 SD). He has a mild brachycephaly, low frontal hairline, thick hair, highly arched eyebrows, mild ptosis and low-set small but normal formed ears. He has a prominent nasal tip, a low nasal bridge and a broad nasal base. He has a short philtrum, thick lips, normal palatum and normal teeth. (See Figure 1 and Table 1). Limb proportions are normal. His hands are long and narrow with absent/shallow dermatoglyphics of the DIP joints, joint movement is normal. He has highly arched feet, hammer toes and a valgus deformity of his left foot. Neurologic examination showed mild peripheral hypertonia with normal muscle strength of hands and feet, biceps and quadriceps. He has stiff movements and a poor coordination. Deep tendon reflexes are normal except for the high reflexes at his Achilles tendon and a clonus of the right ankle.

Written consent was obtained from the parents of both cases for using the clinical and genetic information in this study and for publishing their photographs.

Genomic DNA was isolated from blood samples using standard procedures.

Exome sequencing (case 1)

DNA samples of case 1 and both parents were exome-enriched followed by next-generation sequencing and mapping as described by Neveling et al.[11] We selected candidate *de novo* mutations

Table 1. *Clinical features of the probands in this study and in published cases with the AUTS2 syndrome.*

Clinical features	Proband 1	Proband 2	Published Cases n/total (%)
General			
age at examination	24	20	11m-32y
sex	male	male	18f/13m
De novo occurrence	+	+	18/23 (78%)
Growth and feeding			
low birth weight <p3	+	-	10/26 (38%)
short stature <p10	-	+	16/30 (53%)
microcephaly <p2	+	+	17/28 (61%)
feeding difficulties	+	+	15/27 (55%)
Neurodevelopmental disorders			
intellectual disability/ development delay	+	+	32/32 (100%)
autism/autistic behavior	+	+	13/19 (68%)
sound sensitivity	+	-	5/13 (38%)
hyperactivity/ ADHD	+	-	4/25 (16%)
Neurological disorders			
generalized hypotonia	-	-	13/29 (45%)
structural brain anomaly	-	-	7/21 (33%)
cerebral palsy/spasticity	-	-	10/26 (38%)
Dysmorphic features			
highly arched eyebrows	-	+	10/27 (37%)
hypertelorism	-	-	10/27 (37%)
proptosis	-	+	8/27 (30%)
short palpebral fissures	-	+	10/27 (37%)
up slanting palpebral fissures	-	-	5/26 (19%)
ptosis	+	+	11/27 (41%)
epicanthic fold	-	-	8/27 (30%)
strabismus	-	+	8/28 (29%)
prominent nasal tip	+	+	8/27 (30%)
anteverted nares	-	-	5/27 (19%)
deep nasal bridge	+	+	8/27 (30%)
short/upturned philtrum	+	+	14/29 (48%)
micro/retrognathia	-	+	10/26 (38%)
low set ears	-	+	8/26 (31%)
ear pit	-	-	2/26 (8%)
narrow mouth	-	-	15/26 (58%)
Skeletal abnormalities			
kyphosis/ scoliosis	-	-	5/15 (30%)
arthrogryposis/shallow palmar creases	+	+	4/21 (19%)
tight heel cords	-	-	6/9 (67%)
Congenital malformations			
hernia umbilicalis/inguinalis	-	-	3/30 (10%)
patent foramen ovale/ atrial septum defect	-	-	4/31 (9%)
AUTS2 syndrome severity score	12/32	16/32	

The AUTS2 syndrome severity score [1] is the number of positively scored items in this table, with a maximum of 32. (these items were selected because they occurred in at least two independent AUTS2 syndrome cases).[1-10]

by excluding common variants and variants inherited from either parent as described before. [12] Candidate *de novo* mutations were validated by conventional Sanger sequencing methods in DNA samples obtained from the proband and his parents. Primer sequences and PCR conditions are available upon request.

Array CGH (case 2)

SNP array analysis was performed using a HumanCytoSNP-12 Chip following standard protocols as provided by the manufacturer on an iScan system (Illumina, San Diego, CA). CNV analysis was performed using CNV-WebStore.[13]

RESULTS

Standard diagnostic exome sequencing of case 1 and his healthy parents revealed two *de novo* mutations that could be confirmed with Sanger sequencing. The first mutation was a frame shift mutation in exon 7 of *AUTS2*, NM_001127231.1: c.857_858delAA (p.lys286fs) (hg 19, build 37) (see figure 2). This mutation is predicted to cause haploinsufficiency of the longest *AUTS2* transcript. The mutation lies outside a shorter transcript that starts in exon 9. [1] The other *de novo* mutation

Figure 2. A. *Schematic representation of the AUTS2 gene and the mutations of cases 1 and 2* ***B.*** *Exome sequencing result of case 1. The two-nucleotide deletion at the genomic position g.70227971 is shown in purple. The blue and green lines indicate the reads of the parents. The Sanger sequence result of the forward strand shows the frame shift.* ***C.*** *The array results of case 2 show the deletion of exon 6 as indicated by the log R ratio of -0,5 and the absence of heterozygosity.*

was found in *ABI2,* NM_001282925.1: c.1223C>T, p.Pro408Leu (hg19, build 37). This regards a highly conserved amino acid of the Abl-interacting protein 1 which is a fusion partner of *MLL* in acute myeloid leukemia cells. However, germ line mutations have not been described before and this mutation is unlikely to cause the intellectual disability in the proband.[14]

Array CGH analysis of case 2 revealed a small intragenic deletion encompassing exon 6 of *AUTS2*, (arr 7q11.22(69,985,843x2,69,991,859-70,221,259x1,70,228,020x2)dn (hg19, build 37)). This deletion is predicted to cause a frame shift of the full length transcript and does not affect the shorter 3' transcript.[1] No other rare CNV's were found in this patient. The deletion was not detected in the parents.

Clinical evaluation of both probands resulted in an AUTS2 syndrome severity score of 12 in case 1 and 16 in case 2, see also Table 1.[1]

DISCUSSION

We describe two new adult AUTS2 syndrome cases, one with a two-nucleotide deletion and one with a single exon deletion. To our knowledge this is the first report of a person with a small mutation in *AUTS2* found by exome sequencing. Both probands have comparable age and sex and a genomic defect that causes an early frame shift in the full length AUTS2 protein, likely to cause haploinsufficiency. Both men have intellectual disability, an autism spectrum disorder, feeding difficulties after birth, mild distal joint contractures and mild dysmorphic features. These clinical features are consistent with the phenotypic spectrum reported in the AUTS2 syndrome. As with other AUTS2 syndrome cases, the facial dysmorphisms are subtle, but show clear similarities to each other and other AUTS2 syndrome patients (see Figure 1 and 2 of [1]). Especially the morphology of the eyes (mild ptosis, short palpebral fissures, arched eyebrows), nose (prominent nasal tip and a broad nasal base) and the prominent midface are characteristic. [1; 5; 6] However, both cases have a rather wide mouth with thick lips, whereas almost 60% of the AUTS2 syndrome cases in literature have a narrow mouth (see Table 1).[2] The misalignment of the feet and the mild camptodactyly, with absent or shallow palmar creases that have been described as infrequent features of the AUTS2 syndrome, are present in both cases. Detailed clinical studies of more patients will be necessary to delineate the frequency of these features as they may be very subtle and can easily be missed. Cryptorchidism has not been described before, but as it is a frequent birth defect, this might be an independent finding. The AUTS2 syndrome severity scores of 12 and 16 are similar to the average score of 15 other persons with a genomic rearrangement that affects the C-terminal domain of AUTS2 (average of 12.8).[1]

Both deletion in *AUTS2* described here are likely to cause haploinsufficiency, as the mutations cause a frame shift and are expected to cause nonsense mediated decay. A dominant negative effect of the mutation is unlikely because heterozygous deletions of the entire AUTS2 gene also cause AUTS2 syndrome.[1] Furthermore, 3 cases with similar frame shifting intragenic deletions have been reported: one with a deletion of exon 5-6 and a severity score of 16, and two cases with a deletion of

exon 6 and a severity score of 8 and 9.[1] All these mutations only affect the full-length *AUTS2* transcript and not the shorter transcript starting in exon 9.[1] Nevertheless, this shorter transcript can rescue the microcephaly phenotype of *auts2* zebrafish morphants, strongly suggesting that it contains important information for proper protein function. The typical presentation of AUTS2 syndrome in the two patients described here, where normal levels of the shorter transcript are expected, indicates that the shorter transcript can not compensate for loss of the normal full length transcript in humans. As postulated previously, it is possible that the transcription levels of the short transcript are too low to compensate for frame shift mutations in the full length transcript.[1] Alternatively, in contrast to zebrafish, the full-length human gene product could have unique activities that can not be mediated by the shorter isoform.

In conclusion, our findings confirm the significance of AUTS2 in neurodevelopment and show that a small frame shift mutation in *AUTS2* can cause AUTS2 syndrome.

Acknowledgements

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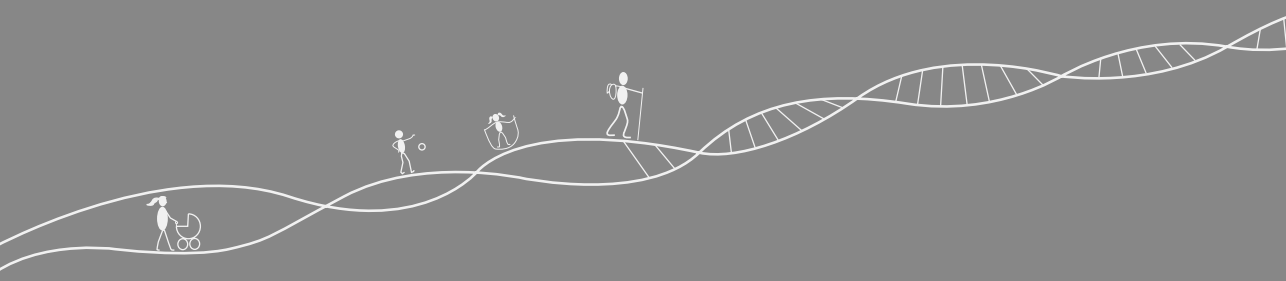
Chapter 5

The AUTS2 syndrome phenotype

A detailed clinical analysis of 13 patients with AUTS2 syndrome further delineates the phenotypic spectrum and underscores the behavioural phenotype.

Beunders G, van de Kamp J, Vasudevan P, Morton J, Smets K, Kleefstra T, de Munnik SA, Schuurs-Hoeijmakers J, Ceulemans B, Zollino M, Hoffjan S, Wieczorek S, So J, Mercer L, Walker T, Velsher L; DDD study, Parker MJ, Magee AC, Elffers B, Kooy RF, Yntema HG, Meijers-Heijboer EJ, Sistermans EA.



J Med Genet. 2016 Aug;53(8):523-32. doi: 10.1136/jmedgenet-2015-103601.

ABSTRACT

AUTS2 syndrome is an 'Intellectual Disability syndrome' caused by genomic rearrangements, deletions, intragenic duplications or mutations disrupting *AUTS2*. So far 50 patients with AUTS2 syndrome have been described, but clinical data are limited and almost all cases involved young children.

We present a detailed clinical description of 13 patients (including six adults) with AUTS2 syndrome who have a pathogenic mutation or deletion in *AUTS2*. All patients were systematically evaluated by the same clinical geneticist.

All patients have borderline to severe intellectual disability / developmental delay, 83-100% have microcephaly and feeding difficulties. Congenital malformations are rare, but mild heart defects, contractures and genital malformations do occur. There are no major health issues in the adults; the oldest of whom is now 59 years of age. Behaviour is marked by it's a friendly outgoing social interaction. Specific features of autism (like obsessive behaviour) are seen frequently (83%) but classical autism was not diagnosed in any. A mild clinical phenotype is associated with a small in frame 5' deletions, which are often inherited. Deletions and other mutations causing haploinsufficiency of the full length *AUTS2* transcript give a more severe phenotype and occur *de novo*.

The thirteen AUTS2 syndrome patients with unique pathogenic deletions scattered around the *AUTS2* locus confirm a phenotype-genotype correlation. Despite individual variations, AUTS2 syndrome emerges as a specific ID syndrome with microcephaly, feeding difficulties, dysmorphic features and a specific behavioural phenotype.

INTRODUCTION

AUTS2 syndrome (OMIM #615834), first described in 2013, is characterized by intellectual disability and microcephaly and is caused by defects of the *AUTS2* gene.[1; 2]

The *AUTS2* gene has several transcripts, the main transcript [NG_034133.1] counts 19 exons and has a highly conserved 3' end. We identified a shorter transcript expressed in human brain, starting in exon 9 of the full-length transcript (NM_015570.2). This shorter '3 transcript can rescue the phenotype in *auts2* zebrafish morphelinos with a knockdown of the main transcript.[2] The function of *AUTS2* has long been an enigma, but recently three papers provided more information on its important function in brain development. *AUTS2* binds to regulatory sequences (eg, to promotors and to brain associated enhancers) of neurodevelopmental genes.[3] *AUTS2* activates gene-expression in the central nervous system by binding to the polycomb repressive complex 1.5 (that normally suppresses gene transcription by chromatin remodelling).[4] The full-length AUTS2 transcript is located in the cytoplasm and in the nucleus, whereas the shorter transcript starting in exon 9 is only located in the nucleus.[2; 5] The cytoplasmic AUTS2 is important in cytoskeletal regulation. Furthermore, knockdown mice studies show that the full-length *AUTS2* transcript, but not the shorter 3' transcript, is important in neurite outgrowth and neuronal migration in the developing mouse brain. [5]

AUTS2 apparently has an important function in neuropsychology, because SNP's in the *AUTS2* region have been associated with schizophrenia, alcohol consumption, and alcohol related suicide.[6-9] Its role in human neurodevelopmental disorders is however even more obvious from the 50 patients with pathogenic disruptions of *AUTS2* that have been described so far. The phenotype described is variable: on the severe side, there is severe intellectual disability and multiple congenital malformations. On the mild side, there are no neurodevelopmental problems or other health issues at all. Forty-one had (intragenic) deletions of *AUTS2*, mostly limited to *AUTS2* but in a few including a maximum of two downstream genes (size 50 kb to 4.5 Mb). Furthermore there were six translocations and two inversions with a breakpoint in *AUTS2*, and finally one '2-base pair deletion' has been described.[1; 2; 10-22] There is evidence for a genotype-phenotype correlation. The complete AUTS2 syndrome phenotype is associated with a disruption of only the 3' end of the full length *AUTS2* transcript or with inactivation of the entire gene. On the other hand, patients with a small in frame 5' deletion (exons 2-5 are all in frame) have a milder phenotype and these deletions were in at least four cases inherited from a mildly affected or unaffected parent.[2; 11]

Little is known about the prognosis and the behavioural aspects of AUTS2 syndrome because most patients described so far are children, and because the phenotype descriptions are very limited. This hampers clinical counselling of newly diagnosed AUTS2 syndrome patients and their parents that have detailed questions about the developmental problems and health issues they might have to expect. Here we describe a detailed and standardized (re)evaluation of ten newly diagnosed and three formerly described AUTS2 syndrome patients including six adults.[1; 2]

PATIENTS AND METHODS

All patients were recruited after diagnostic Array or Whole Exome Sequencing (WES) analysis had shown an AUTS2 defect. After the publication on AUTS2 syndrome in 2013, we were approached by several groups that had found *AUTS2* aberrations. We used this opportunity to start collaborations with these groups to recruit new AUTS2 syndrome patients for this study. Patients with a pathogenic *AUTS2* mutation were included from june 2013 to june 2015 in the study after informed consent.

Clinical evaluation

To gather clinical data as uniformly as possible, the patients with AUTS2 syndrome were seen once by the same clinical geneticist of our research team (G.B.) in the presence of the local physician. In three patients from two families this was not possible and they were seen by the local clinical geneticist only. In all patients, a standardized interview and physical examination was performed. Clinical photographs of the face, body, hands and feet where taken and (where necessary) medical records reviewed with permission of the patients and/or their parents. Three previously described adult cases where re-evaluated by G.B. and were included because their clinical phenotype can now be described in more detail.[1; 2]

Molecular analysis

In all patients, diagnostic array or exome sequencing was performed following local protocols. Where possible parents were tested to analyse the inheritance status. For detailed methods on the array's and exome sequencing protocols see the supplemental methods section.

RESULTS

Ten newly diagnosed AUTS2 syndrome patients from eight families where included. Three previously described patients with AUTS2 syndrome were re-evaluated and included: proband 5 (is proband 2 in reference 2), proband 6 (is proband 9 in reference 1) and proband 7 (is proband 1 in reference 2).[1; 2] The age of the probands varied from 11 months to 59 years. This study includes six adults that are affected and one teenager. A detailed case report of all patients can be found in the supplementary information and a summary is given in table 1 and below.

Genotype

Eight patients (proband 4-11) had an aberration that is likely to cause haploinsufficiency of the full-length AUTS2 transcript, namely: one whole gene deletion, six intragenic deletions (predicted to cause a frame shift) and one nonsense-mutation (see figure 1). Six of these aberrations (in probands 4, 5, 6, 8, 9, 11) occurred de novo and from two the inheritance status is unknown (in proband 7 it is not inherited from the father and in proband 9 it is not inherited from the mother). The other three

deletions were small in frame 5' deletions that where inherited in two patients (probands 1 and 2), both from mildly affected mothers and occurred de novo in one patient (proband 3). (see figure 1) As a clinical evaluation of these affected mothers was performed, and we included them in this study as well. (They are referred to as mother of proband 1 and 2.)

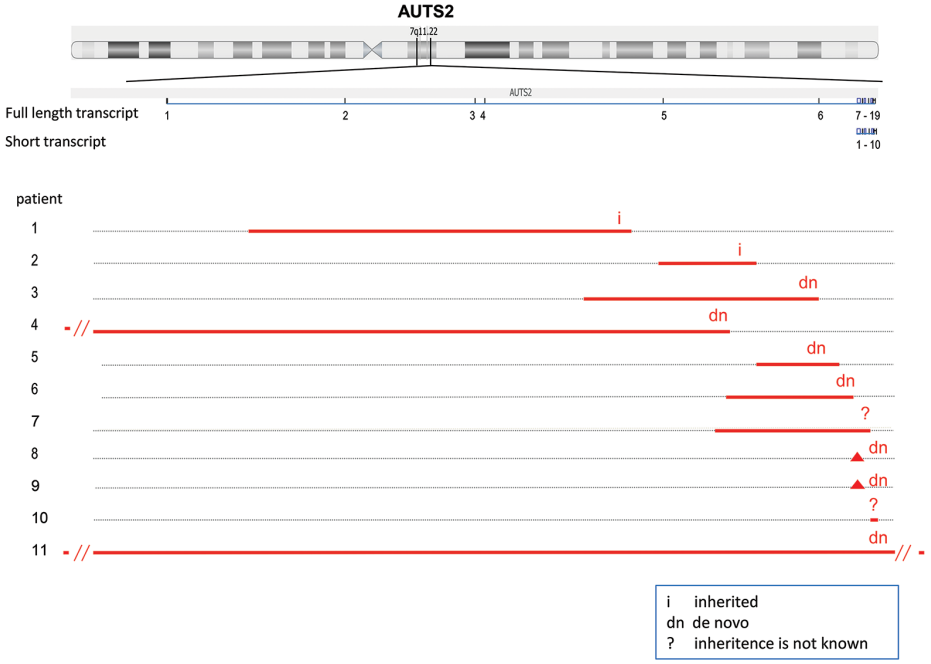


Figure 1. *overview of the deletions and mutations of AUTS2 in the patients described here. Deletions are depicted in red bars, duplications in blue bars, the mutations of one or two nucleotides depicted in red arrowheads. i=inherited, dn= de novo, ?= inheritance status is unknown.*

Phenotype

ID/cognition

All patients have some degree of learning problems and/or developmental delay. Eight have borderline IQ to mild intellectual disability (available Total IQ data range from 61 to 89) and five have moderate intellectual disability (no IQ data are available for these patients).

