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# **A Population Study of Risk Factors for Autism Spectrum Disorders in the Faroe Islands**

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of the degree of Doctor of Philosophy*

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To Elizabeth and Veronica



# ABSTRACT

**Objectives:** To study autism spectrum disorder (ASD) in the Faroe Islands, including prevalence, diagnostic stability and environmental factors that are potentially involved in the aetiology of autism.

**Method: I.** The target group was recruited from the entire population sample of participants with ASD during a two-phase screening and diagnostic process of the entire Faroe Islands population in the relevant school age group born between 1985-1994 (7-16 years, n=7,689) in 2002 and again in 2009 (15-24 years, n= 7,128) using an independent clinical diagnosis and standardised tools. **II.** The diagnostic stability of ASD from childhood to early adulthood over a period of 7 years compared diagnoses in 2002 and 2009. **III.** A literature search of vitamin D and ASD covering the period from January 1 1995 to October 31 2011 was carried out. **IV.** A pilot study involved questioning 20 mothers of young individuals from the target group and 13 mothers of healthy comparisons, regarding mothers' diet habits, health, lifestyle and well-being during their pregnancy with an index child. **V.** 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>) levels were examined in a population based cross-sectional study that involved 219 individuals: 40 participants with a diagnosis of ASD from the target group (31 males/9 females), their 62 typically developing siblings (29 brothers/33 sisters), their 77 parents (40 mothers/37 fathers), and 40 healthy comparisons (28 males/12 females).

**Results: I.** The rate of ASD rose significantly from 0.56% (n=43) in 2002 to 0.93% (n=66) in 2009. Although these results were still within the range of typical findings from other studies, of the 24 newly discovered cases in 2009 nearly half were females thus altering the male/female ratio from 6/1 to 2.7/1. **II.** The stability of clinical ASD diagnosis was perfect for AD, good for "atypical autism"/PDD-NOS, and less than perfect for Asperger syndrome (AS). Stability of the diagnoses made by means of research tools were more variable but still good for AD. Both systems showed excellent stability over the seven-year period for "any ASD" diagnosis, although a number of clear cases (especially in females) had been missed in the original screening in 2002. These results support the notion that a single overarching diagnostic category, 'autism' or ASD, would better suit clinical realities as outlined in the new DSM-5. **III.** The systematic review (in 2010) provided some, albeit very

limited, support for the possible role of vitamin D deficiency in the pathogenesis of ASD: there are three main areas of involvement of vitamin D in the human body that could potentially have direct impact on the development of ASD: (1) the brain, (2) gene regulation and (3) the immune system. The prevalence of ASD has been suggested to be raised at higher latitudes. **IV.** Mothers of individuals with ASD had had during their pregnancy significantly less positive “attitude to sun” ( $p=0.001$ ), consumed fewer vegetables ( $p=0.026$ ) and also less fruit ( $p=0.078$ ). **V.** The ASD case group had significantly lower 25(OH)D<sub>3</sub> levels (24.8 nmol/L) than their typically-developing siblings (42.6 nmol/L,  $p<0.001$ ) and their parents (44.9 nmol/L,  $p<0.001$ ), and also significantly lower than healthy age and gender matched comparisons (37.6 nmol/L,  $p=0.002$ ). There was a trend for males having lower 25(OH)D<sub>3</sub> levels than females. There was no association between vitamin D and age, month/season of birth, IQ or subcategories of ASD. Among the ASD group, 60% were severely deficient ( $<30$  nmol/L) and 84.2% of the whole study sample ( $n=219$ ) had deficient/insufficient levels ( $<50/<75$  nmol/L).

**Conclusions: I.** ASD prevalence in the Faroe Islands increased from 0.56% in 2002 to 0.93% in 2009 mainly due to missed cases in 2002, nearly half of them females.

**II.** There was diagnostic stability for the overall category of ASD over time in the group diagnosed in childhood (7—16) years, but considerable variability with regards to diagnostic sub-groupings. Diagnosing females require novel approach.

**III.** Vitamin D deficiency—either during pregnancy or early childhood—may be an environmental trigger for ASD in individuals genetically predisposed to the broad phenotype of autism. **IV.** There are some interesting differences in the diet and lifestyle habits between mothers with a child with ASD and mothers with a healthy child. The ASD-group’s negative “attitude to sun” may indicate some lifestyle/health differences which may play a role in pathogenesis of ASD, especially in combination with other environmental risk factors.

**V.** The present study, demonstrating an association between low levels of 25(OH)D<sub>3</sub> and ASD, is the first to be based in a total population and to use siblings, parents and general population control groups. It adds to similar findings from other regions of the world, indicating vitamin D deficiency in the population and especially in individuals with ASD. As all groups were exposed to low levels of sunlight, the very low 25(OH)D<sub>3</sub> in the ASD group suggests that some other underlying pathogenic mechanism may be involved.





Faroe Islands. Photo by courtesy of Olavur Frederiksen.

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## Author's Declaration

This thesis represents the original work of Eva Kočovská unless explicitly stated otherwise in the text and is divided into five studies (I-V see Table 2). The research was carried out at the University of Glasgow under the supervision of Dr Helen Minnis, Professor Christopher Gillberg and Dr Eva Billstedt during the period of March 2010 to October 2013. The major part of the work described herein has been published as listed below (including the studies I-V):

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**II. Kočovská, E.,** Fernell, E., Billstedt, E., Minnis, H., & Gillberg, C. (2012). Vitamin D and Autism: Clinical Review. *Journal of Research in Developmental Disabilities*, 33, 1541-1550.

**III. Kočovská, E.,** Billstedt, E., Ellefsen, Á., Kampmann, H., Gillberg, I. C., Biskupstø, R. Andorsdóttir, G., Stórá, T., & Gillberg, C. (2013). Autism in the Faroe Islands: Diagnostic stability from childhood to early adult life. *The Scientific World Journal*, Special Issue on ESSENCE, Article ID 592371, <http://dx.doi.org/10.1155/2013/592371>.

**IV. Kočovská, E.,** Andorsdóttir, G., Weihe, P., Halling, J., Fernell, E., Stórá, T., Biskupstø, R., Gillberg, I.C., Shea, R., Billstedt, E., Bourgeron, T., Minnis, H., & Gillberg, C. (2014). Vitamin D in the General Population of Young Adults with Autism in the Faroe Islands. *Journal of Autism and Developmental Disorders*. E-published June 2014 DOI : 10.1007/s10803-014-2155-1

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# ABBREVIATIONS

## Abbreviation Description

1,25(OH) <sub>2</sub> D	calcitriol 1,25-dihydroxycalciferol, or cholecalcitriol
1,25(OH) <sub>2</sub> D <sub>3</sub>	calcitriol for 1,25-dihydroxycholecalciferol
25(OH)D	calcidiol, or cholecalcidiol.
25(OH)D <sub>3</sub>	calcidiol, or 25-hydroxy-cholecalciferol
A	Average intelligence (IQ 85-114)
AA	Above Average intelligence (IQ≥115)
AD	Autism/Autistic Disorder
ADD	Attention Deficit Disorder
ADHD	Attention-Deficit/Hyperactivity Disorder
ADI-R	Autism Diagnostic Interview-Revised
ADOS	Autism Diagnostic Observation Schedule
ADOS-G	Autism Diagnostic Observation Schedule-Generic
APA	American Psychiatric Association
AS	Asperger's Syndrome
ASD	Autism Spectrum Disorder
ASSQ	Autism Spectrum Screening Questionnaire
ATEC	Autism Treatment Evaluation Checklist
BD	Bipolar disorder
BDNF	Brain-derived neurotropic factor
BISCUIT	Baby and Infant Screen for Children with aUtIsm Traits
BMI	Body Mass Index
Br <sup>-</sup>	Bromide
CARS	Childhood Autism Rating Scale
CH <sub>3</sub> HgX	Methylmercury salt (X is typically a halogen),
CI	CI confidence intervals
Cl <sup>-</sup>	Chloride
CNS	Central nervous system
CNV	Copy number variants
CPA	Commercial Product Assurance

CRC	Clinical Research Comprehensive (diagnosis)
DCD	Developmental Coordination disorder
7DHC	7-Dehydrocholesterol
DEQAS	(Vitamin) D External Quality Assessment Scheme
DISCO	Diagnostic Interview for Social and COmmunication Disorders
DISCO-10	Diagnostic Interview for Social and COmmunication Disorders. 10 <sup>th</sup> version.
DISCO-11	Diagnostic Interview for Social and COmmunication Disorders. 11 <sup>th</sup> version
DNA	Deoxyribonucleic acid
DSM-4	Diagnostic and Statistical Manual of Mental Disorders. 4 <sup>th</sup> Edition
DSM-5	Diagnostic and Statistical Manual of Mental Disorders. 5 <sup>th</sup> Edition
EDTA	Ethylenediamine tetraacetic acid
EPA	The US Environmental Protection Agency
ESSAT	Early Screening of Autistic Traits
ESSENCE	Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations
F	Female
FFQ	Food Frequency Questionnaire
FSIQ	Full Scale IQ
GLM	General Linear Model
GP	General Practitioner
Hg	Mercury
Hg <sup>0</sup>	Mercury – metallic (oxidation state 0)
Hg <sup>I</sup>	Mercury (oxidation state +1)
Hg <sup>II</sup>	Mercury (oxidation state +2)
I <sup>-</sup>	Iodide
ICD-10	International Classification of Diseases. 10 <sup>th</sup> Edition
IDD	Intellectual developmental disorder
IQ	Intelligence quotient
IQR	Inter-Quartile Range
IU	International Unit(s)
LC-MS/MS	Liquid Chromatography-Mass Spectrometry/Mass Spectrometry
LD	Learning Disability

LD/ID	Learning/Intellectual Disabilities
M	Male
MAG	Myelin Associated Glycoprotein
M-CHAT	Checklist for Autism in Toddlers
MeHg	Methylmercury (chemically inaccurate abbreviation)
MLD	Mild Learning Disability (IQ 50-69)
N	North (latitude)
NA	Near Average intelligence (IQ 70-84)
NAMMCO	North Atlantic Marine Mammal Commission
NATO	North Atlantic Treaty Organization
ng/mL	Nanogram per millilitre
nmol/L	Nanomol per litre
NO <sub>3</sub> <sup>-</sup>	Nitrate
°C	Centigrade (degree of Celsius)
OCD	Obsessive Compulsive Disorder
ODD	Oppositional defiant disorder
OH <sup>-</sup>	Hydroxide
PCB	Polychlorinated biphenyl
PCBs	Polychlorinated biphenyls
DDE	Dichlorodiphenyldichloroethylene
PDD	Pervasive Developmental Disorder
PDD-NOS	Pervasive Developmental Disorder Not Otherwise Specified
PGR	Progesterone receptor
PIQ	Performance IQ
PON1	Paraoxonase
prcP	Urinary porphyrin precoproporphyrin
PSI	Processing Speed Index
PRISMA	Preferred Reporting Items for Systematic Reviews
PTH	Parathyroid hormone
PUFA	Polyunsaturated fatty acids
RfD	Reference dose
ROS	Reactive Oxidative Species
SCQ	Social Communication Questionnaire
SD	Standard Deviation

SH	sulfhydryl (-SH)
SIB	self-injurious behaviour
SID	Social Interaction Disorder
SLD	Severe Learning Disability ( $IQ \leq 49$ )
SLI	Speech and language impairment (SLI)
SLOS	Smith-Lemli-Opitz syndrome
SNP	Single-Nucleotide Polymorphisms
Sq km	Square kilometre
TF	Transferrin
UV	Ultra Violet
UVB	Ultra Violet light in B-region (wavelength at 290-320 nm)
VDR	Vitamin D Receptor
Vitamin D	Vitamin D in general.
Vitamin D <sub>2</sub>	Ergocalciferol (derived from ergosterol)
Vitamin D <sub>3</sub>	calciferol for cholecalciferol (derived from cholesterol)
VIQ	Verbal IQ
WADIC	Wing's Autistic Disorder Interview
WAIS-R	Wechsler Adult Intelligence Scale-Revised
WHO	World Health Organization
WISC-III	Wechsler Intelligence Scale for Children. Third Edition.
WISC-R	Wechsler Intelligence Scale for Children-Revised
X <sup>-</sup>	Anion with one negative charge, e.g., chloride (Cl <sup>-</sup> ), bromide (Br <sup>-</sup> ), iodide (I <sup>-</sup> ), hydroxide (OH <sup>-</sup> ), and nitrate (NO <sub>3</sub> <sup>-</sup> ), etc.
μL	microlitre
μg/L	microgram per litre

## A. BACKGROUND

Autism spectrum disorders (ASD) are a heterogeneous group of complex, biologically based neurodevelopmental disorders that undermine optimal brain development and are marked by altered communication and social skills, by cognitive and learning deficits and by stereotypic behaviours. It has been well established that genetic factors play a major role in development of autism (Levy et al., 2009). However, establishing a discrete pathogenesis of autism has proven to be extremely difficult. The common *de novo* point mutations that have been identified are associated with only a small proportion of cases. Recent studies portray the role of signalling pathways in the brain and the synapse structure as crucial to the development of ASD (Gillberg & Coleman, 2000).

There is no agreement as to whether ASD prevalence is genuinely on the rise or if a higher reported rate in recent years might be secondary to better awareness, changing diagnostic trends and more sensitive diagnostic systems (Coleman et al., 2011) and possibly also to diagnosing ASD in females only at older ages, alongside the rising awareness of the existence of ASD in girls (Kopp & Gillberg, 1992; Giarelli et al., 2010; Kočovská et al., 2013).

It is also clear that environmental factors, such as infectious diseases and teratogen exposure in utero, can cause autism and that in some cases there must be an interaction between genetic and environmental factors (Gillberg and Coleman, 2000). Understanding gene-environment interactions in autism (Freitag et al., 2010) is currently a very important topic for research into early neurodevelopment. To study these interactions in genetic isolates with specific environmental exposures presents itself as a model of choice for this type of research.

The Faroe Islands are situated in the heart of the Gulf Stream in the North Atlantic Ocean, half way between Norway and Iceland at 62°00' N. Given the genetic isolate character, the Faroe Islands constitute an interesting environment with many variables being unusually stable, e.g., socioeconomic status, education, health care, familial/genetic history and diet. There are several environmental factors specific to this geographical region, which are likely to be of significance in the interplay between the occurrence of autism and environmental and genetic variables, such as methylmercury poisoning and vitamin D deficiency (Kočovská et al., 2014).

**Mercury:** Methylmercury is a well-established neurotoxicant that can have serious adverse effects on the development and functioning of the human central nervous system, especially when exposure occurs prenatally (World Health Organization, 1990; Zahir et al., 2005; Johansson et al., 2007). The biological plausibility of the hypothesis that mercury (Hg) is linked to neurodevelopmental disorders, including autism, has been demonstrated on a cellular level (Garrecht & Austin, 2011). Methylmercury has the capability of crossing the blood-brain barrier and its lipophilic nature allows binding to neurons and acting as a very potent neurotoxin. Chronic, low-dose prenatal exposure to methylmercury from maternal consumption of seafood/fish has been associated with adverse neurodevelopmental effects (attention, fine motor function, language, visual-spatial abilities and verbal memory) (Grandjean et al., 1997; Debes, 2006). This pollutant is of considerable public concern, because it is found in sea-food and freshwater fish throughout the world (World Health Organization, 1990).

In this small homogeneous Nordic community large variations in seafood intake occur whereas social differences are limited and the potential for other neurotoxic exposures are also limited. Some residents have an excessive exposure to methylmercury, mainly due to the traditional habit of eating pilot whale meat. It has been known for a long time that pilot whale meat contains a large amount of mercury (Juhlshamn et al., 1987).

**Vitamin D:** Calcitriol, the active form of vitamin D, is biosynthesised in the body via a cascade of chemical transformations, initiated by a photochemical reaction in the skin on exposure to the ultraviolet rays of the sun. The Faroe Islands' location and its maritime climate (high rainfall, strong winds, an average summer temperature of 9 °C) reduces the availability of the UVB radiation of sun rays, which has a negative impact on the natural production of vitamin D in the human body. Conversely, the Faroe Islanders have a diet rich in large oily fish containing vitamin D that could possibly, at least in part, compensate for the lack of UVB exposure, which in turn might mean that overall vitamin D status in the Faroe Islands could be adequate (Kočovská et al., 2014).

An apparent worldwide epidemic of vitamin D deficiency is now being recognised (Holick, 2007). The prevalence of ASD has been suggested to be increased at higher latitudes and in children of migrant mothers with darker skin (Dealberto, 2011). Recently, maternal/neonatal vitamin D deficiency has been

proposed as a possible environmental risk factor for ASD (McGrath et al., 2001; Cannell, 2008; Cannell & Grant, 2013; Grant & Soles, 2009; Kočovská et al., 2012a) due to its unique role in brain homeostasis, embryogenesis and neurodevelopment, immunological modulation (including the brain's own immune system), ageing, and also, importantly, in gene regulation (Sigmundsdóttir, 2011; Harms, Burne, Eyles, & McGrath, 2011; Ramagopalan et al., 2010). Interesting results, both at the molecular level and in animal experiments (Fu et al., 1997; Neveu et al., 1994c; Eyles et al., 2003; 2005), begin to indicate the possible mechanisms for this potential risk. Indirect support for the involvement of vitamin D in ASD comes from ecological studies, according to which vitamin D levels vary with season and latitude and with the degree of skin pigmentation (Grant & Soles, 2009; Dealberto, 2011).

The end product of vitamin D biosynthesis is calcitriol (1,25-dihydroxyvitamin D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub>), a neuroactive hormone that signals via nuclear receptors (Eyles et al., 2005; Eyles, Burne & McGrath, 2013). The last 15 years have witnessed great advances in explaining the biochemical mechanisms of the diverse actions of calcitriol in the brain, especially its role in early neurodevelopment and in degenerative processes: (1) cell differentiation and axonal growth; (2) stimulation of neurotrophic factor expression (e.g., cytokines); (3) regulation of calcium signalling directly in the brain; (4) modulation of the production of the brain-derived reactive oxygen species; (5) stimulation of glutathione (a potent anti-oxidant, involved in DNA repair) and thereby down-regulating excitotoxicity (Eyles, Burne & McGrath, 2013; Eyles et al., 2005; Garcion et al., 2002; Cannell, 2013).

Since several of these processes are targeted by the devastating effects of Hg-induced neurotoxicity within the brain, it is tempting to speculate that this diminished protective function of calcitriol during early neurodevelopment (due to vitamin D deficiency at higher latitudes) in conjunction with possible individual genetic predispositions to autism and/or genetic predisposition to Hg toxicity and combined with exposures of varying degrees to a number of other environmental factors, may escalate into neurodevelopment disorder.

Thus studying the interplay of these two environmental factors, i.e., the methylmercury toxicity and vitamin D deficiency, in the ASD population sample in the genetically isolated Faroe Islands offers a unique edge for research.

The present study aims to disentangle the effects of gene-environment interaction in autism in the Faroe Islands. Specifically, this study explores two environmental variables: diet (methylmercury) and vitamin D deficiency. The results were expected to have an influence on dietary counselling in autism spectrum disorders (ASD) and on the development of more sophisticated research protocols for studying gene-environment interaction in ASD.

## B. INTRODUCTION

### B. 1. Autism spectrum disorder: a brief review of past and present concepts

Autism Spectrum Disorders (ASDs) are a heterogeneous group of complex, biologically based neurodevelopmental disorders that undermine optimal brain development and are characterised by altered communication and social skills, by cognitive and learning deficits and by stereotypic behaviours. For most phenotypes of autism there are no reliable biomarkers or discrete pathogenesis.

#### B. 1.1. Clinical presentation of ASD

As Coleman & Gillberg have described, onset, development and phenotypic presentations of autism show a huge variability with two main types: either (a) early manifestation by deviation from the normal progression of early development or (b) sudden regression later, sometimes precipitated by an environmental event (e.g. immune or toxic exposures) after a period of apparently normal development during the first years. However, even in these cases there is usually a pattern of some anomalies or delays detected later. Some *early signs* indicating a possible risk for autism might include slight physical anomalies (e.g., macrocephaly, ears anomalies, muscular hypotonia in new-born, motor function/style and/or abnormal facial expression), difficulties with or non-existent symbolic play or manifestation of odd play habits (e.g., obsessive interest in parts of toys/objects instead of proper functional uses of the whole object) (Coleman & Gillberg, 2012).

Among the most typical *early symptoms* are lack of mimicry and face expressiveness (suggesting early abnormalities of motor functioning) (Teitelbaum et al., 1998) and abnormal responses to sensory stimuli (Ornitz et al., 1978; Ornitz, 1988; Gillberg, 1989). The most frequent *clinical manifestations* of autism are delay, deviation or loss of verbal and nonverbal communication skills, the absence of protodeclarative pointing (an important gesture of the index finger used to draw someone's attention to an object to comment on it or share interest in it) and/or joint attention, impaired eye contact, and unusual responses to environmental stimuli, e.g.

excessive reaction or unexpected lack of reaction to sensory input (extreme sensitivity to certain sounds, bright lights, touching and/or an extremely high threshold to pain and physical injury and/or atypical reaction to pain) (Coleman & Gillberg, 2012).

### **B.1.2. Neuropsychological aspects**

When evaluating ASD from the psychological point of view, most prominent are the symptoms demonstrating lack or deficits in the Central Coherence (Frith, 1996) and Theory of Mind /empathy (Baron-Cohen et al., 1992). ‘Weak central coherence’ can be manifested via (1) a lack of appropriate understanding of the present context; (2) inability to combine various types of information; (3) inability to create a whole from its components; (4) inability to see consequences; (5) inability to be able to predict what will happen next. Instead, in ASD there is usually a marked detailed peripheral processing style (preference for parts vs wholes or greater attention to local information but poor at tasks requiring the recognition of global meaning) (Frith & Happe, 1994; Frith, 1996). ‘Theory of mind/empathy’ refers to a concept of ability to infer what others are thinking/believing/desiring which allows comprehension and prediction of behaviour (Frith, 1989; Baron-Cohen, 1995). ASD is typically associated with limited theory of mind/poor mentalising abilities demonstrated usually by dysfunction in social communication/interaction. Individuals with ASD are often unable to understand why other people behave and react the way they do. They also have difficulties expressing their own feelings, thoughts and desires (Frith, 2012).

The consequences of weak central coherence and impaired theory of mind in ASD impact on interactions with family members and in other social scenarios. Individuals with autism may have problems in making friends and understanding the social intentions of others and may instead demonstrate their preference for attachments to objects. Although they may desire to have friendships with other children/people, their behaviour may result in them being frequently rejected by other children/people. They may also exhibit inappropriate friendliness and lack of awareness of personal space. Thus, all these problems lead to the isolation of children with autism which becomes an even greater problem in adolescence and young adulthood (Liptak et al., 2011; Baron-Cohen et al., 1992).

### **B.1.3. Comorbidities in ASD**

It has been acknowledged that there are a number of comorbidities frequently identified in individuals with autism. These can be of genetic, neurodevelopmental, mental, or behavioural origin or resulting from environmental exposure. Among the most common comorbidities are the learning/intellectual disabilities (LD/ID), epilepsy, tics, Attention-Deficit-Hyperactivity Disorder (ADHD), developmental coordination disorder (DCD), Obsessive Compulsive Disorder (OCD), Bipolar Disorder (BD), anxiety disorders, depressive disorders, Anorexia nervosa, sleep disorders, disruptive behaviour, self-injurious behaviour (SIB), impulse control problems, conduct disorders, feeding problems, catatonia and mutism, foetal alcohol syndrome and very low birth weight (Coleman & Gillberg, 2012).

#### *B.1.3.1. ESSENCE*

There is high co-appearance of symptoms among various neurodevelopmental/psychiatric disorders (Chawarska et al., 2009; Emerson & Einfield, 2010; Einfield et al., 2011; Kantzer et al., 2013). This phenomenon requires a holistic approach of examination and diagnostic process rather than the compartmentalised approach that is common at the moment. Autism belongs to syndromes grouped under the umbrella term ‘Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations’ (ESSENCE) coined by Gillberg (2010), to underscore the importance of examining children with developmental deviations “in the fields of: (a) general development; (b) communication and language; (c) social interrelatedness; (d) motor coordination; (e) attention; (f) activity; (g) behaviour; (h) mood and/or sleep” (Gillberg 2010, p. 1544). Children with deficits in one or more of these areas require a tailored multidisciplinary assessment. ESSENCE is an overarching term for various syndromes which may co-occur and whose symptoms often emerge in early childhood (see Table 1.). It is important to stress that ESSENCE is not a diagnosis in itself.

**Table 1.** ESSENCE Syndromes (modified from Gillberg, 2010).

<b>Syndrome</b>	<b>Prevalence</b>
ASD	1%
Intellectual Disability (ID)	1.5%
ADHD	5%
Developmental coordination disorder (DCD)	5%
Oppositional defiant disorder (ODD)	4%
Speech and language impairment (SLI)	2-4%
Tic disorders/Tourette syndrome	1%
Bipolar Disorder (BD)	1%
Reactive attachment disorder	0.5-1%
Behavioural phenotype syndromes	1%
Rare epilepsy syndromes	0.01%
<b>Total</b> taking overlap into account	<b>7-10%</b>

#### *B.1.3.2. Medical comorbidities*

In addition, there may be co-occurring physical health problems accompanying autism. Recent research has demonstrated that there are several medical conditions that are significantly more prevalent in individuals with autism compared to typically developing populations. Among these are for example eczema, allergies, asthma, ear and respiratory infections, gastrointestinal problems, severe headaches, migraines and epilepsy (Kohane et al., 2012).

Subsequently, mortality is also significantly increased in autism, with death rates being 3-10 times higher than in the general population (Bilder et al., 2012; Woolfenden et al., 2012). The main causes of these deaths tend to be complications arising from medical comorbidities, such as respiratory disorders, gastrointestinal conditions and epilepsy (Shavelle et al., 2001; Pickett et al., 2006; Gillberg et al., 2010; Bilder et al., 2012; Woolfenden et al., 2012).

Adults with autism have been found to be at significant risk for developing diabetes, coronary heart disease and cancer if there is no timely preventative intervention implemented (Tyler et al., 2011). Children with ASD have repeatedly been shown to be at greater risk for rickets, osteomalacia or bone demineralisation

(Neumeyer et al., 2012). Similarly, adults with developmental disorders exhibit a much higher risk for osteoporosis and showing severe degrees of bone demineralisation – a process most probably originating from their childhood when not recognised and treated appropriately (Jaffe et al., 2001; Jaffe et al., 2003).

#### **B.1.4. Neuropsychological aspects of ASD**

The intellectual disability (IQ < 70) is the most common and arguably most debilitating comorbid diagnosis accompanying autism which significantly hinders individuals' chances of improvement and of an independent lifestyle in adulthood. It has been shown that children with combined ASD and ID do not gain as much as would be expected from education and/or therapeutic interventions and a majority of them stay in the same diagnostic category over their lifespan, with a need of high-level support (e.g. living with parents, in institutional facilities or sheltered accommodation) (Coleman and Gillberg, 2010). Only a small proportion of individuals with autism and low IQ achieve independence (Howlin et al, 2004). Even in individuals with high-functioning ASD (IQ > 70) often their ASD deficits (e.g. repetitive behaviours) outweigh their higher cognitive levels (Howlin et al, 2004).

The earliest studies of ASD in adult life showed that many individuals had high dependency for support, with the majority of adults living with parents, in institutional facilities or sheltered accommodation (Rutter et al, 1967). Among individuals with higher levels of cognition – people with high-functioning autism (conventionally defined as having IQ of 70 or over) – deficits or difficulties in the dimensions associated with ASD (and in particular in relation to repetitive behaviour) may outweigh positive outcomes associated with a higher IQ (Howlin et al, 2004).

#### **B.1.5. Impact of ASD on individuals/families/society**

##### *B.1.5.1. Psycho-social consequences*

The social impairments of individuals with autism lead to difficulties in understanding their surrounding environment and consequently results in their inability to deduce from the present the future course of events. This creates states of

insecurity and anxiety that are often expressed through various stress-reducing obsessive behaviours, e.g., flapping, rocking and/or challenging behaviours – kicking, biting, tantrums, etc. (Coleman & Gillberg, 2010).

Due to its physical ‘invisibility’, autism draws the general public condemnation of ‘odd’ behaviour and apparent parental inability to control their child. This leads to social isolation of children with ASD and their families, which has a detrimental effect on their social and emotional wellbeing and physical health and also results in great financial strain (Knapp et al., 2009).

The impact of having ASD for an individual might include a failure in academic, social and work situations, resulting in lack of confidence, low self-esteem, and vulnerability to abuse, high anxiety, depression and impaired mental health and often loss of independence (Knapp et al., 2009).

As origins and phenotypic expression vary enormously, so does the impact and outcomes for individuals. These can range from being odd and/or eccentric and managing to live an independent life to extremely severe autism, often accompanied by other comorbidities and requiring constant help/input from others. Due to this frequent presence of other problems of the ESSENCE group, it has been proposed to shift the clinician’s emphasis more to a person’s individual needs rather than onto diagnostic categories and symptoms (Gillberg, 2010).

#### *B.1.5.2 Monetary impact*

The societal cost of autism has been calculated as treble the price of a typically developing child and equating to a total sum of £28 billion/year in the UK and \$137 billion /year in the USA (Knapp et al., 2009).

### **B.1.6. Diagnosis of ASD and diagnostic instruments**

#### *B.1.6.1. Definition, changing nature and social constructs of ASD*

Stemming from the definition of a neurodevelopmental disorder, ASD presents changes in symptomatology with age according to varying stages of nervous system maturation (Wing, 1980) and this fact has to be strongly emphasised because many children with autism develop in very different directions (Coleman & Gillberg,

2012). The diagnosis thus can be obtained between 20 months to 20+ years but in majority cases the impaired neurodevelopment is due to chromosomal aberrations or genetic mutations in the germs cells prior to conception or *de novo* very early in gestation (Coleman & Gillberg, 2012). Thus, there are numerous patterns of ASD trajectories described in the literature varying from an ASD diagnosis obtained in early infancy or on entry to primary school or at emergence of puberty up to a diagnosis obtained well in adulthood. There are also many variations in diagnostic stability among those on the ASD spectrum – some receive their diagnosis in childhood and still fulfil diagnostic criteria well into adulthood and vary in the degree of required assistance from independent through moderate to severe dependency. For many others there are interchangeable patterns of their symptomatic phenotype: early diagnosis in childhood and then ‘loss’ of their symptoms in adolescence or quite an opposite pattern of worsening with the onset of adolescence (Coleman & Gillberg, 2012). Lorna Wing described three subgroups that present with different symptoms in childhood: the “aloof”; the “active but odd”; and the “passive”; that also correspond to a similar division of cases in adult age (Wing, 1996). She later added and focused her research on the fourth group called the “rigid”. Together with Christopher Gillberg Lorna Wing held a view that all people could be divided into such subgroups, not just those with ASD. Generally, for many cases, there is usually an improvement in behavioural symptoms during early school years, followed by a marked cognitive and behavioural deterioration (10-30%) or improvement (in some high-functioning cases) in adolescence. Interestingly, the Gillberg & Steffenburg’s (1987) study found a rate of deterioration in 50% of girls. The authors have suggested that high maternal age, female sex and the family history of affective disorders might increase the deterioration in puberty. Depression/anxiety is often present during puberty in high-functioning individuals with autism due to their realisation of their difference from peers and to their longing for a friendship that they are unable to establish. This is a more pronounced problem in families with affective disorder (Coleman & Gillberg, 2012).

Any subtle or major symptoms of ASD usually appear in most cases before or around 18 months of age but it is advisable to wait with a final diagnosis until after 30 months of age. The abnormal neurodevelopment may well have started in the prenatal period in some cases but its manifestations may have been obscured for some time. This may be because the nervous system is able to deal with the demands

posed by development up to a certain point, but then the brain is not able to cope with these demands anymore and the autistic symptoms begin to manifest for the first time. Thus even if ASD is of congenital origin, it might appear as having its onset after infancy. Another practical reason for postponing the final diagnosis is the inability to test two major symptoms of ASD – language and peer relationship deficits - before the age of 2 years, as these functions are not yet developed (Coleman & Gillbeg, 2012).

#### *B.1.6.2. DSM-IV and DSM-5*

The new Diagnostic and Statistical Manual of Mental Disorders (DSM-5) has changed its diagnostic system for autism from several separate categories (early infantile autism, childhood autism, Kanner's autism, high-functioning autism, atypical autism, pervasive developmental disorder not otherwise specified, childhood disintegrative disorder and Asperger's disorder) into one overarching category – autism spectrum disorder (ASD). The previous 'triad of impairments' underlying autism in DSM-IV (social interaction, communication and stereotyped behaviours) (American Psychiatric Association 1994) has been reduced to two new diagnostic criteria in DSM-5: social communication and interaction and restricted behaviours (American Psychiatric Association 2013). These two categories are underpinned by behavioural genetic research on a large twin study Ronald et al. (2008).

As it is not possible anymore to cite the DSM-5 criteria/symptoms for a diagnosis of autism without a charge, please see the link below for the detailed description of the diagnostic process for ASD.

[https://depts.washington.edu/dbpeds/Screening%20Tools/DSM-\(ASD.Guidelines\)Feb2013.pdf](https://depts.washington.edu/dbpeds/Screening%20Tools/DSM-(ASD.Guidelines)Feb2013.pdf)  
[www.dsm5.org](http://www.dsm5.org)

#### *B.1.6.3. Diagnostic instruments*

There is now general agreement among clinicians and researchers alike that early detection and diagnosis are necessary to allow early intervention at a very young age in order to optimise the prognosis and outcome. Regarding infants, their parents' and a clinician's/health visitor's observations serve best as the first triage with utilisation

of public awareness and of home videos that might be very helpful to the early screening process. There are numerous instruments for detection of ASD among infants and young children; the most widely used are The Baby and Infant Screen for Children with aUtism Traits (BISCUIT; Matson, Fodstas & Dempsey, 2009) and the modified version of the Checklist for Autism in Toddlers (M-CHAT; Baron-Cohen, Allen & Gillberg, 1992). For older children and adults there are several screening and diagnostic instruments, e.g., Autism Spectrum Screening Questionnaire (ASSQ; Ehlers, Gillberg, & Wing, 1999), (*Autism Diagnostic Interview-Revised* (ADI-R; Couteur, Lord, & Rutter, 2006); Autism Diagnostic Observation Schedule (ADOS; Lord et al., 2000), and Diagnostic Interview for Social and COmmunication Disorder (DISCO; Wing et al., 2002), etc. The last two instruments have been used in the present study.

### **B.1.7. Gender aspects in diagnosing ASD**

Although the ratio of males:females in ASD appears to be quite stable at 2.5-4.1 despite changes in diagnostic systems to broader and more inclusive definitions of ASD, this occurrence and its underlying mechanism remain poorly understood (Zwaigenbaum, et al., 2012). There are gender-linked genetic differences, in particular an increasing number of rare variants involving genes on the X chromosome in association with ASD, including neurologins 3 and 4 (Blasi et al., 2006; Jamain, et al., 2003; Vincent et al., 2004); epigenetic effects on social cognition related to paternally-imprinted X-linked genes (Skuse, 2006); and also biological differences (e.g. testosterone-related effects on brain development in pre- and post-natal periods (Auyeung et al., 2010; Baron-Cohen et al., 2005), resulting in the male brain being more vulnerable during neurodevelopment to the most severe phenotypic symptoms of ASD (Constantino & Charman, 2012) and contributing to the observed sex ratio differences. A decrease in the male:female ratio was recently observed with increasing paternal age (Anello et al., 2009). In addition, cognitive ability was found to have moderating effect on sex ratio in ASD (<2:1 among children with IQ < 50 and up to 8:1 among average or high intellectual functioning) (Nichols et al., 2008).

In school-age children it has been proposed that girls with ASD (especially those with average or high intelligence) may be less likely diagnosed due to their

milder social and communicative symptoms, relatively intact symbolic play skills and less obvious atypicality of their obsessional interests (horses, popular singers, watching soap operas, etc.) (Kopp & Gillberg, 1992; Nyden et al., 2000).

Reasonable assumption would be to expect gender differences among children/adults with ASD to mirror those naturally occurring differences in the non-ASD population and emerging research does support this assumption (Zweigenbaum et al., 2012; Head et al., 2014). Similarly, societal expectations – e.g. being shy/ quiet is acceptable for girls and within the educational settings perhaps attracts less attention from professionals. Conversely, boys, with their more systematic nature and tendency to play with parts of toys/objects as part of their repetitive behaviours, are perhaps more likely to be considered different/odd and thus attract the attention of parents and professionals at much earlier stage. Missed diagnoses place girls at a disadvantage by not being given fair and equal access to adequate intervention, which subsequently leads to possible development of additional comorbid problems. By contrast, studies of very young children report less pronounced gender differences in cognitive abilities (Carter et al., 2007) and suggest a possibility of timely identifying an unanticipated number of higher functioning girls with ASD.

Clinician's expectations, considering boys as an 'at-risk' group and under-identifying girls with ASD, might also play a role in the present diagnostic process and add to still higher ratio of boys with ASD (Nichols et al., 2008).

It has been acknowledged that in the present diagnostic settings females in order to be diagnosed with ASD have to be generally more impaired than males and often to present with an accompanying intellectual disability. In the low IQ range the male:female ratio corresponds to 2:1 (Dworzynski et al., 2012) while in the high functioning range this ratio increases dramatically to around 4:1 (Elsabbagh et al., 2012).

Until recently, it was generally accepted that among high functioning individuals with ASD males and females do not differ behaviourally, emotionally or intellectually (Constantino & Charman, 2012). Recent research is suggesting that this is not the case (Head et al., 2014).

Within the typically developing population many important aspects of development have been shown to be influenced significantly by gender. Thus, in childhood, boys generally display more superior motor skills while girls typically demonstrate more advanced interpersonal and emotional skills. In puberty young

girls usually continue to demonstrate superior social skills while young males more often establish more stable friendships (Rivet & Matson, 2011). Thus, it seems plausible to expect that gender would have similarly significant impact on the expression of ASD (Carter et al., 2007; Dworzynski et al., 2012; Head et al., 2014). The results of a recent study (Head et al., 2014) support the numerous, previously published clinical reports that females with ASD superficially demonstrate better social and emotional skills than males with ASD and that this may camouflage other diagnostic features. This ‘Camouflage hypothesis’ was first proposed by Wing in 1981 and further supported by numerous clinical descriptions (Attwood, 2007) of females, who develop coping mechanisms or an ability to camouflage their social inadequacies by imitating and memorising acceptable social behaviours even though there may be other indications of the condition. These abilities might be superior to those characteristically expressed by males with ASD and this phenomenon may explain, at least partially, the under-diagnosis of females with ASD. Thus, females on the spectrum display social deficits as compared to typically developing females, but demonstrate relative strengths in this area in comparison to males on the spectrum (Wing, 1981; Attwood, 2007). Kopp & Gillberg (1992) also described cases of females clearly demonstrating autistic-like behaviours but not fully meeting criteria for ASD on the then DSM-III rev. These females are reported to be a ‘diagnostic anomaly’.

In the Head’ et al. (2014) study using the ‘Friendship Questionnaire’ (FQ) (designed by Baron-Cohen & Wheelwright (2003) to measure friendship quality, understanding and empathy) among a sample of 101 children/adolescents 10-16 years old with ASD and matched comparisons and their parents, it was found that, independent of the diagnosis, females demonstrated higher scores on FQ than males; regardless of gender, children with ASD demonstrated lower scores than typically developing children; and that the effect of ASD was independent of gender. Interestingly, females with ASD and typically developing males displayed similar scores on the friendship questionnaire (Head et al., 2014). These findings may lend further support to Baron-Cohen’s ‘Extreme Male Brain theory’ (Baron-Cohen, 2002) with females with ASD displaying characteristics of typically developing males.

The Head’ et al. study concentrated on the social domain. Further research should examine also symptom severity between genders. At present, girls usually have to have more severe symptoms in the language and behaviour domains due to

their stronger abilities in social domain, to obtain a diagnosis. The current diagnostic criteria for ASD do not take into account universally accepted gender differences in sociability, friendship and emotionality and this requires reconceptualization. Furthermore, there is a need for social interventions that are specifically tailored for females with ASD based on the fact that their social capacities are qualitatively different to those of males with ASD (Head et al., 2014).

It is possible that these phenomena, stemming from natural gender differences and societal expectations and based on the fact that historically, there were 8 boys out of 11 in the original Kanner's group, might have led to a clinician's bias towards the preferential identification of males with ASD and to some extent exclusion of females from receiving a diagnosis of ASD (Attwood, 2007).

Typically developing girls during their first year of life appear to demonstrate a stronger social orientation and exhibit more eye contact than boys (Lutchmaya & Baron-Cohen, 2002) and consequently, girls with ASD have more problems in the communication arena while boys present more with repetitive/restrictive behaviour problems and hence are diagnosed earlier (Giarelli, 2010). Therefore, it is most likely that the present diagnostic instruments are not sensitive enough for detection of ASD in girls at very early ages. Thus this poses an important challenge to develop new screening and diagnostic instruments, sensitive enough to identify ASD early in girls who do display anticipated symptoms and also to develop interventions specifically for girls. There is good evidence (e.g. Kopp et al., 2011) that girls with ASD are missed or misdiagnosed at early ages and that, in fact, they had the symptoms from a very early age. There is little to indicate that girls develop ASD later than boys, rather it is more probable that the present diagnostic system lacks in sensitivity to detect ASD early in girls.

### **B.1.8. Aetiology of ASD**

There is no agreement as to whether ASD prevalence is genuinely on the rise or if a higher reported rate in recent years might be secondary to better awareness, changing diagnostic trends and more sensitive diagnostic system (Coleman & Gillberg, 2012) and possibly also to diagnosing ASD in females only at older ages, alongside the rising awareness of autism in girls (Giarelli et al., 2010; Kopp et al., 2010; Kočovská, 2013).

There are multiple genetic and environmental risk factors including a variety of mutated and variant genes, advanced paternal age, exposure to toxins and medications in early development, prematurity, and birth complications (Coleman & Gillberg, 2012).

A major role of genetic factors in pathogenesis of autism has been well established (Levy et al., 2009). A new technique, Comparative genomic hybridization (CGH) is a molecular cytogenetic method for analysing copy number variations (CNVs). This involves the isolation of DNA from the two sources to be compared. CGH allows for the exploration of all 46 human chromosomes in single test and the discovery of deletions and duplications, even on the microscopic scale which may lead to the identification of candidate genes (Strachan & Read, 2010; Pinkel & Albertson, 2005). This new technique allows in 8-10% of cases to identify an aetiological diagnosis of ASD. However, geneticists have not identified a common mutation that is involved in most cases of autism. The so far identified common de novo point mutations are associated with only a small proportion of cases (Cannell, 2013). It is also clear that environmental factors, such as infectious diseases and teratogen exposure in utero, can cause autism and that in some cases there must be an interaction between genetic and environmental factors (Gillberg & Coleman, 2000). Understanding the gene-environment interaction in autism is currently a very important topic for research into early neurodevelopment. To study the gene-environment interaction in genetic isolates (e.g., communities living in a relative geographical seclusion resulting in the absence of genetic exchange with other societies/nations) with specific environmental exposures presents itself as a particularly useful model for such research.

Among the most striking aspects of ASD are: (a) much higher monozygotic (60-90%) than dizygotic (0-10%) twin concordance rates (Muhle, Trentacoste, & Rapin, 2004; Lichtenstein, Carlstrom, Rastam, Gillberg, & Ancharsater, 2010); (b) large variability of phenotypic expression (even among monozygotic twins) (Lundstrom et al., 2012); (c) distinct gender ratio (2-4 males to 1 female) (Nygren et al., 2011) due perhaps to the more vulnerable young male brain and/or sex-linked genetic differences; (d) relationship between autism and immune dysfunction (Coleman et al., 2011); and (e) much increased rate of ASD among dark skinned children living at Northern latitudes (Gillberg, Steffenburg, Borjesson, & Andersson,

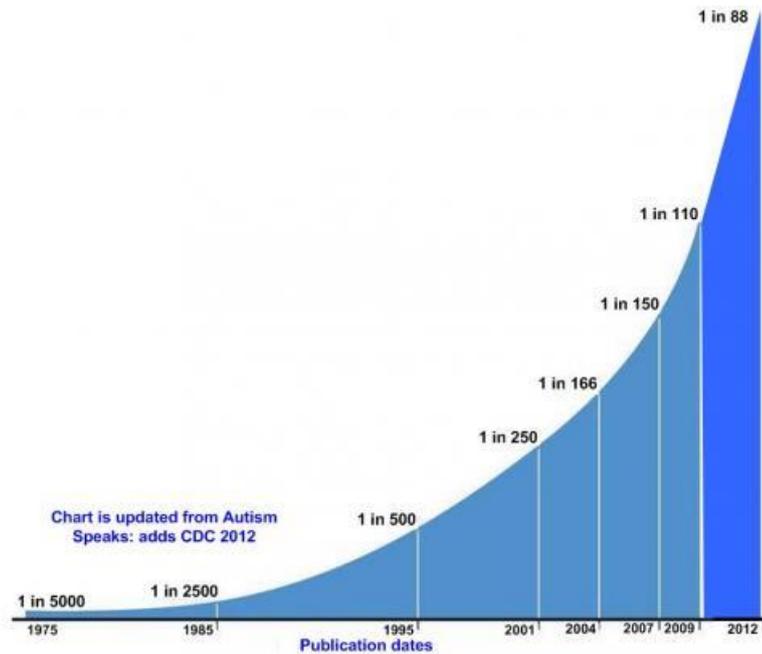
1987; Barnevik-Olsson, Gillberg, & Fernell, 2008; Eyles, 2010). These have led researchers to begin to address the potential role for Vitamin D in autism.

### **B.1.9. Prevalence of ASD (I)**

#### *B.1.9.1. Global trends*

Autism is much more common than previously believed (Gillberg & Wing, 1999; Baron-Cohen et al., 2009). It is clear that the increase in reported prevalence is – to some extent – due to changes in diagnostic criteria, and heightened awareness (Fernell & Gillberg, 2010), but there remains uncertainty as to whether autism “in itself” is on the increase. There is a growing concern that girls with autism might be missed if screening is performed at young ages (Baird et al., 2006; Kopp, Kelly, & Gillberg, 2010; Kim et al., 2011; Zwaigenbaum et al., 2012; Kočovská et al., 2013).

Reported rates of ASD vary worldwide. Generally, it is estimated that ASDs affect up to 10-15 people per 10,000 population (Fombonne, 2008). However, there are reports of an increase in rates from various places around the Globe (e.g., Iceland 120.1 per 10,000 (Saemundsen et al., 2013)). In the U.S.A. the rates vary from 1 in 210 for eight year old children in Alabama to 1 in 47 for children in Utah – there is an overall increase from 1 in 110 in 2009 to 11.3 in 1,000 in 2012 (23% increase) (Figure 1). In a South Korean community an estimated prevalence of ASD of 2.64% has been reported (Kim et al., 2011). The highest rates of ASD so far have been found in Japan; there is a possible explanation that these findings reflect either more careful evaluations by their clinicians or alternatively an impact of more common gastrointestinal and other infections transmitted by seafood (Kurita, 2001). The UK rates of ASD have also been on rise: in 2002, 1 in 200 children were diagnosed (0.5%) while in 2012 this rate increased to 1 in 125 children (Scott et al., 2002). The lifetime cost for someone with high-functioning autism was found to be £3.1 million and £4.6 million for someone with low-functioning autism (Knapp et al., 2007; 2009).



**Figure 1.** Autism Prevalence, Autism Speaks (USA).

<http://www.ncbi.nlm.nih.gov/pubmed/22456193>

Due to reported increased rates of autism world-wide, gene-environment interaction has recently become the focus of intensified ASD research (Freitag, Staal, Klauck, Duketis, & Waltes, 2010) as well as greater awareness of gender discrepancies at various development stages of screening process (e.g., not detecting girls at early stages) (Giarelli et al., 2010).

To this date there are still varying opinions regarding the onset of ASD with most researchers agreeing that ASD develops during gestation – some believe that first 20-24 days of gestation days are crucial – and that symptoms are present from birth onward. However Rogers’ review of developmental literature concluded that ASD is a disorder with a gradual onset of symptoms beginning during the first 2-3 years of life (Rogers, 2009).

### *B.1.9.2. Present study (I)*

Time 1 (2002): Several years ago a population study of prevalence of ASD was performed in the Faroe Islands (Ellefsen, Kampmann, Billstedt, Gillberg, & Gillberg, 2007). All children of 7-16 years of age were screened for autism in the general population, in all schools and all relevant registers. Screen-positive cases were examined in depth, including collateral interviews with the parents using the Diagnostic Interview for Social and COmmunication disorders (DISCO-10) (Wing, Leekam, Libby, Gould, & Larcombe, 2002). A prevalence of 0.56% of the whole age cohort was found, meeting the ICD-10 clinical (and research) diagnostic criteria for childhood autism, atypical autism, or Asperger's syndrome (according to Gillberg & Gillberg, 1989, criteria).

I became involved in the second screening phase at Time 2 (2009): the same cohort has now been re-screened and re-examined in depth by a clinical researcher not involved in the original diagnostic study, with a view to establishing whether: (a) cases might have been missed in the original study, and (b) prevalence rates might have changed significantly over the follow-up period of 7 years. Furthermore, I in particular, endeavoured to test the hypothesis that (c) girls might have been missed out in the previous screening, and that therefore (d) the prevalence rate of autism in females might have gone up drastically over the 7-year period (Kočovská et al., 2012b).

### **B.1.10. Diagnostic stability of ASD (II)**

Almost since the beginnings of its history in clinical medicine, childhood autism/autistic disorder (AD) has been regarded as one of the most, if not the most, stable diagnostic categories applied to young children with psychiatric/developmental disorders (Gillberg, 2010; Coleman & Gillberg, 2012). In the last several years, a number of studies have demonstrated that autism is not a distinct “either/or” phenomenon, but often can be seen as a dimensionally distributed collection of traits in the general population (Constantino & Todd, 2005; Posserud, Lundervold & Gillberg, 2006; Lichtenstein et al., 2010). Recent hypotheses include those that see autism (or Autism Spectrum Disorder/ASD, or Pervasive Developmental Disorder/PDD) as the lowermost portion on a spectrum of “autistic

traits” shading into normally distributed similar traits in the population and that its basis is genetic, regardless of whether one is dealing with “caseness” or “the broader/normal phenotype” (Gillberg, 1992; Lundstrom et al., 2012). However, the question remains as to whether, just as in intellectual developmental disorder (IDD), ASD as a clinical diagnosis, in some cases represents pathological (and qualitatively different) variants that cannot be explained as a normally distributed trait (perhaps associated with brain damage or other non-genetic factors). Diagnostic stability would, hypothetically, be high for ASD under such a model (Kočovská et al., 2013).

Studies reporting on diagnostic stability of ASD from 2005 onwards have concentrated on very young and pre-school age children. Most studies compared the stability of clinical diagnosis over a 2-year period and only one study reported a follow-up interval of 7 years (from age 2 to 9 years) (Woolfenden et al., 2012). The overarching category of ASD (encompassing all the diagnostic subcategories, including Autistic Disorder (AD), Asperger Syndrome (AS) and PDD/Not Otherwise Specified (NOS) DSM-IV (APA, 1994); ICD-10 (WHO, 1993)) has been repeatedly reported as very stable (>90%), and the ‘core autism’ (AD) and AS categories have been found to be more stable than the PDD-NOS category (Daniels et al., 2011; Rondeau et al., 2011).

I have had the opportunity to study the diagnostic stability of ASD from childhood to early adult life in this total population sample in the Faroe Islands, both as regards independent clinical comprehensive diagnosis and in respect of DISCO algorithm diagnosis of ASD at two time points, separated by seven years (see Results, Chapter 2) (Kočovská et al., 2013).

A clinical diagnosis is usually considered the ‘gold standard’. However, for research purposes there has been a demand for some time for a ‘quantified’ diagnostic measure and this has led to the development of some of the frequently used instruments: semi- or highly-structured interviews (the ADI-R (Ehlers, Gillberg & Wing, 1999)), or the Diagnostic Interview for Social and COmmunication disorders/DISCO (Wing et al., 2002)), questionnaires (e.g., the Autism Spectrum Screening Questionnaire (ASSQ) (Berument et al., 1999; Ehlers & Gillberg, 1993; Posserud, Lundervold & Gillberg, 2009), or the Social Communication Questionnaire (SCQ) (Rutter, Bailey & Lord, 2003) and observation schedules (e.g. the ADOS (Lord et al., 2000)). There has also been a need to develop these scales for the purpose of training less experienced, junior clinicians or researchers to assist in

the diagnostic process. This has led to the need for continuous research into diagnostic stability of ASD diagnoses made on the basis of different approaches (clinical ‘best estimate’, or instrument diagnosis) and of compatibility across types of diagnosis made. It is essential that these instruments are compared with the clinical ‘gold standard’.

Clinical diagnosis has consistently been shown to be more stable than any instrument diagnosis (Chawarska et al., 2007), such as diagnoses made using the Autism Diagnostic Interview - Revised (ADI-R) (Moss et al., 2008); the Early Screening of Autistic Traits (ESAT) (Swinkels et al., 2006), Wing’s Autistic Disorder Interview (WADIC) (Wing 1996), and Autism Diagnostic Observation Schedule-Generic (ADOS-G) (Lord et al., 1989; 2000; Gotham et al., 2008; van Daalen et al., 2009); or the Childhood Autism Rating Scale (CARS) (Schopler et al., 1980) and ADOS (Gotham et al. 2009; Kleinman et al., 2008).

The stability over time of a diagnosis of ASD is not only theoretically interesting, but important for a number of clinical reasons. Resources for psycho-education and early intervention in ASD are currently allocated at a relatively high level in many western countries. The same holds for diagnostic services. Often, intervention provision is heavily dependent on availability of diagnostic services, and knowledge about diagnostic stability, therefore, is of particular importance (Kočovská et al., 2013).

## **B.2. The Faroe Islands**

### **B.2.1. Geography**

The Faroe Islands are situated in the heart of the Gulf Stream in the North Atlantic Ocean, north west of Scotland and half way between Norway and Iceland at 62°00’ N. It is a group of 18 islands, several of them now connected by under-sea and through rock tunnels.

The archipelago covers 1400 km<sup>2</sup> (546 sq miles); it is 113 km (70 miles) long and 75 km (47 miles) wide. The distance from the ocean at any point on the islands is at most 5 km.

The climate is maritime and extremely changeable – from frequent hurricanes to misty fog to moments of lovely sunshine. The Gulf Stream buffers the climate - the average temperature ranges from 3 °C in winter to 9 °C in summer (high precipitation, strong winds, the harbours never freeze).

### **B.2.2. Population**

The total population of the Faroe Islands is about 49,000. There are only two towns – the capital Tórshavn (around 19,000 inhabitants; Picture 1) and Klaksvik (around 5,000). The rest of the population live in rural areas and small villages. The most remote places are serviced by helicopters or boats (health care, education etc.).



**Picture 1.** Tórshavn, the capital of the Faroe Islands. Photo by courtesy of Olavur Frederiksen.

### **B.2.3. Genetic isolate**

The Faroe Islands have been an unusually stable community over many centuries and under various foreign rulers and became a self-governing region of the Kingdom of Denmark in 1948. It has always been a strategic military point, first during the

Viking expansion in the medieval times and in recent history during World War II, occupied by the British as part of the defence against Nazi Germany and at present forms part of the NATO alliance.

The Faroe Islands were alone in the heart of the North Atlantic for millions of years before the first settlers came, half-way through the seventh century. These were Irish monks seeking a peaceful place to stay. About a hundred years later, the Norwegian colonisation, developing throughout the Viking Age, made the Faroes a central part of the Viking settlements along the coast of the Irish Sea and the North Atlantic. Shortly following this, Norwegian kings took control of the islands and later the Norwegian crown came under the Danish Monarchy. In accordance with the genetic isolate character, the Faroese community has been living in relative geographical seclusion, resulting in the absence of genetic exchange with other societies/nations. Today the vast majority of the population are ethnic Faroese, of Norse and Celtic descent (Als et al., 2006). Recent DNA analyses have revealed that the Y chromosomes, tracing male descent, are 87% Scandinavian (Jørgensen et al., 2004) and that mitochondrial DNA, tracing female descent, is 84% Scottish/Irish (Als et al., 2006).

The Royal Danish Monopoly ended in 1856 and Faroese businessmen were beginning to discover different connections with other parts of the world. In 1872, deep-sea fishing started, and the Faroe Islanders rapidly became known for being among the best fishermen and sailors in the world. The fishing industry grew rapidly and became the primary source of income for the islands (Bloch, 2007).

#### **B.2.4. Faroese diet and pilot whale**

In this fishing community, there is excessive exposure of some residents to methyl mercury, mainly due to the traditional habit of eating pilot whale meat (Weihe et al., 2003). Methylmercury is a well-established neurotoxicant and previous research demonstrated its multifocal and permanent negative effect on the developing brain (Grandjean et al., 1997; Debes et al., 2006).

The Faroe Islands is still a very traditional Nordic fishing community which traces its roots to early medieval times some 13 centuries ago. This tiny North Atlantic nation occupies 18 tiny grassy islands without any trees or any other possibility for agriculture due to the climate. The secret of the population's survival

and thriving lay in excelling in fishing over the centuries. However, due to the extremely harsh climate of this region, fishing is often impossible and thus since the Norse settlement, the pilot whale has played a central role in daily life. Schools of long-finned pilot whale (*Globicephala melas*) have been periodically appearing at the shores of the Faroe Islands (Picture 2). Mastering the whale drive process ('grind' in Faroese) and long-term storage of the meat (wind-dried, salted or boiled) and blubber offered a solution for survival and in turn also allowed supplies for more productive distant fishing vessels.



**Picture 2.** Long-finned pilot whale (*Globicephala melas*) ([http://en.wikipedia.org/wiki/Pilot\\_whale](http://en.wikipedia.org/wiki/Pilot_whale)).

Centuries ago, the Faroese men developed excellence in this skill. This ability is remarkable, especially prior to the existence of motor boats – first to spot the school, to chase it, drive it to the shore and to kill the animals as quickly as possible by hand. The pilot whale drive is a non-commercial activity involving the entire community. As there are around 800,000 whales in the wild and there are typically just 2-3,000 (0.1-0.6%) catches per year, the Faroese society not only never endangered this species but also they cooperate internationally through the North Atlantic Marine Mammal Commission (NAMMCO) for the conservation of whales and the management of whaling (Bloch, 2007).

The whole process has been a subject to legislation and regulation since very early days. There is meticulous documentation of all catches since 1584, recorded by vicars and assisted later by the chief of police: the particular whaling bay, date, number of whales killed, size of every whale (in traditional ‘skinn’ measurement unit), value of total catch and the allocation of the catch among the community. The pilot whale is no doubt the best studied whale in the world with much valuable information contained in Faroese whale catch records, which most likely represent the oldest records of continuous wildlife utilization anywhere in the world. If there was a generous catch, it was shared with nearby villages (Bloch, 2007).

There were noted 3 periods of rich catches over the past 4 centuries, the last one, interestingly, in the mid 1980s. Mastering the whale drive fulfilled not only the function of mere survival but actually provided an excellent dietary source of all necessary nutrients (e.g., high content of protein, iron, selenium in the skin, vitamins A, B, C, D and E etc., and long-chain polyunsaturated fatty acids, in particular omega-3,6,9 in perfect balance) and the blubber (fat), known for its excellent protective function of cardiovascular health (Dewailly & Knap, 2006). In addition to all of these functions – the whale drive has served over the centuries as a cohesive social activity, in which the whole community is involved, and the tradition and mastery is passed on to the next generation. Nowadays, the habit of eating pilot whale meat and blubber varies among the inhabitants from 3-4 meals/year to 1-2 meals/week.

### **B.3. Environmental Factors**

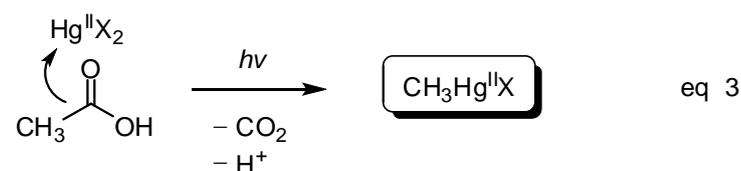
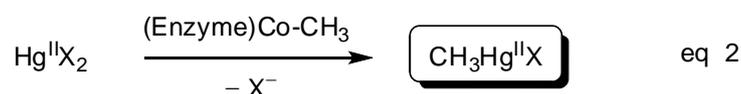
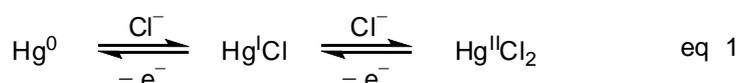
Given the genetic isolate character, the Faroe Islands constitute an interesting environment in which to conduct epidemiological studies. Many variables are unusually stable, e.g., socioeconomic status, education, health care, familial/genetic history and diet (albeit some variations in seafood intake occur). Several epidemiological studies reported to date have focused on the apparently high prevalence of certain diseases in this community – among them Parkinson disease (Wermuth et al., 1997; Wermuth et al., 2000) and autism (Ellefsen et al., 2007). There are several environmental factors specific to this geographical region, which might be of importance for the interplay between the occurrence of autism and environmental and genetic variables, e.g., methylmercury ( $\text{CH}_3\text{HgX}$ , where X is

typically a halogen), polychlorinated biphenyls (PCBs) poisoning and vitamin D deficiency.

Mercury exists in three oxidation states: metallic ( $\text{Hg}^0$ ), and ionic ( $\text{Hg}^{\text{I}}$  and  $\text{Hg}^{\text{II}}$ ), which are interconvertible (Scheme 1, eq 1). Various forms of mercury found in the environment, such as sea water, originate from volcanic eruptions, industrial pollution, and by leaching from mercury-containing minerals. Alkylation of mercury species in the environment can be effected, e.g., by the enzymatic system of aquatic flora and respiring bacteria, such as *Clostridium cochlearium* (eq 2) (Lander, 1972; Shagun et al., 2005) or by photochemical methylation using common chemicals also present in the ecosystem, such as acetic acid (eq 3) (Baudo et al., 1990).

(In the literature, methylmercury (i.e.,  $\text{CH}_3\text{HgX}$ ) is often abbreviated as MeHg. However, this acronym may lead to confusion, since ‘Me’ in chemistry stands for methyl ( $\text{CH}_3$ ), so that MeHg may imply  $\text{CH}_3\text{Hg}$ , in which mercury would be in oxidation state +1 rather than +2 and without the corresponding anion X. Therefore, the MeHg abbreviation has been avoided throughout this thesis).

**Scheme 1.** X = Cl, Br, I, etc.



Understanding the gene-environment interaction in autism is currently a very important topic for research into early neurodevelopment. To study the gene-environment interaction in genetic isolates with specific environmental exposures presents itself as a particularly useful model (Gillberg & Coleman, 2000).

As the search for possible environmental factors involved in gene-environment interaction of autism intensifies, an environment of genetic isolates with specific environmental exposures becomes very useful.

### **B.3.1. A possible connection between methylmercury and ASD**

The biological plausibility of the hypothesis that mercury (Hg) is linked to neurodevelopmental disorders, including autism, has been demonstrated on a cellular level (Garrecht & Austin, 2011). It is not, and never will be, possible for ethical reasons to study a controlled Hg exposure in humans because methylmercury, a potent neurotoxin, can initiate excitotoxicity even at sub-micromolar concentrations (Blaylock & Strunecká, 2009). Thus, the evidence needs to be derived from methods focusing on biomarkers of Hg damage, measurements of Hg exposure, epidemiological data and animal studies.

### **B.3.2. Why might methylmercury be an important environmental factor in the aetiology of autism?**

It is well established that autism has strong genetic roots, but it is unclear what the effects of environmental factors/toxins are. The interplay between genetic susceptibility and exposure to environmental factors at critical moments in development has become the subject of intensified research in the past several years. Furthermore, some of the pathological changes in ASD have been shown to be triggered by excessive immune activation (Blaylock, 2009).

Methylmercury is highly and selectively toxic to the CNS and the prenatal period is believed to be the most susceptible stage of life (Harada, 1968; 1995). A major effect appears to be related to faulty development and not to destructive focal neuronal damage as has been observed in mercury intoxication in adults and children exposed postnatally (Choi et al., 1978).

Methylmercury has the following effects on the brain: (1) increases excitotoxicity by halting endocytosis and exocytosis (Aschner et al., 2007); (2) causes a steep increase in the calcium gradient which initiates apoptosis; (3) halts the production of the antioxidant glutathione and thus causes susceptibility to irreversible radical damage by initiating the apoptotic cascade (Aschner et al., 2000; Ercal et al., 2001; Aschner & Aschner, 2007).

The ability of methylmercury to cross the blood-brain barrier has resulting detrimental effects in the brain. These are profoundly more damaging in a developing brain that has not yet reached complete maturity and thus its protective abilities are

substantially limited. As the majority of the brain growth occurs during the third trimester of the prenatal period, this is also a period of great vulnerability of the developing brain to poisoning by methylmercury which inhibits important biochemical and developmental processes (Sakamoto et al., 2004).

A growing body of data suggests Hg as a possible aetiological agent driving the cellular mechanisms by which Hg-induced neurotoxicity may result in the physiological attributes of autism. Key areas of focus include: (1) route and cellular mechanisms of Hg exposure in autism; (2) possible genetic variables that are linked to both Hg sensitivity and autism; (3) the role Hg may play as an environmental toxin fuelling the oxidative stress found in autism; (4) the role of mitochondrial dysfunction; and (5) the possible role of Hg in abnormal neuroexcitatory and excitotoxicity that may play a part in the immune dysregulation found in autism (Garrecht & Austin, 2011).

Mercury has well-known effects relating to the disruption of the body's sulphur chemistry leading to increased oxidative stress which impacts physiological and organ functioning – especially the central nervous system (CNS). Oxidative stress has been found to be consistently elevated in autism. Naturally, elevated oxidative stress is not unique to autism, never the less it does suggest that autism is more than just a neurological disorder but also a disorder which reflects dysfunction at various metabolic levels (Garrecht & Austin, 2011).

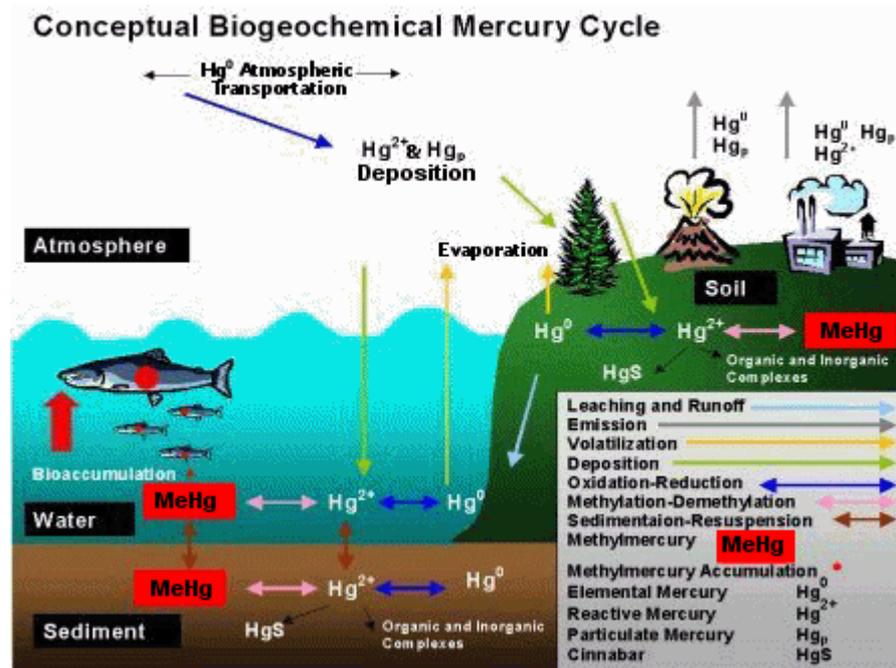
The most important aspect in this hypothesis of Hg being an aetiological factor in neurodevelopmental disorders including autism is recognizing that a critical variable (or unpredictable confound) is the sensitivity of any given individual to Hg, which is likely to have a genetic basis (Julvez et al., 2013).