The age at which the first words were spoken was in the normal range for three patients and slightly delayed (at ages between 18 months and 3 years) in five. Some degree of speech and language delay was observed in almost all patients and two adults still used single words or short sentences only. Four patients stammer. Out of the nine patients of 7 years and older six where able to write and read.

Motor development was slightly delayed in most patients with AUTS2 syndrome; they started walking between 1 and 3 years of age.

Two adults (the mothers of proband 1 and 2) lived independently with some help from family

Table 1 Clinical overview of all probands showing the features present in each patient.

	1	m of p1	2	m of p2	3	4	5	6	7	8	9	10	11	total	%	Total in lit.
AUTS2 defect																
coordinates of breakpoints and mutation at chr7: (hg19)	69225022 69869867	69225022 69869867	69893262 70050442	69893262 70050442	69735528 70162902	65900875 70017343	70049056 70197417	69991859 70221259	69958576 70240310	70227970 c.857_858 delAA p.(Lys286fs)	70228089, c. 317C>T, p(GLN107X)	70246372 70251118	67445895 70356041			
exon del./mut (inherited)	2-4d (m)	2-4d (?)	5d (m)	5d (?)	5d (dn)	1-5d (dn)	6d (dn)	6d (dn)	6-9d (dn)	9fs (dn)	7st (dn)	15-17d (dn)	all d (dn)	9 dn		
General																
age at examination(yrs)	5y	?	38y	59y	10y	5y4m	11m	23y	40y	28y	7y	16y4m	6y4m	11m-59y		
seks	m	f	m	f	f	f	f	m	f	m	f	f	m	8 f / 5 m		
Growth and feeding																
birth weight in grams	3440	ND	2750	ND	2829	3269	3235	3200	3690	2745	2990	3275	3577		0%<p3	
stature for age p value	p25	ND	p10	p0,1	p10	p25	p25	p25	p0,6	p16	p25	ND	p40	p0,1-p40	30%<p10	18/30 (60%)
HC for age p value	p3	ND	p0,1	p 0,6	p1	p25	p0,6	<p0,1	p1	p 0,1	p 0,1	p2	p30	<p0,1-p30	83%<p3	18/30 (60%)
weight for height p value	p50	ND	p85	p80	p0,1	p16	p50	p3	p80	p10	p25	ND	<p2	p0,1-p85	27%<p3	ND
feeding difficulties	+	ND	+	ND	+	+	+	+	+	+	+	+	+	11	100%	15/28 (53%)
Neurodevelopment																
ID/DD	mi/bo	mi/bo	mi	mi/bo	mi	mi/bo	mi	mi/bo	mo	mo	mo	mo	mo	13	100%	36/37 (97%)
walking at age	1y	ND	>2y	<1,5y	2,5 y	3 y	>1y	2y	ND	1,8y	1,5y	1,3y	2y	1y-3y		
first words at age	1,2y	ND	1,5y	<1,5y	2,7 y	1y	>1y	2y	ND	2,5 y	2 y	1 y	2y	1y-2,7y		
speech and language delay	+	+	-	-	+	-	-	+	+	+	+	+	+	9	70%	7/7 (100%)
stammers	-	-	-	-	+	-	-	-	+	+	-	+	-	4	30%	ND
Behaviour																
autism spectrum disorder	+	-	+	+	-	+	ND	+	+	+	+	+	+	10	83%	12/29 (41%)
- obsesive/repetative	+	-	+	-	-	+	-	+	+	+	+	+	+	9	70%	ND
- stereotypic movements	-	-	-	-	-	-	+	+	+	+	+	+	+	7	54%	ND
- social interaction	↓	-	shy	shy	↑	-	↓	↓	shy	↑	↑	↑	↑	5↑/ 6↓	38/46%	ND
hyperactivity/ ADHD	+	-	-	+	-	-	+	-	-	+	+	+	+	7	54%	5/31 (16%)
hypersensitivity	-	-	-	-	-	-	+	+	-	+	+	+	+	6	46%	4/12 (33%)

other behavioural problems		a									b					
Neurological disorders																
generalysed hypotonia	-	-	-	-	+	-	-	-	+	-	+	+	+	5	38%	13/31 (42%)
structural brain anomaly	-	ND	-	-	+	ND	ND	-	ND	-	-	ND	-	1	14%	7/22 (32%)
hypertonia/ hyperreflexia	-	-	-	+	-	+	-	+	+	-	+	+	+	7	54%	11/28 (39%)
epilepsy	-	-	+	+	-	-	-	-	-	-	-	-	-	2	15%	2/27 (7%)
Back and extremities																
kyphosis/ scoliosis	+	-	-	-	+	-	-	-	+	-	-	-	-	3	23%	5/18 (27%)
camptodactyly dig. V	+	-	-	-	+	+	-	-	-	+	-	-	-	4	30%	3/30(10%)
faint extension creases	-	-	-	-	+	-		+	+	+	+	+	-	6	46%	1/20 (5%)
narrow/slender hands	-	-	-	+	+	+	-	+	+	+	-	-	+	7	54%	ND
feet deformities	-	-	-	-	-	+	-	+	+	+	-	+	-	5	38%	ND
narrow hips	-	-	-	-	+	-	-	+	-	+	+	-	+	5	38%	ND
other abn. extremities						c	d				e	f				
Congenital malformations																
hernia umbilicalis/inguinalis	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0%	4/30 (13%)
atrial septum defect	-	-	-	-	-	+	-	-	-	-	-	-	-	1	8%	3/32 (9%)
cryptorchydism	+	NA	-	NA	NA	NA	NA	-	NA	+	NA	NA	-	2	40%	ND
other cong. malformations											g		h			
other																
eczema	-	-	+	-	-	-	-	-	ND	-	+	+	+	4	33%	ND
eyes	-	-	-	-	-	M	S	S	S	M	-	-	-	3		ND
freq. infection in childhood	-	-	-	-	+	-	+	+	+	+	-	+	+	7	54%	ND

The genomic positions are all converted to hg19. The total of patients that were showing a specific feature and the percentage is shown in the last columns. An percentage of the patients in literature showing this feature is depicted in the colum: total in lit. [1,2, 10-22] Abbreviations used are: ..d: deletion including the exon(s) .., 9fs: frameshift mutation in exon 9, 7st: stopmutation in exon 7, m: maternal, dn: de novo, ?: inheritance unknown, y: years, ID: intellectual disability, DD: developmental delay, bo: borderline IQ (TIQ of 70-85), mi: mild ID, mo.: moderate ID, social interaction ↓: poor eye contact, little interaction with peers and/or adults, social interaction ↑: hypersocial behaviour, ND: not determined, NA: not applicable, M: myopia, S: strabismus.

a Anxiety, b sleeping disorder, c pectus excavatum, d temporary contractures, e radio-ulnar synostosis, f clinodactily, g blocked tearduct, h lanryngomalacy

members. They, however, had learning problems at school and did not finish high school and were unable to hold jobs. The other four adults lived in a special needs home with assistance in daily living and three of them worked at a sheltered environment.

Behaviour

A friendly and affably personality was noticed in all patients. Classical autism was not diagnosed in any of the patients with a pathogenic AUTS2 defect. Features of autism or an autism spectrum disorder was however seen in all but two patients. Especially stereotypic movements and obsessions where often observed, whereas social interaction is less affected. In childhood even hypersocial behaviour was reported by parents and/or observed during clinical evaluation including increased social interaction with familiar and unfamiliar adults and lack of inhibition in social contact to strangers (n=6). The adults were all rather shy and quiet, however, and only answered direct questions and made little eye contact. Furthermore, six patients were hypersensitive. These children tend to obsessively smell objects, feel textures of walls, toys and clothes in a stereotypic manner and/or are hypersensitive to sounds.

Seven patients showed hyperactivity and attention deficits. Two children showed a period of mild aggressive behaviour when frustrated.

Neurology

Also, seven of the thirteen patients with AUTS2 syndrome had brisk reflexes in four limbs and/or hypertonia in lower limbs. They tend to walk on their toes and/or where mentioned to have tight Achilles tendons. In four of these patients the hypertonia was preceded by hypotonia. One other patient was reported to be hypotonic in the first year of live, but her muscle tone improved with age and did not evolve to hypertonia. An MRI was made in eight patients. None had structural brain malformations except for a stable arachnoidal cyst in one.

Patient 2 had absence epilepsy, and his mother had mild partial seizures with secondary generalisation. They were both successfully treated with antiepileptic drugs and are seizure free. In two other paternal family members of the mother, epilepsy was diagnosed. It is not known whether they are carrying the *AUTS2* deletion as well because they were not available for testing.

Feeding

All eleven probands of whom data from early childhood were available had feeding difficulties. Severity is variable ranging from failure to breast feed, slow bottle feeding or reflux in the first months of life to severe feeding difficulties needing tube feeding for several months to one year. Young children vomit easily, which mostly resolved after the age of one year. Seven patients had poor weight gain or failure to thrive. Weaning to solids was troublesome in most patients and in some children it took until the age of five to seven years before they were able to chew and swallow solids. Furthermore, parents report frequent choking incidences until young childhood. The feeding

problems did resolve in all patients, although in some it took up to six years.

Growth

We measured a low average to short stature (p25 to p0,1) in 90 % of the patients. Weight varies, four have an average to high average weight, whereas two have a low average weight (p16-10) and four have below average weight (≤ p3). The two patients high weight where both adult women. Also, 83 % (ten out of twelve) of the patients are microcephalic with head circumference ranging from p3 to below p0,6 (~-2 to -3 SD). The other two had a low average head circumference (p25-p30). Microcephaly was not always present from birth, but was non-progressive after the age of 1 year (based on 5 patients of which head circumference data were available from birth).

Figure 2. *dysmorphic features of the probands 1 – 11 and the mother of patient 2ordered from the youngest to the oldest patient. Patient 5 at 11months, patient 4 at 5years and 4 months, patient 1 at 5 years, patient 11 at 6 years and 4 months, patient 9 at 7 years, patient 3 at 11 years, patient 10 at 16 years and 4 months, patient 6 at 23 years, patient 8 at 28 years, patient 2 at 38 years, patient 7 at 40 years and the mother of patient 2 at 59 years of age. (The patient number is depicted in white in the lower left corner of each picture.) A : the frontal views show the arched eyebrows, ptosis, short palpebral fissures, short philtrum and narrow mouth (especially in the younger patients). B : the profile views showing the proptosis, prominent nasal tip, short upturned philtrum and micro-/retrognatia.*

Figure 3. *Age related facial features patients 2 (row A) and his mother (row B), patient 3 (row C), 6 (row D), 7 (row E), 8 (row F), 9 (row G) and 10 (row H) are shown on different ages: in column* ***1****: 1 month - 1 year,* ***2****: 3-5 years,* ***3****: 6-9 years and* ***4****:10-18 years,* ***5****: above 18 years.*

Dysmorphisms

The facial features change with age as is shown in figure 2 and 3. At a younger age the facies are round to square with a low nasal bridge, narrow mouth, a short nose with anteverted nares, proptosis and ptosis. At an adult age the arched eyebrows, proptosis, ptosis, prominent nasal tip, wide nasal base, short philtrum and the long neck are most remarkable. (See also table S1)

Extremities and spine

One or more of the following minor abnormalities of the extremities were seen in 77% of the cases: faint extension creases of the distal interphalangeal (DIP) joints (especially of dorsal side of the fingers and only sometimes on the ventral side as well, digit IV is affected the most severely) (n=6), camptodactyly (of the DIP joint of digit V) (n=4), elbow supination limitation (n=1), pes cavus (n=2) and hammer toes (n=2) (figure 4A and B). One girl (proband 5) had contractures of hips and knees at birth that resolved spontaneously after a few months. Narrow hands with slender fingers (more pronounced in the distal phalanges) were noticed in seven probands (see figure 4 A). Mild kyphosis or scoliosis was seen in three patients from young childhood. No surgery was needed in all three patients.

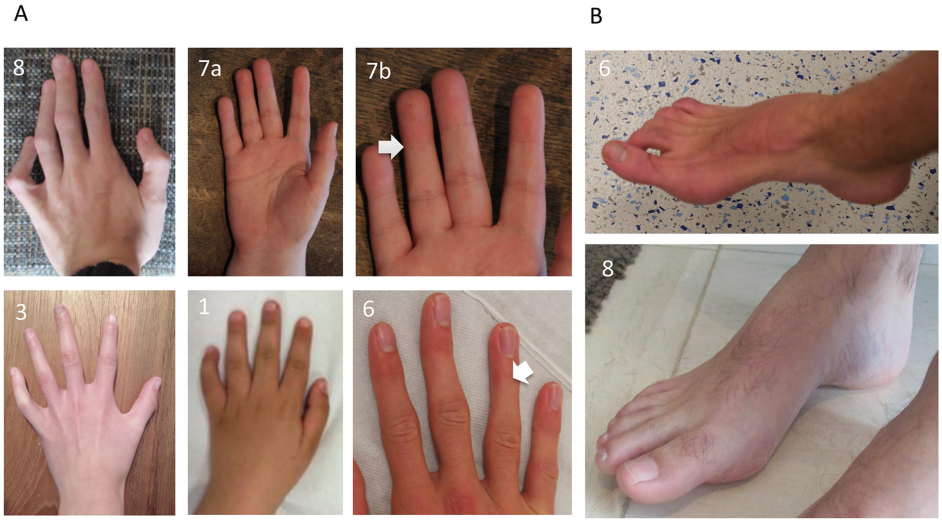


Figure 4. *A The hands of patient 8 (A), patient 7 (B and C), patient 3 (D), patient 1 (E) and patient 6 (F) are showing the typical hand anomalies: the slender hands, the shallow bending folds especially of digit IV (see with arrows in the detail pictures C and F) and the camptodactyly of digit V (A, D and E). B The typical slender long feet with pes cavus and hammer tows as seen in AUTS2 syndrome (proband 6 and 8).*

yes

Six patients have eye problems, three have mild strabismus, two have mild to moderate myopia and needed glasses and one patient had a nasolacrimal duct obstruction.

Birth defects

Birth defects were infrequent. Two of the five boys that were evaluated had cryptorchidism, both where operated. One proband had an atrial septal defect that was clinically monitored and did not need surgery.

General health

Frequent infections occurred up till the age of four to five years (n=8), but there are not many health issues after infancy. One of the adults has Lupus erythematosus.

DISCUSSION

The phenotypic spectrum of AUTS2 syndrome and its pathophysiology

The major features of AUTS2 syndrome are borderline to moderate intellectual disability, microcephaly, feeding problems and/or failure to thrive hypertonia and mild dysmorphic features (arched eyebrows, ptosis, short palpebral fissures, short philtrum, micrognathia/retrognathia, slender hands with faint extension creases).

Subtle but remarkable are the minor abnormalities of the extremities, which were often not noticed by the patients and physicians (and cause no disabilities) but have a specific pattern that can help to recognise AUTS2 syndrome (see figure 4A,B and online supplementary patient reports). Hammer toes and cavus feet were seen in two of the five adults. Cavus feet often have a neurological cause.[23] However, repeated electromyogram in proband 5 with pes cavus from early childhood showed no abnormalities. The faint extension creases and mild contractures of the fingers are both likely to be caused by the aberrant fetal development, primarily caused by the AUTS2 defect or second- ary through lack of movement in utero caused by the AUTS2 defect.[24] As *AUTS2* is highly expressed in the developing brain and the functional clues at the molecular and protein level so far also indicate an important role in neuron development, one could hypothesise that diminished movements in utero (for neurological reasons) are causing these defects.[2-5] However, embryological studies did show more evidence for the theory that the same genetic factors are responsible for flexion crease

formation as well as development for joint movement. This would suggest that AUTS2 could also have a direct effect on hand and joint formation.[24]

Two affected adults from one family had epilepsy diagnosed in childhood and adulthood. Three of their family members of which the *AUTS2* deletion status is unknown had epilepsy as well. There could be another cause for the epilepsy in this family. Epilepsy has however been reported sporadically in patients with AUTS2 syndrome before (n=4, not including the two patients with *AUTS2* deletions described by Mefford et al.[25] as those had intron deletions that we would classify as a variant of unknown significance (VUS)). It can, therefore, not be ruled out that epilepsy is an infrequent feature of AUTS2 syndrome, although in our family the cause may be unrelated to *AUTS2*.

We observed hypersocial behaviour in patients with AUTS2 syndrome. This is also a key feature of patients with Williams–Beuren syndrome (WBS). Interestingly, the *AUTS2* gene is flanking the WBS region and is shown to be downregulated in WBS cells.[26] Furthermore, *AUTS2* is shown to have a lower expression in *Gtf2ird1* knockout mice embryos; this was, however, not a statistically significant finding.[27] *GRF2IRD1* is the candidate gene for the behavioural phenotype in WBS.[28]

Recommendations for clinical practice

One of the major concerns of parents are the feeding difficulties. All patients in this study had feeding difficulties. The problems do resolve spontaneously mostly around the age of 1-2 years but sometimes they can last until the age of 7-9 years before these children can handle solids properly. The problems seem to be caused by absent sucking reflex, low muscle tone and coordination of the mouth muscles. A thorough evaluation by a multidisciplinary team (including a general paediatrician, a speech therapist and a child neurologist) seems valuable. Treatment depends on the specific problems and is not different from patients that suffer from feeding difficulties due to a different cause.

Congenital malformations are not very frequent and mostly mild in patients with AUTS2 syndrome. Because an atrial septal defect was seen in one patient from this series and is reported in 11-25% of AUTS2 syndrome patients in literature (see table 1) we advise an ultrasound of the heart when AUTS2 syndrome is diagnosed especially at a childhood age.[1; 2; 10-22]

Because of frequent infections in infancy, developmental and growth delay in all patients, follow-up by a paediatrician on feeding, growth and development is recommended.

In this study we were able to include six adults and one teenager, the oldest being 59 years of age. This enabled us to get a better view of the natural course of AUTS2 syndrome. The affected adults in this study have mild learning problems to moderate intellectual disability. There are no major health issues (except for the Lupus erythematosus in one of the adults that cannot directly be related to the *AUTS2* aberration and well controlled epilepsy).

Although a periodic control by a general physician or physician for the intellectually disabled is advised in all adults with intellectual disability, there are no specific advises for adult patients with AUTS2 syndrome.

Interpretation of the genetic results

Two of the three newly described probands that carry a small in-frame deletion of exons 2-5 of *AUTS2* have inherited the *AUTS2* defect from a mildly affected parent. All three probands and their family members had mild intellectual disability to borderline IQ and had lower mean AUTS2 syndrome severity scores,[2] indicating that they have less AUTS2 syndrome-specific features. (see table S1) The patients with frame-shift deletions or mutations are more severely intellectually disabled, show higher AUTS2 syndrome severity scores and all have *de novo AUTS2* defects. This is in accordance with the formerly described observation that small in frame 5' deletions cause a milder phenotype

than the 3' in frame deletions or deletions causing haploinsufficiency of *AUTS2*.[2]

In conclusion, AUTS2 syndrome is a variable syndrome, but there is a clearly recognizable pattern with intellectual disability, feeding difficulties, non-progressive microcephaly (head circumference at -2 to -3 SD), mild dysmorphic features, camptodactyly, faint extension creases and a friendly outgoing character in childhood, but a more shy appearance in adulthood as major features.

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SUPPLEMENTAL DATA

Supplemental methods

In proband 1 and her mother array-CGH was performed on genomic DNA from uncultured peripheral blood cells at an average resolution of 35 kb (244K kit; Agilent Technologies, Santa Clara, CA, U.S.A.), following the manufacturer's instructions. Probe alignments were referred to NCBI 37 (NCBI: National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/), UCSC (UCSC: University of California, Santa Cruz, http://genome.ucsc.edu/) hg19 build.