### **B.3.3. Biological impact - Human health effects**

Methylmercury can have serious adverse effects on the development and functioning of the human central nervous system, especially when exposure occurs prenatally. This pollutant is of considerable public concern, because it is found in sea-food and freshwater fish throughout the world (Methylmercury Environmental health criteria 101, Geneva: World Health Organisation; 1990). Due to the commonality of high levels existing in fish, mercury content in hair samples are found to be higher in

populations where fish makes up a large portion of their diet, and lower in populations with little fish consumption (Mahaffey et al., 2004).

Since methylmercury is formed in aquatic systems by microorganisms and algae from elemental mercury (Hg) and is not readily eliminated from organisms (it has a half-life of about 72 days), it is biomagnified in aquatic food chains from bacteria, to plankton, through macro-invertebrates, to herbivorous fish and to piscivorous (fish-eating) fish. At each step in the food chain, the concentration of methylmercury in the organism increases (Figure 2). The concentration of methylmercury in the highest aquatic predators can reach a level million times higher than that in the water. Fish and other aquatic species are the only significant source of human methylmercury exposure (Wiener et al., 2003).



**Figure 2.** The global mercury cycle.

Methylmercury has the capability of crossing the blood-brain barrier and its lipophilic nature allows binding to neurons and acting as a very potent neurotoxin. It denatures enzymes, which can hinder the production of necessary proteins. It promotes the generation of reactive oxidative species (ROS) and reduces the ability of the body to protect itself from and remove these ROS. The increased concentration of ROS can cause cell necrosis. Organic mercury has the ability to initiate cell apoptosis through multiple mechanisms (Sakamoto et al., 2002).



if permanent, could well be of societal significance in the affected populations. (Grandjean et al., 1997).

It has been known for a long time that pilot whale meat contains a large amount of methylmercury (approximately 600 µg in a single meal) (Juhlshamn et al., 1987). Furthermore, blubber contains other organic compounds with a slow rate of decomposition (e.g., PCBs, DDE, etc.) due to the pilot whales roaming all over the oceans and feeding on species from the food chains that now have been contaminated by a variety of industrial chemicals released into the air, ground, rivers and seas all over the world. There were 3 large longitudinal prospective developmental studies conducted in the Faroe Islands, New Zealand and the Seychelles Islands suitable for quantitative analysis that were used for derivation of a new RfD for methylmercury by the U.S. Environmental Protection Agency (EPA) in 2001 (Rice et al., 2000; 2003). Two of these studies from the Faroe Islands and New Zealand (Kjellstrom et al., 1989; Grandjean et al. 1997; Debes et al., 2006) have demonstrated that this chronic, low-dose prenatal CH<sub>3</sub>HgX exposure from maternal consumption of fish has been associated with long-term mercury-related neuropsychological dysfunctions and cognitive deficits - attention, language, memory, fine motor function, and visual-spatial abilities among the most pronounced, and loss of performance IQ points and IQ points generally, and all these negative effects have been found to be multi-focal and permanent (Kjellstrom et al., 1989; Grandjean, 1997; Debes, 2006). The third study from the Seychelles Islands reported no evidence of impairment related to *in utero* methylmercury exposure (Myers et al., 1995; Davidson et al., 1998).

These conflicting findings between these 3 studies were analysed extensively by the National Research Council (NRC) in 2000 and there were some differences outlined as potentially responsible for the opposing outcomes of the Seychelles versus Faroe Islands and New Zealand studies. Among these differences are children's age at testing, differential genetic susceptibility of the populations, differential pattern of exposure to methylmercury (episodic versus relatively continuous) and co-exposure to PCBs in the Faroe Islands. It was not possible at the time to determine which if any of these factors were responsible for the different findings. The power of the Seychelles study to detect the small effects identified in the Faroe Islands study was only about 50% (National Research Council, 2002). In addition, the difference in availability of UVB (due to latitude and/or lifestyle habits)

in these sites may have impacted on the varying genetic susceptibility to methylmercury toxicity.

The results of these New Zealand and Faroe Island studies demonstrate that what was previously considered to be harmless exposure to mercury in conjunction with essential nutrients which might be protective, actually have a detrimental effect on brain neurodevelopment.

This resulted in recommendations that were issued in 1998 in the Faroe Islands by the Faroese Public Health authority, particularly warning all girls and women prior to bearing children not to consume pilot whale meat and the rest of the society to limit their consumption. This recommendation has been followed by the Faroese female population and the daily uptake of both whale meat and whale blubber has been reduced by up to one order of magnitude. While the methylmercury concentration in the umbilical cord blood at births in 1986/1987 (n=1,023) was 24.2 µg/L (median) with 25% exceeding the then 40 µg/L limit, by 2000/2001 the level dropped to 1.4 µg/L with only 2.4% exceeding the new limit of 5.8 µg/L (Weihe and Grandjean, 2013).

Thus, our cohort born between 1985 and 1994 represents one of the last generations of Faroese children whose mothers consumed pilot whale meat during their pregnancies and hence from a research point of view there is no possibility of repeating the procedure with a new birth cohort. Interestingly, this is also a cohort born during the last of the three noted periods of the richest catches of pilot whales in Faroese history (Bloch, 2007).

#### **B.3.4. Genetic sensitivity to methylmercury toxicity**

In order to understand better how mercury may act in any pathogenic process, future research should include among the outcome variables not just behavioural correlates of autism but a wide range of genetic variants and physiological variables known to be associated with Hg damage, including, e.g., urinary porphyrin and precoproporphyrin (prcP), markers of oxidative stress, and genetic variables (Garrecht & Austin, 2011).

The present situation in mercury research is further complicated by the fact that it is most likely that the data from studies on the effects of methylmercury from fish consumption on neurodevelopment are confounded by the dietary benefits of

seafood intake. Confounding factors beneficial to neurodevelopment, such as long-chain polyunsaturated fatty acids and selenium, are found in fish and are likely to cause an underestimation of methylmercury-induced toxicity (Choi, Cordier, et al. 2008).

One recent study, of cognitive consequences at school age associated with prenatal methylmercury exposure, successfully addressed negative confounding heterogeneity (contaminated seafood, content of essential nutrients, socioeconomic factors), in the exposure range and genetic influences on susceptibility in order to prevent underestimation of toxicity (Julvez et al. 2013). In order to identify possible causes of genetic predisposition to methylmercury neurotoxicity, they identified and examined several functional single-nucleotide polymorphisms (SNPs) in 66 genes related to potential gene-methylmercury interactions, including those implicated in brain development, neurotransmitter metabolism, cholesterol metabolism, iron regulation and peroxidative defence (Gundacker et al., 2010). Transferrin (TF) was associated with Hg concentrations and paraoxonase (PON1), progesterone receptor (PGR) and brain-derived neurotropic factor (BDNF) were associated with WISC-III total IQ. Higher methylmercury exposures were associated with seafood consumption during pregnancy, generally healthy nutritional habits and socially advantageous class.

These heterogeneities in several relevant genes suggest possible genetic predisposition to methylmercury neurotoxicity in a substantial proportion of the population (Gundacker et al., 2010; Julvez et al., 2013).

## **B.4. Vitamin D deficiency**

Vitamin D deficiency – either during pregnancy or early childhood – has recently been proposed as a possible environmental risk factor for ASD (McGrath et al., 2001; Grant & Soles, 2009; Cannell, 2008). Interesting results, both at the molecular level and in animal experiments, begin to indicate the possible mechanisms for this potential risk.

Vitamin D deficiency has become common due to an increasingly urbanized lifestyle, rising rates of obesity, and recommendations to avoid sun exposure promulgated since the 1980s (Holick, 2005; Schwalfenberg, 2007; Bosomworth, 2011; Cannell et al., 2008b). Moreover, at northern latitudes (e.g., in Scotland at 55°–61°N or the Faroe Islands at 62°N), sunlight with the ultraviolet B fraction is available only during a limited period of the summer. Dark skinned individuals require about 5 to 10 times longer exposure to sunlight to produce vitamin D compared to fair skinned individuals (Clemens, Adams, Henderson, & Holick, 1982). Therefore, when moving to northern countries, those with dark skin run the risk of not reaching satisfactory vitamin D levels (Kočovská et al., 2013).

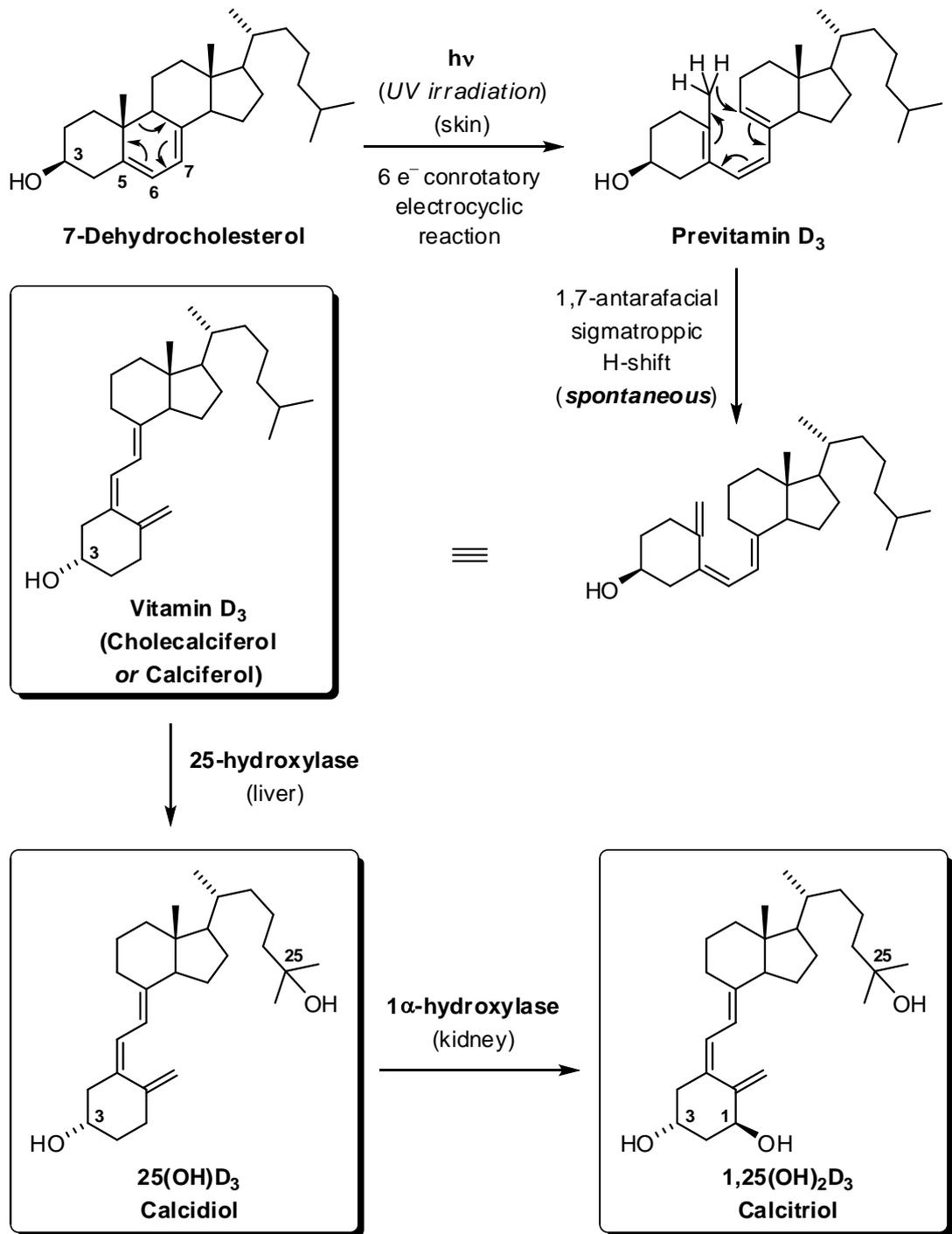
Vitamin D has a unique role in brain homeostasis, embryogenesis, neurodevelopment, immunological modulation (including the brain's own immune system), of ageing, and also, importantly, in gene regulation (Sigmundsdóttir, 2011; Harms, Burne, Eyles, & McGrath, 2011; Ramagopalan et al., 2010). In addition to these effects, vitamin D is now believed to be involved in numerous other functions in the organism. To date, it has been shown to bind to more than 2700 genes and to regulate the expression of more than 200 of them (Ramagopalan et al., 2010). Vitamin D is also known to be involved in healing processes by reducing the risk of cells becoming malignant (Sigmundsdóttir, 2011; Kočovská et al. 2014).

### **B.4.1. Vitamin D: definition, biosynthesis and role in metabolism**

Vitamin D is not really a vitamin, since it is produced in the body by a cascade of chemical transformations, commencing with a key photochemical reaction in the skin on exposure to the ultraviolet rays of the sun, followed by a series of further chemical transformations. Its receptors have been found in many tissues and organs. The biosynthesis of calcitriol, the active form of vitamin D of vertebrates, starts from

its prime precursor 7-dehydrocholesterol, which first undergoes the key photochemical electrocyclicization reaction in the skin, producing an intermediate that is spontaneously converted into **calciferol** (vitamin D<sub>3</sub>), or cholecalciferol to be precise and to emphasize its chemical relation to cholesterol (Scheme 3). Since the first reaction requires irradiation with UV light (at 290-315 nm), it can only proceed in the skin, i.e. within the reach of the UVB rays. Cholecalciferol is then transported to the liver, where it is hydroxylated in the side-chain at position 25 (the latter number refers to the exact position of a substituent group in the molecule, which elicits its highly specific biological properties) to produce **calcidiol** [25(OH)D<sub>3</sub>, or cholecalcidiol]. Finally, the latter compound is transported to the kidneys, where it is further hydroxylated (at position 1 $\alpha$ ) to finally produce **calcitriol** [1,25-dihydroxycalciferol, 1,25(OH)<sub>2</sub>D<sub>3</sub>, or cholecalcitriol], the active compound (Fieser & Fieser, 1959; Feldman et al, 2011). The levels of the enzyme required for the final hydroxylation are controlled by the parathyroid hormone, whose secretion is, in turn, triggered by low concentrations of calcium or phosphate (Holick, Tian, & Allen, 1995; Cheng, Levine, Bell, Mangelsdorf, & Russell, 2004). The latter enzymatic hydroxylation reaction, producing calcitriol, has also been found to occur in lymphocytes and in the brain in microglia (Eyles, Smith, Kinobe, Hewison, & McGrath, 2005) and probably in other locations. It is pertinent to note that while the half-life of calcidiol in the body is approximately 15 days, the half-life of calcitriol is only 15 hours (Feldman et al., 2011). Hence, the closer to the site of action it is produced, the better.

**Scheme 3**



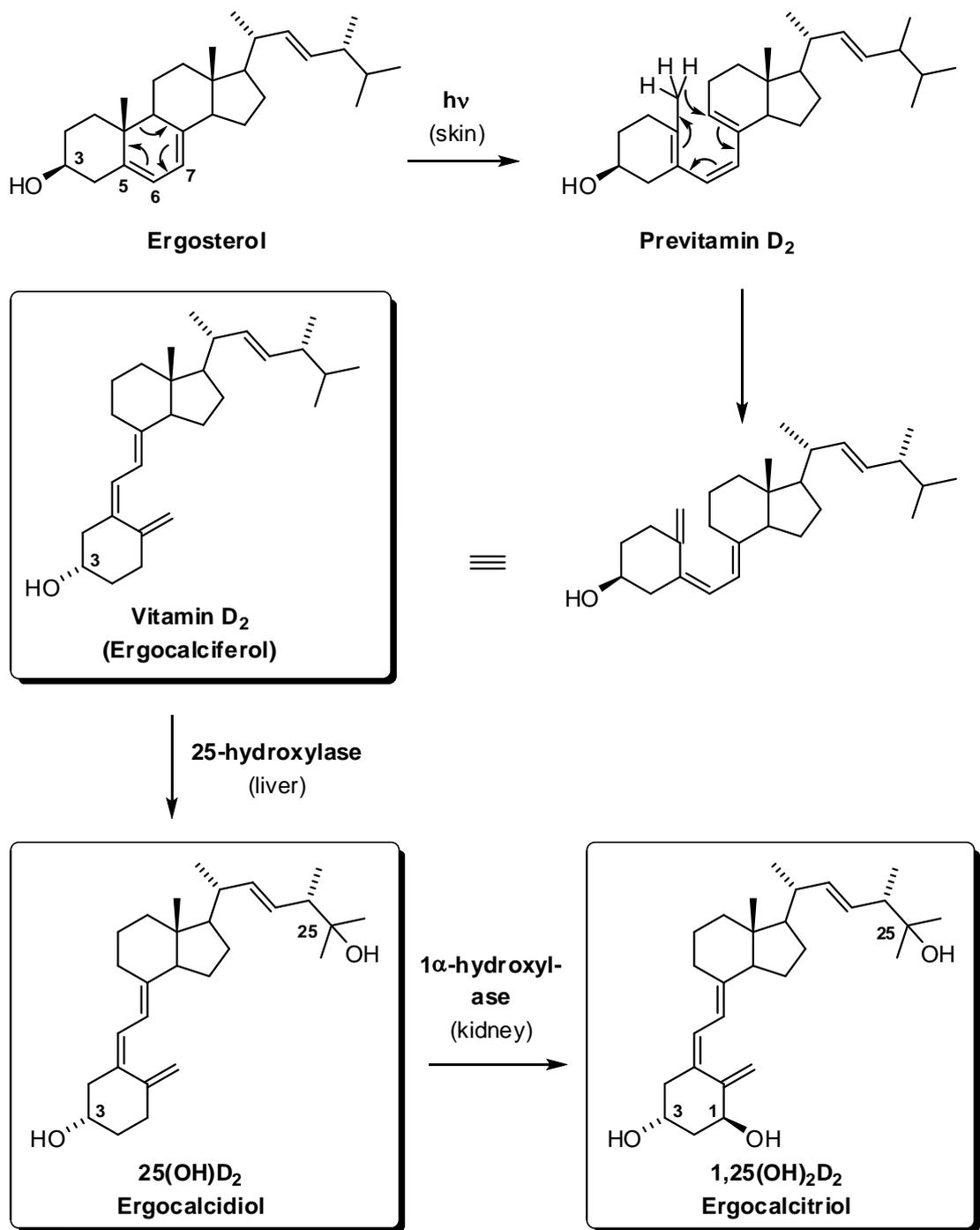
#### B.4.2. Nomenclature

For the sake of simplicity and to avoid confusion, which is widespread in the literature, the following nomenclature will be used throughout the thesis: for the vitamin D family originating from 7-dehydrocholesterol: **calciferol** for cholecalciferol (**vitamin D<sub>3</sub>**), **calcidiol** for 25-hydroxy-cholecalciferol (**25(OH)D<sub>3</sub>**), and **calcitriol** for 1,25-dihydroxy-cholecalciferol (**1,25(OH)<sub>2</sub>D<sub>3</sub>**). Where the literature does not discriminate, I will refer to **vitamin D** in general. Ergocalciferol (**vitamin D<sub>2</sub>**) is an analogue generated from ergosterol, a constituent of yeast and fungal cell membranes, where it serves the same role as cholesterol does in the membranes of animals. Ergosterol differs from 7-dehydrocholesterol in the side-chain (Scheme 4). The same series of reactions commencing with the photochemical transformation thus converts ergosterol into ergocalciferol, which is then hydroxylated in the same way (Fieser & Fieser, 1959; Feldman et al., 2011). Chemical analysis of blood samples can differentiate between the two series and gives the concentration of both 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>, the former being by far the major component.

The best known role of vitamin D is to facilitate calcium and phosphate absorption in the intestine, impacting directly on the formation of the bones and their density. Vitamin D in the body follows first-order mass action kinetics (Holick, 2005), which means that at serum levels lower than 30 ng/mL (75 nmol/L) the majority of ingested or sun-derived vitamin D is immediately diverted to metabolic needs, namely bone formation, leaving nothing to its higher functions within the brain, immune system, or gene regulation.

Therefore the mechanism and actions of the vitamin D group can be regarded as a parallel to a banking system – with calciferol (D<sub>3</sub>) as the retail, calcidiol [25(OH)D<sub>3</sub>] as savings, and calcitriol [1,25(OH)<sub>2</sub>D<sub>3</sub>] as investment ‘banks’, involved in numerous regulatory exchanges that are driven by demand and acute ‘market’ situation. This comparison can make it easier to understand that when we have a supply of ‘currency’ (calcidiol) just enough for a ‘piece of bread for the next day’ (absorption of calcium); we are unable to invest and secure our future (brain homeostasis and neurodevelopment, immune system and gene-regulation).

**Scheme 4**



### B.4.3. Dietary sources of Vitamin D

Furry animals, whose skin is not reached by the UV part of the sunlight, produce cholecalciferol (D<sub>3</sub>) in the greasy surface of their hair and transport it to the body by grooming. Fish, whose body is screened from the UV light by water, get cholecalciferol from plankton, which lives close to the water level, where the

photochemical reaction is still possible, or, in the case piscivorous species, by eating other fish. Fungi, yeast, and other lower organisms differ slightly in that they do not produce lanosterol and consequently 7-dehydrocholesterol; instead, their biosynthetic system produces ergosterol, which has a similar structure to 7-dehydrocholesterol, differing only in the side chain (Scheme 3 and 4). Plant sterols, which also differ in the structure of their side-chain, undergo the same transformations, resulting in yet another analogue of cholecalciferol, known as **vitamin D<sub>1</sub>**. In view of the similar chemical structures, D<sub>1</sub> and D<sub>2</sub> can be considered as substitutes for cholecalciferol (D<sub>3</sub>). Cholecalciferol is industrially produced via a simple irradiation of 7-dehydrocholesterol by UV light, and the same technology offers the production of ergocalciferol (D<sub>2</sub>) from ergosterol, which indeed is being used in medicinal practice as an analogue of D<sub>3</sub>. However, the enzymatic system of humans tuned to the hydroxylation of D<sub>3</sub>, may be less efficient in the case of D<sub>2</sub>. Furthermore, the (minor) difference in the chemical structure suggests that its action may not always be exactly identical to that of D<sub>3</sub>, which is now being debated (Holick, 2010; Feldman et al, 2011). Nevertheless, ergosterol is readily available by isolation from natural sources (e.g., from yeast), whereas 7-dehydrocholesterol has to first be synthesized from cholesterol, which is not entirely trivial on a large scale. Therefore, ergocalciferol has its share of the market owing to the simpler synthetic route.

It is noteworthy that treatment of rickets and other diseases associated with deficiency in vitamin D does not require the active form, i.e., calcitriol. Administration of calciferol (D<sub>3</sub>) is sufficient, as the two remaining steps (Scheme 3), i.e., the introduction of the two additional hydroxy groups, is carried out in the healthy body by its enzymatic apparatus. For a more detailed explanation see (Holick 2005).

The complete vitamin D system - the number of vitamin D receptors (VDR)s, the vitamin D-binding protein that transports vitamin D around the body, the enzymes that metabolize calcidiol in liver, calcitriol in kidney and the enzyme that breaks down vitamin D – is all under genetic control (Cannell and Grant, 2013).

#### **B.4.4. Vitamin D and autism**

There are three main areas of involvement of vitamin D in the human body, which may have direct impact on the development of ASD: (1) the brain (its homeostasis,

immune system and neurodevelopment); (2) gene regulation; and (3) the immune system.

Recently, maternal/neonatal vitamin D deficiency has been proposed as a possible environmental risk factor for ASD (McGrath et al., 2001; Cannell & Grant, 2013; Grant & Soles, 2009; Kočovská et al., 2012) due to its involvement in early neurodevelopment (Eyles et al., 2013), the immune system (Hayes et al., 2003), and gene regulation (Ramagopalan et al., 2010) processes.

Indirect support for the involvement of vitamin D in ASD comes from ecological studies, according to which vitamin D levels vary with season and latitude and with the degree of skin pigmentation (Grant & Soles, 2009; Dealberto, 2011). The prevalence of ASD was found to be greater at higher latitudes and in children of migrant mothers with darker skin (Grant & Soles, 2009; Fernell et al., 2010; Dealberto, 2011).

Calcitriol (1,25(OH)<sub>2</sub>D<sub>3</sub>), the end product of vitamin D metabolism has now been recognized, *inter alia*, as a neuroactive hormone that signals via nuclear receptors (Eyles et al., 2005; Eyles et al., 2013). It has been shown to be required for normal brain homeostasis and brain development (Garcion et al., 2002). The last 15 years have witnessed great advances in explaining the biochemical mechanisms of the diverse actions of calcitriol in the brain, especially its role in early neurodevelopment and in degenerative processes: (1) cell differentiation and axonal growth; (2) stimulation of neurotrophic factor expression (e.g., cytokines); (3) regulation of calcium signalling directly in the brain; (4) modulation of the production of the brain-derived reactive oxygen species; (5) stimulation of glutathione (a potent anti-oxidant, involved in DNA synthesis and repair) and thereby down-regulating excitotoxicity (Eyles et al., 2005; Eyles et al., 2013; Garcion et al., 2002). Outcomes of many of these mechanisms during neurodevelopment might be relevant in a number of Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations (ESSENCE) (Gillberg, 2010) - conditions that are now being linked with deficits of this vitamin/hormone, including ASD (Eyles et al., 2013; Kočovská et al., 2014).

To date, there have only been six clinical studies measuring vitamin D levels of individuals with ASD (Humble et al., 2010 – without a control group; Molloy et al., 2010; Meguid et al., 2010; Mostafa & AL-Ayadhi, 2012; De Souza-Tostes et al., 2012; Gong et al., 2014), four of which showed significantly lower levels of vitamin

D in those with this diagnosis compared to a healthy comparison group. Two studies (Mostafa & AL-Ayadhi, 2012; Gong et al., 2014) also demonstrated a significant inverse correlation between vitamin D levels and severity of ASD diagnosis on the Childhood Autism Rating Scale. Furthermore, Mostafa (2012) identified an autoantibody (anti-MAG) in 70% of autistic individuals and yet again, vitamin D levels had significant negative correlation with serum anti-MAG auto-antibodie levels (Kočovská et al., 2013).

#### **B.4.5. Vitamin D in the General Population of Young Adults with Autism in the Faroe Islands (V)**

An apparent epidemic of vitamin D deficiency is now being recognised (Holick, 2007), and this prompted me to explore the vitamin D levels in a general population cohort of young individuals with ASD (aged 15-24 years) and their siblings and parents, and in a typically-developing comparison group in the Faroe Islands. The Faroe Islands were chosen for this study for various reasons (see INTRODUCTION, Chapter 2).

Since several of the above processes are targeted by the devastating effects of Hg-induced neurotoxicity within the brain, it is tempting to speculate that this diminished protective function of calcitriol during early neurodevelopment (due to vitamin D deficiency at higher latitudes), in conjunction with possible individual genetic predispositions to autism and/or genetic predisposition to Hg toxicity, combined with exposures of varying degrees to a number of other environmental factors, may escalate into a neurodevelopment disorder.

Thus studying the interplay of these two environmental factors, i.e., methylmercury toxicity and vitamin D deficiency, in the ASD population sample in a genetic isolate of the Faroe Islands, offers a unique direction for research.

## **C. AIM**

The present study is a part of a project aiming to disentangle the effects of gene-environment interaction in autism in the Faroe Islands. Specifically, this study investigates the prevalence and diagnostic stability of autism in the Faroe Islands and explores two environmental variables: diet (methylmercury) and vitamin D deficiency as possible risk factors in the aetiology of autism.

## D. METHODS

### D.1. Participants

Prevalence, clinical, and genetic studies of ASD in the Faroe Islands (in the North Atlantic Sea) have been performed by the current research team (comprising members from Sweden, France, Scotland, and the Faroe Islands) for more than a decade.

An outline of all five studies in this thesis is presented in Table 2. To refer to individual sub-studies, Roman numerals are used. The target group in studies I, II, IV and V is an entire age-cohort of individuals born between 1985-1994 in the Faroe Islands, first identified in the whole school age child population through screening and diagnostic assessment for ASD in 2002 (Ellefsen et al., 2007), and then screened and assessed again in 2009 (Kočovská et al., 2012b).

The Faroese population comprised 47,962 individuals on December 31, 2009. Of these, 7,122 belonged to the (then 8-17-year-old) cohort of 7,689 individuals who had been screened by December 31, 2002. In addition to these, 6 individuals had migrated to the Faroe Islands from other countries in the period of 2003-2009. The target group for the new study consisted of those 7,122 from the original cohort plus the 6 “new” individuals.

The Faroe Islands population, in spite of being spread out over 18 different islands (several of which are now connected by tunnels), is a closely-knit community, and it is difficult to remain completely anonymous. Doctors, teachers and psychologists are a small group of professionals who, together, are in contact with virtually every single person in the 7-24-year-old age range of the residents on the islands. Reaching the vast majority of all individuals in this age group, when screening for problems, is therefore usually easier than in most other population settings.

**Table 2.** Study groups and methods used in studies I–V.

Study	I	II	III	IV	V
Type of study	<i>Original research</i>	<i>Original research</i>	<i>Systematic literature review</i>	<i>Original research Pilot study</i>	<i>Original research</i>
Outcome of the study	ASD prevalence longitudinal	Diagnostic stability of ASD diagnosis	Review of Vitamin D in ASD	Dietary and life-style trends during pregnancy	Vitamin D levels in ASD
Target group	67	41	112 articles	20	67
Attrition	14	10	-	0	27
Dead	0	0	-	0	-
Group examined	53	30	80 articles	20 mothers of a child with ASD 13 mothers of a healthy child	40ASD cases 62 siblings 77 parents 40non-related
M:F	49:18 (2.7:1)	25:5 (5:1)	-	All mothers	31:9 33:29 37:40 28:12 15:24
Age range years	15-24	15-24	-	-	15-24
Mental development (n) level	AA (1) A (31) NA (11) MLD (5) SLD (12)	AA (0) A (10) NA (7) MLD (4) SLD (9)	-	-	-
Diagnostic criteria and definitions of ASD and vitamin D deficiency	ICD-10 DSM-IV Gillberg's Asperger syndrome	ICD-10 DSM-IV Gillberg's Asperger syndrome	-	-	Severe deficiency <25 nmol/L Deficiency ≥25-<50 Insufficiency ≥50-<75 Sufficiency ≥75 nmol/L
Instruments and methods used	DISCO ASSQ Wechsler Scales ADOS Psychiatric-medical examination	DISCO ADOS Wechsler Scales Psychiatric-medical examination	-	Dietary and Life-style Questionnaire	LC-MS/MS

M = male, F = female; AA = above average intelligence (IQ >115), A = average intelligence (IQ 85-114), NA = near average intelligence (IQ 70-84), MLD = mild learning disability (IQ 50-69), SLD = severe learning disability (IQ <49). For abbreviations, refer to the Abbreviations section

## **D.2. Screening methods (I, II, IV and V)**

### **D.2.1. Target screening population in 2002(I, II, IV and V)**

In the original study performed in 2002, the entire Faroe Islands population (n=47,704) in the relevant school age group (8-17 years, born between 1985-1994, n=7,689) was screened after a process of systematically organised education (through TV and public lectures) and face-to-face contact with headmasters and teachers of all schools in the Faroe Islands in late 2000 and again in the spring of 2002. Two Faroese clinical psychologists were present at these lectures and parents were encouraged to contact these psychologists in case of any possible signs/symptoms of autism in their child. The two clinical psychologists also contacted all schools (n=65) and visited and lectured in a structured way on autism spectrum disorders for the teachers in all schools with more than 10 pupils (N=52). These lectures included a detailed description of early signs, symptoms and clinical presentations of various ASD diagnostic subgroups (childhood autism, atypical autism and Asperger syndrome). All head teachers/teachers in smaller schools were contacted by one of the psychologists by phone and all children were reviewed individually. In all cases showing any possible symptoms/signs of autism, the child's parents were invited to participate in the study (see Ellefsen et al., 2007 for more details).

*The screening process in 2002* was performed in three stages and produced 56 possible cases. (1) Twelve children were referred by various professionals (clinical psychologists, social services psychologist, educational psychologist, psychologist from a private practice) or parents themselves due to their child symptoms before screening took part in schools. (2) In the only two special schools for children with developmental disorders all children's registers were examined and all staff assessed each individual child in multidisciplinary meetings. There were 19 children considered for subsequent detailed assessment. In the cases of two children of these 19, their parents declined to participate due to their child undergoing recent diagnosing process but agreed to anonymous reporting of their children records. (3) All children who attended mainstream schools and whose parents gave permission were screened by the teachers using the Asperger Syndrome and high-functioning autism Screening Questionnaire (ASSQ) (Ehlers & Gillberg, 1993) and 25 were reported to the research team as possibly meeting some of the criteria for autism.

Four families refused to participate but the teacher's description indicated strongly that the children might be suffering from Asperger syndrome. Seven children (33%) out of these 21 identified by the teacher did not reach the cut-off for autism spectrum disorder and were not further examined. The remaining 14 children underwent the detailed examination.

*Participation rate in 2002:* in the total reported sample (n=56) there were 4 children whose parents declined participation (7% of the total reported sample), 7 children were screened out by their result on ASSQ and 2 children later did not reach the clinical diagnosis after clinical evaluation and DISCO-interview (9 false positive cases) and then there were 2 children previously diagnosed by Danish specialists whose parents refused participation in the study. These 2 children were included in some analyses of *all known diagnosed cases* (n=43) (see Ellefsen et al., 2007 for more details). Thus the corresponding response rate in 2002 was 93%.