In proband 2, his mother and in patient 6 (that is proband 2 in reference 1) an Human CytoSNP-12 Chip (Illumina, San Diego, CA) was performed and analysed (in NCBI 37/hg 19 (2009)) as described in reference 1.[1]In proband 3 and 5 the protocol for the Affymetrix CytoScan HD array platform (Affymetrix Inc., Santa Clara, CA, USA) and data analysis (in NCBI 37/hg 19 (2009)) was used as described before.[2] In proband 4 the 4x180K Oligonucleotide Array by Aligent Technologies was performed and analysed in Genome Build: NCBI 37/hg 19 (2009) by use of analysis software CytoSure Interpret (Oxford Gene Technologies). The deletion found was then confirmed by FISH (TCAG probes were obtained from the Centre for Applied Genomics. The probes were validated and performance characteristics determined by The Hospital for Sick Children Genetics laboratory). In proband 7 (that is patient 9 in reference 3) an Agilent 105K oligo array was preformed and the deletion found in AUTS2 was confirmed with MLPA as described before in NCBI build 36/hg18 (2006).[3]The WES protocol used in proband 8 (that is proband 2 in reference 2) was described before.[1] In proband 9 WES was performed within the DDD project as described before. The de novo AUTS2 mutation was confirmed with Sanger sequencing. Results are reported in Genome Build: NCBI 37/hg 19 (2009).[4]

In proband 10 microarray analysis was completed by Sanger Institute as part of the Decipher Developmental Disorder (DDD) researchproject. An Agilent custom array-CGH (genomic plus 5 probes per exon) and the analysis pipeline version: 2.0.0; DDG2P version: v1.2 was used. Results are in Genome Build: NCBI37/hg 19(2009).[4]

Proband 11 was analysed by Blue Gnome 60K ISCA oligo array, comparing whit sex- matched controls. Using BlueFuse Multi v3.2 and standard analysis protocols with clone position based on genome build GRCh37 (NCBI 37/hg 19 (2009).

Supplemental case reports

Patient 1 (deletion exon 2-4, inherited from mildly affected mother)

This male patient is now five years of age and has a speech and language delay. He is born at a gestational age of 42 weeks after an uneventful pregnancy and delivery. His birth weight was 3440 gram (p15), height 51 cm (p25) and head circumference 33 cm (p0,4). He had a good start with apgar scores of 9/10 after 1 and 5 minutes respectively. He did have mild feeding problems, especially gastro-oesophageal reflux. He had a bilateral cryptorchidism for which he had a orchidopexia. He

had a normal motor development, but his speech development was delayed. He spoke single words until the age of 4 years and started to use two-words-sentences after intensive speech therapy. His expressive language is severely delayed, but understanding of spoken language is significantly better. His IQ was tested at 89 at an age of 3 years and 6 months. He shows stereotypic and hyperactive behaviour and has an attention deficit. He is obsessed with personal cleanness. Physical examination at the age of five years showed a height of 106 cm (p25), a weight of 20 kg (p50) and a head circumference of 48,8 cm (p3). He has mild dysmorphisms, namely arched eyebrows, proptosis, a mild ptosis and a thin upper lip. He has a mild kyphosis and bilateral camptodactily of the 5 finger. Genetic analysis Fragile X testing was negative. Array CGH revealed an exon 2-4 deletion of the AUTS2 gene arr(chr7:68,862,958-69,507,803)x1 (NCBI 36, hg 18). This deletion does not cause a frame shift and is predicted to result in a shorter AUTS2 protein. Parental testing showed this deletion is inherited from his (mildly affected) mother. Her history is described below.

Mother of proband 1 (exon 2-4 deletion of AUTS2, parents not tested)

This mother with learning problems as a child now lives independently. Family history revealed learning difficulties in her sister; that carries the AUST2 deletion as well. The mother of proband 1 was born after an uneventful pregnancy and delivery. There are no details known about birth weight and apgar scores available. She did not have any birth defects. It is not known whether she had feeding difficulties, but she had a speech and language delay and needed special care at school until the age of eight years. After that she could follow normal education and now at the age of 27 years she lives independently. She shows some anxiety, but has no other physical of psychological health problems.

Genetic analysis Array CGH revealed the same in frame exon 2-4 deletion of AUTS2 was found in her son arr(chr7:68,862,958-69,507,803)x1 (NCBI 36, hg 18). This deletion is predicted to give rise to a shorter AUTS2 isoform (missing 87 aminoacids). The parents of the probands mother were not tested, but one of them will carry the deletion as the sister is found to Carrie the deletion as well. A mosaic in one of the parents can however not be ruled out.

Patient 2 (deletion exon 5, inherited from affected mother)

Proband 2 is 38 year old male with mild intellectual disability. He was born at a gestational age of 40 weeks and had a birth weight of 2750 grams (p5) and had a good start. He did not have any congenital malformations. He had feeding difficulties. Breastfeeding did not workout, bottle feeding was slow and he was vomiting frequently. This resolved after starting with buttermilk at the age of 4-5 months. His early development was delayed. He started walking when he was above two years of age and said his first words at 1,5 years. He visited a normal primary school but had learning difficulties and was placed on a special secondary school where he learned how to write and read. He was a rather quit happy boy, did not show stereotypic behaviour (except for some head banging in early childhood) and he made normal contact with peers and adults. He was diagnosed with

Table S1. Overview of dysmorphic features per patient.

Table S1 shows the dysmorphic features per patient and the AUTS2 syndrome severity score as described in reference 3 for which also table 1 from the main tekst is used to calculate total scores. In the last row the percentage of each feature as described in reference 3 is given for comparison. a: alopecia, b: broad forehead high frontal hairline, c: thick helixes ears, e: simple ear.

	1	m of p1	2	m of p2	3	4	5	6	7	8	9	10	11	total:	total in lit. ref 3
Dysmorphic features															
short palpebral fissures	-	-	+	+	+	+	+	+	+	+	+	+	+	11 (85%)	37%
ptosis	+	-	+	-	+	+	+	+	+	+	+	+	-	10 (77%)	27%
(highly) arched eyebrows	+	-	-	-	-	+	+	+	+	+	+	+	+	9 (70%)	37%
short/upturned philtrum	-	-	-	-	+	+	+	+	+	+	+	+	+	9 (70%)	48%
proptosis	+	-	-	-	+	+	+	+	+	-	-	+	+	8 (62%)	30%
micro/retrognatia	-	-	-	-	+	+	+	+	+	-	-	+	+	7 (54%)	38%
prominent nasal tip / broad nasal base	-	-	+	-	-	-	-	+	+	+	+	+	-	6 (46%)	30%
long neck	-	-	+	-	+	-	-	+	+	+	+	+	-	6 (46%)	?
apperantly large median incisors			+	+	+	ND	ND	+	+	-	-	-	-	5 (45%)	?
narrow mouth	+	-	-	-	+	+	+	-	+	-	-	-	-	5 (38%)	58%
deep/broad nasal bridge	-	-	-	-	-	-	+	+	+	+	-	-	-	4 (30%)	30%
low set ears	-	-	-	-	-	+	+	+	+	-	-	-	-	4 (30%)	31%
assymetry of the face	+	-	-	-	+	-	-	-	-	-	+	+	-	4 (30%)	?
upslanting palpebral fissures	-	-	-	+	-	+	-	-	+	-	-	- (down)	-	3 (23%)	19%
brachycephaly	-	-	-	-	-	+	-	-	-	-	+	+	-	3 (23%)	?
hypertelorism	-	-	-	-	+	-	-	-	+	-	-	-	-	2 (15%)	37%
epicanthal fold	-	-	-	-	-	-	+	-	-	-	-	+	-	2 (15%)	30%
anteverted nares	-	-	-	-	-	-	-	-	+	-	-	-	-	1 (8%)	19%
ear pit	-	-	-	-	-	-	-	-	-	-	-	-	-	0 (0%)	8%
other dysmorphic features			a			b	c				e				
AUTS2 syndrome severity score/ total number of scored items	9/31	1/25	9/31	8/29	15/31	15/31	16/30	16/31	23/31	13/31	14/31	16/30	11/31		
seen by:	MZ	MZ	GB	GB	GB	JS and LV	GB	GB	GB	GB	GB	GB	GB		

absence epilepsy at the age of 13 years and started with medication (Carbamazipine). He had no seizures since. MRI of the brain was maid at the age of 30, showing normal brain morphology. He did not face any major heath issues during his life.

Now he works in a sheltered working place and lives at home with his mother. Physical examination showed a height of 162 cm (p1), a weight of 61 kg (p85, weight to length) and a head circumference of 52,5 cm (<p0,4). He has alopecia, ptosis, narrow palpebral fissures, a prominent nasal tip, normal ears, a short philtrum and high palate and carious teeth. He had normal hand and feet and a straight spine. He had high deep tendon reflexes in all extremities, and a high muscle tone in the legs.

Genetic analysis Fragile X test, karyotyping and MECP2 sequencing did not reveal abnormalities. Array CGH revealed an intragenic deletion of the AUTS2 gene on chromosome 7q11.22 arr(chr7:69,893,262-70,050,442)x1 (hg19). This deleted exon 5 causing an in frame deletion of 10 amino acids of the main Auts2 isoform and normal short C-terminal isoforms. Array CGH of his mother showed the same AUTS2 deletion. A report of her history is stated below.

Mother of patient 2 (deletion exon 5, parents unavailable for testing):

This adult woman of 59 years of age (patient 2b) carries the same in frame AUTS2 deletion as her son (patient 2). She had one brother that did not have any learning problems. Her father died. He had a brother who's son had epilepsy and his mother (the paternal grandmother of this patient) also suffered from epilepsy. She was born a term with a normal start and a normal birth weight (exact measurements are unknown) and without congenital malformations. Detailed early medical history is unavailable. It is therefore unclear if there were feeding difficulties or a developmental delay. No hospital administrations at young age are documented. She was described as a hyperactive child. She went to a normal primary school where she had learning problems, but learned to write and read. She did not go to secondary school and has a mild intellectual disability to a borderline intelligence. She is diagnosed with epilepsy around the age of 5 years and had partial, secondary generalized seizures. Seizure control fluctuated, but she did not have seizures in the last five years. Systemic Lupus erythematosus was diagnosed at the age of forty years, which is well controlled by placenile. At 48 years she suffered from a Depakine encephalopathy, which resolved slowly after Depakine was stopped.

She lives independently with her son and does not hold a job. She is quite and shy. Physical examination revealed a height of 151 cm (p0,1), a weight of 72 kg (p80) and a head circumference of 51 cm (p0,6). She has no dysmorphic features, supination of the wrist is limited. Neurologic examination showed high biceps and brachial-radial deep tendon reflexes and no other abnormalities.

Genetic analysis Array CGH revealed the same in frame exon 5 deletion of the AUTS2 gene as was found in her son arr(chr7:69,893,262-70,050,442)x1 (hg19).

Patient 3 (exon 5 deletion, de novo)

This girl of now 10 years old has a mild intellectual disability. She is the second born child of healthy non-consanguineous parents. She has a healthy older brother. She was born after a planned caesarean section because of a breech position at 38 weeks and 3 days of gestation. The pregnancy was normal. She had a good start with apgar scores of 9 and 10 after 1 and 5 minutes respectively and a birth weight of 2829 gram (p25). There where no birth defects noticed. Because of low temperature and feeding difficulties she was admitted to the neonatal care and stayed in an incubator for a few days. She needed tube feeding the first two weeks and after that feeding still was troublesome. She had a no sucking reflex, was hypotonic and bottle-feeding was very slow. As she did not gain weight and at the age of 3-4 months she was admitted to hospital again for evaluation, but no clear cause of the feeding difficulties was found. Tube feeding was started again and remained needed for almost 1 year. Weaning to solids was difficult starting at 1,5 years of age with smooth fruits and at least another year before she could eat soft bread. Now at the age of 10 years she eats normally.

She had recurrent ear infections for which she got grommets. She was operated because of protruding ears and she has a stable arachnoid cyst. She had hypotonia and delayed motor development. She started walking at the age of 2,5 years and said her first words at the age of 2 years and 8 months. She stammers although this has improved after intensive speech therapy. She visits special needs primary school and has learned to write and read. Language development improved dramatically after dolphin therapy at the age of 9 years. Her IQ was measured at 61 (VIQ73 and PIQ 61) (WPPSTIII-NL, 2011).

She has no behavioural problems and makes normal contact with adults and pears, although she seems to lack stranger anxiety. Parents describe her as a cheerful quiet girl that is a bit drawn back. Physical examination at the age of 10 years and 10 months showed a height of 139 cm (p10) a weight of 22 kg (<p0,4) and a head circumference of 49,5 cm (<-p0,4). She has a slender build with narrow hips and broad shoulders. She has a mild brachycephaly, proptosis, ptosis, short palpebral fissures, a narrow mouth and short upturned philtrum. She has slender hands with shallow bowing folds at the DIP joints, she has a bilateral camptodactily of her fifth finger. She has slender long foot without any deformities. She is said to have mild scoliosis that is not clear at physical examination. Neurologic examination did not reveal any abnormalities.

Genetic analysis Array CGH revealed an intragenic AUTS2 deletion, causing a deletion of exon 5 at 7q11.22 (46,XX.arr 7q11.22(69,735,528-70,162,902)x1 dn, hg19).ed This deleted is predicted to cause an in frame loss of 10 amino acids of the main AUTS2 isoform and normal short C- terminal isoforms. Array CGH of both parents did not show any abnormalities, suggesting a de novo deletion. Fragiele X test, karyotyping and MECP2 sequencing did not reveal abnormalities .

Patient 4 (deletion of exon 1-5 of AUTS2, de novo)

Proband 4 (a 4,5 years old girl with a developmental delay) is the third child of non- consanguine

healthy parents. She has two healthy older sisters. During pregnancy there was a positive maternal serum screen for Down syndrome, but the amniocenteses was normal. The further pregnancy was uneventful. She was born at a gestational age of 41 weeks and 3 days. She had a birth weight of 3269 gram (p12) and a good start with apgar scores of 9/9 after 1/5 minutes. She was observed in the nursery for eight hours after birth because of irritability, that resolved spontaneously. She was born with a small atrium septum defect that is monitored. No operation or medication is needed so far. No other birth defects where noticed. She had feeding problems, namely: slow bottle feeding because of a poor suck, swallowing difficulties and choking incidents. At the age of 6 months a swallowing assessment did not reveal any abnormalities. She was diagnosed with a mild cerebral palsy because of increased muscle tone and tight heel cords. No MRI was performed. She has a mild developmental delay. She started walking at the age of three years and started talking at the age of 1 year. Now at the age of 4 years there are no feeding problems any more, she speaks full sentences and is toilet trained. She does not have behavioural difficulties, makes normal contact to adults and peers. She is however rigid and likes follow routines. Physical examination at the age of 4,5 years showed a height of 101,2 cm (p25), a weight of 15 kg (p10-25) and a head circumference of 49 cm (p25). There are dysmorphic features: a broad forehead, high anterior hairline, brachycephaly, ptosis, slender nose, a short philtrum, micrognatia and a high palate. She has a pectus excavatum, camptodactyly of the fifth finger (left more than right), long slender fingers, a single palmar crease (bilateral) and proximally placed thumbs. She has long toes and a sandal gap. Neurological exam revels no abnormalities anymore.

Genetic analysis: Fragile X and Fragile X-E testing was normal. Array CGH however revealed a deletion of exon 1 to 5 of AUTS2, arr 7q11.22(65,900,875-70,017,343)x1 (hg19). As the promoter and the transcription start are both deleted as well, haploinsufficiency of the full length (but not the shorter 3') transcript is predicted to be de result of this deletion. The deletion was confirmed with FISH and was not found in either of the parents.

Patient 5 (deletion of exon 6, de novo)

Patient 5 is a 11 months old girl with severe feeding problems and a mild developmental delay. She is the first child of healthy non-consanguineous parents and was born after a normal pregnancy at a gestational age of 41 weeks and 6 days after a caesarean section. She had a good start and a birth weight of 3235 gram (p10). There where no congenital malformations.

She has severe feeding difficulties. In the first days after birth she had two cyanotic incidences, for which she was observed in hospital. The incidences where thought to be caused by gastrointestinal reflux she was released from hospital, but at 3 weeks of age she started to show severe discomfort during and after feeding, drinking slowed and was refused later. She was admitted to hospital for evaluation and treatment again. An ultrasound of the abdomen did only reveal a mild dilatation of the right pyelum, X-thorax was normal, cranial ultrasound was normal and swallowing X-ray revealed normal swallowing, no reflux and normal anatomy. Tube feeding was started because oral intake

further declined. Discomfort improved after starting continues tube feeding indicating a motility problem. Anti-reflux medication, thickened feeds and Infantrini (high caloric milk from nutricia) has further helped to get her more comfortable. Now at the age of eleven months she is totally fed by tube feeding. She does still vomits very easily and has chocking incidences. Since oral feeding is not expected to be possible soon percutaneous endoscopic gastrostomy will be placed. There where mild contractures of the hips and knees in the first moths of life that have spontaneously resolved. She was a hypotonic but has normal muscle tone now. She reached her motor milestones with a slight delay. She is treated with frequent physiotherapy and now at the age of 11 months has just started to sit without support and to crawl. She has no words jet but does babble. Her development is at the p10 for age. Social- emotional development seems delayed as well. She smiled very little and had poor eye contact. She just started to show clear recognition of her parents and makes better interaction with them now. She was restless, hyperactive and hypersensitive. She Except for strabismus eye examination did not reveal abnormalities. She has frequent upper airway infections and has a lot of mucus. Physical examination at the age of 11 months showed a height of 72 cm (p25) weight of 8150 gram (p16) and a head circumference of 42.5 cm (p1). She has a normal muscle tone and is active. She has a short forehead and a mild asymmetry of the face. We observe a mild ptosis, arched eyebrows, thelecantal folds, a deep nasal bridge, low set ears that have thick helix, a full nasal tip, a short philtrum and micrognatia. There are no abnormalities of the spine, hands or feet.

Genetic testing: SNP-Array showed a small intragenic deletion of exon 6 of the AUTS2, arr7q11.22(70,049,056-70,197,417)x1 (hg19). This deletion is predicted to cause a frame shift from the full length AUTS2 transcript. Array and FISH showed that the deletion did occur de novo and that there was no balanced chromosomal aberrations in the parents that had caused this deletion.

Patient 6 (deletion of exon 6, de novo)

The history of this proband was described in "Two male adults with pathogenic AUTS2 variants, including a two base pair deletion, further delineate the AUTS2 syndrome." By Beunders et al. (2014). He is proband 2 in this article. Re-evaluation at the age of 23 years and a more detailed interview with the parents by GB revealed the following:

As a baby patient 6 had feeding difficulties. He had sucking problems with breastfeeding, and was slow in bottle feeding. Weaning to solids was very difficult and he did not gain weight properly. He only ate when distracted by toys and vomited frequently after a meal. After two years of age the feeding problems resolved spontaneously, although slow weight gain remained.

His motor mile stones where delayed and reached only after intensive physiotherapy. Toilet training took a long time and he had a hyperactive bladder for which he used Minerin with success. He was unable to attend a normal school because of his cognitive developmental delay and behavioural problems (egocentric behaviour, claiming to adults and stereotypic behaviour). Patient 6 was psychologically tested five times in childhood. In all tests his verbal IQ (ranging

from 71 to 101) was significantly higher than is performal IQ (ranging from 55 to 70). The Autism Diagnostic Interview-Revised (ADI-R), Child behavioural Check List (CBCL) and observations by a child psychiatrist showed high scores for anxiety, unsociable behaviour and repetitive behaviour. He showed very little interaction with pears, little emotion, was unable to make a conversation about mutual interests and clingers to adults. He had a disorder in verbal and non-verbal communication and showed clear repetitive behaviour and stereotypic interests. On the last domain of autism, social interaction he scores relatively well. Therefore he was not diagnosed with classical autism but with PDD- NOS. He was helped by clear structure and one to one attention. He needed a lot of stimulation to learn, but has learned to write and read. Now at age of 23 years he is attending a day care where he performs simple jobs (pouring tea for example). He is planning to live on his own soon. He has some pears and his social interaction has improved. He however still
has obsessive interest and shows repetitive behaviours. There are no medical problems at this moment.

Genetic analysis: Array analysis revealed a small intragenic deletion encompassing exon 6 of AUTS2, arr 7q11.22(69,991,859-70,221,259x1)dn (hg19) (submitted to www.LOVD.nl/AUTS2 (patient ID 0016366)). This deletion is predicted to cause a frameshift of the full length AUTS2 transcript and does not affect the shorter 3' transcript. Parental studies did not reveal this deletion suggesting a de novo event. [2]

Patient 7 (deletion exon 6-9, father tested negative, mother not tested)

Proband 7 is a 40 year old woman. She was born at a gestational age of 42 weeks after an uneventful pregnancy, but had a slow start (with an apgar score of 5 after 1 minute). She had a birth weight of 3690 gram (p55). In the neonatal period she was tachypnoeic, hypotonic and inactive. In the first months of her life she had feeding difficulties, failure to thrive and needed tube feeding. Physical examination at that time revealed low muscle tone, an upslant of the palpebral fissures, strabismus and an expressionless face. In childhood (until 7-10 years) she suffered from recurrent upper airway infections. Furthermore autistic behaviour was noticed and she had a developmental delay. At the age of 11 years a scoliosis was diagnosed. In the following years she developed progressive kyphosis as well for which she was treated whit traction and redressing plaster- shells. She used a brace for some time, but now the scoliosis and kyphosis is stable and needs no treatment. She came to our attention at the age of 32 for a diagnostic evaluation of the intellectual disability. At that time she made no eye contact and used single words mainly. She had mild aggressive behaviour (shouting and throwing with things) in situations with a lot of incentives. Physical examination revealed a height of 157 cm (p0.1), a weight of 56 kg (p80) and head circumference of 51.5 cm (p1), low frontal and dorsal hairline, a short forehead, highly arched eyebrows, short palpebral fissures, ptosis, a prominent nasal tip, a short and prominent philtrum, retrognatia and a kypho-scoliosis. Furthermore she has slender hands and feet, a sandal gap and absent creases at DIP joints of digitus IV, the other palmar creases of the DIP joints are very shallow.

We re-examined here at the age of 40 years. She was doing well. She did not show aggression any more and shows better social interaction since she moved to a more quiet sheltered home. She visits day care where she likes to make handicraft and listen to music, she is cheerful and liked to sing. She shows stereotypic behaviour, like asking the same questions over and over again and she fumbles at here clothes. She is very sensitive to incentives and dislikes busy places. She is not able to write of read, she is independent in self care skills, but is not able to perform a job because of her limited social and cognitive development. She has eczema and has had many dental carriers. There are no problems with eating. She is in good health and the last blood analysed did not show signs of diabetes, vitamin deficiency, liver failure or kidney failure. Physical examination did show the same dysmorphic features, and some weight gain, her weight being 61 kg (p90). She had a rather high muscle tone in both legs and normal to low deep tendon reflexes. Her walking pattern is stiff and she does not unroll her feet very well. She has hammer tows and narrow flat feet.

Genetic analysis Chromosomal analysis did not reveal any abnormalities in 30 cells. Further genomic analysis by array CGH revealed an intragenic deletion of the AUTS2 gene on chromosome 7q11.22 (chr7:69,596,512-69,878,246) (hg18). Because of a gap between the deleted and not deleted probes the exact distal breakpoint could not be defined. Further breakpoint analysis by MLPA revealed a deletion of exon 6 to 9, which results in a deletion of 333 amino acids of the Auts2 protein (999 bp) leaving the reading frame of the long transcript in tact and deleting the translation start site of the short transcript starting in exon 9. Array CGH of her normal developed father showed no deletion in AUTS2, her mother was not available.

Patient 8 (frameshift mutation in exon 7, de novo)

Patient 8 was described before in "Two male adults with pathogenic AUTS2 variants, including a two base pair deletion, further delineate the AUTS2 syndrome." By Beunders et al. (2014), where he is proband 1. Re-evaluation and detailed interview with the parents by GB at the age of 28 years of age revealed the following extra information. Proband 8 was very slow in bottle feeding and had a lot of chocking incidences. He had gastroesophageal reflux and vomited a lot. These problems resolved spontaneously after the age of one year. Poor weight gain remained however and he uses high caloric liquid food supplements, but stayed at the a weight of below -2 SD. Behaviour was characterized by hyperactivity and hypersocial behaviour as a child. He showed increased interaction with strangers and approached them like friends. He showed stereotypic movements and had obsessive behaviour. He was diagnosed with PDD-NOS and his IQ was measured at 45. He started stammering as a teenager. Before that he used full sentences, but now stammering hampers him and he mostly uses single words. He has just started with speech therapy again hoping to reduce the stammering. Now at the age of 28 years he lives in a assisted living apartment and works in a sheltered work environment. He is not able to write or read, he can however recognize words and knows his way on the Internet to find information about musicals for which he shows an obsessive interest. He is drawn back and quiet, tidy, friendly and correct. Except for obsessive behaviour he has

no behavioural problems at this moment. Physical examination showed unchanged dysmorphic features. He has shallow dorsal creases at his DIP joints especially of the fourth and fifth fingers and hollow feet with hammer toes that where not noticed before.

Genetic analysis:

Whole exome sequencing revealed a two base pair deletion in exon 7 of AUTS2, c.857_858delAA (p.(Lys286fs)) (hg 19). This mutation causes a frameshift of the full length AUTS2 transcript but does not harm the shorter transcript that starts in exon 9. An other de novo variant was found in ABI2, c.1223C>T, p.(Pro408Leu) (hg19). It is very likely that the AUTS2 variant causes the phenotype in this individual because of the resemblance of the clinical features of proband 8 to individuals with AUTS2 deletions. It can however not be excluded that the de novo variant in ABI2 has an additional effect on the phenotype. [2]

Patient 9 (mutation, c.317C>T, p.(Gln107*), de novo)

Proband 9 is a seven year old girl. She is the third child of healthy non-consanguineous parents. She has two older brothers that are healthy and have no learning problems. During pregnancy there was an elevated risk to Down syndrome with the combination test. Therefore an amniocentesis was performed that did not show abnormalities. She was born á term after a caesarean section on maternal indication. She had a birth weight of 2990 gram (p9) and a head circumference of 43 cm (<p0,4). She had mild hypotonia, feeding problems and a failure to thrive in the first year of live. bottle feeding was slow, weight gain poor and she got extra caloric milk. Weaning to solids was very difficult, as she did not want to eat from a spoon. With a lot of effort from parents this was overcome and she eats well now.

She has lacrimal duct stenosis for which she underwent surgery. There were no other congenital malformation. She had recurrent otitis media, but hearing was normal. Her motor development was normal. She learned to walk at the age of 17 months. She did however walk on her toes. Although she spook single words from the age of 1,5 years, she has a severe expressive speech delay now. Language perception is better. She still uses single words mostly and she has dysarthria. She is not toilet trained yet and can not dress herself yet. Her developmental age is estimated at 3,5 years. She is hyperactive, has an attention disorder, is impulsive and shows repetitive behaviour. She is hypersocial to adult strangers. Furthermore she is hypersensitive to touch and inappropriately smells or touches objects and foods. Brushing therapy was started with a positive effect. There where severe sleeping problems, and still falling a sleep and staying asleep is a problem. Installing a stable door in her room so she can play in her room in the middle of the night, reduced the impact for the family.

At physical examination at the age of seven years we see a hyperactive curious girl. She has mild dysmorphic features, namely: brachycephaly, mild asymmetry, ptosis, short palpebral fissures, a broad nasal base, a short philtrum and a simple protruding left ear. Her hands are slender, she has no campotodactily but does have shallow bending folds of the dorsal side of the PIP joints and a

supination limitation on both wrists. There is a high muscle tone in both legs and her deep tendon reflexes are all low. She has a broad based gait.

She was seen again at the age of 9 years. Her speech has improved, as she is now using hundreds of words and puts together some short sentences. She is completely toilet trained during the days. She remains very sociable and active with no sense of danger. She was formally assessed for autism, but she does not fulfil the criteria for classical autism, but she has autistic traits and is diagnosed as having an autistic spectrum disorder. She is diagnosed with ADHD. Physical examination revealed the same dysmorphic features as before and a height of 129 cm (p25), a weight of 28,1 kg (p25) and a head circumference of 47,8 cm (<p0,6).

Genetic analysis Array had not shown abnormalities. Whole exome sequencing of this girl and her parents showed a de novo mutation in exon 7 of the full length AUTS2 transcript (ENST000004164) at position 70228089, c. 317C>T, p(GLN107X) (hg19). No other abnormalities where found.

Patient 10 (intragenic AUTS2 deletion of exon 15-17, not maternal)

This young woman of now 16 years old was referred for genetic testing because of intellectual disability. She is born at a gestational age of 41 2/7 week and had a good start. Except for proteinuria the pregnancy was normal, as was delivery. She had a birth weight of 3275 gram (p9). She was hypotonic as a baby and she had feeding difficulties. At the age of two months she had a length of 56 cm (p50), a weight of 4,6 kg (p2) and a head circumference of 38.5 (p40). She had a poor suck, was slow in bottle feeding and had poor weight gain. Parents fed her frequently with small portions for the first year. Eating solids was better, but she vomited a lot. Feeding problems have resolved but she still eats small portions, eats slow and she prefers soft foods. General health problems where: frequent upper airway infections until the age of 7 years, vitamin D deficiency and eczema. She had a few bladder infections. She has a normal regular period since she was 13 years. She has normal hearing and normal vision. She has a mild to moderate intellectual disability with a developmental age of about 8 years now at the age of 16. Her milestones where not delayed: she started walking and spoke her first words at the age of 15 months. She had poor pronunciation for a long time and stammered. She was diagnosed with an autism spectrum disorder. She showed a lot of stereotypic hand movements in childhood that are far less now. She has repetitive behaviour, has trouble to sympathize and favours routines. She makes good contact though and is a considerable, sweet and friendly. She shows hypersocial behaviour and can act to strangers as if they are friends. She is hypersensitive to loud noises and smell. She sleeps well now but had a period in which she frequently wake up at night. She is fearful and has nightmares frequently. Physical examination at the age of eleven she had a height of 135,2 cm (p9), a weight of 29,5 (p50) and head circumference of 50,5 (p2). She was brachycephalic, has a round face, highly arced eyebrows, proptosis, ptosis, downslanting palpebral fissures, broad nasal base, short philtrum and micro-/retrognatia. Now at the age of 16 years physical examination revealed a head circumference of 52 cm (p2). The facial dysmorphisms are unchanged. Her hands show clinodactily of both little fingers and normal dermatoglives. She

has shallow bending folds of her dorsal DIP joints. She has a scoliosis, tight heel cords a high muscle tone of both legs, mild pes cavus and a normal to low muscle tone of both arms.

Genetic testing: Array revealed a small intragenic deletion within AUTS2 at 7q11.22 (70,246,372-70,251,118) that deletes exon 15-17 of AUTS2 (hg19). This deletion is predicted to cause a frameshift of the full length and short '3 transcript of AUTS2. The deletion was not found in the mother. Her father was not tested.

Patient 11 (whole gene deletion, de novo)

This six year-old boy was referred to Clinical Genetic because of developmental delay. He is the first child of healthy non-consanguineous parents; he has a younger brother and sister who are healthy and have no developmental problems. The pregnancy was complicated by first trimester bleeding. The combination test showed a low risk on Down syndrome. Delivery was uneventful, at 42 weeks gestation, with a birth-weight of 3577 grams (p15). There where no congenital malformations accept for a laryngomalacy for which he had a supraglottoplasty aged 18 months. He was hypotonic, but this is improving now. He had an admission for bronchiolitis aged seven months.

He had feeding difficulties: bottle-feeding took a long time and weaning to solids was difficult. He was over two years-old before he could eat solids; at the time of writing, he still has difficulty chewing and frequent chocking incidences. He has had episodes of rectal prolapse. He had frequent otitis media, requiring three grommet insertion procedures. He had eczema and he had amblyopia, which has has improved now.

He has a developmental delay: he could stand alone at the age of 12 months and walked without support at the age of two years. He spoke his first words between 18 and 24 months
of age. Now, at the age of six years, he seems to have a developmental age between four and five years. He visits a normal school with extra help and learns to write and read. He has concentration problems and can be hyperactive and impulsive. He has no sense of danger and had a period of severe tantrums, especially at school, which were apparently unprovoked and involved throwing things. He shows little to no interaction with peers, but makes good contact with adults. However, he does show hypersocial behaviour, as he can show similar behaviour to strangers as to familiar people and can inappropriately hug strangers. He has repetitive speech, but does not show obsessive/compulsive behaviour and he can cope with sudden changes of routine. He is hypersensitive and constantly smells and feels new objects or surroundings. Physical examination at the age of six years and four months revealed a cheerful boy, who made good eye contact. He showed his difficulty in sensing appropriate distance to strangers and gave intense cuddles to the physician on seeing for the first time. He has a slender build: height of 114 cm (p25), weight of 15 kg (p0,1) and a head circumference of 51 cm (p25). He has mild facial dysmorphisms with arched eyebrows, proptosis, a broad nasal bridge, short philtrum, small teeth and mild retrognatia. His hands are slender and he has long fingers with normal bending folds. Orientating neurologic exam did not show any abnormalities, although his movement is a bit immature. He has normal muscle tone and his deep

tendon reflexes are normal.

Genetic analysis: Array CGH showed a male profile with an interstitial deletion on 7q11.22 from 67,445,895 to 70,356,041, deleting the whole AUTS2 gene and no other genes (hg19). FISH analyses confirmed the deletion and showed no abnormalities in the parents, indicating a de novo deletion.

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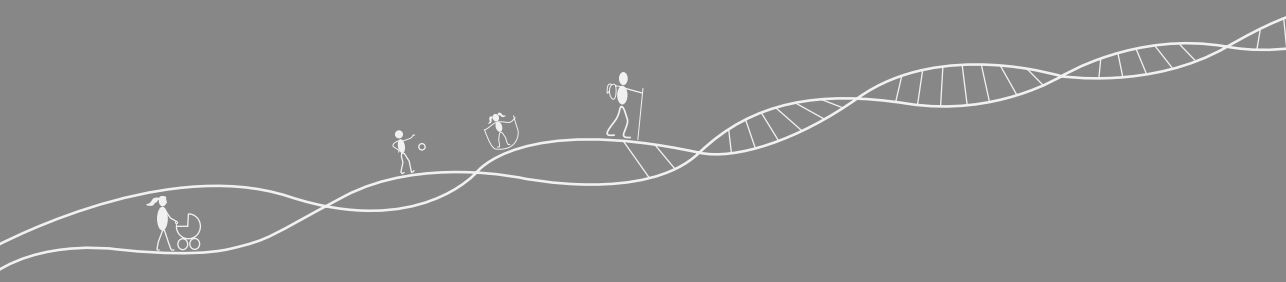
Chapter 6

How to inform parents of patients with intellectual disability about new technical possibilities?

Recontacting in light of new genetic diagnostic techniques for patients with intellectual disability: Feasibility and parental perspectives.

Beunders G, Dekker M, Haver O, Meijers-Heijboer EJ, Henneman L



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ABSTRACT

A higher diagnostic yield from new diagnostic techniques makes re-evaluation in patients with intellectual disability without a causal diagnosis valuable, and is currently only performed after new referral. Active recontacting might serve a larger group of patients. We aimed to evaluate parental perspectives regarding recontacting and its feasibility in clinical genetic practice. A recontacting pilot was performed in two cohorts of children with intellectual disability. In cohort A, parents were recontacted by phone and in cohort B by letter, to invite them for a re-evaluation due to the new technologies (array CGH and exome sequencing, respectively). Parental opinions, preferences and experiences with recontacting were assessed by a self-administered questionnaire, and the feasibility of this pilot was evaluated.

47 of 114 questionnaires were returned. In total, 87% of the parents believed that all parents should be recontacted in light of new insights, 17% experienced an (positive or negative) emotional reaction. In cohort A, approached by phone, 36% made a new appointment for re-evaluation, and in cohort B, approached by letter, 4% did.

Most parents have positive opinions on recontacting. Recontacting might evoke emotional responses that may need attention. Recontacting is feasible but time-consuming and a large additional responsibility for clinical geneticists.

INTRODUCTION

One of the goals of a clinical genetic evaluation is to make a causal diagnosis in patients with intellectual disability. This can be helpful for parental acceptation of having a child with a disorder and to fulfill their 'need to know', to oversee the prognosis for the child, to guide follow-up and management, and to assess the recurrence risk in future pregnancies.

Technological improvement in genetic tests makes it increasingly possible to make a causal diagnosis. Where ten years ago karyotyping and directed resequencing of single genes was common practice, now high-resolution SNP array and trio exome sequencing have made their entrance into the clinical genetic practice. This has increased the diagnostic yield from 10% to about 30%.[1]

As these new techniques tend to develop every few years, re-evaluation and additional genetic testing using these new techniques is valuable for children with intellectual disability without a diagnosis and their parents.[2] Currently, the initiative for a new appointment to re-evaluate a child with these new techniques often resides by the patients and their families. Active recontacting does take place ad hoc, but structural recontacting is rare.[3] A number of ethical, legal and (psycho)social issues have been raised considering active recontacting patients. A recent review on 'the duty to recontact' addresses these topics.[4] Ethical and legal issues were often proposed as arguments in favor of the duty to recontact, whereas (psycho)social issues and practical barriers were proposed as counterarguments.[4; 5]

Very little is known about the opinions of patients or their parents about recontacting, and about the experiences in practice.[4] Empirical studies that explored how to perform recontacting and patient or parental opinions on the recontacting that had taken place were performed in only a few different patient cohorts: Fragile X families,[6] cancer genetics patients,[7-9] and families that lost a child with a mitochondrial disorder.[10] These studies, including one study on patients (or parents of patients) with different conditions,[11; 12] revealed a generally positive attitude towards recontacting in genetics among patients or their parents.

Since our aim was to get a better understanding of parental perspectives on recontacting, we informed parents of patients that had visited our clinic because of their intellectual disability about the availability of new technical diagnostic tools, and offered re-evaluation and additional genetic testing. We evaluated parents' general opinions about recontacting, and were especially interested in their experiences with and preferences concerning the way of recontacting, and whether recontacting was experienced as burdensome. Additionally, we wanted to study the feasibility of recontacting and the effect of recontacting by looking at the percentage of parents that could be reached and the percentage of patients that indeed made a new appointment.

MATERIALS AND METHODS

A pilot study in recontacting patients was performed and parents' experiences, opinions and preferences were evaluated using a self-administered questionnaire. For this study, we recontacted two cohorts: cohort A was contacted in 2010 by phone and cohort B in 2015 by a letter to inform them about new techniques (array CGH and exome sequencing, respectively). Both techniques result in a higher diagnostic yield when compared to other techniques already in use. We invited the parents in cohorts A and B for re-evaluation of their child at the Clinical Genetics Department of VU University Medical Center (VUMC) (Fig. 1). Ethical approval for this study was granted by the Medical Ethical Committee of the VUMC Amsterdam.