#### **D.2.2. Target screening population in 2009 (I, II, IV and V)**

Members of the research group continued to appear on TV, radio, and in the newspapers, sharing information about ASD, during the period from 2003 through 2009. They also gave a series of widely attended public lectures about ASD during the same period. In the follow-up study, which was performed during the whole year of 2009, all previous participants were invited to take part. Hospital doctors, GPs, teachers and psychologists (including those in private practice) were encouraged to refer any cases diagnosed with ASD or undiagnosed but raising some suspicion of suffering from ASD, to members of the research team. Thus, these individuals diagnosed with ASD represent the entire age-cohort population sample with ASD in the Faroe Islands, although it needs to be noted that this was not a uniform screening process of the entire population with a standardised screening instrument and there might have been a certain element of subjectivity present (Kočovská et al., 2012b).

The screening population consisted of the 7,128 individuals (3,590 males, 3,538 females) born in the 10-year period from 1985 to 1994, aged 15-24 years on December 31, 2009 (Time 2). This should be contrasted with the screening population of 7,689 (3,895 males, 3,794 females) residing in the Faroe Islands on December 31, 2002 (Time 1), when the original screening had been performed. In the present study, it has not been possible to specifically document the reason for the

drop in the number of males (8%) or females (7%) in the age-specific population, even though it is likely that a proportion of the reduction in numbers is accounted for by temporary migration for educational purposes. As it turned out, none of those in the out-migration group had been detected as having ASD at the time of the population study in 2002.

*In the screening process in 2009*, similarly to 2002, all mainstream and special schools, hospitals, clinics, private clinics, GP surgeries and social services were contacted to refer any possible cases of ASD. The original cohort (n=41), diagnosed by the research team in 2002, were contacted and interviewed by phone to confirm their original diagnosis and they were also invited to take part in the more in-depth research study. Thirty one of those 41 from the original study of 2002 agreed to participate. Thus the *response rate in 2009* was 76%.

### **D.2.3. Instruments at screening**

The screening at Time 1 (2002) included the use of the Autism Spectrum Screening Questionnaire (ASSQ) (Ehlers & Gillberg, 1993), which has been shown to be highly reliable and valid for screening autism across the range of intellectual functioning (Posserud, Lundervold, & Gillberg, 2006, 2009). The screen-positive children were examined in detail with a number of instruments including the Diagnostic Interview for Social and Communication Disorders (DISCO-10) (Wing et al, 2002) and the Wechsler Intelligence Scale for Children – Third Edition (WISC) (Wechsler, 1992) or Wechsler Adult Intelligence Scale – Revised (WAIS) (Wechsler, 1981).

### **D.2.4. ASD study group Time 1 (2002)**

There were 56 children aged 8-17 years identified at screening with a suspicion of ASD from the population of 7,689 at Time 1; 43 of these (36 males, 7 females) met the DSM-IV PDD/ASD diagnostic criteria or, in the case of ‘Asperger syndrome’, they met criteria for this condition operationalised by Gillberg (1991). The parents of two of the 43 children did not wish for their child to participate in the in-depth assessment study, but both these children had been ‘worked up’ comprehensively and diagnosed with ASD by Faroese or Danish clinicians and members of the research group prior to the study.

### **D.2.5. ASD study group Time 2 (2009)**

All 41 participating individuals with ASD at Time 1 (2002), now aged 15–24 years were contacted at Time 2 (2009). In addition, all individuals in the same birth cohort with a suspicion of ASD that had been previously undiagnosed were also invited to take part in the Time 2 study.

### **D.3. Student's role (EK)**

First of all, there was a need to find new collaborators and to establish a new team as most Faroese members of the original research team from 2002 have left the project due to retirement, promotion or moving abroad. This required a lot of liaison work during the 7 work journeys undertaken by EK and almost 7 weeks spent in the Faroe Islands over a period of 2 years between November 2009 and October 2011. The task was successfully accomplished and our research team has been joined by several new Faroese members in various positions, ranging from the head of research and manager of the Biobank of Faroes (GA) to the Chief Physician and highly active researcher in mercury research (PW), to researchers, medical doctors and research assistants.

Secondly, at the point of EK joining this study in late 2009, the data collection on prevalence (I) and diagnostic stability (II) research was unfinished, incomplete and some data misplaced so that these had to be traced. EK's assistance in the data completion was versatile - in some cases by an active participation or by observation in diagnostic screening. Some data required scoring (WAIS, WISC-III); other data, namely DISCO interviews had to be entered into the computer algorithm, to obtain computerised diagnoses, which was done manually. Subsequently, all results had to be entered into the Faroese database 'Progeny' and EK actively participated in all these manual processes before being able to delegate the rest of the process to a suitable person(s).

EK brought the new idea of studying vitamin D deficiency as one of the environmental factors in this region potentially involved in the aetiology of autism. At that time of 2009 to early 2010 there were no clinical studies published on this topic. EK performed a thorough, systematic literature review (III) and

conceptualised study V with a special design for the control group involving the siblings and parents of young individuals with ASD (unlike any other study published since). EK coordinated the blood samples preparation, shipping, and laboratory analysis.

The ASD cohort of this PhD study is unique not only for its location and its screening of the entire population but also due to the fact that this cohort of individuals with ASD, born between 1985 and 1994, represents one of the last generations of Faroese children whose mothers consumed pilot whale meat during their pregnancies. Regarding exploration of the possible impact of mercury on the aetiology of autism during the prenatal and antenatal period, there was therefore no possibility of repeating the procedure with a new birth cohort.

Thus, only retrospective research methods were possible. For the study of retrospective estimates of mercury levels of participants with ASD during the prenatal/antenatal period (IV) EK suggested an analysis of mercury via neutron activation analysis, assuming that milk teeth of individuals with ASD and the controls would be available, and identified two labs in Europe capable of carrying out these analyses. As the prenatal development of the primary dentition stretches over a period of several months and the half-life of mercury in the human body is around 72 days, milk teeth have been considered a good source of information on the exposure of the foetus to mercury levels during the prenatal period. Previous research has demonstrated differences in levels of mercury in milk teeth of children with ASD compared to healthy controls (Adams et al., 2007). However, it transpired that the custom of keeping children's milk teeth is not prevalent in the Faroe Islands and thus this method could not be used. Instead, EK suggested using a dietary questionnaire and actively participated in its design.

There was a particular technical challenge in study V to be overcome. The blood collection had already been done but its analysis proved to be a complicated process. The Faroese laboratory does not hold accreditation for research purposes of vitamin D analysis, so another laboratory had to be found. The analytical laboratory at the Karolinska Hospital in Stockholm, Sweden, was suggested but it transpired later that the method used there was based on chemi-luminescence, which is unsuitable for analysis of the haemolysed whole blood samples. During the work on the vitamin D review (III) EK came across a study comparing 3 different analytical methods for vitamin D analysis, one of which was suitable for haemolysed whole

blood samples. This method was performed by a laboratory in Oslo. EK made an enquiry but found that the price would be rather high (NOK328/sample). After further searching various labs throughout Europe, eventually it was agreed on collaboration with a lab in Birmingham, UK where the offered deal was acceptable (£20/sample).

EK performed statistical analyses for all studies I, II, IV and V under the guidance of professional statisticians and all statistical methods used were approved by them. EK actively participated in the process of interpretation of all the results (I, II, III, IV and V).

#### **D.4. Measures and instruments (I, II, IV and V)**

The instruments used in the different studies are briefly outlined in Table 2.

##### **D.4.1. Diagnostic criteria (I, II, IV and V)**

All the clinical data (including the data from the DISCO-11 and the ADOS) was reviewed by one of the clinicians and Clinical Research Comprehensive (CRC). Diagnoses of childhood autism/autistic disorder, atypical autism/pervasive developmental disorder not otherwise specified (PDDNOS) and Asperger syndrome were assigned. The same categories and diagnostic criteria that had been used in the original diagnostic study were applied, i.e., the ICD-10 and DSM-IV criteria for childhood autism/autistic disorder (APA 1994), ICD-10/DSM-IV-similar Billstedt, Gillberg, & Gillberg (2005) criteria for atypical autism - i.e., 5 or more childhood autism/autistic disorder symptom criteria met, at least two of which from the social interaction domain, and not meeting criteria for childhood autism/autistic disorder or Asperger's disorder (Billstedt et al., 2005) - and Gillberg (1991) criteria for Asperger's syndrome. Whenever there was clinical doubt about the diagnosis, individuals were seen by the leader of the project CG ( $n=12$ ). The final diagnosis was then assigned by him. DISCO-11 diagnoses were separately assigned in accordance with the computer algorithm of this instrument. This included a new "proposed DSM-5 diagnosis for ASD" (see [www.dsm5.org](http://www.dsm5.org)) according to an algorithm suggested by Hallerbäck, Billstedt, Johansson, & Gillberg (2010).

#### **D.4.2. Diagnostic Interview for Social and COmmunication Disorders (DISCO)**

The DISCO is an investigator-based and semi-structured instrument developed with a view to serving as a research and clinical interview with a collateral informant (usually one of the parents, as in the present context) for differential diagnosis within the spectrum of autism and other social communication disorders (Wing et al., 2002; Nygren et al., 2010). It has been used in a large number of studies (for a recent overview, see Leekam (2011)) and has been shown to have good to excellent psychometric properties including excellent inter-rater reliability and good validity for diagnoses within the autism spectrum (Nygren et al., 2009). The DISCO takes 2-4 hours to complete. It is currently available in its eleventh version (DISCO-11) but the difference between the tenth (DISCO-10) and the eleventh version is marginal. The DISCO-10 was used at Time 1, and the DISCO-11 at Time 2.

The DISCO provides a computerized diagnostic algorithm that allows the following (mutually not exclusive) diagnoses to be made: ‘childhood autism/autistic disorder’, ‘atypical autism/PDD-NOS’, ‘Asperger syndrome according to ICD-10/DSM-IV, Asperger syndrome according to Gillberg (1991), ‘social impairment’ and ‘ASD’ according to Wing and Gould (2001). Thus, the diagnosis is made by computer analysis on the basis of the clinical information given by the collateral informant and coded by the interviewer, and is *not*, at this “algorithm diagnostic stage” influenced by clinical comprehensive assessment, nor was the clinical diagnosis influenced by the DISCO-algorithm diagnosis.

DISCO-interviews were carried out at both time points. DISCO-10 interviews were performed by one of two clinical psychologists at Time 1. DISCO-11 interviews were performed by a third clinical psychologist in the majority of cases (“old” and “new”) at Time 2. In 9 cases, for practical purposes, one of the two Faroese psychologists active at Time 1 performed the DISCO-11 interviews. In these cases, at Time 2, they each met with the parents whom they had interviewed personally at Time 1.

#### **D.4.3. Other diagnostic instruments**

**Wechsler intelligence scales** were used age-appropriately for the cognitive assessment: The Wechsler Intelligence Scale for Children, Third Edition (WISC-III)

(Wechsler, 1992) in a majority of cases at Time 1, and Wechsler Adult Intelligence Scale (WAIS) (Wechsler, 1981) at Time 2. Those who were not tested at Time 1 were tested at Time 2. WISC-III and WAIS are individually administered intelligence tests which are organized into Verbal and Performance scales and provide scores for Verbal IQ (VIQ), Performance IQ (PIQ), Processing Speed Index (PSI), and Full Scale IQ (FSIQ).

Mental development levels were divided into 5 broad categories: (i) Above Average intellectual-developmental capacity (AA:  $IQ \geq 115$ ) (ii) Average intellectual-developmental capacity (A:  $IQ = 85-114$ ) (iii) Near Average intellectual-developmental capacity (NA:  $IQ = 70-84$ ) (iv) Mild Learning Disability (MLD:  $IQ = 50-69$ ) and (v) Severe Learning Disability (SLD:  $IQ \leq 49$ ).

**ADOS-assessment** was performed at Time 2. The ADOS is an instrument used for diagnosing and assessing autism. The protocol consists of a series of structured and semi-structured tasks that involve social interaction between the examiner and the subject. The examiner observes and identifies segments of the subject's behaviour and assigns these to predetermined observational categories. Categorized observations are subsequently combined to produce quantitative scores for analysis. Research-determined cut-offs identify the potential diagnosis of autism or related ASD, allowing a standardized assessment of autism symptoms.

#### **D.4.4. Medical examinations and medical record data (I, II, IV and V)**

Parents, or in a few cases older siblings, were interviewed regarding their child's pre- and perinatal periods and early development in accordance with a structured pro-forma used in the PARIS project (Philippe et al., 1999). The individuals themselves were examined in accordance with the pro-forma. All psychiatric and medical records of ASD suspected cases were retrieved and any relevant risk factors, diseases, and disorders were noted down.

#### **D.4.5. Vitamin D and autism: Clinical review (III)**

In order to study one of the environmental factors – vitamin D deficiency - possibly involved in the aetiology of autism in this region, a comprehensive literature review was performed. The plan was to: (a) access all papers in English regarding vitamin D

since 1947 and to process the results according to the expected various sub-topics; (b) to review all titles since 1947 in order to check that there are not any sub-topics missed and to review the latest papers on these sub-topics; (c) to filter the resulting papers based on quality, for example, excluding (or only giving a very brief mention to) non-quantitative studies, such as single case reports, small series, animal research, etc.; (d) to check abstracts of papers in other languages in order not to miss anything important; (e) to include all papers since 1995 in the main body of the review. The period from 1995 to 2011 was chosen based on the initial research indicating presence and/or involvement of calcitriol in the brain/neurodevelopment.

As the extensive review progressed, it became clear that there was no need for a general review on vitamin D, as there were several recent ones of very good quality. However, it also became apparent that there were several emerging areas of the involvement of vitamin D in the human body, apart from bone metabolism, namely the brain, gene regulation and the immune system, which play a significant role in the aetiologies of autism. This all suggested that it would be of interest to look into the results of these vast areas of research more closely, with the aim of exploring any potential relation of vitamin D to ASD. Thus, subsequently, a combined search of vitamin D and ASD was carried out covering all of these sub-topics.

A literature search covering the period January 1 1995 through October 31 2011 was carried out using PubMed, the Web of Knowledge, EBSCO OVID, MEDLINE, PsycARTICLES, Psychology and Behavioral Sciences Collection, PsycINFO, SocINDEX databases with Full Text Number of Hits.

The search strategy was as follows: vitamin d or vitamin D or ergocalciferol or vitamin d2 or vitamin D2 or vitamin d 2 or vitamin D 2 or cholecalciferol or vitamin d3 or vitamin D3 or vitamin d 3 or vitamin D 3 or calcitriol or vitamin 1,25 D3 or vitamin 1,25 d3 or vitamin 1,25 D 3 or vitamin 1,25 d 3 or calcidiol or vitamin 25 D or vitamin 25D or 25 hydroxy vitamin d or 25-OHD or 25- hydroxyvitamin D or 25 hydroxyvitamin D or 25 hydroxy vitamin d or 25 hydroxyvitamin D **AND** autism or autism spectrum disorder or ASD or Asperger.

The review presents the results of this systematic search and a narrative overview of additional literature regarding the role of vitamin D in the human body as it could potentially relate to ASD.

The review was conducted according to PRISMA guidelines (Moher et al., 2009).

#### **D.4.6. Dietary and life-style trends during pregnancy (IV)**

There was a need to better understand the notable distinction/differences of the Faroese dietary and life-style traditions that might possibly impact on the aetiology of autism (e.g. high content of mercury in the pilot whale meat, high content of vitamin D and omega-3 fatty acids in the blubber of pilot whale, lack of sunshine, etc.). The present ASD cohort of this PhD study, born between 1985 and 1994, represents one of the last generations of Faroese children whose mothers consumed pilot whale meat during their pregnancies. From the research point of view, it was therefore not possible to repeat the procedure with a new birth cohort. Also, there was no option to analyse mercury in dried blood spots back in 2009-2010 due to unavailability of the necessary technological advancement in the field of mercury analysis at that time. Therefore another method of estimation of mercury levels prenatally or around birth in individuals with ASD and their comparisons was needed.

##### *D.4.6.1. Choice of method*

Instead, a *dietary questionnaire* was designed as a proximal method to evaluate varying levels of mercury during prenatal development of individuals with ASD. There is ample experience with this type of questionnaires in the Faroe Islands research team for scientific, clinical or public health research purposes, yielding good results over the past 40 years (Grandjean & Weihe, 1993; Budtz-Jørgensen et al., 2007; Petersen et al., 2008; Dalgård et al., 2010; Grandjean et al., 2011).

This questionnaire (see Appendix 1) was designed for the purpose of this pilot study by Dr Weihe (the leader of the Faroese research team) and by myself. The dietary part of the questionnaire was based on the questionnaires that had been used repeatedly in his previous studies (Budtz-Jørgensen, Grandjean, & Weihe, 2007), the health-related, socio-demographic and life-style items were suggested by me and the particular questions were formulated in the Faroese language by Dr Weihe. The aim was to identify any dietary, health-related and life-style differences during pregnancy between mothers of a child from the ASD cohort born between 1985-1994 and diagnosed in 2002 or 2009, and mothers of age and gender matched typically developing control children (V).

#### *D.4.6.2. Study population*

Twenty mothers of young individuals with ASD and 20 mothers of healthy comparisons from the study V were selected to match as closely as possible on gender and age of a child and also on their address/geographic location as the access to pilot whale meat varies locally (e.g. less available in the capital Tórshavn than in the countryside). They received a letter explaining the purpose of the study – to see whether there were any dietary, health-related and life-style differences during pregnancy between mothers with a child with ASD or with a typically developing child - signed the consent form agreeing to answer the questions and also agreeing to their own and their child's medical databases to be checked. Later, the mothers who agreed to take part were called by the interviewer and a visit or a telephone call interview was arranged. The interviewer was an experienced Faroese clinician. The whole questionnaire took about an hour to complete. The diagnoses and treatments, e.g. miscarriage, Caesarean sections, etc., as well as any use of medication or supplements were verified through the Faroese medical database. There was a 100% response rate in the ASD group and a 65% response rate in the comparison group.

In the 'ASD group' there were 20 mothers of young individuals who were screened and diagnosed with ASD in 2002 and 2009 from a total population sample in the Faroe Islands, recruited from the original study group for vitamin D levels (V) (n=20, 11 sons/9 daughters). In the 'control group' there were 13 mothers of healthy comparisons originally matched as closely as possible on gender and age to form the control group of study V (n=13, 6 sons/7 daughters).

In addition, 3 individuals with ASD from the present study took part in a large study by Dr Weihe during 1986-87 (Grandjean et al., 1997), measuring prenatal exposure to methylmercury from maternal consumption of pilot whale meat in cord blood at birth. For these 3 individuals with ASD, diagnosed in 2002 or 2009, their results were available for levels of exposure to mercury during the prenatal period as measured at birth by my Faroese colleagues and kindly shared with me for the purposes of this thesis (Grandjean et al., 1997; Debes et al., 2006).

#### *D.4.6.3. Procedure*

This small pilot/feasibility study involved questioning 33 mothers of young individuals born between 1985-1994 in the Faroe Islands (from the target group of study I.), regarding the mothers' eating habits during their pregnancy with an index child and also regarding their life-style, health and wellbeing prior to and during their pregnancy. Later, they were either visited by a Faroese researcher in their home or travelled to the nearest health centre to meet with the researcher or when there were geographical/travel difficulties they answered the questions via a telephone call interview. All mothers were selected to cover a geographically wide area of the Faroe Islands.

#### *D.4.6.4. Measures*

The dietary and life-style habits questionnaire (see Appendix 1) contained 54 main questions (and for some of them up to 3 supplementary questions) regarding not only the mother's eating habits during her pregnancy but also her age, general health, mental health, use of contraception, miscarriages, timing and course of delivery, parity of an index child, use of medication/supplements, alcohol consumption and smoking, living conditions, general well-being, holidays and/or place of living during pregnancy, attitude to sun, etc. The majority of questions were taken from already existing questionnaires previously successfully used by my Faroese colleagues and for the purposes of this study were combined with questions of particular interest to the vitamin D area as suggested by EK. The questionnaire was prepared by EK in collaboration with the Faroese colleagues.

### **D.4.7. Vitamin D levels (V)**

#### *D.4.7.1. Study population*

As the 67 individuals diagnosed with ASD (I) represent an entire age-cohort of individuals with ASD in the Faroe Islands, the present study (V) represents the first ever entire population sample of vitamin D levels in ASD population. This cross-sectional population-based study involved 219 individuals, all of white European

origin: 40 participants (this number was given by availability of the blood samples) with a diagnosis of ASD (31 males/9 females), their 62 typically developing siblings (29 brothers/33 sisters), their 77 parents (40 mothers/37 fathers), and 40 healthy comparisons (28 males/12 females).

In 2008-2009, 40 of the 67 individuals with ASD from the general population - 24 participants (56%) from the 2002 screening phase cohort and 16 (67%) from the 2008-2009 cohort - and their close family members agreed to have blood drawn for the purposes of the genetic analysis. These blood samples were subsequently also used for an analysis of various environmental factors (informed consent was obtained either from the individual or, if younger than 18, from the parent). The 40 participants with ASD were 15-24 years old (Mean 18.9 (SD 2.9)) at the time of blood sampling, and 31 were male. The comparison group was matched as closely as possible for age (Mean 18.5 (SD 2.5)), season of birth and gender. The reasons for non-participation in blood sampling among the ASD group (n=27) were as follows: non-participation in the follow-up study in 2009 of those with ASD first diagnosed in 2002 (n=10); participation but not willingness to give blood sample (n=14); and participation but practical difficulties in blood drawing (n=3). The group of 40 with blood samples had a similar gender profile and Autism Diagnostic Observation Schedule (ADOS) (Lord et al., 1989) scores to those from whom blood samples were not obtained (77%/73% and 12.5/9.4 respectively,  $p=0.1$ ).

Although some degree of genetic relatedness in the Faroe Islands can be assumed - due to the small population and its genetic isolate character, the prevalence of autism in the Faroe Islands has been found to be similar to other western nations, namely 0.56% in 2002 (Ellefsen et al., 2007) and 0.94% in the follow up study in 2009 (Kočovská et al. 2012b). The fact that in the follow up screening process an additional 24 individuals were diagnosed with autism, who were originally missed, and nearly half of these were females (n=11), supports the findings of other studies, suggesting that girls are often missed at a young age and that screening and diagnostic processes need to address this phenomenon in the future (Giarelli, 2010; Kočovská et al., 2013). There were only two families with an index child with ASD with another sibling also diagnosed with ASD but these siblings were not part of our study due to the age restriction (our participants had to be born between 1985 and 1994). During the diagnostic process in 2009, several

families had siblings with some ASD traits but without an ASD diagnosis. Thus, in this study there were no multi-ASD families.

#### *D.4.7.2. Blood sampling and assay of 25-hydroxyvitamin D levels*

Over a period of a year during 2008-2009, all participants had their blood drawn (using EDTA in monovette) at Tórshavn Hospital, Faroe Islands, for the purposes of genetic analysis. These samples were later also used to determine the levels of 25(OH)D<sub>3</sub>. Therefore, it was not possible to match sampling times for ASD and comparison groups, nor was it possible to duplicate the vitamin D analysis. More than three quarters of the families with an index child with autism (78%) had their blood drawn in summer (Jun-Aug) and autumn (Sep-Nov). The rest of the families were blood sampled in spring (Mar-May). No ASD participants had their blood sample drawn during the winter months. Thus it can be expected that the ASD group might demonstrate a certain seasonal elevation in their levels of vitamin D. In contrast, all healthy comparisons had their blood drawn in schools during a relatively short period between February and April 2009. In some participants with ASD, the blood samples were drawn at their homes due to needle phobia and/or other behavioural/care-taking problems.

Samples were then frozen at -80 °C and stored at the Department of Biochemistry of the Biobank of Faroes. The long-term stability of 25(OH)D<sub>3</sub> serum concentrations for more than 10 years has been demonstrated under similar storage conditions (Agborsangaya et al., 2010). The stored, frozen whole blood samples were thawed in early 2013 and the required amount of 0.5 ml of haemolysed full blood separated, packed, and posted to the Department of Clinical Biochemistry, City Hospital, Birmingham, UK, where the laboratory analyses were performed by using the “gold-standard” method - Liquid Chromatography – tandem Mass Spectrometry (LC-MS/MS), details of which have been described by Schöttker et al (2012). The laboratory staff were blind to the identity and diagnostic/comparison status of the individuals. The assay is accredited by the Vitamin D External Quality Assessment Scheme (DEQAS) and the laboratory is CPA accredited. (Available at: <http://www.deqas.org/>). There were some remnants of the original blood samples and these were later returned to the Faroe Islands laboratory for storage.

By using the haematocrit (hct = a measure of the packed cell volume of red cells, expressed as a percentage of the total blood volume; the normal range is 43-49 % in men and 37-43 % in women) of the sample, the concentrations of 25(OH)D<sub>3</sub> were calculated after measurement of the haemolysed sample (Shea & Berg, 2013) which enabled the use of the haemolysed whole blood samples. All samples from all participants and the entire control group were haemolysed.

Ergocalciferol (often called vitamin D<sub>2</sub>), a direct analogue of cholecalciferol (vitamin D<sub>3</sub>), is made from ergosterol (obtained from yeast) and has been used for food fortification and supplements. The LC-MS/MS assay in blood (used in this study, see below) can differentiate between and determine levels of both 25(OH)D<sub>2</sub> and vitamin 25(OH)D<sub>3</sub>. The 25(OH)D<sub>2</sub> level reflects vitamin D<sub>2</sub> intake from supplements and the 25(OH)D<sub>3</sub> level reflects the vitamin D<sub>3</sub> intake from diet, supplements or sun exposure. The overall result is a sum of both circulating forms and it was this overall sum that this study has used (Feldman et al. 2011).

#### *D.4.7.3. Chemicals and reagents*

18.2 MΩ water was obtained from a Millipore Milli-Q water system (Billerica, USA) located at the Department of Toxicology at City Hospital, Birmingham, UK. Ammonium acetate and zinc sulfate monohydrate were obtained from Sigma-Aldrich (St. Louis, USA). Formic acid (100%, Aristar) was obtained from VWR (Lutterworth, England). LCMS grade methanol was obtained from Fisher (Loughborough, England) and hexane and isopropanol from Rathburn (Walkerburn, Scotland). Internal standard (IS) 26,27-hexadeuterium-25-hydroxyvitamin D<sub>3</sub> was obtained from Synthetica (Oslo, Norway). Mobile Phase A contained 154 mg/L (2 mM) of ammonium acetate in 0.1% formic acid in 18.2 MΩ water. Mobile Phase B contained 154 mg/L (2 mM) of ammonium acetate in 0.1% formic acid in methanol.

Serum calibrators and controls were prepared from lyophilized material (25-OH-Vitamin D<sub>3</sub>/D<sub>2</sub>, Level I and Level II controls), obtained from Chromsystems (Munich, Germany). An additional control was made from pooled patient serum.

#### *D.4.7.4. Sample preparation*

To a glass tube containing 150  $\mu\text{L}$  of sample (serum or plasma) was added 20  $\mu\text{L}$  of internal standard (250 ng/mL in an 80:20 methanol-isopropanol mixture), 150  $\mu\text{L}$  of 0.2M  $\text{ZnSO}_4$ , 300  $\mu\text{L}$  of methanol and 700  $\mu\text{L}$  of hexane. The tubes were vortex mixed for 30 s and then centrifuged at 4500 rpm for 10 minutes. 550  $\mu\text{L}$  of supernatant were transferred to labelled high recovery glass vials (Waters, Hertfordshire, England) and dried under nitrogen for 6 minutes at 25  $^\circ\text{C}$ . The samples were reconstituted in 80  $\mu\text{L}$  of loading solvent (70:30 methanol-water), capped and mixed for 30 s.

#### *D.4.7.5. Sample analysis*

A 'Waters Acquity Ultra Performance Liquid Chromatograph' (ACQUITY UPLC) and 'Quattro Premier XE' MS/MS instruments (Hertfordshire, England) with an electro-spray ionisation interface were used to analyse the samples. A liquid-liquid extraction was used to remove protein with calibrators Chromsystems (Germany). MS/MS Nitrogen was supplied from a Peak Scientific NN30LA-MS nitrogen generator (Peak Scientific Instruments, Renfrewshire, Scotland). An Acquity UPLC BEH C18 1.7  $\mu\text{m}$  2.1x50 mm column supplied by Waters was used, with a Waters Acquity UPLC Column in-line filter unit and a Waters assay frit (0.2  $\mu\text{m}$ , 2.1 mm). Sample injection volume was 20  $\mu\text{L}$ . Column oven temperature was 45  $^\circ\text{C}$  and a gradient elution was used (Table 3).

Detection of the analytes and internal standard was performed in positive ionisation mode using multiple reaction monitoring. Source temperature was set to 120  $^\circ\text{C}$  and de-solvation temperature at 400  $^\circ\text{C}$ . Capillary and cone voltage was set at 1 kV and 22 V respectively. Cone and de-solvation gas flow was 50 L/h and 1000 L/h respectively. The following MRM transitions were used: 25(OH) $\text{D}_3$  401.5>159, 25(OH) $\text{D}_2$  413.5>83 and IS 407.5>159.

The method is linear from 7.5-200 nmol/L and 2.8-200 nmol/L with a limit of quantitation of 7.5 nmol/L and 2.8 nmol/L for 25(OH) $\text{D}_3$  and 25(OH) $\text{D}_2$  respectively. The inter-assay coefficient of variation is 11.7% at 25(OH) $\text{D}_3$  of 37.8 nmol/L and 11.7% at 25(OH) $\text{D}_2$  of 10.8 nmol/L. The assay is accredited by the Vitamin D

external Quality Assessment Scheme (DEQAS) and the laboratory is CPA accredited. (Available at: <http://www.deqas.org/>)

**Table 3.** HPLC analysis of blood samples.

Time (min)	Flow rate (mL min <sup>-1</sup> )	% mobile	
		Phase A <sup>a</sup>	Phase B <sup>b</sup>
Initial	0.6	27	73
1.5	0.6	27	73
3	0.6	2	98
3.2	0.8	2	98
3.5	0.6	27	73

<sup>a</sup> Mobile phase A: 2 mM ammonium acetate in 0.1% aqueous formic acid made using ultra pure water (18.2 Ω). <sup>b</sup> Mobile phase B = 2 mM ammonium acetate in 0.1% formic acid made using LC/MS/MS grade methanol.

#### *D.4.7.6. Relationship between ideal sample type (serum) and haemolysed whole blood*

The ideal sample type for measuring 25-hydroxyvitamin D 25(OH)D is serum and plasma is an acceptable alternative. This is because 25(OH)D is found only in the serum component of blood. Therefore, when a haemolysed whole blood sample is used, the sample is effectively diluted by the proportion of the sample that is made up of red blood cells. In theory, by using the haematocrit (hct) of the sample, the concentration of 25(OH)D can be calculated after measurement of the haemolysed sample.

Forty-two whole blood samples were collected and the hct for 40 of these samples was known. The paired serum of these samples had previously been measured for 25(OH)D, so that samples were selected to cover a range of 25-hydroxyvitamin D<sub>3</sub> and 25-hydroxyvitamin D<sub>2</sub>. Full blood samples were gently mixed and an aliquot removed and centrifuged to obtain a plasma sample. The rest of the whole blood was left to freeze overnight at -80°C. The next day the samples were defrosted and mixed. Haemolysed full blood samples and plasma samples were analysed for 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>. In addition, the hct after freezing was also measured (Shea and Berg, 2013).

A regression line was used to calculate the 25(OH)D that would have been obtained for the sample had it not been haemolysed.

#### *D.4.7.7. Vitamin D status*

I resolved to use the same reference range as Holick et al. (2011), since this range has been used in several recently published studies of vitamin D levels in patients with autism (e.g. Humble et al., 2010; Meguid et al., 2010; Dalgård et al., 2010; De Souza Tostes et al., 2012). The Swedish reference range for 25(OH)D<sub>3</sub> levels awaits revision and the cut off for deficiency and insufficiency are expected to correspond to 50 nmol/L and 75 nmol/L respectively. This scale, based on the latest research, seems plausible and practical to adopt (Bischoff-Ferrari et al., 2009; Priemel et al., 2010; Heaney, 2011; Murad et al., 2011). The cut off for ‘severe deficiency’ (25 nmol/L~10 ng/ml) fulfils a practical function in clinical settings for the prescribed supplementation of vitamin D.

According to current UK recommendations, the reference ranges used by the Birmingham laboratory related to total 25(OH)D concentration (D<sub>2</sub> + D<sub>3</sub>) and were as follows (Table 4).

**Table 4.** The reference ranges used by the Birmingham laboratory, UK.

<b>Total 25(OH)D concentration (D<sub>2</sub> + D<sub>3</sub>) nmol/L</b>	<b>Clinical status</b>
< 15	severe deficiency
15 - 30	deficiency
30.1 - 50	insufficiency
> 50	adequate

However, as the present UK guidelines are outdated and awaiting an update and because several recently published articles on vitamin D levels in patients with autism were using the reference ranges according to Holick et al (2011), I decided to use Holick’s ranges for clarity and to allow comparison of my results with the literature (Table 5):

**Table 5.** Reference ranges used in this PhD study.

Total concentration (D <sub>2</sub> + D <sub>3</sub> )		Clinical status
nmol/L	ng/mL	
<25	<10.0	severe deficiency
25-50	10-20	moderate deficiency
50-75	20-29	mild deficiency/insufficiency
>75	>30	sufficiency/adequate
~150	~60	optimal

## D.5. Statistical analyses

In the Follow-up prevalence study (**I**), Poisson-distributed 95% confidence intervals (ci) were calculated for the population absolute rates and overall prevalence rates. Chi-square-tests were applied when comparing group frequencies. Means were compared using Fischer's permutation test (Bradley, 1968).

In the Diagnostic Stability study (**II**), all statistics were calculated by using the SPSS 17.0 software on anonymised data with two-tailed p-values. The p-values <0.05 were considered statistically significant. An agreement between diagnostic raters at two time points in 2002 and 2009 was quantified by using Kappa statistics. Kappa score was assigned according to the Landis and Koch scale (Landis and Koch, 1977) using 95% confidence intervals.

In the dietary and life-style habits pilot study (**IV**) the association between categorical variables and cases/controls was investigated using chi-squared tests, or Fisher's exact test where criteria for chi-squared tests were not met. Mann-Whitney tests were used to compare continuous variables between the two groups. All analyses were done using Minitab (version 16.0) at a 5% significance level.