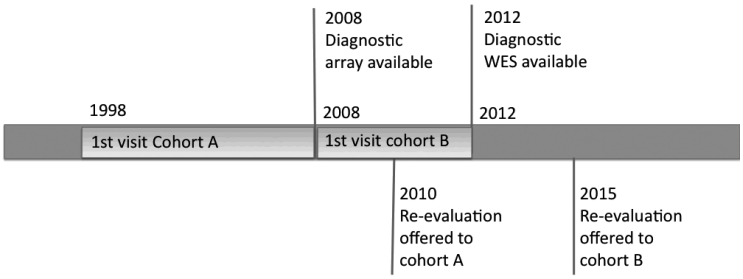

Figure 1. *The timeline gives an overview of the timing of the first visit, the introduction of new techniques and moment of recontacting during this study for the cohorts A and B.*

Cohort A

We selected a patient cohort, A, by using a Fragile X diagnostic DNA laboratory registry at our hospital DNA diagnostic laboratory. Fragile X testing was routinely performed in all boys and most girls with intellectual disability. All children that tested negative for Fragile X and in which clinical genetic counseling was performed between 1998 and 2008 were selected. A total of 297 children were selected and the medical records were reviewed to see if they indeed had intellectual disability, if another causal diagnosis was identified and if an array Comparative Genomic Hybridization (CGH) had been performed already. Inclusion criteria were: age of the patient between 4-18 years, intellectually disability or developmental delay, negative Fragile X testing result, and normal karyotyping result. Exclusion criteria were: a causal diagnosis and a previously performed array.

The parents of eligible patients (n=151) were approached by telephone by a clinical geneticist in training (G.B.) to inform them about the availability of a new technique with a higher diagnostic yield (array CGH) and were invited for re-evaluation at our outpatient clinic. The information about the higher diagnostic yield, the chance of finding variants of unknown significance, and the small chance of unsolicited findings were mentioned. Furthermore, practical information about the appointment and test was given.

If during the telephone call informed consent for the questionnaire study was given, a

questionnaire was sent to the parents, irrespective of their wish for a genetic re-evaluation of their child.

Cohort B

Worldwide, clinics have started to use next generation sequencing (NGS) on a more routine basis. The Department of Clinical Genetics of the VUMC added whole exome sequencing (WES) to their range of genetic testing resources for diagnostics at the beginning of 2012.[13] We again performed a recontacting pilot study in 2015. We now contacted parents by sending them an information letter about the possibility of re-evaluation of their child with unexplained intellectual disability and the possibility of additional genetic testing using WES. The written information on WES was comparable to the information about array CGH given in cohort A: it informed parents about the higher diagnostic yield, the chance of finding variants of unknown significance, and the small chance of unsolicited findings. The questionnaire to evaluate recontacting and a consent form for the questionnaire study were also included. Parents were asked to send back the filled-out questionnaire or an answer card if they did not want to participate in the questionnaire study.

The selection of cohort B was made based on the array requests from 2008 up to and including 2012 because of intellectual disability (n=139). Medical records were analyzed to see if patients met the inclusion criteria: mild syndromic intellectual disability or moderate to severe syndromic or non-syndromic intellectual disability, normal array result, normal Fragile X testing result. They were excluded when a causal diagnosis was known, WES had already been performed, or when they had been included in cohort A of this study. In total 52 patients were selected to be recontacted by letter, presenting the number of patients included in cohort B after application of exclusion criteria. We chose not to maintain the age-related inclusion criteria in 2015, as the sample size was already very small. All addresses were checked to ensure that the letter was sent to their current address.

Evaluation of recontacting (feasibility)

Notes were made to evaluate feasibility of the study on, among others, the attainableness of parents, time effort to select suitable cases, and the number of tries before parents could be reached by phone. Moreover, records were made in Excel of parents who responded to the invitation for re-evaluation, allowing us to calculate the proportion of parents that made a re-evaluation appointment.

Survey instrument

The questionnaire was specifically developed for this study. Topics addressed were based on literature data on recontacting. The questionnaire consisted of 21 questions and it took approximately 15 minutes to complete. Respondents were questioned about demographic characteristics including the age of the patient and the respondent, information on siblings, and the severity of the child's developmental delay as perceived by the respondent. Questions on parents' opinions and experiences regarding recontacting in genetics in light of new insights and technological advances

were included. These were two general statements "All (parents of) patients should be recontacted in light of new insights" and "I would like to be recontacted when new insights emerge", and three statements about the recent approach (by phone or letter) to inform them on the possibility of additional genetic testing: "I was pleased to be recontacted for follow-up genetic testing"; "I was pleased with the method of recontact (by letter/by phone)"; and "the recontact letter/phone call regarding additional genetic testing evoked emotional feelings". All these items were answered on a five-point Likert scale (strongly disagree (1) to strongly agree (5)). To get a better view of the impact of recontacting, parents were asked what emotions they felt when being recontacted and what would be reasons to appreciate or not to appreciate being recontacted. To learn about the effect of recontacting, parents were asked whether they were planning to make a new appointment, and for what reason they would accept or decline further genetic testing (these questions were multiple choice and giving more than one answer was possible).

Two general questions on motives for genetic testing were included, asking for the reason for genetic testing in the past and for their estimate of the chance of finding a cause of the intellectual disability in their child. Moreover, they were asked in which situation it would be appropriate to be recontacted, who they thought was responsible for informing parents on new insights or technological possibilities in genetics, and their preferred method of recontact. Finally, parents were asked in an open question whether they had suggestions to improve the way of contacting parents to inform them about new insights.

Statistical analyses

Descriptive statistics were used to describe the characteristics and the answers to the questions. Answers to 5-point Likert scales were compressed into a 3-point scale: (1) (strongly) disagree; (2) neither disagree nor agree; and (3) (strongly) agree. Levene's test, student's t-test, χ^2 and Fisher's exact test were used to compare study population characteristics. If no statistically significant differences in were found between the cohorts, cohorts A and B were combined and considered to be one study population for further analysis. The Mann-Whitney U test was used to test if the positive parental attitudes towards recontacting in genetics was associated with the fact that they had planned re-evaluation or not, and if there were differences in attitude between the two cohorts. We chose this test because of the negatively skewed outcome variables. In all analyses, a p value of < 0.05 was considered to indicate statistical significance. Data were analyzed using IBM SPSS for Windows Version 22.0.

RESULTS

Feasibility

The active recontacting was very time-consuming, especially selecting the suitable patients took a lot of time. For 49% and 63% of the selected patients (146/297 in cohort A and 87/139 in cohort B, respectively) recontacting did not make sense, for example because they did not have intellectual

disability, they already had a causal diagnosis or because they had recently been re-evaluated (Fig. S1 and S2). Furthermore one-third (54/151) of the parents in cohort A could not be reached after calling three times and/or did not use the available telephone number anymore or were not available because the child lived with other caregivers or they had passed away. In cohort B eight out of 52 addresses were no longer correct and the right address could not be found.

More than half (57%) of all 94 eligible parents that we were able to inform by phone about the new technological possibilities for genetic testing in cohort A wanted re-evaluation for their child with intellectual disability. For these 54 patients, an appointment was planned. The phone call in cohort A enabled tailored information to be given to parents about their new appointment. Parents asked questions about the duration or location of the consultation, the need for taking a new blood sample, details about the new technique and the possible test results. In cohort B, six out of 44 parents (14%) that were recontacted by letter indicated in the questionnaire that they intended to make an appointment, but only two of them did so in the year after the information was received.

The total yield of our effort to actively recontact parents for re-evaluation at the clinic (number of patients that made a re-evaluation appointment) was 36% when parents were informed about new techniques (array) via a phone call (54 of 151 included in the pilot cohort A), and about 4% when recontacted by letter (WES) (two out of 52 included in the pilot cohort B). See Fig. S1 and S2. Parents often had more than one reason to want re-evaluation or additional genetic testing; the reasons most frequently mentioned were: the hope that a diagnosis would result in better organized care for their child (n=27), or because of a desire for an addition to the family on the part of parents or siblings of the child with intellectual disability (n=20).

Response rates and demographic characteristics of respondents

Of the 94 parents reached by phone (cohort A), 70 consented to receiving a questionnaire (this was not influenced by the wish for re-evaluation of their child). In cohort B, 16 consent forms were returned, ten parents consented to participate in the questionnaire study and six did not. Of all the parents that were recontacted, 39% (37/94) in cohort A and 23% (10/44) in cohort B returned a questionnaire (see Fig. S1 and S2). Characteristics of respondents to the questionnaires are shown in Table 1. Overall, the mean age of respondents (parent) was 45.8 years. As both cohorts were selected on different criteria, the average age of patients in cohort B was higher than in cohort A and more patients in cohort B were severely affected.

Reasons for not participating in the pilot study were: parents were unavailable or unable to participate, they felt no need for a causal diagnosis at that moment as the child with intellectual disability was doing well, or there was no active wish to have an additional child, because of the perceived burden of a new appointment for the child with intellectual disability, or because parents had accepted the situation. Of all 47 parents who completed the questionnaires, 32 made an appointment for re-evaluation.

Table 1. *Characteristics of the respondents in cohort A and cohort B. **

	Total N = 47	**Cohort A N = 37**	**Cohort B N = 10**
Mean age of respondent, years (range)	45.8 (26 – 69)	44.9 (35 – 54)	49.5 (26 – 69)
Mean age of patient, years (range)*	15.3 (7 – 55)	13.1 (7 – 18)	23.3 (7 – 55)
Does the patient have siblings, n (%)			
Yes	39 (83)	30 (81)	9 (90)
No	8 (17)	7 (19)	1 (10)
Living situation patient, n (%)*			
With both parents	32 (68)	28 (76)	4 (40)
With father only	1 (2)	1 (3)	0 (0)
With mother only	8 (17)	6 (16)	2 (20)
Supported housing	6 (13)	2 (5)	4 (40)
Severity of developmental delay of child as perceived by parents, n (%)* (missing answer n=1)			
Mild	10 (22)	9 (25)	5 (50)
Moderate	21 (46)	19 (53)	1 (10)
Severe	12 (26)	5 (14)	4 (40)
No longer applicable	3 (7)	3 (8)	0 (0)

Significant difference between cohort A and B, p<0.05.

Parental opinions on recontacting

Overall, 87% of respondents thought that all parents should be recontacted when new information or techniques became available, and 89% wished to be recontacted themselves (Table 2). Only one respondent did not want to be recontacted him/herself. Eighty-three percent of the parents were pleased to be recontacted in this pilot. Eighty-five percent of the parents were pleased with the method of recontacting (phone call or letter) to inform them about follow-up genetic testing. Parental attitude towards being recontacted was not influenced by the cohort (p=0.9). Parents that did come for a re-evaluation of their child seemed more positive than the parents that did not make an appointment for re-evaluation. These differences are however not significant (Mann-Whitney U test: p=0.7) (see Table 3).

Seventeen percent of parents reported that they had responded emotionally by the unexpected recontact (Table 2). Emotions that were mentioned were positive (hopeful n=5; cheerful n=3; relief n=2) as well as negative (grief n=3; anger n=2; fear n=2, frustration n=1).

Parental preferences

Most parents of patients with intellectual disability (69%) believed that the clinical geneticist is responsible for keeping them informed on new diagnostic possibilities. Only 11% of the respondents felt that it is their own responsibility (Table 4).

Table 2. *Parental opinions on and experiences with recontacting, n=47.*

	(Strongly) disagree n (%)	Neither agree nor disagree n (%)	(Strongly) agree n (%)
All (parents of) patients should be recontacted in light of new insights	0	6 (13)	41 (87)
I would like to be recontacted when new insights emerge	1 (2)	4 (9)	42 (89)
I was pleased to be recontacted for follow-up genetic testing (missing answer n=1)	1 (2)	7 (15)	38 (83)
Cohort A	1 (3)	5 (14)	30 (83)
Cohort B	0	2 (20)	8 (80)
I was pleased with the method of recontact (missing answer n=1)	1 (2)	6 (13)	39 (85)
Cohort A	1 (3)	4 (11)	31 (86)
Cohort B	0	2 (20)	8 (80)
The recontact letter/phone call regarding additional genetic testing evoked emotional feelings	24 (51)	15 (32)	8 (17)
Cohort A	19 (51)	11 (30)	7 (19)
Cohort B	5 (50)	4 (40)	1 (10)

Table 3. *Parents' experience with recontacting in relation to the wish for further genetic evaluation, n=47.*

	I was pleased to be recontacted for follow-up genetic testing		
	(Strongly) disagree n (%)	Neither agree nor disagree n (%)	(Strongly) agree n (%)
Accepted follow-up genetic evaluation (missing answer n=1)	1 (3)	3 (10)	27 (87)
Did not accept follow-up genetic evaluation	0 (0)	4 (27)	11 (73)

The differences between those that did accept follow-up genetic evaluation and those that did not are not significantly different (Mann-Whitney U test: p=0.6).

Seventy percent of parents believed that a recontact is valuable only when new techniques and/or new information becomes available. Only one respondent felt that regular contact with their clinical geneticist would be valuable. Most parents preferred a letter (49%), while 42% of the respondents found an email a good way to be recontacted as well (see Table 4).

Table 4. *Parental preferences on recontacting, n=47.*

	n (%)
Who is responsible for recontacting? (missing answer n=2)	
Clinical geneticist	31 (69)
General practitioner	16 (36)
Pediatrician (or other specialist)	15 (33)
Patient's (or parent's) own responsibility	5 (11)
In which situation? (missing answer n=1)	
New (technical) methods	32 (70)
New available information	32 (70)
Regularly, even if there is no news	1 (2)
Which is the preferred method? (missing answer n=2)	
Letter	22 (49)
Email	19 (42)
Telephone	14 (31)
Website	8 (17)

Results of cohorts A and B were combined. Percentages do not add up to 100% as more than one answer could be given.

DISCUSSION

Most parents we recontacted to inform them about new technical possibilities and to invite them for follow-up genetic testing were very positive about recontacting in general, and had positive experiences with being recontacted in this pilot. As in our study, Sexton et al.[10] also showed a positive attitude towards recontacting among parents of (deceased) children affected by mitochondrial disorder because of new technical possibilities with a higher diagnostic yield. Most studies reporting views of participants on recontacting that had taken place for this same reason involved adult research participants rather than minors in a clinical setting, however these studies indicate a similar positive attitude towards recontacting.[7-9] In a recent interview study, Dheensa et al. [12] studied the general opinion of adult patients and parents of minors with a (potential) genetic disorder about recontacting in clinical genetics for updates on variant classification, new technological possibilities or additional screening or treatment advices. Most of the 41 interviewed patients or parents had a positive attitude towards recontacting [11].

In a survey among US and Canadian genetic professionals, Fitzpatrick et al.[14] found that anxiety, stress and worries concerning health and life insurance, and intrusion of privacy were identified as possible burdens for patients if a formal system for recontacting were to be implemented. In our study, 17% of the parents indeed experienced an emotional reaction when being recontacted, most of which were positive (cheerfulness and hope) and some negative (frustration, anger, fear and grief). These emotions should be taken into account when thinking about recontacting on a more regular basis. Because of the small numbers involved, we were unable to analyze whether, for

example, long time intervals between the last visit and recontacting, or adult age of the child, might lead to more negative emotions. In contrast to Fitzpatrick et al.[14] no concerns regarding privacy or insurance issues were expressed by parents in our study. In the Netherlands, all citizens are obliged to have a healthcare insurance,[15] which is why this issue may not be considered that important to parents. Further studies on this subject may be helpful to obtain more insight into potential harm caused by recontacting.

The results of our study show that parents generally do not consider themselves as being responsible for recontacting. The vast majority reports the clinical geneticist to be responsible. In a systematic literature review by Otten et al.[4] on recontacting in the clinical genetic practice, 33 of the 61 articles included discussed the patients' duties to recontact. The general conclusion was that the responsibility to recontact is shared between healthcare professionals and patients. From empirical studies that studied both patients' and counselors' opinions in the UK, US and Canada, it can be observed that patients mostly held their healthcare professional responsible for recontacting, while healthcare professionals see a responsibility for the patients or families as well.[4] However, in the latest empirical studies from the UK, both patients and caregivers valued a 'joint venture' model, sharing responsibility [11; 12; 16] which is in line with a general tendency toward shared decision making in healthcare.[17] Earlier, Hirschhorn et al.[18] for the Social Ethical and Legal Issues Committee of the American College of Medical Genetics considered the role of primary care physicians in informing patients or families on the need to recontact their clinical geneticist, while only 36% of respondents in our study and 19% in the study of Griffin et al.[7] felt that general practitioners should be involved in recontacting in genetics. No clear overall preference was found for the way parents wanted to be recontacted in our study, although recontacting by letter or by email was preferred the most. The use of email might be a good alternative but we did not test this.

The process of active recontacting appeared to be very time-consuming. Especially the selection procedure to find patients for which recontacting was thought to be sensible was labor-intensive, as no direct data were available on referral reasons, unsolved cases and parental willingness to receive new information. We feel active recontacting can be feasible if those data were readily available by collecting and storing them centrally after each counseling session. Hampel[8] suggested keeping a detailed clinical database and DNA banking linked with social security numbers to keep track of potential address changes. However, this may lead to privacy issues and has a lot of practical concerns as well.

In addition to patient selection, reaching patients and/or their parents and providing them with suitable information is another challenge. Recontacting by sending an information letter is faster than informing parents by phone. A phone call however gave parents the opportunity to ask questions directly and to make an appointment. Parents had practical questions and questions regarding content, for example: 'When can the appointment take place?'; 'Is a new blood sample necessary?'; 'How long will an appointment take and where will it be?'. This approach seems more effective as the effect rate (percentage of follow-up appointments after recontacting attempts) was

36% in cohort A (parents that were approached by phone about array) and 4% in cohort B (parents approached by letter about WES). These last results are in line with former pilots in recontacting by information letter, where 7%[8] and 4%[9] made a new appointment or contacted the genetic services for a new test.

Parents find it burdensome for their child with intellectual disability to visit the outpatient clinic especially if there are behavioral problems or when the intellectual disability is severe. They therefore might be more willing to perform additional genetic testing if their child with intellectual disability would not have to visit the outpatient clinic again. We did not formally test this in this study, but this was one of the reasons given on the telephone by parents to decline the invitation for additional genetic testing.

This study did not focus on cost and effectiveness. Before introducing active recontacting on a larger scale, these issues need to be addressed, as introducing recontacting programs will be a large additional responsibility for the clinical geneticist. As indicated by Carrieri et al.[19] we need to discuss recontacting with patients and caregivers to find a viable solution.

There are some limitations to this study. Firstly, the study population is quite small and the response rate to the questionnaires was low. It is likely that parents who were not interested in recontacting, who did not wish to be recontacted, who did not want to have their child re-evaluated, or who for whatever reason adopted a rather negative attitude towards recontacting, were also not motivated to return the questionnaire. These reasons might especially be applicable for cohort B, as the parents of cohort A were asked whether they would like to receive a questionnaire beforehand during the recontacting phone call. Consequently, this might have influenced the results in favor of recontacting, and selection bias cannot be ruled out. The same holds true for other empirical studies on this subject that had response rates in the same magnitude or did not have data available on the response rate.[6; 7; 9; 12] Although the positive attitude assessed here should be interpreted with some care, our data do not indicate a more negative opinion for parents that did not want re-evaluation of their child. Secondly, especially in cohort A the moment of recontacting could be several years after the last visit to the Clinical Genetics Department. Recontacting after a long period of time might evoke a different emotional reaction among parents compared to recontacting after a shorter period of time. This might have influenced the outcome of the study.

In conclusion, we present here the first empirical data on parental opinions about active recontacting because of new technological possibilities for a child with intellectual disability. Parents are generally positive about recontacting and report positive experiences. They believe that the clinical geneticist is responsible to recontact. To make recontacting feasible and efficient, clinical practice and registration of patient data should be adapted accordingly.

Acknowledgements

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SUPPLEMENTAL DATA

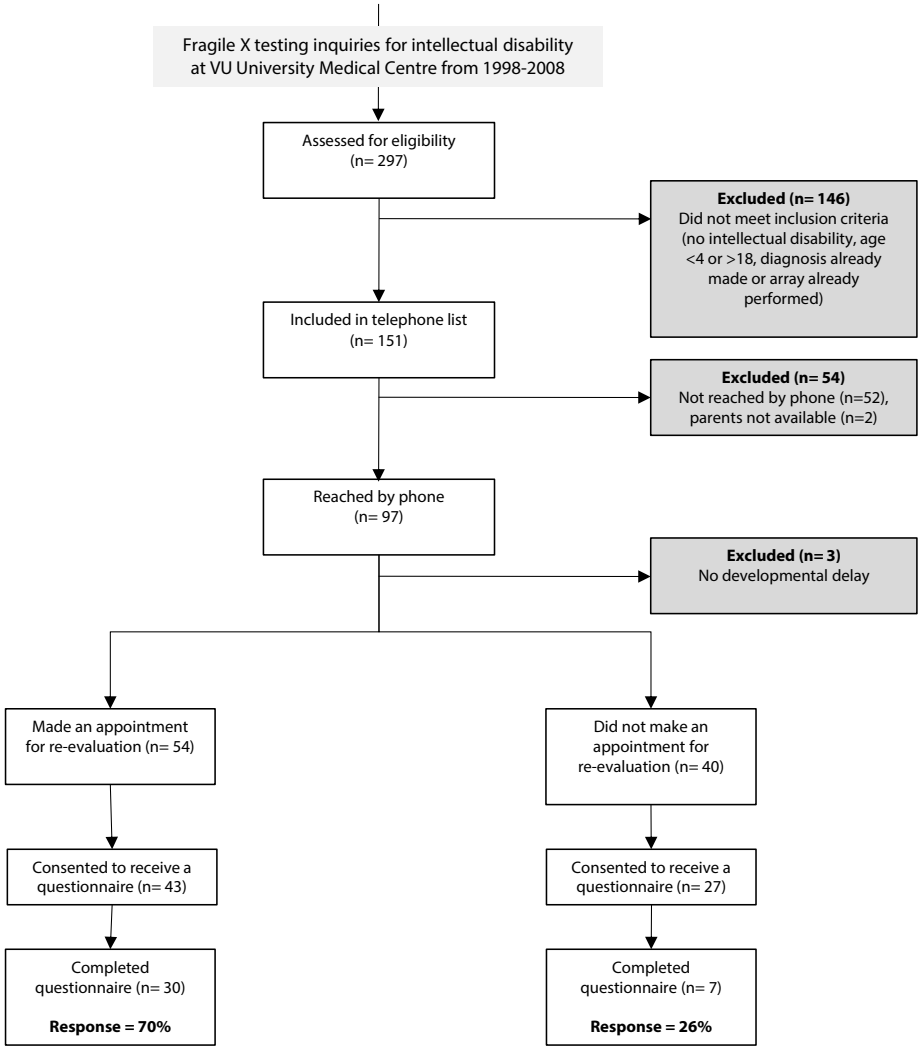


Figure S1. *Flow diagram of the recontacting pilot and questionnaire study process in cohort A. The number of patients included and excluded are shown, as well as response rates to the questionnaire and the number of patients for which a new appointment was made for re-evaluation and additional genetic testing.*

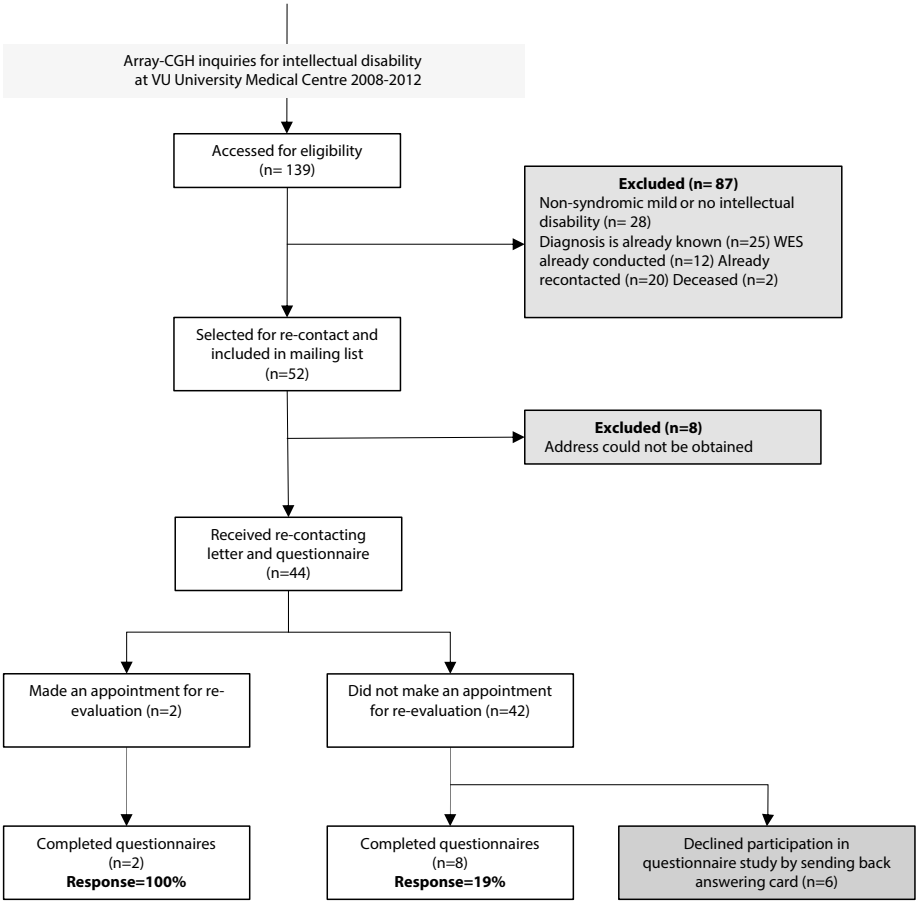
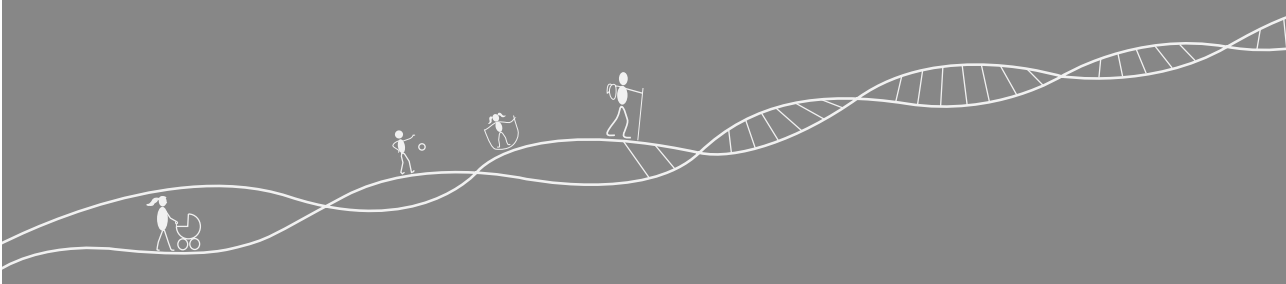


Figure S2. *Flow diagram of the recontacting pilot and questionnaire study process in cohort B. The number of patients included and excluded in cohort B are shown, as well as response rate to the questionnaires and number of patients for which a new appointment was made for re-evaluation and additional genetic testing. Array CGH: array Comparative Genome Hybridisation, WES: Whole Exome Sequencing.*

Chapter 7

General discussion and Future prospects



GENERAL DISCUSSION AND FUTURE PROSPECTS

Intelligence is a term referring to our ability to sense and react to our surroundings and to solve problems. The variation in intelligence is normally distributed and highly genetic. The genetics of intellectual ability and disability is complex and interconnected. Variation in the normal range is influenced by the sum of many genetic and environmental factors, each with small individual effects, and mild intellectual disability can be seen as the lower end of this variation. Whereas severe or syndromic intellectual disability seems to be a different genetic entity, even here the same biological pathways could be involved. Learning more about the genetic aspects of intelligence and intellectual disability will bring us closer to understanding the complex brain processes involved in learning, memory and other cognitive abilities that make humans so unique as a species. However, translating this knowledge to patients and clinical genetic practice is equally important. With our studies on *SNAP25, AUTS2* and recontacting, we have contributed to both of these aspects.

Generalist genes

A complex interplay between common genetic variants with a small effect, rare variants with a small or moderate effect, and environmental factors seem to cause the variation in intelligence. Genetic variations with a large effect on protein function are likely to have a large effect on the phenotype, driving it to the ends of the Gaussian curve. In the area around the mean of the Gaussian curve, an interplay of genetic variants with small effects will be more likely. Mild intellectual disability can be seen as the lower end of the normal distribution often with a multifactorial cause. CNVs shown to be risk factors for intellectual disability, such as the 15q13.3 deletion, illustrate this multifactorial model: these CNVs can be found in controls, but are more often seen in cases. They are often inherited from a normal or mildly affected parent, most have mild intellectual disability, but there is a large variation in the cognitive and physical phenotype in patients carrying such a deletion. Those are all indications that, next to this CNV, more causal factors play a role.[1] From a biological point of view, it is likely that the pathways involved in variation in intelligence in the normal range are also involved in intellectual disability and the other way around as suggested in the 'Generalist genes theory'.[2] This way of thinking might be helpful in finding new genetic factors for variation in intelligence and/or intellectual disability. In this thesis, we present several lines of evidence supporting this idea.

We found evidence that the 'risk allele' of one of the genetic factors associated with intellectual ability is also associated with mild intellectual disability, namely the minor allele (G) of SNP rs363050 in *SNAP25*. Although our study could not find direct proof for an influence on SNAP25 gene function by this SNP, such an effect cannot be excluded either. Other evidence that the *SNAP25* gene is important for intellectual ability and disability came from a recent paper describing a *de novo* variant in *SNAP25* in a girl and a boy with intellectual disability and epilepsy.[3;4] Proteins coded by intellectual disability genes are enriched in synapses, thereby supporting the 'synapse-based theory for intellectual deficits'.[5] SNAP25 is a synaptic protein of the SNARE complex that is important

in regulation of neurotransmitter release. Another SNARE complex protein that is involved in intellectual disability is Syntaxin binding protein-1 (STXBP1). Mutations in *STXBP1* are associated with early infantile epilepsy and intellectual disability. [6; 7]

A large group of IQ-variation-associated genes (52 in total) were found in a recent GWAS meta-analysis. Some of these were known intellectual disability genes, for example *SHANK3, JMJD1C* and *MEF2C*. [8] This is another example of howgenes related to variation in intelligence in the normal range can cause intellectual disability when more severely disrupted by mutations or deletions. On the other hand, knowledge of biological pathways and genes, gained from research in intellectual disability, can help to better understand variation in intellectual ability as well. Franic et al.[9] show that *ELP2, TMEM135, PRMT10* and *RGS7* (autosomal recessive intellectual disability genes) are associated with variation in intelligence within the normal range.

All together the same genes and biological pathways seem to be involved in intellectual ability and disability, supporting 'the generalist genes theory'. However, this theory not only suggests an effect of the same genes in the mean and extremes of one quantitative trait, but also a pleiotropic effect for different intellectual processes. The genes I have studied in this thesis, *SNAP25* and *AUTS2*, both show evidence for this theory. *SNAP25* has been suggested to be associated not only with intellectual disability and intellectual ability, but also with related cognitive and behavioural traits such as autism [10], ADHD [11] and working memory capacity [12]. There is a lot of evidence that the AUTS2 protein is important in several neurodevelopmental disorders such as intellectual disability, autism, addiction and schizophrenia.[13-17] SNPs and (non-coding) CNVs are associated with ADHD.[18; 19] 5' in frame deletions cause mild non-specific intellectual disability and are found in normal to very mildly affected parents as well. However, mutations causing haploinsufficiency of the full-length AUTS2 transcript causes AUTS2 syndrome with mild to moderate intellectual disability and syndromic features.[20; 21] These cognitive and behavioural traits seem to be caused by the influence of AUTS2 variations on neurodevelopmental processes, like neuronal growth and migration.[13; 20; 22] The fact that the highest signal for a selective sweep between modern-day humans and Neanderthals is within the '5 end of *AUTS2* suggests a role in human evolution as well, and might indicate that small changes in this gene triggered positive selection, presumably via an effect on intelligence.[23] This nicely illustrates that *AUTS2* and *SNAP25* are a generalist genes associated with the total spectrum of evolution of intelligence, with variation in intellectual ability and disability, and with many other cognitive and behavioural traits.

AUTS2 and AUTS2 syndrome

Humans with AUTS2 disruptions

We described phenotypic details of 27 unrelated patients and 6 of their affected family members who have disruptions of the *AUTS2* sequence, ranging from mutations at the nucleotide level to exon or whole gene deletions, translocations and inversions (figure 1 in Beunders et al 2013 (chapter 3) and figure 1 in Beunders et al 2016 (chapter 5)). [20; 21; 24]

In literature, approximately 35 other cases have been described with disruptions of the *AUTS2* coding sequence.[25-40] For some of these, extensive phenotypic data are available, but in most cases the clinical data are limited. The phenotypic spectrum caused by pathogenic AUTS2 disruption is variable but recognizable, especially when patients with known AUTS2 disruptions are compared. Next to intellectual disability, feeding problems and microcephaly, patients with AUTS2 syndrome can be recognized in the consulting room by the arched eyebrows, ptosis, proptosis, short palpebral fissures, short philtrum, narrow mouth and micrognatia (figure 1 and 2a/b), the shallow bending folds of the fingers (figure 3c), the kyphosis or scoliosis and outgoing friendly, and/or stereotypic and obsessive behaviour.

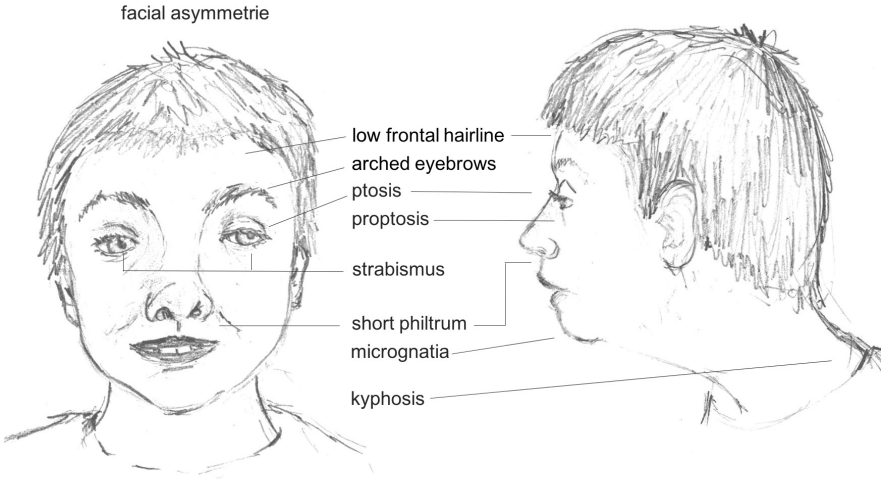


Figure 1. *Common facial features seen in AUTS2 syndrome patients: facial asymmetry, low frontal hairline, arched eyebrows, ptosis, proptosis, strabismus, short philtrum, micrognatia and kyphosis.*

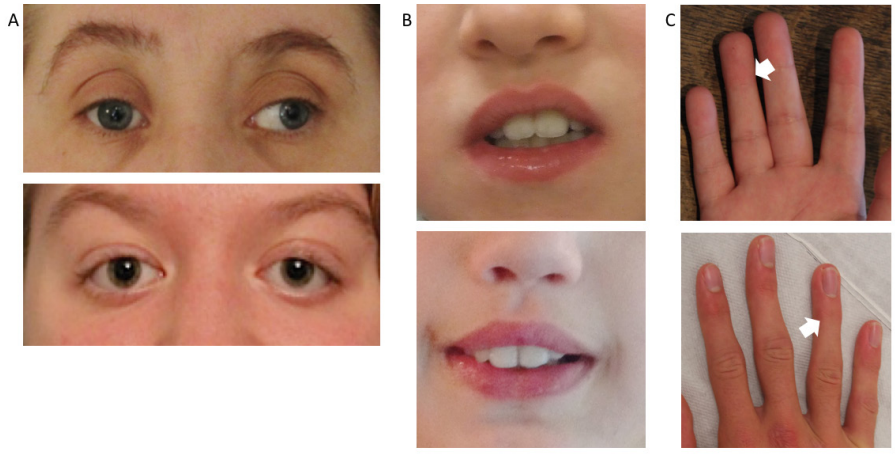


Figure 2. *Specific recognizable details in the facial features (ptosis, arched eyebrows, short palpebral fissures and narrow mouth with a short and upturned philtrum) and hand phenotype (absent DIP creases) of AUTS2 syndrome.*

The clinical phenotype of AUTS2 syndrome shows overlap with several other intellectual disability syndromes. Differential diagnostic considerations are:

1. Mild forms of Cornelia de Lange syndrome, especially in young patients with prominent arching of the eyebrows, microcephaly, short stature and mild hand abnormalities.[41]
2. KBG syndrome, because of the feeding problems in early childhood, relatively small head size, hypotonia, strabismus and overlap in dysmorphic features: prominent, sometimes arched, eyebrows, ptosis and a broad nasal base can be seen in both syndromes. The short-upturned philtrum of AUTS2 syndrome patients can give the illusion of rather large upper frontal incisors as seen in KBG syndrome.[42]
3. Coffin Siris syndrome, with microcephaly, arched eyebrows, hypotonia and behavioural characteristics like those seen in AUTS2 syndrome. Mostly, the dysmorphic features will help to differentiate, as sparse scalp hair and hirsutism, coarse facial features with thick lips and the fifth fingernail hyperplasia are not seen in AUTS2 syndrome patients.[43]
4. Kleefstra syndrome, with microcephaly, arched eyebrows, hypotonia, speech delay and behavioural problems. The prognatia mid facial hypoplasia and birth defects seen in children with Kleefstra syndrome are not common in AUTS2 syndrome patients.[44]

Large clinical variability

The phenotypic features seen in AUTS2 syndrome patients are very variable and the single features of the AUTS2 syndrome are aspecific. This makes it hard to recognize the syndrome. Recent advances in diagnostic techniques, array CGH or SNP array and Whole exome sequencing, are very helpful as they allow screening for CNVs or variants at the nucleotide level in this gene and many other genes in the same experiment. When variants of unknown significance in *AUTS2* are found, the phenotype can help to decide how likely the pathogenicity of those variants is. It, however, will never enable a definitive conclusion because of this large variation and the aspecific features seen in AUTS2 syndrome.

Parents of children diagnosed with AUTS2 syndrome do want to know the prognosis for their (young or even unborn) child, especially concerning the level of independence they can reach. Like many other syndromes, the variation in intellectual ability in AUTS2 syndrome patients is large, making predictions about future development of individual patients difficult. The most important indicator still is the degree of the developmental delay of the individual child, although this can be misleading when determined at a very young age. It is not even possible to use this indicator if the *AUTS2* variant was found in a prenatal setting. Some prognoses can be made based on the cases we have described and those described in literature. As described in chapter 5, the patients we clinically evaluated were all verbal above 4 years of age. Hypotonia and feeding problems improve with age. Adults do not seem to have major health issues.[21; 33; 37]

The deletions, translocation or inversion breakpoints and single nucleotide variants causing AUTS2 syndrome are scattered throughout the gene (figure 1 in Beunders et al 2013 (chapter 3)

and figure 1 in Beunders et al 2016 (chapter 5)). [20; 21] Nonsense and frameshift mutations causing haploinsufficiency of the full length *AUTS2* transcript can cause AUTS2 syndrome as we described for patients 8 and 9.[21] Up to now, no pathogenic missense or intron deletions have been described. Ropers et al.[45] found 11 loss-of-function mutations within *AUTS2* in the ExAC database of 60,706 healthy controls. Although individuals with severe childhood disorders are removed from this database, mild AUTS2 syndrome phenotypes in those 'controls' cannot be excluded. It could also indicate that there is a reduced penetrance for mutations causing haploinsufficiency of *AUTS2*.

When counselling parents of AUTS2 syndrome patients it is important to keep their child's specific mutation in mind because a genotype-phenotype correlation is emerging as described in chapter 3, 4 and 5. Less severe intellectual disability and a less specific phenotype is seen in patients with in frame 5' deletions compared to mutations disrupting the whole gene or the 3' end.[20]

AUTS2 function

When we started our studies of the *AUTS2* gene and the AUTS2 syndrome, there was very little known about the function of AUTS2. Much more has now become clear through our studies and those of others. AUTS2 seems important in (early) neuronal development through its effect on neuronal migration, histone modification and transcription regulation.