In the Vitamin D study (**V**), statistical analysis was performed in Minitab (version 16.0) and SPSS (version 19). Continuous data are presented as 'mean' and 'standard deviation' (SD) if normally distributed or as 'median' and 'inter-quartile range' (IQR) if not normally distributed. All data on vitamin D levels for all groups were not normally distributed, therefore we used non-parametric tests. We treated the groups of individuals with ASD and their siblings and parents as related.

The comparison group was selected to match as closely as possible in terms of gender, season of birth and age to give comparable groups for the statistical analysis.

Group comparisons of normally distributed data were made using Student's t-test. For continuous data that were not normally distributed (~all vitamin D levels in all groups), Mann-Whitney tests and Kruskal-Wallis tests were used when performing pairwise-comparisons and several-group-comparisons respectively. A significance level of 0.05 was considered significant for all analyses.

A general linear model (GLM) was used to correct for season of sampling. The Vitamin D levels were not normally distributed (Anderson-Darling  $p < 0.005$ ) however, a log transformation of the Vitamin D levels was ( $p = 0.525$ ). The GLM model included a term for group (case/control) and a term for the season of sampling. For season,  $p = 0.009$ , indicating that there was a difference in Vitamin D between the seasons of samples. The group factor had  $p < 0.001$ , indicating that there was still a difference between the groups when adjusted for season of sample.

Chi-squared test or the Mantel-Haenszel linear-by-linear association chi-squared test for trend was used to assess categorical variables. Pearson correlation and logistic regression were used for analysis of the association between vitamin 25(OH)D<sub>3</sub> levels and certain background variables.

EK performed statistical analyses for all studies I, II, IV and V under the guidance of professional statisticians and all statistical methods used were approved by them.

## **D.6. Ethics**

The study was approved by the Faroe Islands Board for Ethics in Medicine. All families provided informed consent (parents or, in the case of individuals 18 years or over, by the individuals with a diagnosis of ASD (Time 1) or with suspected autism spectrum problems (Time 2) themselves.

Since the Faroe Islands are such a small community, there were several special ethical requirements on the conduct of our studies. For example, in study I it was not possible to incorporate the years of birth and IQ values into the published article as a diagnosis and the year of birth would be sufficient to identify the patient in the Faroese society.

This cohort of young Faroese people born between years 1985-1994 is one of the last whose mothers consumed pilot whale meat during their pregnancy. Based on the findings by Faroese researchers, a warning by the Faroese Public Health authorities was issued in 2006 not to consume pilot whale meat due to its high content of mercury. This recommendation was addressed mainly to young girls and women prior to bearing children, as methyl-mercury is a well-established potent neurotoxicant. The mothers of our cohort were the last generation not to be generally aware of the danger of consuming pilot whale meat, which they had eaten all their lives and even during their pregnancies. Thus, our retrospective research had to be carried with extra sensitivity and care by our Faroese colleagues who have ample experience with this type of research.

There was an unexpected delay during this PhD study due to the requirements of a new approval by the Faroe Islands Board for Ethics in Medicine required because of the change of several members of the research team. This delay stretched over 9 months, from October 2011 until July 2012, which paralyzed all the research activities over that period and in practice it meant that collection of 50% of the data was completed just around or after the official end of EK's 3 year PhD.

## E. RESULTS (I–V)

### E.1. Prevalence of autism in the Faroe Islands (I)

#### E.1.1. Overall number of suspected and definitive cases identified

In the original Time 1 diagnostic study performed in 2002, 43 individuals with clear ASD diagnoses had been identified (two of whom had not been assessed by the research team, but by Danish clinicians). In the new study in 2009 (Time 2), all of these 43 minus the two who had not wanted to be part of the in-depth study at Time 1, were contacted again and confirmed by telephone interview (for one exception see below) to have diagnoses within the autism spectrum. They were all invited to take part in the in-depth clinical research follow-up study – 10 declined participation and 31 underwent the in-depth clinical examination (Time 2 study). There was one interesting exception: one male (22 years old) with a clear diagnosis of atypical autism at Time 1 did not meet the criteria for a clinical ASD diagnosis at Time 2 even though he still demonstrated some ‘autistic traits’ (based on a clinician’s examination in agreement with his results on DISCO-11 and ADOS tests).

In the follow-up study in 2009 (Time 2) there were altogether 55 (22 newly examined/31 re-examined) cases with a CRC diagnosis. Two additional cases (females) did not take part in the in-depth study but had been referred to the research team from the Faroe Islands main hospital, and diagnosed by other clinicians. When combined, including the 43 cases from 2002 (36 males, 7 females), those 12 who received their diagnosis in 2002 and declined participation in the follow-up study, and the 24 cases newly diagnosed in 2009 (13 males, 11 females), *the total number of cases of “ever” ASD was 67 (49 males, 18 females) and the number of “current” ASD was 66.*

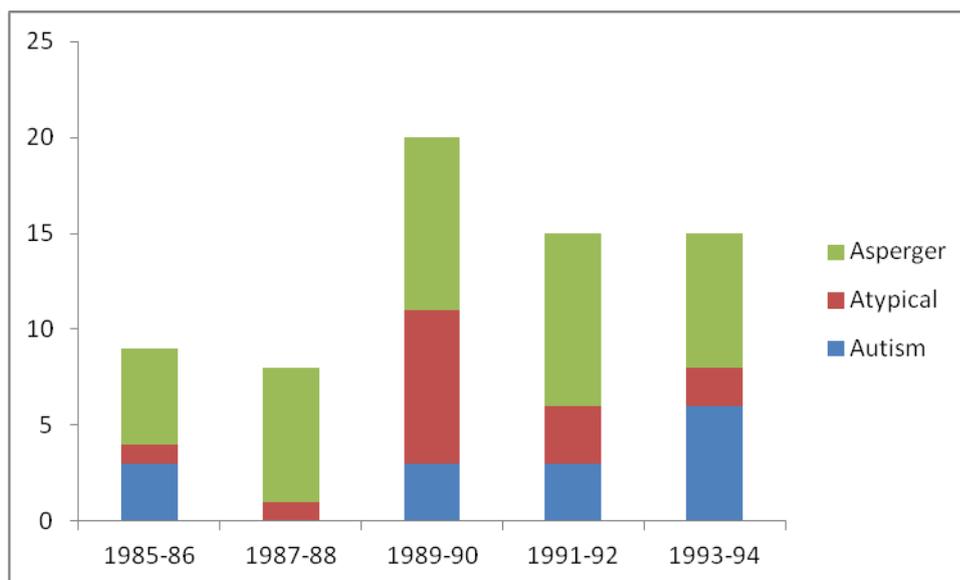
The total number of individuals who had ever been diagnosed with ASD was 67 (49 males, 18 females), with 66 currently thought to have ASD (autism, atypical autism/PDDNOS, or Asperger syndrome) at the end of the survey in 2009. This corresponds to total population prevalence for ASD in 15-24-year-old young adults in the Faroe Islands of 0.93% (Kočovská et al., 2012b). The relative contributions to this total of the various “types” of autism was: autistic disorder 23%, Asperger’s

syndrome 56%, and atypical autism 21%. Of these 66 comprising the final Time 2 sample, 24 were “new cases”, not found in the study at Time 1. Unfortunately, it has not been possible to ascertain whether some of these “new cases” were among those 13 children of the target group in 2002, who did not fulfil the diagnostic ASD criteria then (56 children were identified in 2002 with a suspected ASD and 43 of these were then diagnosed as shown in Table 6).

**Table 6.** Comparison of rates of clinical diagnoses of ASDs and gender ratios in the Faroe Islands in the original (2002) and the follow-up (2009) studies.

Diagnosis	Original study 2002 <i>n</i> = 43 (ratio 5:1)		Follow-up study 2009 <i>n</i> = 24 (ratio 1.2:1)	
	Males	Females	Males	Females
<b>Childhood autism</b> ( <i>n</i> =15)	9*	4	1	1
<b>Asperger’s syndrome</b> ( <i>n</i> =37)	18	3*	8*	8*
<b>Atypical autism</b> ( <i>n</i> =15)	9	0	4	2
<b>Total</b> ( <i>n</i> = 67)	36 (83.7%)	7 (16.3%)	13 (54.2%)	11 (45.8%)

\* Five individuals within these groups did not receive their clinical diagnoses from the research team.



**Figure 3.** Number of ASD cases in the Faroe Islands according to year of birth.

There was a non-significant trend towards higher prevalence rates of ASD among participants born in the second half of the 10-year period between 1985 and 1994 (Figure 3).

### **E.1.2. “New” cases referred with possible symptoms of ASD**

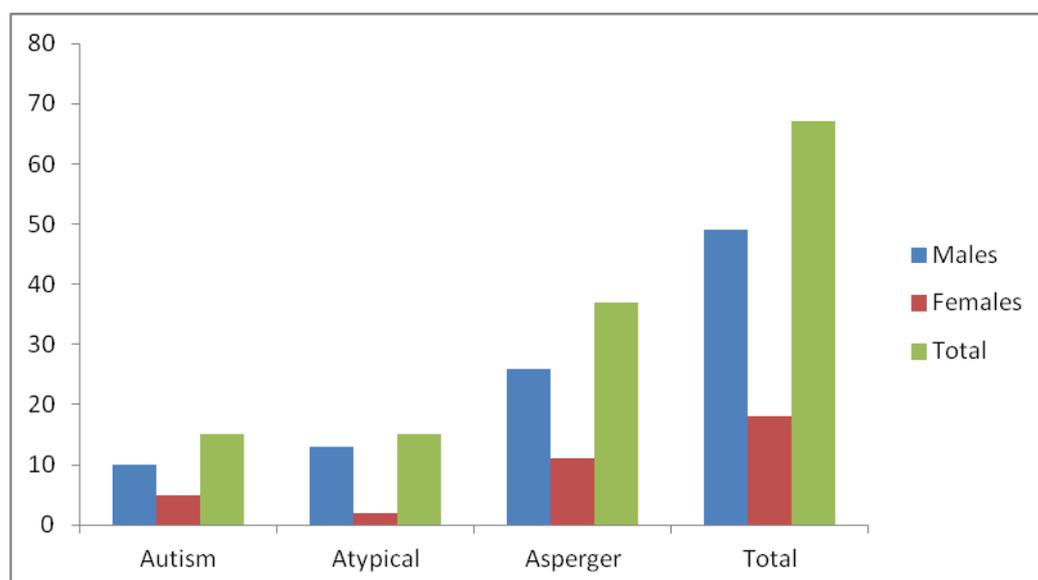
In 2009 (Time 2) 30 individuals from the same birth cohort as the target group were referred to the research team yet again by educational and clinical psychologists, teachers/head-teachers, special schools’ staff, clinicians, social workers etc. with symptoms suggesting that they might be suffering from a previously undiagnosed ASD. Of these, 22 (13 males and 9 females) actually did meet criteria for an ASD: 2 cases of childhood autism (1 male, 1 female), 6 cases of atypical autism (4 males, 2 females), and 14 cases of Asperger’s syndrome (8 males, 6 females). In addition, two further females with Asperger’s syndrome, who had already received a clinical diagnosis of ASD elsewhere, were referred to the research team, leading to a total number of newly diagnosed ASD cases of 24, 16 of whom (8 males, 8 females) had Asperger’s syndrome (Table 7).

The newly discovered cases of ASD in the age cohort examined differed from the originally diagnosed cases in the following ways (Table 6). The newly discovered cases were, of course, significantly older at the time of receiving a diagnosis of ASD (mean age at diagnosis 18.2, SD 3.5 versus 11.2, SD 3.6,  $p < 0.001$ ).

There were relatively more females among the “new” cases (16.3% of the original cohort versus 45.8% in the newly diagnosed,  $p < 0.001$ ). In the 2002 study, 36 of the total number of 43 with ASD were male and 7 female. Among the new 24 cases identified in the 2009 study, there were 13 male and 11 female cases. The newly diagnosed males constituted 27% of the whole 2009 group of males with ASD, whereas the newly diagnosed females constituted 61% of the total female group ( $p < 0.05$ ), chi-square test with Yates’s correction as shown in Figure 4. There were more female cases of atypical autism in 2009 compared to 2002 (22% versus 0%,  $p < 0.001$ ) (Table 6 and Table 7).

**Table 7.** Rates of prevalence of clinical diagnoses of ‘ASDs ever’ and gender ratios in the Faroe Islands in the 2002 and 2009 combined study.

<b>Diagnosis 2009</b>	Males	<b>Males</b>	Females	<b>Females</b>	Total	<b>Total</b>	95% ci Lower Upper
	<i>n</i>	population prevalence	<i>n</i>	population prevalence	<i>n</i>	population prevalence	
Childhood autism	10	0.28%	5	0.14%	15	0.21%	0.12% 0.35%
Asperger’s syndrome	26	0.72%	11	0.31%	37	0.52%	0.37% 0.72%
Atypical autism	13	0.36%	2	0.06%	15	0.21%	0.12% 0.35%
<b>Total</b>	<b>49</b>	<b>1.37%</b>	<b>18</b>	<b>0.45%</b>	<b>67</b>	<b>0.94%</b>	<b>0.73%</b> <b>1.19%</b>



**Figure 4.** Rates of clinical diagnoses of ASDs and gender ratios in the Faroe Islands in the 2009 Follow-up Study.

The source of referral to the study for ASD diagnostic assessment of the 24 new cases at Time 2, who were missed at Time 1, included the following: (a) the Tórshavn Hospital Adolescent Psychiatry Outpatient Unit where they had been treated for other mental health problems: anxiety (n=2), depression (n=2), ADHD (n=2), other problems (n=5); (b) Adult psychiatrists who had treated them as outpatients for other mental health problems: depression (n=1), psychosis (n=1), other problems (n=6) or (c) the Tórshavn Hospital Adult Psychiatry Inpatient Unit

where they were hospitalised for other psychiatric disorders: depression (n=4), OCD (n=1).

Among all 67 cases (49 males, 18 females) there were 15 cases with childhood autism (10 males and 5 females), 15 cases with atypical autism (13 males, 2 females), and 37 cases with Asperger's disorder (26 males, 11 females). These diagnoses and the DISCO-11 diagnoses are outlined in Table 8.

**Table 8.** Clinical and DISCO diagnoses, year of birth, gender and IQ-level in 67 individuals diagnosed in 2002 (1-43) and 2009 (44-67).

	Year of birth	Gender	IQ-level	Clinical diagnosis	DISCO-diagnosis
<b>1</b>	1994	M	SLD	autism	childhood autism
<b>2</b>	1994	M	SLD	autism	childhood autism
<b>3</b>	1993	M	SLD	autism	childhood autism
<b>4</b>	1993	M	SLD	autism	PDD-NOS
<b>5</b>	1992	M	SLD	autism	childhood autism
<b>6</b>	1992	M	SLD	autism	childhood autism
<b>7</b>	1990	M	SLD	autism	childhood autism
<b>8</b>	1986	M	SLD	autism	childhood autism
<b>9</b>	1986	M	SLD	autism	childhood autism
<b>10</b>	1993	F	SLD	autism	childhood autism
<b>11</b>	1990	F	SLD	autism	childhood autism
<b>12</b>	1985	F	A	autism	childhood autism
<b>13</b>	1992	F*	-	autism	childhood autism
<b>14</b>	1992	M	NA	atypical autism	atypical autism, PDD-NOS
<b>15</b>	1991	M	NA	atypical autism	atypical autism
<b>16</b>	1990	M	NA	atypical autism	**
<b>17</b>	1990	M	NA	atypical autism	Asperger's syndrome
<b>18</b>	1994	M	A	atypical autism	atypical autism
<b>19</b>	1994	M	A	atypical autism	atypical autism
<b>20</b>	1990	M	A	atypical autism	atypical autism, PDD-NOS
<b>21</b>	1989	M	A	atypical autism	atypical autism
<b>22</b>	1989	M	A	atypical autism	atypical autism
<b>23</b>	1990	M	MLD	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>24</b>	1986	M	MLD	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>25</b>	1994	M	NA	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>26</b>	1986	M	NA	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>27</b>	1994	M	A	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>28</b>	1992	M	A	Asperger's syndrome	Asperger's syndrome
<b>29</b>	1991	M	A	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>30</b>	1991	M	A	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>31</b>	1990	M*	-	Asperger's syndrome	**
<b>32</b>	1990	M	A	Asperger's syndrome	Asperger's syndrome
<b>33</b>	1989	M	A	Asperger's syndrome	Asperger's syndrome, PDD-NOS
<b>34</b>	1988	M	A	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>35</b>	1988	M	A	Asperger's syndrome	PDD-NOS
<b>36</b>	1988	M	A	Asperger's syndrome	childhood autism, Asperger's syndrome
<b>37</b>	1987	M	A	Asperger's syndrome	atypical autism
<b>38</b>	1985	M	A	Asperger's syndrome	**
<b>39</b>	1985	M	A	Asperger's syndrome	childhood autism, Asperger's syndrome

40	1985	M	AA	Asperger's syndrome	Asperger's syndrome, PDD-NOS
41	1992	F	A	Asperger's syndrome	Asperger's syndrome
42	1989	F	A	Asperger's syndrome	**
43	1988	F	A	Asperger's syndrome	Asperger's syndrome, PDD-NOS
44	1989	M	A	childhood autism	atypical autism, childhood autism
45	1994	F	SLD	childhood autism	atypical autism, Asperger's syndrome
46	1991	M	MLD	atypical autism	atypical autism, childhood autism
47	1990	M	NA	atypical autism	atypical autism, childhood autism
48	1989	M	MLD	atypical autism	**
49	1986	M	-	atypical autism	childhood autism
50	1989	F	MLD	atypical autism	atypical autism, childhood autism
51	1987	F	NA	atypical autism	Asperger's syndrome, childhood autism
52	1993	M	A	Asperger's syndrome	Asperger's syndrome
53	1993	M	A	Asperger's syndrome	atypical autism, Asperger's syndrome
54	1993	M	NA	Asperger's syndrome	Asperger's syndrome, childhood autism
55	1992	M	A	Asperger's syndrome	atypical autism, childhood autism
56	1992	M	A	Asperger's syndrome	childhood autism
57	1989	M	A	Asperger's syndrome	childhood autism, Asperger's syndrome
58	1989	M*	-	Asperger's syndrome	**
59	1988	M	A	Asperger's syndrome	childhood autism
60	1994	F	A	Asperger's syndrome	atypical autism, childhood autism
61	1993	F	A	Asperger's syndrome	**
62	1992	F	NA	Asperger's syndrome	atypical autism, childhood autism
63	1992	F	NA	Asperger's syndrome	atypical autism, Asperger's syndrome
64	1991	F*	-	Asperger's syndrome	**
65	1990	F*	-	Asperger's syndrome	**
66	1989	F	-	Asperger's syndrome	Asperger's syndrome
67	1987	F	A	Asperger's syndrome	atypical autism, Asperger's syndrome

\* Did not participate in the study.

\*\* Did not participate in the DISCO interview

M = male, F = female; AA = above average intelligence (IQ >115), A = average intelligence (IQ 85-114), NA = near average intelligence (IQ 70-84), MLD = mild learning disability (IQ 50-69), SLD = severe learning disability (IQ <49)

## E.2. Diagnostic stability (II)

Naturally, there were no Time 1 assessments, clinical, or DISCO-algorithm ASD diagnoses available for these 24 “new cases”. Only in the case of those individuals who had been assessed both at Time 1 and Time 2 was it possible to study diagnostic stability over time; for clinical diagnosis, (n=31) and for DISCO-algorithm diagnosis (n=30). The reasons for refusal to participate in the study at Time 2 among the original group diagnosed at Time 1 included (as recorded by the DISCO interviewer): (a) parent’s denial of any problems related to autism’ (n=2); (b) individuals with autism own denial of any problems’ (n=2); (c) parent blaming the health system for not offering enough help (n=2); (d) parents’ refusal due to very low general functioning of the person with autism (n=1); (e) involvement of genetic analysis in the study (n=1); and (f) other ‘unspecified reasons’ or ‘no information available’ (n=3).

The mean age of the DISCO-algorithm diagnostic stability study group (n=30) in the follow-up study was 19.5 years (SD 3.1). There were 25 males (83%) and 5 females (17%). IQ sub-categories were defined as follows: IQ  $\leq$ 49 (SLD) n=9 (30%); IQ 50-69 (MLD) n=3 (10%); IQ 70-84 (NA) n=7 (23%); IQ 85-114 (A) n=11 (37%).

Eleven of the Time 1 sample and six of the Time 2 sample individuals failed to take part in the DISCO-11 assessment, leaving 50 cases at Time 2 for whom there was a DISCO-11-algorithm diagnosis.

When combining the AD, AS, and atypical autism/PDDNOS into a collapsed ASD group, 30 of 31 (97%) remained in this overarching clinical diagnostic category. When separating them into specific ASD diagnostic subcategories (Table 9), those with an AD diagnosis in 2002 (n=10) all maintained their diagnosis, whereas in the group with an original diagnosis of AS in 2002 (n=15), 5 were no longer diagnosed in this category (4 with atypical autism/PDDNOS and 1 with no ASD diagnosis at all at Time 2). All but one of those with an atypical autism diagnosis in 2002 (n=6) were still diagnosed in this category at the follow-up (one male in this subgroup was diagnosed with AS at Time 2).

**Table 9.** Stability of clinical diagnosis from 2002 to 2009 (n=31).

<b>Clinical diagnosis 2002</b>	<b>Clinical diagnosis 2009</b>			
	no diagnosis	atypical autism	Asperger syndrome	autistic disorder
atypical autism, n=6	0	5 (83%)	1 (17%)	0
Asperger syndrome, n=15	1 (7%)	4 (27%)	10 (67%)	0
autistic disorder, n=10	0	0	0	10 (100%)

### **E.2.1. Clinical subgroup characteristics according to change/no-change of diagnostic category**

The 6 individuals (5 males), whose original clinical diagnosis of AS or Atypical autism/PDDNOS had changed at Time 2, had a mean age at Time 2 of 21.0 years (SD 3.6); their IQ ranged from 50–102; 5 individuals had low scores (0-1) on the ADOS ‘Stereotypical/Repetitive Behaviour’ scale. This was markedly different from the group of 10 individuals (8 males) with an original clinical diagnosis of AD (all of whom were again diagnosed clinically as AD at Time 2). Their mean age was 19.3 years (SD 3.6); all but 1 had IQ<50 ( $p<0.001$ ), and all had ADOS Stereotypical/Repetitive Behaviour scores of 2 or more ( $p<0.01$ ). However, the AS/Atypical autism/PDDNOS group that remained stable (n=15, 12 males) did not differ from those that changed, in terms of ADOS Stereotypical/Repetitive Behaviour scores, but they were younger at Time 2 (18.7, SD 2.8 years,  $p<0.05$ ) and IQ tended to be a bit higher (range 73-114).

### **E.2.2. CRC diagnoses versus DISCO-11 diagnoses in diagnostic and follow-up studies**

There was good correspondence between CRC and DISCO diagnoses both in the diagnostic and in the follow-up study (Table 9). In the original study, DISCO-10 was used. The minor changes from DISCO-10 to DISCO-11 did not affect the diagnostic algorithms used in the present study. The figures are therefore presented as “DISCO-11” findings.

### E.2.3. DSM-IV and DSM-5 diagnoses compared

There was an excellent correspondence between DSM-IV and DSM-5 diagnoses as regards to the collapsed group of DSM-IV autistic disorder, atypical autism and Gillberg's Asperger's disorder cohort on one hand, and the ASD category of the DSM-5 on the other. All cases with DSM-IV autistic disorder, atypical autism and Gillberg's Asperger's syndrome qualified for a DSM-5 ASD diagnosis.

### E.2.4. Stability of DISCO-algorithm diagnosis

Of all five DISCO-algorithm diagnoses, the category of AD was the most stable between 2002 and 2009 (8 of the 10 individuals remained in the same category), as shown in Table 10. The DISCO-algorithm-diagnoses of AS and Atypical autism displayed considerable variability; however, no individual moved out of the overarching ASD category altogether.

**Table 10.** Stability of DISCO-algorithm diagnosis from 2002 to 2009 (n=30).

DISCO-algorithm diagnosis 2002	DISCO-algorithm diagnosis 2009				
	SID*	ASD**	atypical autism	Asperger syndrome**	AD
No diagnosis n=1	0	0	0	1	0
SID* n=2	1	0	1	0	0
ASD** n=0	0	0	0	0	0
Atypical autism n=5	2	0	1	2	0
Asperger syndrome*** n=13	2	3	2	4	2
Autistic disorder n=10	1	0	1	0	8

\*Social Interaction Disorder according to Wing and Gould criteria.

\*\*ASD according to Wing and Gould criteria.

\*\*\*Asperger syndrome according to Gillberg and Gillberg criteria.

### E.2.5. Correspondence between clinical diagnosis and DISCO diagnosis in the follow-up study

The highest agreement/stability between the Clinical ICD-10/DSM-IV diagnosis and DISCO-algorithm-diagnosis in 2009 was noted for AD (67% complete agreement) and AS (52% complete agreement) (Table 11).

**Table 11.** Correspondence between clinical diagnosis and DISCO diagnosis at follow-up (n=50).

Clinical ICD-10 diagnosis in 2009 (n=50)	DISCO diagnosis in 2009					
	No diagnosis	Atypical autism	Asperger syndrome	AD	SID *	ASD**
No diagnosis n=1	0	0	0	0	1 (100%)	0
Atypical autism n=14	0	5 (36%)	1 (7%)	0	4 (28%)	4 (28%)
Asperger syndrome n=23	0	5 (22%)	12 (52%)	2 (9%)	1(4%)	3 (13%)
Autistic disorder n=12	0	2 (17%)	2 (17%)	8 (67%)	0	0

\*Social interaction disorder according to Wing and Gould criteria.

\*\*ASD according to Wing and Gould criteria.

### E.2.6. Gender effects

Among the females (n=6) who participated in the follow up study, 5 of them remained in the original diagnostic category whereas 1 woman, earlier diagnosed with Asperger syndrome, now received the diagnosis of atypical autism.

There were more females identified at Time 2 (n=11, ~46%): 1 with childhood autism, 8 with Asperger syndrome and 2 with atypical autism diagnosis, in comparison to the original study at Time 1 (n=7, ~16%): 4 with childhood autism and 3 with Asperger syndrome diagnosis, indicating that more females were missed at younger ages.

### **E.2.7. Diagnosis and IQ levels**

The IQ levels/status was available for 60 out of 67 participants in 2009. Five did not take part in the in-depth study and the remaining two were unable to undergo the testing for other reasons.

There were altogether 26 participants (39%) with IQ levels below average (<85): 9 (13%) with IQ levels near average (NA), 5 (7.5%) with mild learning disability (MLD: IQ levels >70<85) and 12 (18%) with severe learning disability (SLD: IQ levels <50).

Eleven of those with SLD (IQ < 50) obtained a diagnosis of childhood autism in 2002 and all of them retained this diagnosis throughout the period between their childhood/teenage years into adolescence/early adulthood in 2009.

Those with MLD (IQ >50<69) had either a diagnosis of atypical autism (n=3) or Asperger syndrome (n=2) and for those of them taking part in both the original (2002) and follow-up (2009) studies, the stability of their diagnosis was more variable (often interchanging these two diagnoses).

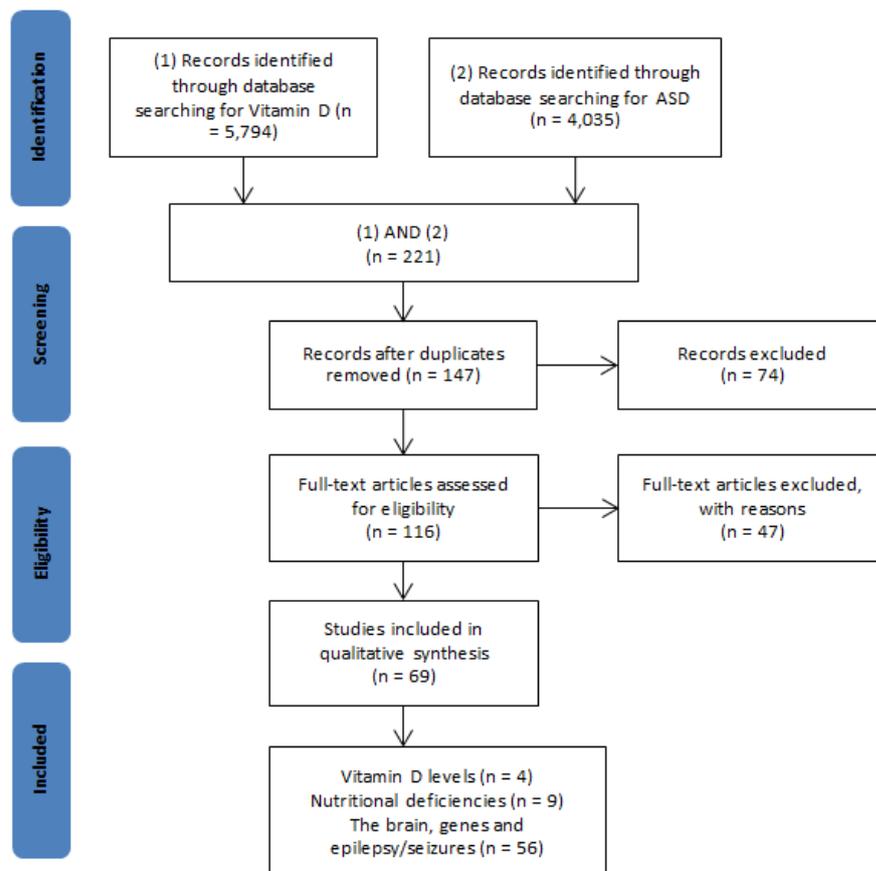
Among those with the IQ levels near average (NA: 70-84) four had a diagnosis of atypical autism and 5 had a diagnosis of Asperger syndrome and, apart from one case of 'no diagnosis' in 2009, they all retained their original diagnosis.

### **E.2.8. No diagnosis at follow-up**

There was only one case of 'growing-out' of the ASD diagnosis between childhood and young adulthood in this Faroese cohort. A male with previous diagnosis of 'atypical autism' in the original study in 2002 received 'no ASD diagnosis' ('autistic traits' only) in the follow up study in 2009 (at age 21 years). He had NA intelligence and his ADOS score was 1. Unfortunately, any detailed information regarding his family situation/upbringing or educational settings that might possibly shed light on the reasons for his remarkable improvement was not available.

### E.3. Vitamin D and autism: clinical review (III)

Of the existing 69 articles dealing directly with autism and vitamin D, only four studies have determined vitamin D serum/plasma levels in human subjects with diagnosed ASD or their mothers. Nine further studies reported on nutritional deficiencies (including vitamin D) in ASD. The remaining articles are covering (a) genetics and gene regulation; (b) the brain (homeostasis, neurodevelopment and the brain's own immune system); (c) epilepsy/seizures; and (d) medication in pregnancy. The 47 full-text articles excluded 'with reasons' were regarding the possible impact of vitamin D on the immune system which was not considered at the time relevant to ASD. The review was conducted according to PRISMA guidelines (Figure 5; Moher et al., 2009).



**Figure 5.** PRISMA flow diagram for the vitamin D review (Moher et al., 2009)

### **E.3.1. Systematic review**

#### *E.3.1.1. Plasma vitamin D-levels in individuals with autism and their family members*

Three studies investigated plasma levels of vitamin D directly in individuals with autism (Table 12).

Meguid et al (Meguid, Hashish, Amwar, & Sidhom, 2010) reported on a cohort of Egyptian children with ASD having significantly lower levels of both calcidiol (28.5 ng/mL) and calcitriol (27.1 ng/mL) as well as lower calcium serum values compared to healthy controls. The season of birth in relation to vitamin D and autism was also taken into account but no significant difference was found for the month or season of birth in either group. This was the only study that measured plasma levels of vitamin D in both children with autism and a suitable control group.

Molloy et al (Molloy, Kalkwarf, Manning-Courtney, Mills, & Hediger, 2010) compared the actual plasma calcidiol levels in a cohort of Caucasian boys with ASD diagnosis (4-8 years old) and a group of age-matched typically developing comparison boys having intravenous catheters placed for outpatient tonsillectomies. No differences were observed in the levels between participants with ASD and controls, but the majority of all children in this cohort (61%) had very low vitamin D levels (<20 ng/ml or <50 nmol/L). A limitation of this study was that the control group children were all suffering from some form of acute inflammation (Molloy et al., 2010).

Humble et al (Humble, Gustafsson, & Bejerot, 2010) tested vitamin D levels in adult outpatients with a range of psychiatric disorders and found that those with a diagnosis of autism or schizophrenia had significantly lower levels than other groups. This study demonstrated a considerable improvement in several patients of some of their psychiatric symptoms, e.g., psychosis and depression, on vitamin D treatment. However, there are limitations of this study as little information was given on details of the treatment, and there was no control group.

The fourth study - of mothers of Somali origin with children with autism in Sweden (Fernell et al., 2010) has shown that mothers of children with autism had the lowest levels of vitamin D. The differences between mothers of Somali origin with and without a child with autism were not statistically significant, but those with a

child with autism had approximately 30% lower mean value of vitamin D during spring (the time of lowest availability of vitamin D) compared to those whose child did not have autism. All Somali mothers had vitamin D levels in the deficient range.