Zebrafish auts2 knockdown studies by ourselves and Oksenberg et al. show a smaller body length, smaller head size, smaller jaw size and less movement after tactile stimulus in auts2 morpholinos; features very similar to the human phenotype of AUTS2 syndrome patients.[13; 20] Studies with transgenic zebrafish lines with a fluorescent signal of specific cell types show fewer developing and proliferating neurons, fewer motor neurons and fewer sensory neurons in the spinal cord. [13; 20] Oksenberg et al. reported increased apoptosis and increased proliferation of neurons, whereas we saw fewer proliferating neurons in auts2 MO zebrafish. This seems a contradiction, but it might be the result of different stainings showing different cellular stages. The results of Oksenberg et al.[13] could suggest that there is apoptosis of neuron precursor cells or early developing neurons that fail to develop to mature neurons, leaving fewer proliferating neurons in a later developmental stage and thus explaining our results.[20] Altogether these data show that AUTS2 is important for neuronal development and the presence of fewer neurons might explain the microcephaly. Fewer motor and sensory neurons are likely to explain the reduced movement after tactile stimuli in zebrafish and could play a role in hypotonia of AUTS2 syndrome patients. [13; 20] We have shown that the zebrafish MO phenotypes could be rescued with the human full-length as well as with the shorter transcript. This indicates that the effects seen in the auts2 MO fish are AUTS2-specific and that the shorter transcript contains the major functional elements of the gene.[20]

Mouse knockdown studies show that Auts2 is expressed in mouse cerebral cortex, hippocampus and Purkinje cells of the cerebellum. There is a shorter isoform expressed in early embryo development, but not after birth and only in the nucleus. The long transcript is shown to be present from early embryonic development throughout adulthood and can be found in the nucleus and the

cytoplasm. [46; 47] *Auts2* knockout mice are neonatal lethal. *Auts2* conditional knockouts show smaller body size, a deficient righting reflex (a reflex turning mice back on their feet when laid down on their back) and less ultrasonic vocalization after separation from their mother in a dose dependent matter. [46; 47] Heterozygous mutant mice show less exploratory behaviour and less fear. The latter might be analogous to the ebullient social behaviour with lack of fear of strangers seen in AUTS2 syndrome patients. The impaired novel object recognition and associative memory deficits seen in those mice are indicative of learning and memory deficits. This seems similar to the impaired cognitive abilities seen in AUTS2 syndrome patients.[48] At a cellular level, *Auts2* knockout mice embryos, with both the full-length and the short transcript or only the full-length transcript knocked out, show neuronal migration disorders and shorter axon length and less neurite outgrowth. Different from zebrafish, there is no indication of an important function in proliferation of neurons or its precursors in the ventricular zone in these mutant mice.[22; 48]

The biological pathways of AUTS2

Although the exact function of AUTS2 is not yet unravelled, there are some pathways emerging in which AUTS2 has an important role. AUTS2 and TBR1 are co-expressed in mouse brain, especially in glutamatergic neurons.[49] SATB2 regulates TBR1, which in turn directly regulates AUTS2 expression as well as RELN expression (important for neuronal migration).[13] Benitez-Burraco [50] describes how these genes might have had an important role in modern human evolution and speculates on their function in neuronal development and skull/bone formation. They discuss connections of SATB2, TBR1 and AUTS2 to two other proteins that have been indicated as harbouring a selective sweep when comparing ancient human races with modern humans: FOXP2 and RUNX2. [50] An exact pathway in which AUTS2 functions cannot be deduced from these data yet, but again a relation to neuronal migration and to histone modification (transcription regulation) pathways seems likely.

There are several lines of evidence for an activating role in transcription regulation by histone modification. AUTS2 binds to a polycomb transcription complex together with RYBP and CK2 (polycomb transcription 1.5-Ring1B-RYBP-AUTS2-CK2 complex). This is remarkable as polycomb transcription complexes normally keep transcription low through histone acetylation and methylation. [46] AUTS2 binds to active transcription start sites in mice and in zebrafish.[51] There is an overrepresentation of neuronal developmental genes in the total group of genes bound by AUTS2. Next to these transcriptional regulation functions, there are clues that suggest a function in neuronal migration and neurite outgrowth. This seems to be mediated through a positive effect of AUTS2 in the Rac1 pathway that is important in the maintenance of the cytoskeleton by actin regulation.[47]

Interestingly, some of the proteins mentioned as interacting with AUTS2 are known to be encoded by intellectual disability genes as well. The *SATB2* gene is related to Glass syndrome, which can be caused by deletions, translocations or other mutations disrupting *SATB2*. Glass syndrome is characterized by a moderate to severe intellectual disability, tooth abnormalities, feeding problems and dysmorphic features.[52] *RELN* disruptions cause dominant epilepsy or an autosomal

recessive neuronal migration disorder called Norman-Roberts type lissencephaly.[53; 54] *De novo TBR1* (likely pathogenic) mutations are found in intellectual disability and autism patients.[55; 56] Mouse studies have shown neuronal migration defects when *TBR1* is disrupted.[57] In addition, some of the syndromes that show clinical overlap with AUTS2 syndrome, such as Cornelia de Lange syndrome and Kleefstra syndrome, are caused by genes that have a histone-modifying roll as well.[41; 44]

Informing patients and their families

Keeping parents updated on new technological possibilities and diagnostic tools and information about specific diseases in Genetics is challenging. General discussions about 'the duty to recontact' have concluded that recontacting patients or families would be desirable. [58] Moments to recontact are: when there is new information on a specific syndrome (especially when screening or treatment advice changes), when technological possibilities with higher diagnostic yield become available or when reanalysing (NGS) sequence data reveals a new diagnosis or reclassification of a variant of unknown significance. [68] In our study and recent studies of Carrieri et al.[59] a positive attitude of parents and patients towards recontacting was found. There are however different opinions on who is responsible for the update in genetic knowledge or on new technical possibilities, patients/parents, healthcare providers or both. Objections to recontacting are the practical issues associated with doing so and the possible psychological burden of new information. Altogether, these drawbacks make it hard to introduce recontacting in the general practice. In the pilot we performed we show in particular that it took a lot of time to select patients in retrospect for whom recontact would be valuable. Next to that, re-establishing contact posed practical problems as often phone numbers and/or addresses had changed.[58; 60 61] Our study showed an emotional reaction to a sudden phone call about the possibility of further genetic testing. These were positive and negative emotions, but the real impact of such a call or letter cannot be extracted from our study.[62] Many of the issues above might be overcome in the future when genetic testing and counselling is not only provided by the geneticist but becomes more and more integrated in general health care. Many advertise the gains from this approach, called: 'mainstreaming' of genetic care, bringing genetics closer to the patients that benefit from it. There are however large struggles to get pilots of this approach working in clinical practise as was shown by an integrative analysis of multiple projects by the IGNITE (Implementing GeNomics In pracTicE) network.[63] In my opinion 'recontacting' and 'mainstreaming' share many goals and challenges. Both care for 'up to date' genetic information reaching the people that benefit from it most. In 'recontacting' the geneticist is however mostly placed in a central role, whereas the primary care doctors and patients have a key role in the projects that advocate 'mainstreaming of genomic medicine'. I hope the national, international and local debates on these topics keep their goals clear and that the geneticist keep working together with patients, the general public and other (health care) professionals to overcome these challenges.

Future prospects

Better and more causal diagnosis in intellectual disability.

Our small candidate gene association study of variants in SNAP25 has provided a little more understanding about the nature of the genetic variance causing part of the variation in intelligence and mild non-syndromic intellectual disability. A recent large association study using new statistical methods such as MAGMA for per gene analysis has been very successful in finding new genes associated with variation in intelligence. If we could find more proof that those genes and pathways are also important for intellectual disability this would be useful in our area of genome-wide analysis. When a diagnostic WES experiment reveals a *de novo* (loss-of-function) variant in such a gene in a 'cognition pathway', it would be suggestive of a pathogenic effect.

Studying RNA profiles might be helpful to get closer to the core biological 'cognition pathways' as different genetic variants are likely to cause effects on the same downstream genes leading to comparable RNA profiles. Even environmental factors might have an effect on transcription regulation of the same genes and therefore cause comparable RNA profiles. Until now, working with RNA was very difficult because the presence of RNA transcripts is tissue-dependent and the abundance of a transcript varies in time. A statistical technique that filters out this noise might enable us to study RNA profiles of traits such as intelligence.[64] One of the big issues to overcome is, however, that the cells most often available for research are lymphocytes and not neurons. This could be overcome by the techniques of induced pluripotent stem cell and neuron differentiation. [65] Many intellectual disability syndromes have recognizable facial features, as seen in AUTS2 syndrome. Dysmorphologists use these facial features to recognize specific syndromes in patients with intellectual disability. Computer face-matching technology can match 2D pictures of an individual patient to faces of other individuals with a specific syndrome. These systems can therefore be trained to also recognize intellectual disability syndromes and have proven to be more successful than clinicians in recognizing the correct syndrome in a recent pilot study. [66] This concept is also used by the face2gene program. As these techniques advance and are trained by uploading large numbers of pictures of individuals with intellectual disability and known molecular diagnoses, they could become a major support in the clinical genetic practice especially to improve the objective knowledge of the phenotypes caused by defects of certain genes. One could hypothesize that the genetic variation with small effect associated to variation in intellectual ability in the normal range could have a small effect on facial features too. It would therefore be interesting to find out if minor facial anomalies are associated with variation in intellectual ability.

We are still far from predicting IQ for individuals and this is, in my view, not a goal of research on the genetics of intelligence, nor even desirable. It might however be a 'side effect' of the growing knowledge on intellectual ability and disability and its association to genetic and environmental factors (in the far future). Ethical debate on this is therefore crucial. Researchers and others are responsible together for using it in a decent ethical and human way.

Improving information and 'treatment' for patients and their families after a diagnosis

One of the main goals of clinical geneticists is to inform patients and their families about genetic diseases so they can make educated decisions about their own life and health. During my research and clinical work as a clinical geneticist I was struck by how little we know about the rare syndromes we diagnose. I also experienced how seeing several patients with AUTS2 syndrome and interviewing their parents enabled me to recognize the clinical pattern that make AUTS2 syndrome patients recognizable. This pattern was described in chapters 3, 4 and 5 of this thesis as objectively as possible. It should however be mentioned that not all details can be captured in a paper. The experience of meeting these children and adults adds pieces to the puzzle that help me to counsel parents, but these are not easily objectified or explained in words. This made me realize the importance of experts. For these experts, it is crucial to connect with patients and their families to gather information from them and to keep them updated about what we have learned. Modern information and communication tools (such as internet sites, WebEx and facebook) help in doing this. Some research projects have shown how this concept can be very effective, for instance the chromosome 6 study of the UMCG (https://www.chromosome6.org). We also have to keep searching for the best way to inform families now and in the future. An international expert network which can be asked for professional advice to inform health care providers or for direct counselling of families (via WebEx) about a specific syndrome or rare disease would be very helpful for all rare diseases, including AUTS2 syndrome. One of the keys for this concept is knowing who the experts are on what syndromes. Within the Netherlands the society of clinical genetics is working on a model to register the expertise of Dutch geneticists. This would enable doctors/clinicians to refer patients with a specific syndrome or to discuss difficult cases and will therefore be a valuable first step towards sharing knowledge nationally in a manner that would also facilitate clinical research and maintain expertise. Internationally the European Reference Network on congenital malformations and rare intellectual disability (ERN ITHACA) is an initiative to facilitate diagnostics, guideline development and research for rare intellectual disability syndromes and congenital malformations.

Patients with AUTS2 syndrome seem to have a specific behavioural pattern and neurocognitive profile. It would be valuable to investigate this further in a larger group of patients as these patterns can help to advise clinicians, physicians, care-givers and parents on the specific needs of individual patients with AUTS2 syndrome. Training programs like 'DIR/floortime'[67] or 'pivotal response training' [68] might be especially helpful when a diagnosis is made at a young age because these approaches specifically address the inappropriate social behaviour with more or less contact and language developmental delay seen in AUTS2 syndrome patients. As many children with AUTS2 syndrome are hypersensitive to sound or other sensory experiences, it would be worthwhile studying whether sensory integration therapies are beneficial. These symptomatic treatments are especially interesting as it is not very likely that a curative treatment will become available in the near future. Functional studies have shown that AUTS2 defects cause early embryonic neuronal developmental aberrations. However, as the diagnosis is made after this period and because of the difficulties

in reaching neurons, it will be hard to design drugs or gene therapy to treat or even cure AUTS2 syndrome.

Interpretation of a VUS in AUTS2

One of the major clinical enigmas for clinical geetics was and is the interpretation of variants of unknown significance (VUS). Finding a VUS introduces uncertainty. When a VUS can be classified as pathogenic or benign, this enables better counselling and, in case of a pathogenic mutation, gives the parents more reproductive options. For the interpretation of variants found in the AUTS2 gene (as for other genes) several lines of research will be helpful. Clinical analysis of more patients might enable the development of a more specific clinical score. For this purpose, our AUTS2 syndrome severity score can be used as a basis.[20] In this scenario it would be interesting to investigate if computer face-matching technology could be helpful. These techniques may allow us to compute a score for the similarities between a face of an individual with a VUS in AUTS2 and faces of individuals with pathogenic AUTS2 variants. If the resulting similarity-score is greater for faces of individuals with a VUS in AUTS2 versus controls, it could indicate pathogenicity. Secondly, RNA/cDNA analysis and RNA expression studies (for example Quantitative Fluorescence-Polymerase Chain Reaction, QF-PCR) helps the interpretation of the effect on splicing and expression of a variant AUTS2. Finally, studying the variant in a cell or animal model would also be very helpful. Recent developments such as the introduction of the Clustered Regularly Interspaced Short Palindromic Repeats, Cas9-enzyme (CRISPR-cas) and the possibility of using induced pluripotent stem cells are valuable. CRISPR-cas enables a relatively easy introduction of specific mutations or copy number variants to specific locations in the DNA of cells. This has made the development of animal or cell models much more straightforward. [69] Induced pluripotent stem cells refers to techniques that enable resetting the differentiation of, for example, fibroblast to (pluripotent) stem cells that can be differentiated into multiple other celltypes.[70] This would enable researchers to 'grow' neurons derived from a patient's fibroblasts that would carry the variant of interest. For AUTS2 variant analysis, dendrite outgrowth or changes in expression profiles of neurodevelopmental genes could be readouts to see if there is an effect on the cellular level, and microcephaly and small body size can be readouts in mice or zebrafish disease models.[13; 20; 46-48] Hopefully high-throughput techniques to perform functional studies will be developed to support the interpretation of VUS analysis, making it feasible for clinical use.

Although the effect of AUTS2 haploinsufficiency is becoming more and more clear, the effect of whole gene duplications remains to be elucidated. There are several loci where both deletions and duplications are associated with a phenotype, sometimes characterized by opposite features. This is illustrated by the Williams-Beuren syndrome region where deletions cause 'Williams-Beuren syndrome' with a relative strength in expressive language, and where the duplication or triplication of this region causes severe language deficits. [71] There is currently too little data on duplications in human, or on higher AUTS2 expression in animal models, to speculate about the effect of

AUTS2 gene duplications. As these duplications have been found in humans and are now variants of unknown significance, this is an important topic for future research. Furthermore, as AUTS2 is located on 7q11.22 next to the Williams-Beuren syndrome region, large deletions and duplications could include both these loci. Indeed, we know of patients with these large deletions (personal communication) and it would be interesting to analyse the phenotype in these patients as well.

How does new information reach patients?

Recontacting, when new technical possibilities for making a causal diagnosis are available, is appreciated by parents. Topics for further research on recontacting could be cost effectiveness , and the emotional burden (If there are negative emotions, what is the impact and can they be predicted and/or prevented?). However the first question should be: "Does recontacting fit in our 'genetic clinic of the future' where we hope to integrate genetics in general healthcare by 'mainstreaming project'?" This is a topic we need to discuss in our national and international genetics societies and in our communities before deciding to do futher research on this topic or change the current practice.

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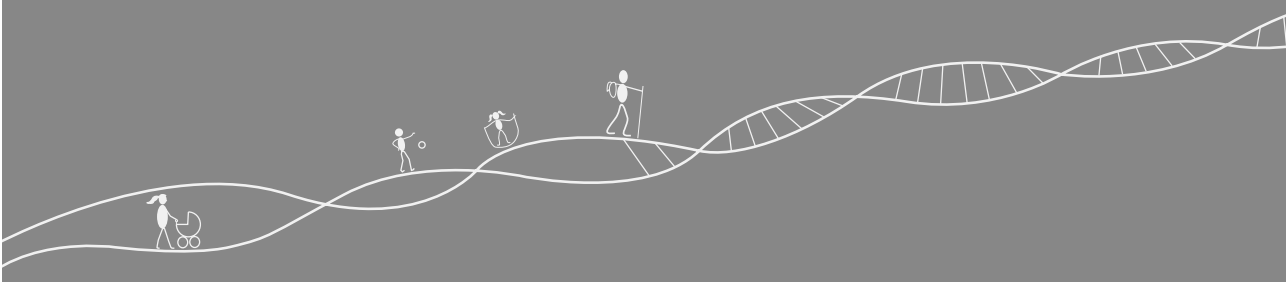
Addendum

summery/samenvatting
list of publications
about the author
dankwoord



SUMMARY

Variation in intellectual ability is normally distributed and partly caused by genetic variation. We wanted to test the hypothesis that mild intellectual disability (IQ 70-50) is at the lower end of this distribution and often has a multifactorial cause, whereas moderate to severe (IQ>50) and/or syndromic intellectual disability often has a monogenetic cause by studying *SNAP25* and *AUTS2*. Keeping patients informed about all advances in knowledge and diagnostic techniques in a clinical setting is challenging. We hope to contribute to improvement on that area by two different studies: first our study on recontacting the patients and their families and second our detailed clinical overview of the AUTS2 syndrome phenotype.

To demonstrate the multifactorial model for mild intellectual disability we compared the risk allele frequency of SNP rs363050 in *SNAP25* in cases with mild intellectual disability to controls with a higher than average IQ in Chapter 2. This SNP was already associated with variation in cognitive ability in general by Gosso et al. We show that there is a significantly higher number of minor alleles (G) (the risk allele) for this SNP in cases versus controls. The SNAP25 gene has an important function in modulation of neurotransmitter release and is thought to have a role in learning and memory by its involvement in hippocampal long-term potentiation of neurons. The rs363050 SNP is in high linkage disequilibrium with two SNP's in intron 2 of SNAP 25 that are located in a predicted transcription binding site.

In chapter 3 we describe a new intellectual disability syndrome, now named AUTS2 syndrome (autosomal dominant mental retardation-26 (MRD26, OMIM nr. # 615834). Array analysis of 49,684 individuals with intellectual disability and/or multiple congenital malformations revealed 24 exon deletions in AUTS2, but no exonic deletions were found in 16,784 controls. The frequency of exonic deletions that we found was 1 in 2,000 cases, comparable with some of the recurrent deletions such as the 10q23 deletion (NRG3 [MIM 605533] and deletions causing Sotos syndrome (MIM 117550) described by Cooper et al. (2011)

The syndrome is recognizable by microcephaly, feeding problems, hypotonia evolving to hypertonia and in some patients also mild dysmorphic features like micrognatia can be identified. The more severe phenotype in patients with C-terminal deletions has led to further analysis of the gene structure, conservation and alternative transcripts. This revealed an alternative transcription start site in exon 9 (a highly conserved area of the gene) that is transcribed in human brain. Translation of this alternative transcript would lead to a protein only containing the C-terminal part of AUTS2. Zebrafish studies confirmed the importance of this part of the protein for at least the dysmorphological part of the phenotype as the microcephaly and smaller jaw size in zebrafish treated with auts2 morpholino's could be rescued with the human short transcript starting in exon 9.