**Table 12.** Systematic review: Studies measuring plasma levels of vitamin D.

<b>Study Year Country</b>	<b>N (Case/Control)</b>	<b>Design (Limitations)</b>	<b>Mean/Median Plasma Levels of Vitamin D (ng/mL)</b>
Meguid et al. 2010 Egypt	112 (70/42)	Case - Control, Cross - Sectional Children - Egyptian boys and girls with autism 2-8y	Significant difference <u>Cases:</u> 28.5 ng/mL <u>Controls:</u> typically developing children 40.1 ng/mL
Molloy et al. 2010 USA	89 (49/40)	Case - Control, Cross - Sectional Children -Caucasian boys with autism 4-8y	No significant differences <u>Cases:</u> 20 ng/mL <u>Controls:</u> boys with acute inflammation 17 ng/mL* All participants <31 ng/mL
Humble et al. 2010 Sweden	117 (117/0)	No Controls, Cross – Sectional Adults - European white males and females with autism and other psychiatric disorders	Adequate values in only 15% of all patients <u>Cases:</u> 12.6 ng/mL**
Fernell et al. 2010 Sweden	80 (40/40)	Case - Control, Cross -Sectional Adults – mothers of Somali or Swedish origin with a child with or without autism	Significant difference Somali vs Swedish mothers Trend lower values autism vs non-autism <u>Cases:</u> Somali mothers with autism child 6.7 ng/ml; Swedish mothers with autism child 24.8 ng/mL <u>Controls:</u> Somali mothers with non-autism child 9.6 ng/ml; Swedish mothers with non-autism child 20.7 ng/mL

Note: Severe deficiency <10 ng/mL; deficiency 10-20 ng/mL; insufficiency 20-30 ng/mL; recommended 30-100 ng/mL (Humble et al. 2010).

\* No significant differences most probably due to the problematic control group - see Nseir et al., 2012.

\*\* Other psychiatric patients had also deficient levels but higher than the ASD group.

### *E.3.1.2. Autism – vitamin D: Nutritional status, clinical and case studies*

Vitamin D deficiency and insufficiency is common during pregnancy (Hollis, 2007; Johnson et al., 2011) and it has been suggested (but not based on empirical research) that nutritional supplementation before and during pregnancy, including vitamin D, may prevent some cases of autism (Johnson, 2001).

Lindsay et al. (Lindsay et al., 2006) used a quantitative 'Food Frequency Questionnaire' (FFQ) to study prospectively the nutritional intake of 20 children (5-13 years old) with autism. The results of this questionnaire study suggested that 50% of these children with autism were likely to have inadequate vitamin D intake.

Several observational and clinical studies reported vitamin D deficiency in children with autism as a consequence of the highly selective eating behaviour that is typical for this group (Schreck, Williams, & Smith, 2004; Clark, Rhoden, & Turner, 1993; Noble, Mandel, & Patterson, 2007; Stewart & Latif, 2008; Weig, 2009). Several studies reported an association between vitamin D status and nutrition in children or young people with autism (Schreck et al., 2004; Lindsay et al., 2006; Herndon, DiGuseppi, Johnson, Leiferman, & Reynolds, 2009; Sadowska & Cierebiej, 2011; Shamberger, 2011). Thus, Shamberger (Shamberger, 2011) in an ecological study across the 50 United States, showed that infants who were solely breast-fed had diets low in vitamin D and other nutrients and that in the states with high rates of exclusive breast-feeding, autism rates were also higher. This might imply the need for vitamin D supplementation during the breast-feeding period.

By contrast, Herndon et al. (2009) found few differences in average nutritional intake between children with autism and typically developing children in Colorado, USA. However, a large proportion of children in both groups did not meet the national recommendations for daily intake of fibre, calcium, iron, vitamin E and vitamin D. Very similar results have been reported recently by Sadowska & Cierebiej (2011) in Poland (Table 13).

There are several case studies reporting an extreme form of inadequate nutritional status due to food selectivity and food-avoidant behaviours in children/young people with autism, leading to symptomatic nutritional rickets or painful leg weakness. In all cases, severe vitamin D deficiency was diagnosed and the rickets and pain were successfully treated with adequate diet and vitamin D supplementation (Noble et al., 2007; Weig, 2009; Stewart et al., 2008; Clark et al., 1993; Cannell & Hollis, 2008a) (Table 13).

**Table 13.** Clinical and other studies of vitamin D and autism.

<b>Vitamin D and Autism – Clinical Studies</b>	<b>First Author</b>	<b>Type of Study</b>	<b>Year</b>
Strong effect of latitudinal increase on Childhood Autism prevalence (seasonal variations, latitude)	Grant	Ecological	2009
Nutrition – inadequate vitamin D intake in children with ASD due to their selectivity with food	Sadowska	Clinical	2011
	Schamberg	Clinical	2010
	Herndon	Clinical	2008
	Lindsay	Clinical	2006
	Weig	Case	2009
Extreme cases of inadequate nutritional intake resulting in co-morbidity of autism and rickets	Stewart	Case	2007
	Noble	Case	2007
	Clark	Case	1993
Medication in pregnancy – antiepileptic drugs: Lower levels of vitamin D observed in babies born to mothers on anti-epileptic drugs during pregnancy	Bromley	Clinical	2009
Ethnicity:			
Observed low vitamin D in Somali origin mothers of children with ASD in Sweden	Fernell	Clinical	2010
Increased rates of ASD among dark-skinned immigrant mothers at high latitudes	Dealberto	Review	2010
Plasma levels in children with ASD:			
	USA	Molloy	Clinical
Egypt	Meguid	Clinical	2010
Plasma levels of vitamin D measured in a group of adult out-patients with various psychiatric illnesses – lowest levels in groups with ASD and schizophrenia	Humble	Clinical	2010

### *E.3.1.3. Autism – vitamin D: Latitudinal effects and ethnicity, clinical studies*

Wintertime solar UVB radiation varies with latitude and Grant and Soles (Grant et al., 2009) found a strong latitudinal increase in infantile autism prevalence. This finding is consistent with the hypothesis of maternal vitamin D deficiency being a risk factor for autism (Cannell, 2008).

Gillberg, Schaumann and Gillberg (Gillberg et al., 1995) reported on 3 boys with autism, born in one area of Sweden and with mothers coming from Uganda, and discussed possible reasons for the high autism rate in this particular ethnic subgroup.

Due to a higher prevalence of autism in children of Somali origin living in Sweden, and the evidence that low vitamin D impacts adversely on brain development, serum levels of calcidiol were analysed in mothers of Somali origin with and without a child with autism (Fernell et al., 2010a). Since the availability of vitamin D differs across the year, plasma levels were measured in both spring and autumn. Both groups of mothers of Somali origin had significantly lower values of calcidiol compared to Swedish mothers, in both spring and autumn. The difference in the levels of calcidiol between mothers of Somali origin with a child with or without autism was not statistically significant, but the lowest values of all in the study were found in mothers with a child with autism.

A review by Dealberto (Dealberto, 2011) summarized several studies reporting on increased rates of autism among dark-skinned immigrant mothers, especially those who moved to high latitudes. The results of this review are consistent with the hypothesis that maternal vitamin D deficiency (or insufficiency) levels may be associated with autism (Table 13).

### **E.3.2. Narrative Review**

The remaining articles covering (a) genetics and gene regulation and (b) the brain (homeostasis, neurodevelopment and the brain's own immune system) are discussed in the Narrative Review in Article III.

Table 14 provides an overview of some of the most important papers published in recent years on the role of vitamin D in genetics, brain development and brain functions.

**Table 14.** Overview of studies discussed in the Narrative Review (III).

<b>Possible Vitamin D Involvement in Autism via:</b>	<b>First Author</b>	<b>Type of Study</b>	<b>Year</b>
The Brain	Harms	Review	2011
	Blaylock	Review	2009
	Lucas	Review	2008
	Lawrence	Original Article	2008
Foetal Brain Development	Eyles	Review	2011
Genetics	Currenti	Review	2010
	Ramagopalan	Original Article	2010
De Novo Genetic Mutations	Kinney	Review	2009
Hypomelanosis	Bakare	Hypothesis	2011

*E.3.2.1. Autism – vitamin D: Epilepsy and seizures*

Animal and clinical studies have demonstrated the neuroprotective role of vitamin D in epilepsy (Harms et al., 2011). As epilepsy and convulsions are very common in autism (about 25% (Coleman & Gillberg, 2012), it is pertinent to note that vitamin D has also been shown to increase the electroconvulsive threshold for seizures, to decrease the severity of seizures, and to enhance the effect of the antiepileptic medication (Siegel, Malkowitz, Moskovits, & Christakos, 1984; Kalueff, Minayan, & Tuohimaa, 2005; Borowicz et al., 2007). However, antiepileptic drugs are known to decrease vitamin D levels, which further complicates research on the potential link between vitamin D deficiency and increased risk of seizures (Berquist, Schall, & Stallings, 2007).

*E.3.2.2. Autism – vitamin D: Medication during pregnancy*

Bromley et al. (Bromley, Mawer, Clayton-Smith, & Baker, 2008) (Table 13) reported on low plasma levels of vitamin D and an increased incidence of autism among infants of mothers taking antiepileptic medication, especially sodium valproate, during pregnancy. An inverse relationship between the antiepileptic drugs and calcidiol (an intermediate in calcitriol biosynthesis, produced in liver) levels have been demonstrated in animal research (Borowicz et al., 2007).

## E.4. Dietary and life-style trends during pregnancy (IV)

The results of the dietary and life-style questionnaire are summarised in Tables 15 and 16. For a complete questionnaire see Appendix 1.

In the ASD group there were 20 mothers of young individuals with ASD (100% of the target group of the pilot study). In the control group there were 13 mothers of young healthy comparisons (65% of the planned control group). All 37 mothers completed the dietary questionnaire between September 2012 – June 2013.

### E.4.1. Dietary differences

#### *E.4.1.1. Vegetables, fruit and potatoes*

There were some differences in self-reported dietary intake during pregnancy between mothers of children who were diagnosed with autism in 2002 or 2009 ('ASD group') and mothers of healthy, typically developing children ('control group'). Namely – in agreement with traditional dietary habits - the mothers in the ASD group reported consuming significantly fewer **vegetables (p=0.02)** and less **fruit (p=0.07)** than the control group. There was a trend, although not significant, for the mothers in the ASD group to consume fewer vegetables (apart from potatoes) and less fruit also during their childhood (0-12 years) and teenage years (Table 15 and 17).

There were no differences in reported consumption of **potatoes** between the two groups, as these are traditionally consumed daily by all inhabitants of the Faroe Islands.

#### *E.4.1.2. Pilot whale meat (a Faroese specific), blubber, sea bird and fish*

The US Environmental Protection Agency (EPA) derived the reference dose (RfD) for methylmercury of 0.1 µg/kg/day. As there is approximately 600 µg of methylmercury in a single meal of pilot whale meat and methylmercury half-life in the human body is about 72 days, consumption of more than 4 meals per year (e.g. more frequently than once in every 3 months) for a person weighing ~60 kg would lead to an increase of methylmercury level above the recommended amount. In other

words, a portion of pilot whale meal containing 600 µg of methylmercury could be consumed by a person weighing 60 kg once in 100 days (ca. every 3 months) without exceeding the recommended RfD.

One has to bear in mind that there is a cumulative effect for methylmercury concentrations in blood arising from consumption of other sea food species, especially large types of fish also containing quite high levels of methylmercury.

In addition, it has been demonstrated that the infants' levels at birth are up to twice the maternal levels due to methylmercury ability to cross the blood-brain barrier (Morrissette et al., 2004 - great Lake and St Lawrence River; Schoeman et al., 2010 - Ontario; Weihe & Joensen, 2012 - Faroe Islands).

For the reported consumption of **pilot whale meat** meal during mothers' childhood there was no difference between the ASD group and the control group with both groups' medians corresponding to a reported fortnightly consumption. There was a difference, although not significant, in consumption of pilot whale meat during teenage years up to pregnancy with the ASD group reporting higher consumption ( $p=0.65$ ) corresponding to a frequency of a meal every 3 weeks as opposed to a monthly rate in the control group. The reported rate of consumption during pregnancy for the ASD group then dropped to a 6-weekly interval as opposed to a sustained monthly rate in the control group.

The ASD group consumed less **blubber** than the control group during all three periods but these differences were not statistically significant. There were no differences between the two groups in consumption of **sea birds** (very low in both groups) and **fish** (consumed almost daily by all population) (Table 15).

## **E.4.2. Life-style and health differences**

### *E.4.2.1. Alcohol and smoking*

There was a difference - close to significant – in the use of **alcohol** ( $p=0.07$ ) and **nicotine** ( $p=0.07$ ) during pregnancy with lower rates among the ASD group and also lower consumption/quantity (e.g. a number of units of alcohol or a number of cigarettes consumed per week during pregnancy) among those who reported using alcohol and/or smoked as compared to the control group (Table 16).

#### *E.4.2.2. Mental health, miscarriage, Caesarean section, prematurity and birth weight*

There was a significant difference in reported **mental health disorders** experienced ‘ever’ prior to pregnancy with the ASD group having more mental health problems than the control group (**p=0.03**). Reported mental health issues included depression and/or autism traits. During pregnancy this difference between the two groups in reported mental health disorders was not significant (p=0.62).

There was also a close to significant difference in mother’s experience of **miscarriages** ‘ever’ with nearly a half of the control group experiencing miscarriages (**p=0.05**) in contrast to just 15% among the ASD group.

There were no differences between the two groups in reported rates of **Caesarean sections** or **prematurity** or in **birth weight** of an index child (Table 16).

#### *E.4.2.3. Life-style differences*

There was a highly significant difference in mothers’ **attitude to sun**. When asked “Were you often seeking sunlight during your pregnancy?”, all but one mother from the control group (92%) answered ‘yes’ in contrast to just 35% of the ASD group (**p=0.001**) (Table 16).

**Table 15.** Dietary trends: differences of chi-square ratios/proportions of the medians of reported portions/meals per year of pilot whale meat, blubber, sea bird, fish, fruit and vegetables consumed in childhood, teenage years up to confirmation of pregnancy, and pregnancy periods by mothers of a child with ASD and by mothers of a healthy child.

	<b>Pilot whale meat</b>			<b>Blubber</b>			<b>Sea bird</b>			<b>Fish</b>			<b>Fruit</b>			<b>Vegetables</b>		
	Child	Teen	Preg	Child	Teen	Preg	Child	Teen	Preg	Child	Teen	Preg	Child	Teen	Preg	Child	Teen	Preg
<b>ASD</b>	24.0	18.0	6.0	0.5	2.0	1.0	1.0	1.5	0.0	156.0	156.0	84.0	67.4	104.0	<b>105.0</b>	59.5	78.0	<b>84.0</b>
<b>Control</b>	24.0	12.0	9.0	12.0	4.0	3.0	3.0	2.0	1.0	156.0	156.0	84.0	88.5	52.0	210.0	82.3	104.0	126.0
<b>p</b>	0.39	0.65	0.55	0.23	0.32	0.50	0.20	0.65	0.20	0.51	0.79	0.83	0.91	0.83	<b>0.07</b>	1.00	0.28	<b>0.02</b>

**Table 16.** Life-style and health trends: differences of chi-square ratios/proportions of mothers with a child with ASD and mothers with a healthy child who were smoking and consuming alcohol in pregnancy; who experienced miscarriages ever, Caesarean sections with an index child and mental health problems ever and during pregnancy; and ratios of mothers according to their attitude to sun.

	Alcohol in pregnancy		Smoking in pregnancy		Miscarriage	Cesarean section	Prematurity	Birth weight (kg)	Mental health		Positive attitude to sun
	Yes	N° of units per week (median)	Yes	N° of cigarettes per week (median)	Ever	with an index child	of an index child	of an index child	Ever	Preg	Actively trying to get lots of sun
<b>ASD n=20</b>	3 (15%)	<b>0</b>	11 (55%)	<b>0</b>	3 (15%)	5 (25%)	4 (20%)	3.50	4 (20%)	4 (20%)	<b>7 (35%)</b>
<b>Control n=13</b>	5 (39%)	1	8 (62%)	1	6 (46%)	2 (15%)	2 (15%)	3.75	0	1 (8%)	12 (92%)
<b>p</b>	0.12	<b>0.07</b>	0.71	<b>0.07</b>	<b>0.05</b>	0.67	0.73	0.32	<b>0.03</b>	0.62	<b>0.001</b>

As a result of the existing substantial cross-cultural difference in the dietary style of this Nordic community in comparison to other European countries, there were a number of mothers within our cohort who had never eaten fruit or vegetables (except potatoes) in their childhood or adolescent years and only changed their diet during pregnancy (Table 17). Potatoes are consumed daily in the Faroe Islands. All mothers reported taking multi-vitamin supplements during pregnancy.

**Table 17.** Chi-square test for trend: proportions of participants reporting consumption of less than one portion of vegetables (except potatoes) or fruit per week during their childhood, teenage years up to pregnancy, or during pregnancy.

	<b>Vegetables</b> < 1 portion per week			<b>Fruit</b> < 1 portion per week		
	Childhood	Teenage	Pregnancy <b>p=0.02</b>	Childhood	Teenage	Pregnancy <b>p=0.07</b>
<b>ASD (n=20)</b>	75%	50%	35%	75%	45%	15%
<b>Controls</b>	54%	38.5%	8%	62%	54%	8%

### E.4.3. Mercury exposure

#### *E.4.3.1. Mercury exposure data available from the previous studies*

For 3 individuals with ASD, diagnosed in 2002 or 2009, their results were available for levels of exposure to mercury during the prenatal period as measured at birth by my Faroese colleagues and kindly shared with me for the purposes of this thesis (Grandjean et al., 1997; Debes, 2006;). These 3 individuals took part in a large study in 1986-87, measuring prenatal exposure to methylmercury from maternal consumption of pilot whale meat in cord blood at birth. In all 3 participants their mercury levels were very high at birth as compared to the geometrical average (22.9 µg/L) of the whole sample (n=894). The mercury levels were measured again later at age of 7, 14 and 22 years (Grandjean et al., 1997; Debes et al., 2006; Weihe & Grandjean, 2013) (Table 18).

**Table 18.** Methylmercury exposure levels ( $\mu\text{g/L}$ ) at birth (0), 7, 14 and 22 years (Grandjean et al., 1997; Debes et al., 2006). The geometric average of the cord blood concentrations of the whole sample ( $n=894$ ) was  $22.9 \mu\text{g/L}$  (IQR: 13.4-41.3).

	<b>Hg level (<math>\mu\text{g/L}</math>)</b>		
	<b>M1</b>	<b>F1</b>	<b>F2</b>
<b>Birth</b>	46.9	47.0	33.9
<b>7 years</b>	19.3	4.85	-
<b>14 years</b>	15.47	1.85	2.95
<b>22 years</b>	38.99	2.13	0.65

Table 18 demonstrates that all three individual's levels were very high at birth as compared to the geometric average of the whole sample and thus a comparison of their methylmercury levels with all their other available data from studies I, II, IV and V is of great interest, although not statistically significant.

As a consequence of the serious and significant outcomes of the above mentioned studies of prenatal exposure to methylmercury from maternal prenatal consumption of pilot whale meat (Grandjean 1997; Debes 2006) the Faroese Public Health issued recommendations, targeted especially at girls and women of childbearing age, who were urged not to consume pilot whale meat at all and the rest of the Faroese society was advised to reduce its consumption.

Table 18 also shows that this programme was efficient and resulted in a dramatic drop in females' levels of methylmercury in blood between 1980s and 2009 (no changes for the male).

#### *E.4.3.2. Comparison of mercury levels with available data*

The male (M1) and the female (F1) with mercury levels at birth greater than  $40 \mu\text{g/L}$  had more severe diagnosis: M1=childhood autism; F1=atypical autism. The male (M1) had severe learning disability (SLD), the female F1 had an average IQ (A).

At age of 22 years the male (M1) had a severely deficient vitamin D level (<20 nmol/L, i.e. ~8 ng/mL) and the female (F1) had deficient vitamin D level (<50 nmol/L, i.e. ~ 20 ng/mL), although both of them were sampled in July.

The female (F2) with lower levels of mercury at birth 33.9 µg/L had a diagnosis of Asperger syndrome and near average IQ-level (NA). She had severely deficient vitamin D levels at age 22 (<20 nmol/L, i.e. ~ <8 ng/mL), although she was sampled in July.

Mothers of females F1 and F2 with high levels of methylmercury at birth, took part in the ‘dietary and life-style trends during pregnancy’ pilot study (IV) and both reported ‘negative attitude to sun’ (not seeking sun actively) during pregnancy.

## **E.5. Vitamin D in the general population of young adults with autism in the Faroe Islands (V)**

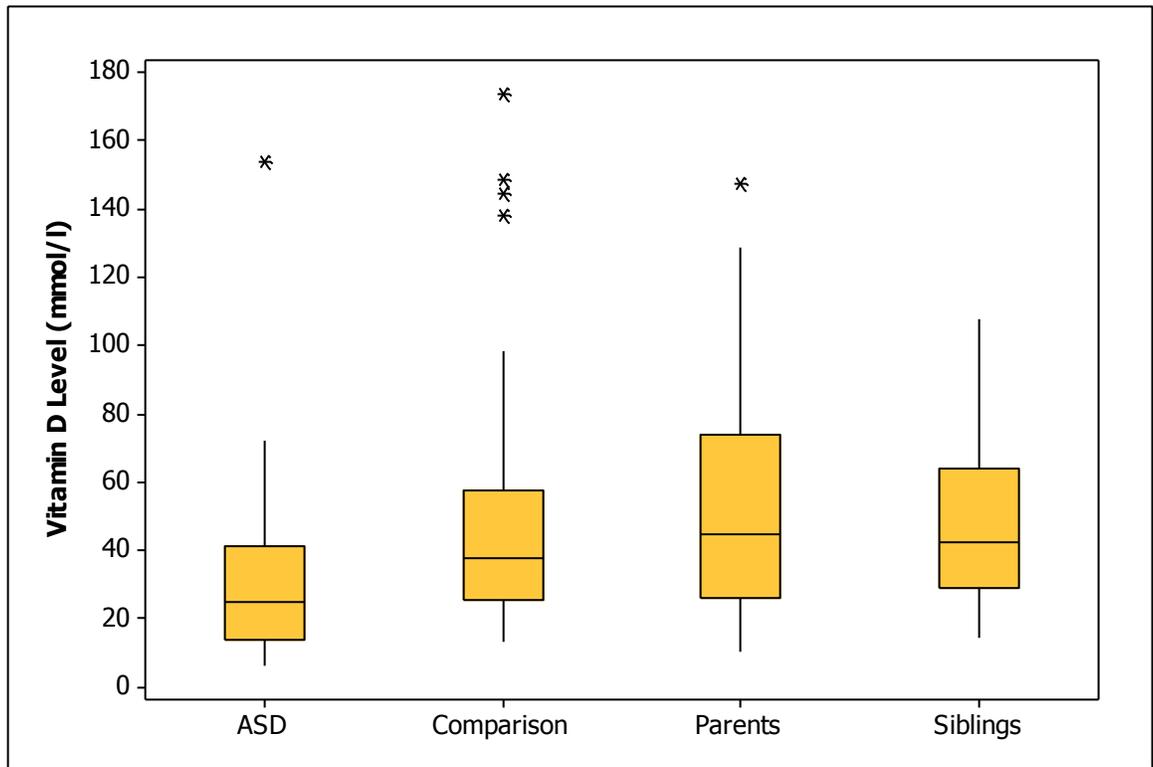
### **E.5.1. 25(OH)D<sub>3</sub> Levels**

As there were several outliers with very high levels in all groups apart from siblings, all the statistical analyses were re-calculated with all outliers removed. I consider however, inclusion of the outliers important. Since the results remained unchanged and significant after exclusion of all the outliers, I present the original results including all the outliers unless specifically stated otherwise.

The ASD group had significantly lower levels of 25(OH)D<sub>3</sub> (median (IQR) = 24.8 (27.5) nmol/L] compared to the healthy comparison group [median (IQR) = 37.6 (32.3) nmol/L], (95% CI 5.0 to 22.5), **p=0.002** to their siblings [median (IQR) = 46.1 (28.3) nmol/L] (95% CI 9.4 to 24.8), and parents [median (IQR) = 46.7 (36.2) nmol/L] (95% CI 11.0 to 27.74), **p<0.001** (in both instances) (Figure 6). The confidence intervals (CI) relate to the difference between the median values.

In the ASD group, 88% were vitamin D deficient (Figure 7). Among their siblings, parents, and the comparison group, the corresponding rates were 58%, 58%, and 65% respectively (**p<0.001**) (Table 19): for clarity and comparability of my results with several recently published studies of vitamin D levels in patients with autism I

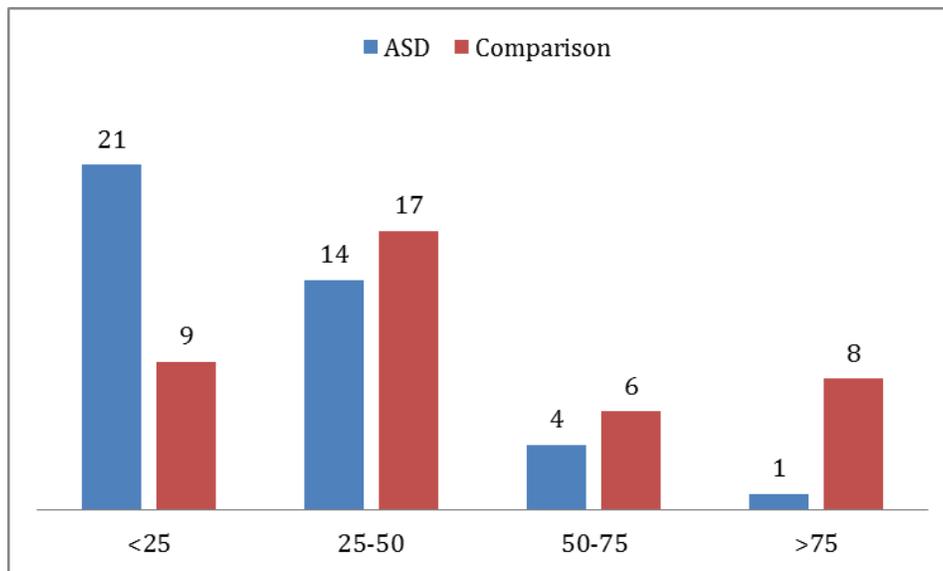
resolved to use the same reference ranges as Holick et al., (2011) and I used values of nmol per litre (nmol/L).



**Figure 6.** Box plots for 25(OH)D<sub>3</sub> levels for ASD, comparison (**p=0.002**), ASD, parent (**p<0.001**) and sibling (**p<0.001**) groups, (95% CI) (\*outliers).

**Table 19.** 25(OH)D<sub>3</sub> status: Chi squared test or table of percentages of participants with severe deficiency, deficiency, insufficiency and sufficiency of vitamin D (nmol/L) in the comparison group, ASD group and in siblings and parents of the individuals with ASD (Pearson Chi-Square = 26.730, DF = 9, p=0.002).

25(OH)D <sub>3</sub> status (nmol/L)	Count	% within Groups	Groups			Total
			Comparison	ASD	Sibling	
Severe deficiency <25	Count	9	21	10	18	58
	%	22.5%	52.5%	16.1%	23.4%	25.5%
Deficiency 25-50	Count	17	14	26	27	84
	%	42.5%	35.0%	41.9%	35.1%	38.4%
Insufficiency 50-75	Count	6	4	17	13	40
	%	15.0%	10.0%	27.4%	16.9%	18.3%
Sufficiency >75	Count	8	1	9	19	37
	%	20.0%	2.5%	14.5%	24.7%	16.9%
Total	Count	40	40	62	77	219
	%	100%	100%	100%	100%	100%



**Figure 7.** 25(OH)D<sub>3</sub> Status among ASD and comparison groups (95% CI, p=0.003).

### E.5.2. Analysis of season of blood sampling

Median 25(OH)D<sub>3</sub> D levels of individuals with ASD varied according to season of the year in which they were sampled: spring (n=9) = 13.80 nmol/L, summer (n=18) = 39.90 nmol/L and autumn (n=12) = 22.95 nmol/L (p=0.017), reflecting a similar trend that was previously found in the study of Faroese elder population (Dalgård et al., 2010). Therefore, the vitamin D data was adjusted for month of sampling in our analysis (see p 85 in section D. METHODS).

### E.5.3. Comparison of 25(OH)D<sub>3</sub> levels across and within gender

The trend for males having lower vitamin D levels was observed in the ASD and sibling groups, although this gender difference was statistically significant only in the sibling group (**p=0.03**). In the parent group both mothers and fathers had comparable levels (Table 20).

**Table 20.** 25(OH)D<sub>3</sub> Gender differences in individual groups.

GROUP (n)	Vitamin D level Median (nmol/L)		P (CI 95%)
	MALE	FEMALE	
ASD (40)	24.7	42.00	0.1 (-5.6-31.1)
Comparison (40)	37.4	43.95	0.6 (-12.7-23.5)
Siblings (62)	34.60	54.00	0.03 (1.2-27.3)
Parents (77)	44.90	44.45	0.9 (-11.7-11.29)

Because of the variability in male:female ratio in all groups (ASD group ~ 3:1; sibling and parent groups ~ 1:1 and the comparison group ~ 2:1), a combined comparison of vitamin D levels was also carried out for ‘males only’ in all groups. There was a significant difference between the groups (**p=0.001**). Males with ASD had significantly lower levels of 25(OH)D<sub>3</sub> [median (IQR) = 24.7 (20.6) nmol/L] compared to their brothers [median (IQR) = 34.6 (25.2) nmol/L] (**p=0.004**) and fathers [median (IQR) =

44.9 (49.8) nmol/L] (**p<0.001**) and also to the healthy comparison males [median (IQR) = 37.4 (31.0) nmol/L] (**p=0.002**).

#### **E.5.4. Comparison of 25(OH)D<sub>3</sub> levels in individuals with ASD recruited in 2002 and 2009**

As there were only 2 females diagnosed in 2002 and one of them was ‘an outlier’, i.e., her 25(OH)D<sub>3</sub> level was much higher than all other participants’ at 153 nmol/L (she was on treatment for osteoporosis), it was not meaningful to compare 25(OH)D<sub>3</sub> levels across the gender in this 2002 group. In the 2009 group there was no difference between males [median 24.9 nmol/L (IQR = 20.3)] and females [median 22.2 (IQR = 27.1)] (**p = 0.37**).

For comparison between the 2002 and 2009 groups this outlier was removed and there was no difference between the 25(OH)D<sub>3</sub> levels of the 2002 group [median (IQR) = 24.7 (28.80) nmol/L] and the 2009 group [median(IQR) = 23.6 (24.38) nmol/L] (95 % CI -9.10–11.40) (**p = 0.965**).

Among the 24 participants (22 males/2 females) recruited in 2002, 12 (50 %) had severely deficient, eight (30 %) deficient, three (13 %) insufficient, and one (=female) (4 %) sufficient levels of 25(OH)D<sub>3</sub>.

Among the 16 participants (9 males/7females) recruited in 2009, nine (56 %) had severely deficient, six (38 %) deficient, one (=female) (6 %) insufficient, and zero (0 %) sufficient levels of 25(OH)D<sub>3</sub>.

Although the 2002 and 2009 cohorts were diagnosed at two different time points, it should be noted that both groups were blood sampled for vitamin D analysis during the same period in 2008–2009.

#### **E.5.5. 25(OH)D<sub>3</sub> levels and other variables**

There was no association between 25(OH)D<sub>3</sub> levels and age, IQ, subcategories of ASD or ADOS score. When investigating ‘season of birth’ in the ASD group, those born in the spring season (March – May) had the lowest levels of 25(OH)D<sub>3</sub> (median 13.8 nmol/L; **p=0.17**) in agreement with the literature (Grant & Soles, 2009). Among the

ASD group there were 13 (32.5%) spring births (the comparison group was matched for season of birth; therefore, it included the same number of spring births). There was no correlation between the 25(OH)D<sub>3</sub> levels and ADOS scores (p=0.3). There was also no correlation between the 25(OH)D<sub>3</sub> levels and diagnosis - Asperger syndrome (n=18): 23.55 nmol/L (20.2); atypical autism (n=11): 24.7 nmol/L (29.6); autism (n=11): 30.3 (51.8) (p=0.3).

## **F. DISCUSSION (I-V)**

### **F.1. Prevalence of Autism in the Faroe Islands (I)**

#### **F.1.1. ASD Prevalence in the Faroe Islands**

The prevalence of ASDs of 0.94% of the general population in the Faroe Islands in 2009 (as compared to 0.56% in 2002) is within the range of “typical” findings of 0.6-0.7% (or one child in about 150 children) (Fombonne, 2008; 2009) up to 1% (Baird et al., 2006) in Europe and the rest of the western world.

This finding of relatively high rate of ASD in the genetic isolate of the Faroe Islands is very interesting and to some extent surprising from a genetic point of view. A somewhat higher rate in the Faroe Islands might have been expected, given the high rate of inbreeding. However, it is also possible that a much smaller “autism gene-pool” exists in the isolate, and this could explain a lower than “average” rate of the disorders. Maybe both types of factors are in operation and outweigh each other (Ellefsen et al., 2007). The overall Faroese rates could have possibly been higher and there might be a number of ASD cases that were not identified in the present study due to some limitations mentioned on p 124.

##### *F.1.1.1. The representativeness of the sample*

Even though relatively small, the groups studied are representative of the total population of young people with ASD in the Faroe Islands, as has been argued in more detail in a previous publication by our group (Kočovská, et al., 2012b). The fact that they were recruited in a genetic isolate could, by some, be taken to indicate that they might be atypical, and findings therefore not generaliseable to other populations. Even though this cannot be absolutely excluded, several members of the research group have experience of working with thousands of individuals with ASD, and their conclusion is that the Faroe Islands ASD groups are typical of similar age groups with ASD in other countries as regards their clinical presentation.

### *F.1.1.2. Agreement between the diagnostic instruments*

As in previous psychometric research on the DISCO (Wing et al. 2002; Nygren et al. 2009), agreement between interview and clinical diagnosis for the overall category of ASD was very good. Before drawing any conclusions in this respect one has to bear in mind that clinical (CRC) diagnosis took findings at DISCO-interview (but not the DISCO algorithm generated diagnoses) into account. Nevertheless, the DISCO algorithm diagnoses were computer-generated, and the clinician making the final CRC diagnosis was not influenced by the specific DISCO ASD subgroup diagnosis. The study findings demonstrated that, even though the overall rate of ASDs was not greatly influenced by CRC or DISCO-11 ASD status, subgroup diagnosis was clearly affected by clinical judgement as compared with DISCO algorithm diagnosis. This is partly stemming from the fact that the DISCO generates at least ten possible diagnostic subgroups (many of which overlap), whereas the number of CRC diagnoses was limited to three. However, even when like was compared with like (clinical autistic disorder with DISCO autistic disorder, Gillberg's Asperger's disorder with Gillberg's Asperger's disorder, and atypical autism with atypical autism) and the clinical hierarchy method was applied to the DISCO as well as the CRC diagnoses (autistic disorder taking precedence over Asperger's disorder etc.), very substantial discrepancies were identified. This, clearly, could be taken to support the stance of the DSM-5, in which only one major category of autism ("ASD") is acknowledged with sub-grouping recommended only on the basis of non-autism measures, such as IQ and adaptive functioning.