In chapter 4 we describe the first AUTS2 syndrome patient with a mutation at the nucleotide level picked up by Whole Exome Sequencing. This adult male has a 2-nucleotide deletion in exon 7 of AUTS2 and is compared to an adult male patient with an exon 6 deletion. Both patients show

many similarities that can be catagorized as full blown AUTS2 syndrome. As the mutations in both men do not affect the shorter 3' transcript starting in exon 9, we conclude that in humans there is no rescue of the phenotype by this transcript.

Chapter 5 is dedicated to the further evaluation of the clinical phenotype of AUTS2 syndrome. Thirteen cases, all clinically analysed by the same physician, helped to further delineate the phenotype of AUTS2 syndrome and confirmed the observation that haploinsufficiency of the long transcript of AUTS2 is causing AUTS2 syndrome, and that there is no rescue by the shorter transcript. Common clinical features of AUTS2 syndrome are: mild to moderate intellectual disability with speech delay and stammering, hypotonia at a young age sometimes evolving to hypertonia and tight heel cords later in life, feeding problems until childhood age, microcephaly, low weight and stature between p1 and p25. Birth defects or general health problems are rare. A behavioural phenotype emerged, showing hyperactive and hypersocial behaviour in childhood and rather shy, drawn back behaviour in adulthood. Classical autism is rare but stereotypic movements and obsessive behaviour is frequently seen, while social interaction is less affected.

In chapter 6 we describe a pilot study on recontacting parents of patients with intellectual disability to inform them about new diagnostic techniques (array Comparative Genome Hybridization and Whole Exome Sequencing, WES). This pilot showed that recontacting is time consuming especially if there is no database with patients suitable for recontacting. The yield of recontacting is rather low but seems higher when contact is made by phone. The parental attitude towards recontacting in general is very positive as is the feeling about the recontacting, although an ascertainment bias cannot be excluded.

Some concluding remarks are described in chapter 7. The biological pathways important for cognitive ability and disability are largely overlapping. There are many different processes interacting with each other involved in normal and abnormal brain function. Next to that, many different cognitive traits are influenced by the same genetic factors that are in line with the large correlation between different cognitive traits when tested in IQ tests. These observations support Pearson's idea of a 'general intelligence factor's called g. and the 'Generalist genes theory' of Plomin. From humans with AUTS2 syndrome, zebrafish and mouse knockdown or knockout experiments we learned that the AUTS2 protein has an important function in neuronal development by transcription regulation through histone modification and neuronal migration, and by its effect on the cytoskeleton and dendrite growth. Defects of the *AUTS2* gene cause the above described AUTS2 syndrome.

Recontacting parents of patient's with intellectual disability to inform them about new diagnostic possibilities was appreciated. It can be debated who is responsible for recontacting and there are practical barriers that need to be overcome before a general introduction into clinical practice. Next to this 'mainstreaming of genomic medicine' might be another way to get the up to date information to the patients in an efficient way.

Future research on genes in 'cognition pathways' that effect intellectual ability and disability

would be valuable. As is research on symptomatic treatment for AUTS2 syndrome, on variants of unknown significance in *AUTS2*, on the phenotype of patients with whole gene duplications of *AUTS2* and on patients with large deletions or duplications of *AUTS2* and the Williams Beuren syndrome region. We suggest an expert network with national or international registration to improve information on rare syndromes. Further studies on recontacting mainly focusing on cost effectiveness and the emotional burden are necessary before introducing it into general clinical genetics practice.

SAMENVATTING

Wat intelligentie is, is moeilijk te omschrijven. Kort (2002) noemde intelligentie het vermogen om doelgericht te handelen, rationeel te denken en effectief met de omgeving om te gaan. Er bestaan echter ook veel uitgebreidere definities, waarin termen als 'abstract denken', 'plannen', 'gebruik van taal' en het 'oplossen van problemen' gebruikt worden. Helemaal eens zijn we het dus niet over de definitie, maar we weten wel dat er een grote variatie in intelligentie is en dat deze variatie voor een groot deel erfelijk bepaald is. De variatie in intelligentie zoals die gemeten wordt met een IQ test is normaal verdeeld, wat betekent dat veel mensen een gemiddeld IQ of een IQ dat net iets hoger of lager is hebben en dat veel minder mensen een heel laag of juist heel hoog IQ hebben. Een verstandelijke beperking wordt gekenmerkt door een IQ onder de 70 en daarbij beperking in verschillende vaardigheden, zoals: sociale, educatieve en/of fysieke vaardigheden. Milde verstandelijke beperking (IQ 50-70) kan gezien worden als het uiterste van deze normale verdeling en is net als IQ in de normale range multifactorieel bepaald. Ernstige verstandelijke beperking of syndromale verstandelijke beperking (waarbij er ook aangeboren afwijkingen of uiterlijke kenmerken zijn) wordt vaak veroorzaakt door een monogenetische verandering in de DNA-code of een chromosomale afwijking. De huidige technieken van DNA en chromosoomonderzoek zijn erg verbeterd en de kennis over de genetica van verstandelijke beperking neemt toe, hierdoor kan steeds vaker een oorzaak voor verstandelijke beperking worden aangetoond.

Er zijn verscheidene varianten bekend die geassocieerd zijn met de variatie in intelligentie in de normale range (IQ 70-130). Eén van deze varianten is het single nucleotide polymorfisme (de SNP) rs363050 in het *SNAP25* gen. In hoofdstuk 2 laten we zien dat het allel van deze SNP, dat geassocieerd is met een lager IQ (G), vaker voorkomt bij mensen met milde verstandelijke beperking vergeleken met mensen met een relatief hoog IQ. Het *SNAP25* gen heeft een belangrijke functie in de synaps van zenuwcellen bij het vrijkomen van neurotransmitters. We konden geen direct bewijs vinden dat de SNP rs363050 de SNAP25 functie beïnvloedt. Wel ligt deze SNP heel dicht bij (en in linkage disequelibrium met) twee andere SNP's die beide op een plek liggen waarvan voorspeld is dat het een transcriptie-eiwitbindingsplek is. Een kleine verandering op een dergelijke bindingsplek zou de eiwitproductie mogelijk kunnen veranderen, in dit geval van respectievelijk het eiwit MEF2A en FOXL1. Met name van dit eerste eiwit wordt ook gedacht dat het een belangrijke functie beoefent in de synapsen van zenuwcellen. De processen in synapsen zijn belangrijk voor leren en geheugen.

In hoofdstuk 3 beschrijven we voor het eerst een nieuw syndroom met verstandelijke beperking, veroorzaakt door veranderingen van de erfeigenschap (het gen) *AUTS2*. Een verfijnd chromosomenonderzoek (arry-CGH) toont bij 24 van de 49.684 onderzochte kinderen met een verstandelijke beperking en/of aangeboren afwijkingen dat er een (eiwit coderend) stukje van het *AUTS2* gen mist. In 16.784 controles (mensen/kinderen zonder verstandelijke beperking of aangeboren afwijkingen) worden dergelijke AUTS2 gen defecten niet gevonden.

We zien dat kinderen en volwassenen waarbij het *AUTS2* gen niet goed kan functioneren naast de verstandelijke beperking een relatief kleine hoofdomvang, voedingsproblemen op de baby- en jonge kinderleeftijd en een lage spierspanning hebben. Ook zien we overeenkomsten in de uiterlijke kenmerken, onder andere: een kleine kin, nauwe ooglidspleten en gebogen wenkbrauwen. Door de gegevens van 17 patiënten met AUTS2 syndroom nauwkeurig te vergelijken hebben we aanwijzingen dat er een ernstigere vorm van het AUTS2 syndroom ontstaat als het einde van het AUTS2 eiwit defect is. Dit deel van het AUTS2 gen is ook nauwelijks veranderd in de evolutie, een aanwijzing dat dit een belangrijke functie heeft. Met een zogenaamd RACE experiment (een manier om te zoeken naar de start van een gen) tonen we een alternatieve start in het AUTS2 gen aan, namelijk in exon 9 van het volledige transcript. Als de alternatieve start van AUT2 gebruikt word ontstaat een korter AUTS2 eiwit, met alleen het laatste deel van het volledige eiwit, de C-terminus. Om na te gaan of het einde van het AUTS2 eiwit inderdaad een belangrijke functie heeft hebben we een zebravis model gebruikt. Zebravisjes waarin het AUTS2 gen uitgeschakeld is (d.m.v. zogenaamde auts2 morfolinos) hebben een kleiner hoofd en een kleinere kaak, ook waren er minder delende zenuwcellen in hun hersenen. Door het toevoegen menselijk AUTS2 transcript konden deze effecten van het uitschakelen van het auts2 gen teruggedraaid worden, zowel bij het toevoegen van het lange als bij het korte AUTS2 transcript. Hieruit blijkt dat het laatste deel van het AUTS2 eiwit, de C-terminus een belangrijke functie heeft. Mogelijk verklaart dit waardoor er een ernstiger AUTS2 syndroom ontstaat als dit deel van het eiwit niet goed functioneert, terwijl mensen met een klein defect van alleen het eerste deel van het lange AUTS2 eiwit een mildere verstandelijke beperking hebben met minder bijkomende problemen.

In hoofdstuk 4 beschrijven we dat ook een verandering op het niveau van de letter code van het *AUTS2* gen het AUTS2 syndroom kan veroorzaken. In dit hoofdstuk wordt het klinisch beeld van twee jonge mannen vergeleken. Eén van hen mist twee letters van de *AUTS2* code, wat gevonden werd met whole exome sequencen (WES, een techniek waarbij de eiwit coderende delen van alle genen wordt afgelezen). De ander mist een groter stuk van het *AUTS2* gen. Dit werd gevonden met een verfijnd chromosomen onderzoek (array-CGH genaamd). Bij beide mannen is er een defect in het laatste deel van het lange AUTS2 eiwit, maar is het korte AUTS2 eiwit intact. Zij hebben beiden een heel vergelijkbaar klinisch beeld, passend bij het eerder beschreven AUTS2 syndroom. Dit wijst erop dat het korte AUTS2 eiwit in de mens niet in staat is om het effect van een defect aan het lange AUTS2 eiwit te corrigeren.

In hoofdstuk 5 wordt het klinisch beeld van het AUTS2 syndroom nader beschreven. Door 13 kinderen en volwassenen met het AUTS2 syndroom te bezoeken hebben wede kenmerken van het AUTS2 syndroom in kaart gebracht. Deze kenmerken zijn: milde tot matige verstandelijke beperking, met een spraak-taal ontwikkelingsachterstand en stotteren. Vaak hebben kinderen lage spierspanning (hypotonie) op jonge leeftijd die bij sommigen overgaat in een hoge spierspanning (hypertonie) op oudere kinderleeftijd. Voedingsproblemen komen veel voor, kunnen tot de leeftijd van 6-8 jaar duren en zijn soms ernstig. Bijna alle kinderen en volwassenen hebben een

kleine hoofdomvang (microcephalie), vaak is er een laag gewicht en vaak is de lengte onder het gemiddelde. Aangeboren afwijkingen en gezondheidsproblemen komen niet vaak voor. De mensen met AUTS2 syndroom zijn vriendelijk, kinderen kunnen druk zijn en zijn vaak erg makkelijk in contact ook naar vreemden, terwijl volwassenen wat meer verlegen en teruggetrokken zijn. Klassiek autisme is zelden vastgesteld, maar kenmerken van autisme zoals stereotype bewegingen en obsessief gedrag komen wel geregeld voor.

In hoofdstuk 6 beschrijven we onze ervaringen met het her oproepen van kinderen met een verstandelijke beperking vanwege nieuwe technische mogelijkheden in de genetica. Deze nieuwe technieken verhogen de kans op het stellen van een diagnose. We informeerden ouders (die eerder bij de klinische genetica waren geweest met hun zoon of dochter met een verstandelijke beperking) over de mogelijkheid van array en WES onderzoek, respectievelijk telefonisch of per brief. Hierbij werd een nieuwe afspraak op de afdeling klinische genetica aangeboden om hun zoon of dochter opnieuw te onderzoeken en om aanvullend erfelijkheidsonderzoek met deze nieuwe techniek te verrichten. We evalueerden de haalbaarheid en de mening van ouders over het opnieuw benaderen. Het was veel werk, met name om de kinderen te selecteren waarvoor her evaluatie nuttig leek en het was moeilijk hun ouders te bereiken, door het ontbreken van recente adres gegevens. Bij het telefonisch informeren van ouders over de mogelijkheid van array onderzoek als aanvullende test maakte 36% van de ouders een nieuwe afspraak. Na de informatie over WES die we per brief toestuurden, maakte 4% een nieuwe afspraak. Ouders waren positief over het feit dat zij opnieuw benaderd werden ook al was dit ongevraagd en onverwacht. Wel had het telefoontje of de brief bij 17 % een emotionele reactie tot gevolg, soms positieve emoties zoals blijdschap of hoop, maar ook negatieve reacties zoals verdriet of angst. We kunnen niet helemaal uitsluiten dat dit overall positieve beeld vertekend is doordat slechts 47 van de 114 verstuurde vragenlijsten ingevuld zijn, en mogelijk juist de mensen met een negatiever beeld over het opnieuw benaderen niet hebben meegedaan aan dit onderzoek.

Enkele concluderende opmerkingen worden in hoofdstuk 7 beschreven. De biologische processen en hersenfuncties die belangrijk zijn voor het ontstaan van verstandelijke beperking en voor de variatie in intelligentie in de normale range lijken grotendeels te overlappen. Er zijn vele verschillende factoren die in interactie met elkaar bepalen hoe goed het brein functioneert en hoe intelligent iemand is. Er zijn verschillende erfelijke factoren (waaronder het SNAP25 gen en het AUTS2 gen) die niet alleen geassocieerd zijn met intelligentie, maar ook met bijvoorbeeld de mate waarin iemand zich kan concentreren en met de gevoeligheid voor het krijgen van psychiatrische ziekten zoals een depressie of een verslaving. Dit alles ondersteunt de 'generalist gene theorie' van Plomin.

Het AUTS2 gen heeft een belangrijke functie in het ontstaan van zenuwcellen, door transcriptie regulatie van andere genen (door middel van histon modificatie), door zijn effect op de migratie van zenuwcellen tijdens de ontwikkeling van het brein en door de functie in het cytoskelet en in de dendriet uitgroei. Als het AUTS2 eiwit niet voldoende wordt gemaakt door een gen defect of een

chromosoom afwijking ontstaat het AUTS2 syndroom, met onder andere verstandelijke beperking, hypotonie, voedingsproblemen en dysmorfie zoals hierboven beschreven.

Het opnieuw benaderen van patiënten of hun ouders met als doel hen te informeren over nieuwe technische mogelijkheden door de klinische genetica is arbeidsintensief, maar wordt wel gewaardeerd door ouders. Wellicht is in de toekomst de genetische zorg anders georganiseerd en zullen behandelend specialisten of zelfs huisartsen aanvullend genetisch onderzoek aanvragen. Hierover wordt veel gediscussieerd in het kader van 'mainstreaming van de genetische zorg'. Het is de vraag of her oproepen dan nog wel zinvol is. We zullen samen met patiënten, specialisten, huisartsen en anderen moeten blijven nadenken over de beste manier om belangrijke informatie over erfelijke aandoeningen op de juiste plek te krijgen.

Verder onderzoek aan genen die variatie in intelligentie beïnvloeden is waardevol, ook voor het beter interpreteren van WES onderzoek bij mensen met een verstandelijke beperking. Vervolg onderzoek naar de symptomen van AUTS2 syndroom, met name naar het ontwikkelingsprofiel en het gedrag zou waardevol zijn om tot goede behandel- en begeleidingsadviezen te kunnen komen. Geregeld worden varianten van onbekende betekenis (VUS) in het AUTS2 gevonden en we weten nog niet wat een verdubbeling van het AUTS2 gen voor een effect heeft, onderzoek naar de betekenis hiervan is nodig.

Een netwerk van experts evenals het samenwerken met patiënten of hun ouders en het gebruik van 'social media' kan helpen om de informatie over zeldzame aandoeningen te vergroten en beter te verspreiden. Verder onderzoek naar de kosteneffectiviteit en het psychologische effect van het opnieuw benaderen van patiënten door de klinische genetica is belangrijk. Daarnaast moeten we nadenken in hoeverre deze methode de beste manier is om up to date informatie op de juiste plek te krijgen.

LIST OF PUBLICATIONS

Beunders, G., Dekker, M., Haver, O., Meijers-Heijboer, H.J., and Henneman, L. (2018). Recontacting in light of new genetic diagnostic techniques for patients with intellectual disability: Feasibility and parental perspectives. Eur J Med Genet 61, 213-218.

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ABOUT THE AUTHOR

Gea Beunders was born on the 26th of June 1980 in Hengelo, the Netherlands, and soon thereafter left with her parents for Embu, Kenia for three years. Back in the Netherlands she grew up and went to school in Scherpenzeel. In 1998 she graduated secondary school (VWO, Christelijk Lyceum Veenendaal) with a cum laude degree and in the same year she started medical school in Nijmegen, the Netherlands. In 2003 she got her masters degree cum laude. She did her scientific internship at the department of child neurology in the Radboud University Medical Centre where she studied the efficacy of the multidisciplinary outpatient clinic for children with a brain tumour. And obtained her degree of medical doctor in January 2005. After some wandering around (at sea, in the pulmonology and paediatrics) she felt to have reached the right place to start her medical and scientific career in 2006 at the clinical genetics department in the VU University Medical Centre (VUMC). Here she started the first research projects in 2008 that later resulted in this thesis. From 2009 to 2015 she was in training to become a clinical geneticist. Gea was registered as a clinical geneticist in 2015 and started to work in the University Medical Centre of Groningen where she became a member of the staff specialised in dysmorphology and genetics of intellectual disability. Together with Wido Stam she lives in Alkmaar and they've got three children: Juriën, Amber and Wessel.

DANKWOORD

'Het gaat niet om de bestemming, maar om de reis er naartoe'

Het is een lange en avontuurlijke reis geweest (soms letterlijk, soms figuurlijk) die mij tot dit eindpunt bracht. Het ging niet snel, maar ik ben onderweg, naast pittige beklimmingen en hoge golven, zoveel moois tegen gekomen.

Graag wil ik iedereen die voor mij nieuwe wegen opende, mij weer op koers bracht of een stukje samen met mij opliep heel erg bedanken, zonder jullie had ik mijn reis niet kunnen voltooien. Zonder iemand te kort te doen wil ik graag een paar mensen in het bijzonder bedanken.

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Allereerst de kinderen en volwassenen met een verstandelijke beperking en hun ouders die ik ontmoette, want deze ontmoetingen inspireerden mij voor deze reis en gaven mij steeds de nodige energie en focus onderweg.

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Hanne, jij hebt mij de hoofdwegen laten zien, maar ook gewezen op vele prachtige zijpaadjes, vaak sloeg ik ze in, soms keerde ik toch weer om en uiteindelijk koos ik voor een zelf ontdekt zijpad. Ik heb veel van je geleerden en misschien nog wel het meest om mijn eigen pad te kiezen.

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Wegwijzers
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Jiddeke, wanneer ik de weg kwijt dreigde te raken in alle dysmorfologische details wees jij in de juiste richting zodat ik mijn pad weer vond.
Liedewij, je hielp mij te navigeren op onbekend terrein, met altijd aardige aanmoedigende aanwijzingen, een leuke en leerzame ervaring.

Leden van de promotiecommissie: prof. dr. M.C. Cornel, prof. dr. P. de Knijff, Dr. B.A. de Vries, prof. dr.

B. Wieringa, Dr. A.S. Brooks, Dr. P. Troost, Dr. G. van Haaften, prof. dr. F. Baas, prof. dr. M.H. Breuning voor de kritische blik aan het eind en de laatste richtinggevende aanwijzingen.

Reisgenoten:
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