### **F.1.2. Exploration of reasons behind growing incidence/prevalence of ASD world-wide**

#### *F.1.2.1. Diagnostic criteria, type of assessment, inaccuracies and research methodologies*

Since 1980 when autism was first included in an official classification of mental disorders of DSM-III (1980) the ASD criteria were modified several times, changed and

expanded to its present form in DSM-5 (2013) and this broadening of diagnostic criteria, in conjunction with simultaneous better awareness of the disorder over time, became generally accepted as two of the most common factors impacting on growing ASD prevalence (Fombonne, 2009).

There is also a potential increase of prevalence stemming from the difference between: (a) Assigning the diagnosis solely derived by a clinician as ‘clinical diagnosis’, possibly based on just brief observations and/or biased by a personal specialty/style (experience and/or training) and/or diverse variety of clinical professions (paediatricians, psychiatrists, neurologists, psychologists, educational psychologists, speech and language therapists, etc.). (b) Assigning the ASD diagnosis by using any of the numerous instruments for ‘clinical research comprehensive (CRC) diagnosis.’ (c) Assigning the ASD diagnosis by combination of both – clinical diagnosis and CRC - that might be the most rigorous diagnostic system (Fombonne, 2009).

Clinically derived ASD diagnoses generate greater increase in ASD prevalence than research-identified ASD (Barbarese et al., 2009). This finding seems further supported by a recent study of the largest young twin sample in the world (Lundström & Gillberg, personal communication) that found an increase in the rates of ASD diagnoses, while the number of symptoms remained the same. Here, the original diagnoses data were retrieved from the National Register and parents were interviewed when the twins were 9 years old on the phone using the Autism Treatment Evaluation Checklist (ATEC) (Rimland & Edelson, 1999) that has been well validated for clinical diagnoses of ASD and ADHD (Geier, Kern & Geier, 2013). This increased rate of ASD diagnoses could be interpreted as a result of the changed diagnostic criteria, better awareness, a better service provision and/or diagnosing style of the clinicians.

There may even be some practical reasons behind the raised diagnostic rates of ASD due to an increased international attention to ASD and to some extent its preferential treatment when allocating resources. However, this aspect is not relevant to the Faroese situation and will require more rigorous exploration.

#### *F.1.2.2. Gender aspects*

Another recently proposed factor possibly impacting on increasing rates of autism is an appearing trend of girls being missed out in the very early years, most probably due to varying phenotypic presentation and the (then) available instruments not being sensitive enough to detect these differences and also due to less severe problems in school in girls with ASD (Baird et al., 2006; Kopp, Kelly, & Gillberg, 2010; Kim et al., 2011; Zwaigenbaum et al., 2012; Mandy et al., 2012; Kočovská et al., 2013).

Females with autism are very often diagnosed as having other disorders, and the autism aspect of their impairment may be missed altogether (Kopp et al. 2010). The trend (statistically significant) in our study was for missed autism cases (i.e., missed at the time of the original diagnostic study) to be relatively much more frequently female than male. Almost two-thirds of all the females with ASD in the study were only recognised at or after the age of 15, whereas almost three quarters of all males with ASD had been diagnosed before that age. Also, there was a significant trend towards older (previously unrecognised) females with ASD having an “atypical” presentation, even though the majority of them actually met full Gillberg and Gillberg (1989) diagnostic criteria for Asperger’s disorder.

#### *F.1.2.3. Better perinatal care*

The higher prevalence of autism may be increased by recent highly improved perinatal and neonatal care leading to much higher survival rates of pre-term babies, including extremely premature ones. These surviving infants present later with higher rates of ASD than the general population (Matson & Kozlowski, 2011).

#### *F.1.2.4. Cultural factors*

It has been suggested that culture and related social factors also play an important role in the identification of children with ASD and in the increase of regional rates of ASD. A higher prevalence of ASD has been observed in the countries of Asia over the past two

decades. Some of the sharpest rises in ASD have been noted in immigrant minorities (Matson & Kozlowski, 2011) and apart from the differences in cultural norms, there might be other reasons for this phenomenon (e.g. an impact of stress due to the move and/or the factor of latitude and the possibly associated vitamin D deficiency) (Fennell et al., 2010a).

#### *F.1.2.5. Environmental factors*

For some time it has been accepted that the rising rate of ASD world-wide could be attributed to a combination of all the above mentioned factors. However, the dramatic increase in the number of diagnoses of ASD during the past few decades, demonstrated by the rise from 1: 5,000 children in 1975 to 1: 88 children (1: 54 boys ~ prevalence rate among boys only) in the USA, 20.5: 10,000 in Sweden and >1: 100 children in the UK in 2009 (Gillberg et al., 2006; Baron-Cohen et al., 2009; Elsabbagh et al., 2012), became one of the most puzzling aspects of ASD.

Genetics is unable to explain this large increase because gene mutations, gene deletions, copy number variants (CNVs) and other genetic anomalies so far detected in conjunction with autism are responsible only for a minority of ASD cases (30%) (Schaaf & Zoghbi, 2011). Some geneticists attribute this soaring incidence of such a highly heritable disorder to a genetic-environmental interaction (Hallmayer et al., 2001; Coleman & Gillberg, 2012).

An aetiology study of ASD, searching for environmental factors may thus offer an explanation for this intriguing increase in the incidence and prevalence of ASD worldwide. In a rather simplistic way, Cannell (2013) argued that a broad autism genotype underpins the broad autism phenotype (Losh et al., 2007). Sasson et al. (2013) found that about 5-10% of the adult population demonstrate the broad autism phenotype (impaired social and communication skills, aloofness, rigidity and an extent of obsessiveness). If this genotype were stable in incidence then there may be environmental exposures (e.g. toxic chemicals) interacting with the broad autism genotype to result in this increased incidence of ASD and the observed heterogeneity in the autism phenotype (Landrigan, 2010; Cannell, 2013; Grandjean & Landrigan, 2014).

There are several reasons to suggest that one of the possible environmental risk factors might be vitamin D deficiency: (1) vitamin D deficiency has increased dramatically world-wide (due to sun avoidance, change of life-style and diet with no compensatory recommendations for higher oral intake) (Holick, 2005) during the same period of the last 40 years as ASD incidence increased; (2) vitamin D is involved not only in early brain development and brain function but also in gene regulation via a vitamin D receptor (VDR) that resides on up to 10% of the genes in the coding human genome (Carlberg et al., 2012) (some form of genetic aberration of the steroid system might be likely in ASD) and in the body's immune system regulation (Noriega & Savelkoul, 2014) (immune disorders are often present in wider families of individuals with ASD); (3) the epidemiology of ASD includes its higher prevalence in urban areas, high air pollution areas, cloudy areas and at higher latitudes (including the higher prevalence of ASD in dark-skinned populations living at higher latitudes (Cannell, 2010; 2013; Dealberto, 2011; Eyles, 2010; Atladóttir et al., 2009).

Because of the unusual characteristics of the Faroese region, e.g. the high latitude and consumption of pilot whale meat contaminated with high levels of mercury, these two environmental factors became an object of the present study related to the prevalence of autism in the Faroe Islands.

### **F.1.3. Strengths and limitations**

#### *F.1.3.1. Limitations*

In spite of the fact that this was a total population study, and screening was achieved in the total group of young people in the Faroe Islands, the target population was, by default, relatively small, and the number of cases identified correspondingly limited. The confidence limits for the reported prevalence were therefore relatively wide, particularly for females who were represented by very small numbers indeed. Because the screening process was not performed in a fully systematic manner with a standardised instrument, and there might have been a certain element of subjectivity present, there is a real possibility that some cases could have been missed. The refusal rate of 7% (4 families) of strongly suspected cases also possibly reduced the overall number of identified cases.

Another major limitation of this study was the fact that it was not possible to explore the characteristics of the 561 children lost to follow-up in 2009 among the original school age cohort of 2002 as there might have been some new cases of ASD among them. In addition, the literature suggests that the instruments used might not have been sensitive enough for detection of autism in girls at younger age.

#### *F.1.3.2. Strengths*

The total population character of the sample and the very good coverage plus the comprehensive assessment including gold standard instruments (with well documented good-excellent psychometric properties) for the diagnosis of ASD were major assets, counterbalancing the limitations to some extent. The follow-up over a period of many years from school age into late adolescence and early adult life, using the same instruments at both time points is a unique feature of the study.

#### **F.1.4. Conclusions**

Autism is as common in the Faroe Islands—a genetic isolate—as in the rest of the western world. The present prevalence rate of 0.93% (0.94% of ‘ever’ diagnosed cases) has been identified in the follow up study in 2009 (as compared to 0.56% in the original study in 2002). This increase in prevalence rates revealed that about 36% of the total cohort had been missed in the original screening study performed in school-age years. Due to 49% of the newly diagnosed cohort in 2009 being females (as compared to 16.3% of the original cohort in 2002), the most possible explanation for this omission might be the unfamiliarity with the clinical presentation of autism in females at an early age.

## **F.2. Autism in the Faroe Islands: Diagnostic Stability from Childhood to early Adult Life (II)**

### **F.2.1. Diagnostic stability of clinical and DISCO diagnoses**

#### *F.2.1.1. Comparison of clinical and DISCO diagnostic stability*

Interestingly, the stability of clinical ASD diagnoses was perfect for AD, good for Atypical autism/PDD-NOS, and less than perfect for AS. Stability of the DISCO-algorithm subcategory diagnoses was more variable but still good for AD. In terms of “any ASD” diagnosis, both systems showed excellent stability over the seven-year period with only one case of ‘clinical ASD’ at Time 1 receiving ‘no clinical diagnosis’ at Time 2 and one case of ‘No DISCO ASD-diagnosis’ at Time 1 receiving a ‘DISCO-ASD diagnosis’ (AS) at Time 2.

#### *F.2.1.2. Evaluation of the diagnostic process*

Before going on to discuss the implications of the findings, several things need to be addressed. First, was the clinical diagnostic process sufficiently expert and in-depth to allow generation of valid comprehensive clinical ASD diagnoses? I would argue that indeed it was. The individuals in the study were examined for many hours, and on several different occasions, by experienced psychologists and psychiatrists. These experts were working in the context of an internationally well-known and clinically highly experienced research group that has demonstrated excellent reliability for autism diagnoses (Steffenburg & Gillberg, 1986).

#### *F.2.1.3. DISCO and its psychometric properties*

Second, is the DISCO an instrument with established psychometric properties? The DISCO has excellent inter- and (short-term) intra-rater reliability, and is valid for ASD diagnoses, both as derived from clinical assessment and after interview using an

alternative investigator-based collateral informant interview, the ADI-R (Nygren et al., 2009). The DISCO generates much more information about early development and ASD-associated (not just “ASD-diagnostic”) symptoms and problems than the ADI-R, and so it is our contention that it is at least as useful in ASD diagnostics.

#### *F.2.1.4. Diagnosticians*

Third, were the diagnosticians independent of each other and in relation to the DISCO-algorithm diagnoses when they made their clinical diagnosis within the spectrum of autism? All clinical diagnoses were made on the basis of all available information obtained by each Faroese clinician (sometimes with the help of the Swedish clinical researchers) without any knowledge of the DISCO algorithm ASD diagnoses delivered by the computer. It could not be ruled out that information obtained at DISCO-interview might have influenced the Faroese clinician when assigning a clinical diagnosis, but the algorithm diagnosis (a complex combination of a very large number of items from all the many areas covered by the DISCO) and its constituent parts were not known to the clinician when the diagnosis was made. On balance, therefore, I conclude that the findings obtained are highly relevant as a basis for discussion of the stability and interrelationship of clinical and DISCO diagnoses of ASD in a long-term perspective.

#### *F.2.1.5. Possible reasons behind a number of missed cases in 2002*

I found a significant number of “missed” cases in the follow-up study, individuals with unequivocal ASDs who had not been identified as having autistic disorder, Asperger’s disorder, or atypical autism when they were younger. Superficially, the findings, if treated as the results of two separate cross-sectional studies performed with a time gap of seven years, could have been interpreted as support for the notion that the prevalence of ASDs has gone up with time. This would be supported particularly in view of the generally held notion that it is easier to recognise autism in young (school age) children — and therefore more likely that the prevalence would be higher — than in young adults, producing a lower rather than a higher estimate at the older age. However, this was a longitudinal study following (almost exactly) the same cohort of individuals over

time. This means that the “higher prevalence” was an artefact of insufficient coverage and diagnostic precision at the time of the original study. The group of ASD cases missed in the original screening had all shown autism symptoms in childhood, but these had not been recognized by the teachers as abnormal. If parents had also been included as informants in that screening, it is possible that many of the missed cases would have been detected. A number of studies have shown that there are large differences across informants (Posserud et al., 2006; Ronald et al., 2008), suggesting the need to gather information both from families and from schools when screening for ASD. There was virtually no migration to the Faroe Islands over the time period covered, in fact there were indications of some migration from the islands in the studied age cohort, and so the higher prevalence rates could not have been produced by influx of new cases. Conversely, the rather limited migration from the Faroe Islands in the original target population and none in the autism sample, meaning that the “base rate” contributed by cases identified in the first study remained stable over time. Instead, our findings indicate that insufficient knowledge about the phenotype of ASDs, particularly about the clinical presentation in females, is the main reason for the apparent increased/higher prevalence of autism in the Faroe Islands between 2001 and 2009.

#### *F.2.1.6. Gender effects*

There were no significant gender effects as regards stability/change of diagnosis, either in respect of clinical or DISCO-algorithm diagnoses. However, the number of female cases included in the study was low (even though several previously undiscovered cases were identified at the second study), meaning that conclusions can only be tentative in this respect. In effect, one might argue that the relatively high number of “new” female cases emerging at Time 2 could be seen as an indication of the poor “diagnostic stability” of ASD in females (non-caseness turning into caseness at a considerable rate over a seven-year-period, in spite of the “true” onset of the ASD having been in early childhood in all the “new” female cases).

#### *F.2.1.7. Outcomes*

It appears, then, that the take home message from this study is that both clinical and DISCO-algorithm diagnoses are stable over the period from school age through late adolescence and early adult life so long as one is referring to ASD and not to individual categories within the ASD umbrella concept. For autistic disorder/childhood autism the clinical diagnosis is very stable, and the DISCO-algorithm diagnosis fairly stable over a 7-year-period from school age to early adult life. Asperger syndrome “caseness”, on the other hand had relatively poor predictive ability for the same diagnosis at Time 2, with a “hit rate” of 67% for clinical and only 27% for DISCO-algorithm identical diagnosis at follow-up.

#### **F.2.2. No diagnosis on follow-up**

##### *F.2.2.1. A case of the present study*

There was only one case of ‘growing out’ of the ASD diagnosis during the period between childhood and young adulthood in this Faroese cohort. A male with a previous diagnosis of atypical autism in the original study in 2002 received no ASD diagnosis (‘autistic traits’ only) in the follow up study in 2009. Unfortunately, I do not have any detailed information regarding his family situation/upbringing or educational settings that could shed some light on the reasons for his remarkable improvement. However, this case raises the question of how much/to what extent his pertaining ASD traits will impact on his personal/professional life and whether he will be able to live independently without any service provision, etc.

##### *F.2.2.2. Additional research findings*

There is a recent study from Gothenburg, Sweden that followed 100 male cases of ASD diagnosed 30 years earlier in their childhood and followed twice at age 22 and 31 years (Helles et al., 2014). There was a decline in the stability of AS over time, the rate dropping from 82% at 22 years to 44% at 31 years. There was also a significant decrease

in the cases reaching any ASD diagnosis from 91% at 22 years to 76% at 31 years. Those who had a stable diagnosis had presented with more symptoms of greater severity in their adolescence/young adulthood. The authors concluded that there is, on the one hand, a number of individuals, who were diagnosed in childhood with ASD and later in adulthood do not meet the criteria of an ASD diagnosis any longer, who function well in the society and do not experience any major limitations in their everyday life. On the other hand, there are others with AS or PDD-NOS diagnoses, who in adulthood do not meet the new ASD diagnosis in the DSM-5, although they display clear ASD symptoms and their everyday functioning is markedly impaired (Helles et al., 2014). If supported by subsequent research, this finding may pose a challenge for the future – either there needs to be a service provision established and based on practical needs of individuals without an official diagnosis, or the present diagnostic instruments need to be fine-tuned to the adult patients' population.

Another study (Barnevik-Olsson et al., submitted) followed up 17 children first diagnosed with ASD at age 2.5 years and then re-examined at age 4.5 years when a subgroup of these children did not meet the criteria for a diagnosis any more (their parents believed that they improved due to the training programmes, etc.). At age 10.5 years, several children of the 'diagnosis-free group at age 4.5 years' received the diagnosis again or met the criteria for another impairment (e.g. language disorder, ADHD, etc.) (Barnevik-Olsson et al., submitted). If repeated in future studies, there might be a need for personally tailored service provision and/or education.

### **F.2.3. ASD and intellectual disability/age**

Lower IQ scores have been reported to commonly accompany ASD diagnosis with rates varying in the range of 71-88% (Steffenburg & Gillberg, 1986). In recent studies, the number of children with ASD receiving lower than average IQ scores varies hugely between 10-90 % (Coleman & Gillberg, 2012).

In the present study the rates of lower IQ categories (< 85) were 39%, with severe and mild learning disability at 18% and 7.5% respectively. All cases with SLD received the diagnosis of childhood autism while the cases with MLD received either the diagnosis of atypical autism (5%) or Asperger syndrome (3%).

Among those 24 young individuals diagnosed only later in their adolescence/early adulthood in 2009 was only one case with SLD (a young female) diagnosed with autism, 3 cases with MLD diagnosed with atypical autism, and 5 cases of NA diagnosed with either atypical autism or Asperger syndrome. The rest (54%) were high functioning individuals and received either diagnosis of atypical autism or Asperger syndrome. This finding supports the notion of high functioning cases often being missed in early childhood.

It has been acknowledged that with the change of diagnostic criteria over the period of the last 4 decades, at present a child with ASD and intellectual disability would be more likely to receive an ASD diagnosis while in the past it was quite the opposite (Leonard et al., 2010). This may partly explain the omission of the 5 ASD cases in the present study, possibly ‘masked’ by their mild/severe intellectual disabilities in early childhood.

The participants with learning disabilities of the Faroese cohort were placed during their school years in a well suited local special educational setting, with a multidisciplinary team including a speech and language therapist and led by an educational psychologist (two of the researchers of the original study in 2002) and received an excellent personally tailored educational program and care with an input from special needs assistants (including regular play-time outside in the sun when weather permitted). Arguably, these children with mild/severe learning disabilities and ASD received more systematic and intense care than those with less demanding needs, placed in a mainstream class. However, the MLD/SLD group were the most stable group regarding their diagnosis of childhood autism, possibly also due to their extensive special needs (Fennell et al., 2010b; Coleman & Gillberg, 2012).

#### **F.2.4. Strengths and limitations**

##### *F.2.4.1. Limitations*

In spite of the fact that this was a total population study, and screening was achieved in the total group of young people in the Faroe Islands, the target population was, by default, relatively small, and the number of cases identified correspondingly limited. The

confidence limits for the reported prevalence were therefore relatively wide, particularly for females who were represented by very small numbers indeed. The unavailability of ADOS scores for the original cohort screened in 2002.

#### *F.2.4.2. Strengths*

The total population character of the sample and the very good coverage plus the comprehensive assessment including gold standard instruments (with well documented good-excellent psychometric properties) for the diagnosis of ASD were major assets, counterbalancing the limitations to some extent. The follow-up over a period of many years from school age into late adolescence and early adult life, using the same instruments at both time points is a unique feature of the study. The use of DSM-IV, Gillberg, and DSM-5 criteria in one and the same study is a first, and clearly an additional strength. The fact that the original cohort was seen at time 1 by two clinicians and at time 2 by one other clinician adds to the validity of the findings obtained.

#### **F.2.5. Conclusions**

In terms of “any ASD” diagnosis, both the clinical and DISCO diagnostic systems showed excellent stability over the 7-year period from school age through early adult life. Stability of clinical diagnoses was perfect for AD, good for atypical autism/PDD-NOS and less than perfect for AS. Only 1 case of ‘clinical ASD’ with no other comorbidities in 2002 received ‘no clinical diagnosis’ in 2009. Stability of DISCO-algorithm subcategory diagnoses was more variable but still good for AD. A large number of females were missed in childhood and diagnosed only later in their adolescence years. These results support the notion that a single overarching diagnostic category, ‘autism’ or ASD, would better suit clinical realities as outlined in the new DSM-5.

## **F.3. Autism – vitamin D: Clinical Review (III)**

### **F.3.1. Overview of the systematic literature review**

Vitamin D deficiency – either during pregnancy or early childhood – has recently been proposed as a possible environmental risk factor for ASD (Cannell, 2010). Although at the time of conducting the literature review the number of clinical studies of vitamin D in individuals with ASD was limited, a large number of studies was found to support the role of vitamin D in numerous cellular functions, in particular, cell differentiation, neurotrophic factor expression, cytokine regulation, neurotransmitter synthesis, intracellular calcium signalling and anti-oxidant activity. In animal studies its ability to partially reverse brain damage has been demonstrated (Burne et al, 2004), as well as its ability to increase cellular levels of glutathione (Garcion et al., 2002) and the expression of genes and proteins involved in neuronal differentiation, structure, and metabolism (Eyles et al., 2011).

### **F.3.2. Hypotheses of involvement of vitamin D in aetiology of ASD from the narrative review**

The results of these studies have been translated into hypotheses regarding a possible involvement and role of vitamin D in aetiology and/or phenotype expression of ASD, some more plausible than others. Most of these hypotheses support the notion that neither genetic nor environmental exposure operate alone in the development of autism. In fact, the risk factors are likely to involve a combination of genetic susceptibilities and environmental exposures (Noriega & Savelkoul, 2014; Gentile et al., 2013; Patrick & Ames, 2014; DeLuca et al., 2013).

#### *F.3.2.1. An autoimmune hypothesis of ASD*

There is now growing support for an autoimmune hypothesis of ASD based on immune system dysregulations observed in ASD. Hypotheses involving vitamin D deficiency are stemming from two observed processes evolving in parallel over the past several

decades: (a) a higher prevalence of autism - 1: 5,000 in 1975 to 1: 88 (1:54 among boys only) - in the USA in 2012 that cannot be explained by genetics, and (b) an increase in prevalence of vitamin D deficiency worldwide during the same period due to the increased indoor urbanised lifestyle, sun avoidance/use of sunscreen. Dietary changes also seem to play a significant role, e.g. reducing vitamin D intake by more than 50% from fish (a food with the highest content of vitamin D) by frying instead of boiling, which removes most of the oil-soluble vitamin D (Chen et al., 2007; Chaplin & Jablonski, 2013), etc.

#### *F.3.2.2. Other hypotheses*

The discovery of increased rates of autism in people with darker skin at higher latitudes indirectly provides support for the hypothesis of vitamin D deficiency in the pathogenesis of autism. It has been concluded that the findings regarding very low vitamin D levels generally in Somali mothers in Stockholm (Fernell et al., 2010a) have considerable consequences from a public health perspective, and that more research regarding the role of vitamin D in autism is warranted.

Results from on-going research in schizophrenia might provide possible clues to the pathogenesis of ASD as there are some parallels between the aetiology of both disorders and hypothetically both may be related to prenatal and developmental vitamin D deficiency (Grant et al., 2009; Humble et al., 2010).

#### **F.3.3. Vitamin D and epilepsy**

The hypothesis of involvement of vitamin D deficiency in autism is further supported by the observation of low plasma levels of vitamin D and an increased incidence of autism among infants of mothers taking antiepileptic medication, especially sodium valproate, during pregnancy (Bromley et al., 2008). This finding may suggest low vitamin D levels as a possible mechanism warranting further investigation. An inverse relationship between the antiepileptic drugs and vitamin D levels has been demonstrated in animal research (Borowicz et al., 2007).

Animal and clinical studies have demonstrated the neuroprotective role of vitamin D in epilepsy (Harms et al., 2011). As epilepsy and convulsions are very common in autism (25% (Coleman & Gillberg, 2012)), it is pertinent to note that vitamin D has also been shown to increase the electroconvulsive threshold for seizures, to decrease the severity of seizures, and to enhance the effect of the antiepileptic medication (Siegel et al., 1984; Kalueff, Minayan, & Tuohimaa, 2005; Borowicz et al., 2007). Calcitriol exerts its neuroprotective effects via the inhibitory effect on calcium ( $\text{Ca}^{2+}$ ) influx. Calcitriol up-regulates the expression of the calcium-binding proteins calbindin and parvalbumin in motor neurons (Alexianu et al., 1998), which chelate (i.e. bind) intracellular  $\text{Ca}^{2+}$  and thus limit its excitotoxicity (Harms et al., 2011). However, antiepileptic drugs are known to decrease vitamin D levels, which further complicates research on the potential link between vitamin D deficiency and increased risk of seizures (Berquist et al., 2007; Borowicz et al., 2007). Vitamin D deficiency thus might lead to disruption in calcium signalling and homeostasis, which might account for some of the pathologies and disrupted information processing typical for autism.

#### **F.3.4. Vitamin D and nutrition**

When the nutritional history of children with ASD was studied, they were repeatedly shown to have a probably inadequate intake of vitamin D due to their highly selective intake of food. The nutritional studies highlight the possibility of acquired hypocalcaemia and hypovitaminosis D in subgroups of children and adolescents with autism and suggest the importance of a comprehensive history-taking, careful diet assessment, and when appropriate, screening for vitamin D deficiencies as an integral part of every child with autism's medical care (Noble et al., 2007; Cannell et al., 2008a; Holick et al., 2011) (Table 13).

### **F.3.5. Strengths and limitations**

#### *F.3.5.1. Limitations*

The literature is very limited as regards clinical studies of individuals with autism or their close relatives and provides only weak support for the hypothesis of the modulatory role of vitamin D in the pathogenesis of autism specifically.

#### *F.3.5.2. Strengths*

This was the first review of this kind at the time, linking exclusively vitamin D and autism.

### **F.3.6. Conclusions**

Vitamin D deficiency – either during pregnancy or early childhood – has recently been proposed as a possible environmental risk factor for ASD. The findings obtained over the past 15 years, including animal studies, human molecular, cellular and physiologic research, post-mortem brain, neuro-imaging, and genetic studies, suggest that vitamin D plays numerous roles in various processes in the human body. However, the literature is very limited as regards clinical studies of individuals with autism or their close relatives and provides only weak support for the hypothesis of the modulatory role of vitamin D in the pathogenesis of autism specifically. Therefore, there is an urgent need for intensified research in this important area. If pursued with greater detail as regards other environmental variables, medical and family history and genetic analysis, such studies might offer a very valuable insight into this intriguing interplay of genetics, environmental factors, and the proposed role of vitamin D in foetal neuro-development. This approach will require large and comprehensively diagnosed cohorts with ASD, preferably sampled in a population context. There is also a need for more in-depth longitudinal research on chronic latent vitamin D insufficiency and deficiency.

It is important to consider that autism is a very heterogeneous condition with many aetiologies and that vitamin D might be one important factor in the aetiological

panorama among other operating elements (Lappe, 2011; DeLuca et al., 2013; Cannell & Grant, 2013; Patrick & Ames, 2014).

Despite the rather limited data available at the time of the review, there appears to be a trend indicating that individuals with ASD may be at risk for vitamin D deficiency/inadequacy, and that low vitamin D levels in utero or early postnatal life might interact with other factors to increase the risk for ASD.

#### **F.4. Dietary and life-style trends during pregnancy in 1980s-1990s in the Faroe Islands – pilot study (IV)**

This pilot study, as part of a large epigenetic study of autism in the Faroe Islands, has aimed to explore the dietary, life-style, health and socio-demographic co-factors that might possibly be influencing prenatal methylmercury exposure in pathogenesis of autism and also to aid future in-depth research regarding gene-environment interplay in the aetiology of autism in the advantageous conditions of the genetic isolate of the Faroe Islands.

In particular, the research interest of this pilot study was directed to explore reported dietary differences during pregnancy, specifically mothers' consumption of pilot whale meat as a source of methylmercury exposure, blubber as a rich source of protective omega unsaturated fatty acids (PUFAs) and a high content of vitamin D and other essential nutrients; consumption of vegetables and fruit as a source of the antioxidant vitamin C, folic acid, etc.; smoking and/or use of alcohol; presence of mental/health problems and certain life-style choices (e.g. actively seeking the sun).

##### **F.4.1. Outcomes of the dietary questionnaire pilot study**

The reported differences in consumption of pilot whale meat, blubber, fruit (close to significant) and vegetables (significant) between mothers of the ASD and control groups, with lower consumption of blubber, fruit and vegetables in the ASD group, may tentatively indicate enhanced risk of one factor in the presence of reduced levels of several other protective factors. However, the whole picture is more complicated due to the Faroese society still being close in their dietary style to the traditional well balanced

diet high in protein and fat with all necessary nutrients/vitamins and a perfect balance of PUFAs protecting against heart disease and cancer (Dewailly & Knap, 2006). Thus it is not possible to imply that fruit or vegetables are more nutritionally valuable than the traditional sea food sources of vitamins in the Faroe Islands especially without more precise/detailed data. The last 2-3 decades, as documented by research (Dewailly & Knap, 2006) and also by the answers of mothers in this pilot study, have witnessed a clear phase of transformation of this very traditional Faroese dietary style to the more western one (with all its pitfalls of sugar, fizzy drinks, fried fish robbed of its greater part of vitamin D content, etc.).

The reported significant difference in an experience of miscarriages represents another interesting issue. The result of the ASD group mothers reporting smoking less and consuming less alcohol during pregnancy is in line with previous research reporting individuals with ASD using less of these and other substances (Hallerbäck et al., 2012). It can be hypothesised, that an impact of alcohol/smoking may cause more substantial impairment to the developing foetus resulting in a miscarriage, while perhaps the onset of ASD in an early neurodevelopment is more 'subtle' and not leading to a miscarriage.

Most interestingly, mothers of our ASD group reported significantly less positive 'attitude to sun' during pregnancy than mothers of children with ASD ( $p=0.001$ ). This is a very important finding, indicating some kind of underlying problem that might play a crucial role in this complex interplay of genetic and environmental factors involved in the pathogenesis of autism and possibly supporting the notion of involvement of vitamin D deficiency in the aetiology of autism.

#### **F.4.2. Faroese studies of prenatal mercury exposure**

In the present ASD cohort, there were 3 participants who were also part of one of the largest and most intensive Faroese studies, measuring levels of prenatal exposure to methylmercury from maternal consumption of pilot whale meat. The study followed 1,022 infants born during 1986-87 in the Faroe Islands, whose methylmercury exposure was investigated from the concentrations of mercury in umbilical cord blood and maternal hair at birth. The geometric average of the cord blood concentrations ( $n=894$ ) was 22.9  $\mu\text{g/L}$  (IQR: 13.4-41.3) and the median maternal hair concentration was 4.3

µg/L. At the age of 7 years these children underwent detailed clinical, neuro-physiological and neuro-psychological examinations. Negatively mercury-related neuropsychological dysfunctions were most prominent in the domains of language, attention and memory (highly significant) and slightly less in visuospatial and motor functions (Grandjean et al., 1997). These examinations were repeated again at the age of 14 and 22 years. Analysis of the test differences between the results at 7 and 14 years indicated that mercury-associated deficits had not changed between the two examinations. Thus, the impact of prenatal methylmercury exposure appears to be multifocal and permanent (Debes et al., 2006).

The 3 individuals from the present study, whose methylmercury levels of prenatal exposure were available, had highly elevated concentrations of methylmercury at birth (M1: 46.9 µg/L and F1: 47 µg/L; F2: 33 µg/L), as compared to the geometrical average of the whole cohort (22.5 µg/L). Among the three individuals, the higher levels of prenatal exposure to methylmercury of the two individuals (M1 and F1) interestingly corresponded to the more severe form of ASD diagnosis (autism and atypical autism respectively) as compared to the lower (albeit still highly elevated) levels of the third individual (F2) and her diagnosis of Asperger syndrome.

The mothers of the two females (F1, F2) took part in this dietary questionnaire pilot study. There were some differences in their reported consumption of pilot whale meat, blubber, fruit and vegetables, although these are of course, not significant. All three young individuals (F1, M1, M2) presented with severe vitamin D deficiency in 2009 at the age of 22-23 (< 25 nmol/L) and their mothers with insufficiency (<75 nmol/L). Interestingly, the mothers of both females (F1, F2) also reported negative attitude to sun during pregnancy.

Although these results are not statistically significant, they may be important clinically and possibly indicate the direction of future research. They also document (here only illustratively due to the small sample of only 3 individuals) the effectiveness of the Faroese Public Health advisory strategies related to the dietary changes implemented between 1997-2006 (the recommendation targeted exclusively at girls and women of childbearing age and not men). However, this effectiveness was demonstrated by rigorous follow-up studies conducted by my Faroese colleagues (Weihe & Grandjean, 2013).

### **F.4.3. Methylmercury**

As mentioned earlier, methylmercury negatively impacts the brain: (1) increases excitotoxicity (Aschner et al., 2007); (2) causes a steep increase in the calcium gradient which initiates apoptosis and; (3) halts the production of the antioxidant chelating agent glutathione (Aschner et al., 2000; Ercal et al., 2001; Aschner & Aschner, 2007).

Due to the ability of methylmercury to cross the blood-brain barrier, the resulting detrimental effects in the brain are profoundly more damaging in a developing brain that has not yet reached complete maturity and thus its protective abilities are substantially limited. As the majority of brain growth occurs during the third trimester of the prenatal period, this is also a period of great vulnerability of the developing brain to poisoning by methylmercury which inhibits important biochemical and developmental processes (Sakamoto, 2004).

As mentioned in study III, vitamin D deficiency – either during pregnancy or in early childhood – has recently been proposed as a possible environmental risk factor for ASD (Grant & Soles, 2009; Cannell, 2008). The end product of vitamin D biosynthesis, the neurosteroid hormone calcitriol, that signals via nuclear receptors (Eyles et al., 2013) and via its multiple diverse actions in the brain, plays an important role especially in early neurodevelopment and in degenerative processes: (1) cell differentiation and axonal growth; (2) stimulation of neurotrophic factor expression (e.g., cytokines); (3) regulation of calcium signalling directly in the brain; (4) modulation of the production of the brain-derived reactive oxygen species; (5) stimulation of glutathione (a potent antioxidant, involved in DNA synthesis and repair and exhibiting a particularly strong affinity for the methylmercury ion) (6) and thereby down-regulating excitotoxicity (Eyles et al., 2013; DeLuca et al., 2013).

Therefore, it is logical to speculate that this diminished protective function of calcitriol during early neurodevelopment due to vitamin D deficiency in higher latitudes, in conjunction with possible individual genetic predispositions to autism and/or to Hg toxicity and combined with exposures of varying degrees to a number of environmental factors, may escalate into a neurodevelopment impairment (Grandjean & Landrigan, 2014).

#### *F.4.3.1. Balancing the risks and benefits of seafood consumption*

To date, the consumption of fish has been recommended world-wide, since omega-3 fatty acids from fish are known for their protective function: e.g. the reduced risk of premature birth and regulation of desirable birth weight (World Health Organisation; 1990). However, with the increasing pollution of earth, rivers and oceans, a considerable body of research was devoted to finding a solution to the present problem of balancing the benefits of n-3 polyunsaturated fatty acids and the risk of methylmercury exposure from fish consumption (Mahaffey et al., 2011). Numerous studies have demonstrated beneficial effects of PUFAs via their involvement in (1) cell membrane formation and functions; (2) functioning of brain, retina, liver, kidney, adrenal glands and gonads; (3) regulation of blood pressure and immune and inflammatory responses on cognitive development, eye-hand coordination, verbal IQ, pro-social behaviour, fine motor skills, social development, reduced risk of hyperactivity, higher verbal IQ, better psychomotor development, higher verbal and full IQ at 48 months (Mahaffey et al., 2011). A number of studies have also unravelled the negative impact of methylmercury on neurodevelopment: language, attention, memory, visuospatial and motor functions, lower full IQ scores, language development and gross-motor skills and clearly showed that this damage is multifocal and permanent (Grandjean, 1997; Debes et al., 2006).

While the ultimate goal is to control pollution and clean up the environment, the timely solution meanwhile is the newly developing advisory service on nutritional suitability of certain marine species for vulnerable subgroups of young children, pregnant women and women of childbearing age. There are species differing in both PUFAs and mercury content due to varying level of environment contamination, species-specific physiological factors such as metabolism and growth rate and concentration of mercury in their diet (depending on type of prey) (Mahaffey et al., 2011).

There are species with low PUFAs and high mercury levels (that may negate the beneficial aspects of PUFAs) which need to be avoided: shark, tilefish, swordfish, large bluefin tuna and whale; and there are marine species with high PUFAs content and low mercury content that need to be promoted: mackerel, salmon, sardines. Other low

methylmercury sea food includes pollock, herring, sole flounder, crabs, shrimp and oysters (Weaver et al., 2008). An additional option might be to produce omega-3 fatty acids synthetically (from non-fish sources preferably, such as algae) and use them in combination with vitamin D as diet supplements (Weaver et al., 2008).

Specific conditions in each country need to be taken into account. In the case of the Faroe Islands, without any reservations, the Public Health recommendation to children and women of child-bearing age to avoid pilot whale meat and to the rest of the society to reduce its consumption significantly is the only right and absolutely necessary solution in order to protect healthy neurodevelopment of the future generations (Grandjean, 1997; Debes et al., 2006). However, at the same time, it poses a huge concern for this particular society that has to import everything from the mainland, except for electricity and fish. There is no domestic production of fresh fruit, vegetables (except for potatoes for personal use), meat (except for lamb for personal use); and dairy production is self-sufficient only for milk and yogurts (no cheese). Fish, blubber, sea birds and potatoes have been the sole dietary source over the centuries until very recently. Thus, it will be an extremely challenging task to substitute completely this rich dietary component once proclaimed “the freshest fish from the cleanest seas in the world” with imported products that cannot arrive truly fresh and nor be affordable to the whole community. The locally-caught species with low mercury and high PUFAs content and supplementation seem to be the most suitable solutions at present. The mercury levels of our three participants with ASD strongly suggest, and the rigorous Faroes follow-up studies of mercury levels in children of the 1986-87 original cohort documented without doubt (Weihe & Grandjean, 2013), that the recommendations were followed as females’ levels dropped dramatically between 1980s and late 1990s.

For research purposes greater precision, as regards not only the absolute amount of the fish consumed but also its frequency and specific types of fish that will allow calculation of the Hg: PUFAs ratio, is necessary.

#### *F.4.3.2. Methylmercury toxicity and genetic factors*

In addition, when conducting research on prenatal MeHg<sup>+</sup> exposure, there is a need to take into account nutritional and socio-demographic co-factors as well as relevant

genetic polymorphisms (Julvez et al., 2013). Heterogeneities in several relevant genes suggest possible genetically predisposed enhanced sensitivity to methylmercury neurotoxicity in a substantial proportion of the population (Julvez et al., 2013). A new way to analyse mercury levels has recently become available in freeze-dried umbilical cord tissue.

It has been demonstrated that the cord-blood mercury concentration - so far considered the best biomarker in regards to developmental methylmercury neurotoxicity – may be affected by the binding of methylmercury to haemoglobin. Therefore even greater precision can be achieved by adjustment of mercury concentrations both in maternal and cord blood for haemoglobin (Kim et al., 2014).

An exploration of Hg-induced neurotoxicity combining relevant genetic polymorphisms with timing of exposure to MeHg<sup>+</sup>, haemoglobin, latitude/vitamin D deficiency/sufficiency in individuals with ASD and combinatorial effect of protective confounders, may shed light into this complex interplay of genetic and environmental factors in the pathogenesis of ASD.

#### *F.4.3.3. Special features of the present study cohort*

In view of the recent recommendation in the Faroe Islands (*vide supra*), the cohort of this study, born there between 1985 and 1994 during the last of the three noted periods of the richest catches of pilot whales in Faroese history, represents one of the last generations of children whose mothers consumed pilot whale meat during their pregnancies.

As demonstrated in the study on the prevalence of ASD in the Faroe Islands study I (Figure 3), there was a non-significant trend towards higher prevalence rates of ASD among participants born in the second half of the 10-year period between 1985 and 1994. And this particular increase was represented especially by cases of Asperger syndrome and atypical autism, suggesting that this might be the result of increased occurrence of one of the environmental risk factors in the Faroese region during the 1980s period.

Maternal serum vitamin D levels during pregnancy have also been shown to impact on the neurocognitive development of her offspring. A recent study from

Australia demonstrated that mothers deficient in vitamin D levels (< 46 nmol/L) were significantly associated with offspring language impairment at ages 5 and 10 years as compared to mothers whose vitamin D levels were > 70 nmol/L (Whitehouse et al., 2011).

Therefore, it seems reasonable to include into this ASD pathogenesis interplay the latitude factor/aspect as the combined impact of mercury and PUFAs in a sunny region can be influenced by an additional protective factor ‘vitamin D sufficiency’ with calcitriol ability among its other functions in the brain to up regulate the glutathione (= a powerful chelating agent with an extra strong affinity for the methylmercury ion), while at higher latitudes in the presence of yet another additional risk factor ‘vitamin D deficiency’ the detrimental effect of Hg may be actually accentuated. The follow up studies should also concentrate on research of available biomarkers for autism.

#### **F.4.4. Strengths and limitations**

##### *F.4.4.1. Limitations*

The serious limitations of this pilot study are first of all the small numbers and especially lower number of controls. It is hoped, that it will be possible to complete it with the whole cohort of study V in the near future. Also, additional limitation of this study is the fact that all of the results were univariate.

Another limitation is the fact that the dietary questionnaires were administered a long time after the actual pregnancy. The collected information regarding the reported dietary consumption lacks detail regarding the actual size/weight of portions and also in details of species of the consumed fish. Therefore, a better estimate of confounding beneficial effects of PUFAs could not be made at this stage.

Yet another limitation might be an artificial ‘reduction’ in the reported quantity of ‘consumed pilot whale meat during pregnancy’ by mothers of the ASD group as a result of possible feeling of guilt. This might be a common ‘real-life effect’ influencing the statistical outcome of some of the studies concerning/exploring people’s life/health habits/choices. In view of the advertising campaign and recommendations issued by the Public Health authorities in the Faroe Islands during the past two decades (based on the

available data), the mothers are now fully aware of the situation and related risk and thus it must psychologically be an extremely difficult topic for them, although it cannot be viewed as their fault or wrong-doing.

#### *F.4.4.2. Strengths*

Among the main strengths of the study are the exclusive features of the geographic region of the Faroe Islands that differentiate it from other countries: (1) the genetic isolate character; (2) high latitude position; (3) specific environmental exposures (e.g. consumption of pilot whale meat); and (4) certain socio-demographic conditions (e.g. an extremely cohesive society). The historical systematic approach to distribution of the pilot whale catches, the traditional habits of individual families and the homogeneity of dietary customs in the Faroe Islands, that vary so greatly from other western countries and at the same time are so uniform within the Faroese society, allow a reasonable degree of credibility to these results.

#### **F.4.5. Conclusions**

The results of this pilot study are mostly not significant due to very low numbers and - at best - of only indicative value, suggesting possible directions of future research. Involvement of Hg-induced neurotoxicity in the aetiology of autism and similarly exploration of several additional environmental factor(s) in this complex panorama of ASD pathogenesis in the Faroe Islands warrants further investigations (e.g. timing of exposure to methylmercury, combinatorial effect of protective confounders, latitude and vitamin D deficiency/sufficiency). Therefore, it was suggested by the Faroese researchers that completion of the dietary questionnaire with the whole cohort would be a valuable component of future research.

Among the mechanisms by which mercury induces neurotoxicity (damage to the brain) there are some that vitamin D - or rather its active form, the neuro-steroid hormone calcitriol - helps to counteract in the process of brain homeostasis and the brain's own immune system. In light of the most interesting outcome of the pilot study -

the difference in mothers' reported 'attitude to sun' - it would be desirable to conduct future research involving both Hg and vitamin D.



Photo by courtesy of Olavur Frederiksen.

## **F.5. Vitamin D in the General Population of Young Adults in the Faroe Islands (V)**

### **F.5.1. The present study in the context of the existing knowledge**

This first-ever population-based study of vitamin D in ASD showed significantly lower levels in young adolescents/adults with ASD than in their siblings and parents, and also than in healthy comparisons.

These findings are consistent with those of several studies published since 2010, although some of these had methodological problems - e.g., no comparison group (Humble et al., 2010) or an inadequate comparison group undergoing tonsillectomy and finding no difference in vitamin D levels (Molloy et al., 2010) – it has been since demonstrated that individuals with recurrent tonsillitis had significantly lower vitamin D levels than healthy controls (Nseir et al., 2012). In addition, all these studies were cross-

sectional and thus causation cannot be inferred from their results. Never the less there are now 5 studies, including the present one, demonstrating significantly lower levels of vitamin D in a population with ASD. These studies come from wide-ranging regions of the world - Egypt, Saudi Arabia, Brazil, China and the Faroe Islands - thus, signalling the gravity of this problem in ASD and leading Gong et al. to propose vitamin D deficiency as a possible independent risk factor for ASD (Gong et al., 2014). (Table 22).

**Table 22.** Overview of studies of vitamin D levels in individuals with autism.

Author Year	Country Study Participants (n)	ASD Group		Comparison Group		p
		nmol/L	ng/mL	nmol/L	ng/mL	
Humble et al. 2010	Sweden Adults (121)	31.5	12.6	-	-	-
Molloy et al. 2010	USA Boys 4-8ys (89)	49.4	19.8	44.0	17.6	0.4
Meguid et al. 2010	Egypt Children 2-8ys (112)	71.3	28.5	100.3	40.1	<b>&lt;0.001</b>
Mostafa et al. 2012	Saudi Arabia Children 5-12ys (80)	46.3	18.5	82.5	33.0	<b>&lt;0.001</b>
De SouzaTostes et al. 2012	Brazil Children ±7ys (48)	66.2	26.5	101.3	40.5	<b>&lt;0.001</b>
Gong et al. 2014	China Children ±4ys (96)	49.8	19.9	56.5	22.6	<b>0.002</b>
Kočovská et al. Present study	Faroe Islands Young Adults 15- 24ys (219)	24.8	9.9	37.6	15.0	<b>0.002</b>

The Humble et al., 2010 study did not have a control group. For more details see Kočovská et al. (2012a).

### **F.5.2. Seasonal variations of vitamin D deficiency**

The finding that 80% of the comparison group (which can be considered representative of the general population in the Faroe Islands) were in the ‘deficient/insufficient’ range and 22.5% in the ‘severely deficient’ range (<25 nmol/L ~ <10 ng/mL) that is indicative of the risk of osteomalacia/rickets (Holick, 2007), adds to the mosaic of perceived global vitamin D deficiency in various regions of the world. It is of note that the levels of 25(OH)D<sub>3</sub> found in ASD participants of the present study from the Faroe Islands are even lower than those in other regions. (For personal experience of the Faroe climate, see Appendix 2.) It might be that the severity of this region’s climate and its high Northern latitude, both diminishing the production of vitamin D by UVB rays, cannot be compensated for by a diet albeit rich in large oily fish.

The only other report on vitamin D levels in the Faroe Islands was the Faroese elder study (Dalgård et al., 2010) that found a small seasonal variation in the 25(OH)D<sub>3</sub> levels in Faroese elders, with a winter nadir of 42.6 nmol/L and a summer peak of 56.5 nmol/L (females 51.0 nmol/L; males 44.6 nmol/L). Our study found a similarly small, significant seasonal variation in our ASD group corresponding to the season of blood sampling.

The significant difference between the ASD and comparison groups is even more striking when taking into account that, while the ASD group’s blood samples were drawn over a period of a year during 2008-2009 with 27.5% of the samples drawn during the summer period (Jun-Aug) when vitamin D availability from the sun is highest, the comparison group’s blood samples were drawn in early spring between March and April, the period of the year when vitamin D levels are lowest (Grant & Soles, 2009). This significant difference was confirmed also when comparing only males in the ASD and comparison groups.

Unlike in Meguid’s (2010) and Gong’s (2014) studies the Faroese group with the most severe diagnosis of autism had the highest levels of vitamin D (30.30 nmol/L;  $p=0.3$ ). This could be partially explained by the fact that 9 out of 11 individuals with autism diagnoses were blood sampled during the summer holidays while for the two other diagnoses there was a greater variability in season of sampling.

The spring births among the ASD group (32.5%) and corresponding lowest level of vitamin D in this group (22.69 nmol/L;  $p=0.6$ ) might indicate a risk for life-long vitamin D deficiency if not treated. To date the Faroese population generally has not been supplementing vitamin D, even in pregnancy (apart from ordinary multivitamins with no more than 400 International Units (IU) of cholecalciferol or ergocalciferol per day), and the use of solaria in the Faroe Islands is also rare.

### **F.5.3. Vitamin D deficiency and gender aspects**

The trend towards lower levels of vitamin D in males from all groups (apart from the parent group) presented another interesting finding. There are no acknowledged sun exposure differences between males and females among Faroese adolescents. Previous research has demonstrated various gender differences in vitamin D metabolism (Spach & Hayes, 2005; Orton et al., 2006; Novakovic et al., 2009; Feldman et al., 2011). Thus, future research is warranted, as it would shed light on this phenomenon. Some studies have found a higher prevalence of vitamin D deficiency among men than women (Aasheim et al., 2008; Lagunova et al., 2009). In another study morbidly obese Norwegian men seeking weight loss treatment have significantly higher odds of vitamin D deficiency than women (Johnson et al., 2012).

### **F.5.4. Vitamin D deficiency and Body Mass Index (BMI)**

High 'Body Mass Index' (BMI) has been associated with vitamin D deficiency (Holick, 2007). I did not have exact BMI values available for either of the comparison groups. In the ASD group, eight participants (7 males and 1 female) ~ 20% were noted by diagnosing clinicians as overweight/obese. Thus, this factor itself is unlikely to offer an exhaustive explanation for our overall result. It is in line with the above mentioned Johnson's et al., study (2012).

### **F.5.5. Vitamin D deficiency and possible underlying hormonal imbalance**

Here I only aimed at cross-sectional comparison of vitamin D levels and I did not investigate mechanisms involved in the differences found. It can be expected that both diet and availability of UVB rays can influence vitamin D levels. I did not measure calcium, parathyroid hormone (PTH), or phosphate, which might reveal more about the nature of the deficiency either from inadequate sun exposure and/or inadequate dietary intake (Holick, 2007). An individual's underlying hormonal imbalance could also play a role (Cannell & Grant, 2013; Holick, 2007; Feldman et al., 2011). The apparatus regulating the hormone calcitriol (e.g. vitamin D receptors, vitamin D binding protein, associated enzymes) is all under genetic control and might exacerbate environmentally determined low vitamin D status or amplify its consequences to elevate ASD risk (Fu et al., 2009; Ahn et al., 2010; Hiraki et al., 2012; Cannell & Grant, 2013). Thus future research is expected to shed light on a possible role of genetic factors. The Faroe team intends to follow up and treat the diagnosed hypo-vitaminosis D among participants with ASD and try to obtain further information on the origin of low vitamin D status.

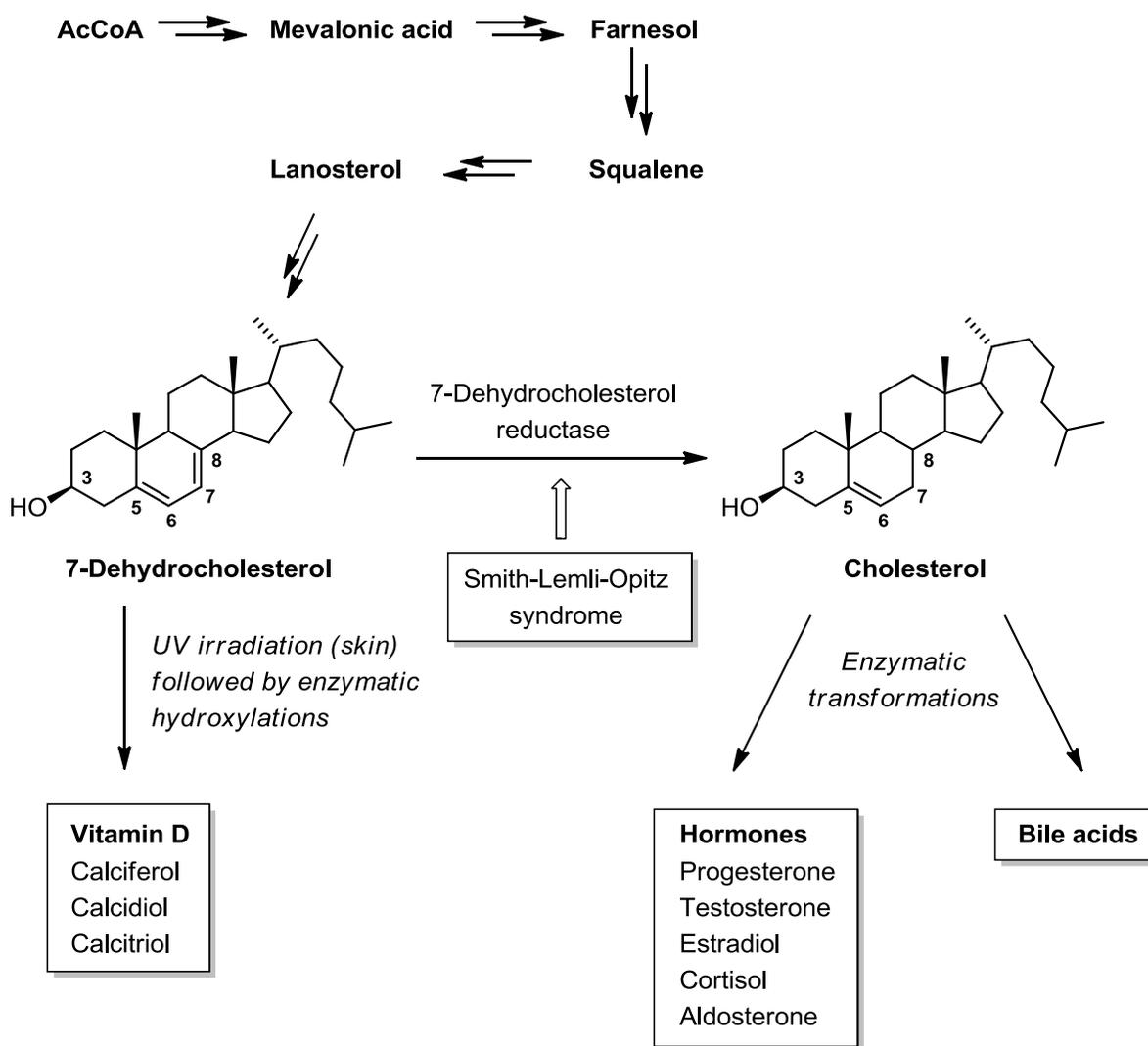
As all groups were exposed to low levels of sunlight, the very low 25(OH)D<sub>3</sub> in the ASD group suggests some other underlying pathogenic mechanism may be involved.

I have, however, no evidence regarding the direction of causality as the blood samples of the ASD group were drawn when they were 15-24 years of age. The low vitamin D levels in the ASD group could be either a result of autism impacting on a family/child's lifestyle and/or diet (indoor activities, selective eater, etc.) or the underlying biology of autism altering the metabolism of vitamin D in some way or vitamin D deficiency itself contributing to the pathogenesis of ASD. Children with Williams syndrome very often have high 25(OH)D<sub>3</sub> levels in early infancy due to a single gene mutation, which also involves abnormal vitamin D metabolism (Feldman et al., 2011; Stamm et al., 2001).

Children with Smith-Lemli-Opitz syndrome (SLOS) were found to have co-morbidity of ASD in 50-86% (Coleman & Gillberg, 2012). They have mutations in the DHCR7 gene that codes for the enzyme 3 $\beta$ -hydroxysterol- $\Delta$ (7)-reductase, the catalyst for the final step in cholesterol biosynthesis, namely the reduction of 7-

dehydrocholesterol (7DHC) to cholesterol (Scheme 5) (Witsch-Baumgartner et al., 2008). As a consequence, patients with SLOS present with hypo-cholesterolemia and often with higher levels of 7DHC. 7DHC is a prime precursor of the calcitriol biosynthesis and it undergoes the key photochemical electrocyclic reaction in the skin by a reaction that requires irradiation with UVB light; the biosynthesis is then completed via a couple of intermediate steps in liver and kidney (Scheme 3). The genetic impairment impacting on the cholesterol synthesis in patients with SLOS should not, theoretically, affect the biosynthesis of calcitriol and this should be possible to explore via analysing the actual blood levels of 25(OH)D<sub>3</sub> (calcidiol).

**Scheme 5**



Interestingly, a recent study reported elevated foetal steroidogenic activity (levels of sex hormones and cortisol) in children later diagnosed with autism in comparison to typically developing children (Baron-Cohen et al., 2014). This finding suggests a possible synergic mechanisms – increase in concentrations of sex hormones and cortisol levels and decrease in 25(OH)D<sub>3</sub> levels - in autism, stemming from the impaired steroid metabolism. The nature of this potential impairment warrants future research.

### **F.5.6. Multi-functional character of the hormone calcitriol**

#### *F.5.6.1. Vitamin D deficiency and possible immune dysregulations*

An increasing body of research indicates that ASD may be associated with a variety of complex immune dysregulations (Noriega & Savelkoul, 2014), including autoimmunity, and may have a neuro-immune component (at least two anti-neural autoantibodies are directly and strongly associated with autism severity (Walker et al., 2013; Mostafa & Al-Ayaghi, 2012; Cannell, 2013; Cannell & Grant, 2013; Gentile et al., 2013)).

Patrick & Ames (2014) argue that there is a growing body of evidence suggesting that calcitriol regulates serotonin synthesis via vitamin D response elements (VDREs) and its relevance for autism (serotonin in the brain promotes pro-social behaviour and correct assessment of emotional social cues (Crockett, 2009)). This hypothesis could account for the four most typical characteristics of ASD: (1) the lower levels of serotonin in the brain and its higher levels outside the blood-brain barrier in ASD; (2) lower levels of vitamin D in ASD; (3) the higher male prevalence of autism; and (4) the presence of maternal antibodies against foetal brain tissue (Patrick & Ames, 2014). Similarly, two peptide hormones, oxytocin and vasopressin, are also associated with autism and their encoding genes contain VDREs for activation (Patrick & Ames, 2014). In addition, alexithymia (inability to identify and describe emotions in the self (Sifneos, 1973)) that shows high comorbidity with autism, has recently been associated with low vitamin D levels in young healthy adults (Altbäcker et al., 2014).

#### *F.5.6.2. Vitamin D and the ESSENCE syndromes*

As stated earlier (pp.27-28), this phenomenon suggests co-existing developmental deficits in the areas of general development, communication and language, social interrelatedness, motor coordination, attention, activity, behaviour, and mood and/or sleep (Gillberg, 2010). To date, Vitamin D deficiency has been shown to be implicated in: epilepsy (i.e., increasing the threshold for seizures) (Borowicz et al., 2007; Bromely et al., 2008; Harms et al., 2011); cognitive/language development (Whitehouse et al., 2012); motor coordination (muscle tone/strength/body sway and balance) (Boland, 1986; Bischoff-Ferrari et al., 2009), activity/fatigue (Kennel et al., 2010); and mood/sleep disorders (Czeisler et al., 1998; Lansdowne & Provost, 1998; Berk et al., 2014). Thus, the vitamin D deficiency appears to be a very relevant element in this panorama of ESSENCE syndromes.

#### *F.5.6.3. Multifaceted character of the hormone calcitriol*

Thus, a substantial body of research suggests the importance of the multi-functional character of the hormone calcitriol with its ability to up/down-regulate a number of metabolic, genetic and immunologic processes and exert some level of protective function. On the other hand, its absence or deficiency results in the disruption of a number of metabolic/physiologic processes. These disruptions may subsequently lead to an unfavourable outcome of illness/disorder, with autism being one of the possibilities, depending on circumstances (genetic predispositions, timing, severity of vitamin D deficiency, and co-occurrence of other risk factors, etc.). It transpires from the entire body of research that the global problem of vitamin D deficiency is actually not merely a simple 'hypo-vitaminosis' but rather a substantial hormonal imbalance.

As noted previously the growing body of research suggests that autism is more than just a neurological disorder but also a disorder which reflects dysfunction at various metabolic levels (Garrecht & Austin, 2011) and this work indicates that dysfunction or impairment of the steroid metabolism might be one of these.

Thus, future simultaneous investigation utilizing a combination of genetic, epigenetic, and mechanistic studies of this complex interplay between autism, vitamin D deficiency, steroid metabolism, and autoimmunity will be desirable.

Calcitriol, the vitamin D active form, is a hormone with an exceptionally wide range of roles in metabolic, physiologic, immunologic and genetic processes. As research teams world-wide continue to search for explanations for the intriguing mechanisms of action of calcitriol and provide approaches for optimizing vitamin D status, dramatic improvements in the prevention and treatment of a wide range of human disorders - possibly including autism – can be expected in the future. Meanwhile, corrected vitamin D status in those with autism might improve their wellbeing (Lappe, 2011; Cannell, 2013; Gentile et al., 2013; DeLuca et al., 2013).

### **F.5.7. Strengths and limitations**

#### *F.5.7.1. Limitations*

The main limitations of the present study were the modest size of the sample, a lack of data regarding Body Mass Index (BMI) – only known in the some of the ASD group and lacking in all other control groups - and a lack of information about the participants' habits with regards to indoor/outdoor activities or their vitamin D supplementation. Also there was an inconsistency of blood sampling across seasons among various groups. These research findings require replication with larger numbers. Because it would be challenging for any one centre to produce data on sufficient number of children with ASD, a multicentre international collaboration would be desirable in order to achieve more conclusive results.

#### *F.5.7.2. Strengths*

This was the first study using a total population and that is one of its major assets. The choice of the geographical region, increasing the potential for possibly low vitamin D exposure was another strong point. The special design of the control group, using a sibling group to attempt to control for certain genetic factors, when studying an

environmental exposure, is yet another unique feature of the study and has not been used before.

### **F.5.8. Conclusions**

This first-ever population study of vitamin D levels in ASD showed significantly lower vitamin D levels in participants with ASD (aged 15-24 years) living in the Faroe Islands, as compared to their siblings, parents, and typically developing comparisons. A trend towards lower levels in males compared to females in the ASD, comparison and sibling groups, was also observed.

The findings could reflect the consequences of ASD per se impacting on a person's lifestyle and diet or the underlying biology of ASD impacting in some way directly on the metabolism of vitamin D. Alternatively, the low vitamin D levels could be an indication of life-long vitamin D deficiency in ASD, and this hormone deficiency could, at least theoretically, have been involved in early aberrant development of the brain in these individuals, leading to the development of ASD.

### **F.5.9. Future directions**

It would be interesting to run a new prevalence screening in the Faroe Islands, this time including very young children, whose mothers do not consume pilot whale meat anymore, and to apply standardized instruments across the whole population, while trying to improve the identification of girls with the newly adapted diagnostic measures.

Regarding mercury, there is a need to establish its levels in our cohort at the time of birth, retrospectively and relate them to genetic analysis of mercury toxicity and to vitamin D levels at birth.

In vitamin D research, the first step to be taken is to treat vitamin D deficiency in our cohort and to observe any possible difficulties in correcting their levels as an indicator of potential steroid metabolism impairment. The next step would be a comparison of vitamin D levels at birth of the children later diagnosed with ASD with those of their siblings and typically developing children. Subsequently, there will be demand for a large birth cohort, starting with monitoring vitamin D levels in women

before they become pregnant. These investigations should run in parallel with genetic studies focused on steroid imbalance, including sex and corticoid hormones. All these efforts are likely to present a mosaic that would combine the occurrence of ASD, mercury toxicity, steroid imbalance and the role of gender.

#### **F.5.10. The research and clinical implications of the work**

I. Prevalence of ASD in the Faroe Islands: The exploration of prevalence rates of ASD in the Faroe Islands is a highly valuable addition to the mosaic of similar findings from several other regions of the world. These current soaring global ASD rates might have multiple reasons. This work offers clear support to indications that one of these reasons might be unfamiliarity with the clinical presentation of autism in females at an early age and/or insensitivity of the present diagnostic system for the female ASD phenotype thus preventing timely recognition of this diagnosis in girls at young age.

II. Diagnostic stability: clinical implication - the outcome of the present study supports the new trend of DSM-5 with its single over-arching diagnosis of ASD.

III. Systematic review of vitamin D deficiency and ASD aided to draw attention to the possibility of connection between vitamin D deficiency and autism. This may alert clinicians to the importance of checking vitamin D levels in children with ASD.

IV. Diet and lifestyle study of pregnant mothers in the Faroe Islands uncovered a very interesting trend of negative attitude to sun of mothers with a child with ASD, suggesting some possible underlying problems worth of future exploration. Clinicians should ask about attitudes to the sun in patients with ASD and their parents as part of normal history taking.

V. Vitamin D study: the majority of the whole study population has been found in the deficient range of 25(OH)D<sub>3</sub> levels in agreement with the literature on global epidemic of vitamin D deficiency. The ASD group had significantly lower levels of 25(OH)D<sub>3</sub> in comparison to their siblings, parents and also to healthy comparisons, indicating some underlying pathological mechanism in the ASD group. This is in line with similar results of other studies from all over the world, suggesting that in clinical settings vitamin D deficiency might be an independent symptom for ASD and thus

indicating the need to examine vitamin D levels/intake in pregnant women and in patients with ASD.

An overview of the results of all these 5 studies suggests a possibility of interplay of all these three factors – ASD, mercury toxicity and vitamin D deficiency – which warrants future research interest.

## G. SUMMARY

**(I)** Autism is as common in the Faroe Islands – a genetic isolate – as in the rest of the western world. The follow-up study in late adolescence-young adult age (15-24yrs) revealed that about 36% of the total cohort of clinically clear ASD cases had been missed in the first screening study performed in childhood. The reasons for this failure to identify the whole autism population at an earlier age remain partly obscure but it is possible that the unfamiliarity with the clinical presentation of autism in females may have played a significant role in this context.

**(II)** There was diagnostic stability for the overall category of ASD over time in the group diagnosed in childhood (7-16 years), but considerable variability as regards to diagnostic sub-grouping. These results support the notion that a single over-arching diagnostic category ‘autism’ or ‘ASD’ would better suit clinical realities as outlined in the new DSM-5 for neurodevelopmental disorders (and most likely the corresponding ICD-11 also) than the current subdivision into autistic disorder, Asperger syndrome, childhood and PDDNOS/atypical autism.

**(III)** Vitamin D deficiency – either during pregnancy or early childhood – has recently been proposed as a possible environmental risk factor for ASD. The findings obtained over the past 15 years, suggest that vitamin D plays numerous roles in various processes in the human body. However, the literature is very limited as regards to clinical studies of individuals with autism or their close relatives and provides only weak support for the hypothesis of the modulatory role of vitamin D in the pathogenesis of autism specifically. Therefore, there is an urgent need for intensified research in this important area. Despite the limited and inconclusive results of this review, there are indications that individuals with ASD may be one of the populations at risk for vitamin D deficiency/inadequacy, and that low vitamin D levels in utero or early postnatal life might interact with other factors to increase the risk for ASD.

(IV) Despite the limited numbers, the pilot study of dietary questionnaires yielded some very interesting results. Although these results are mostly not statistically significant and of only indicative value, they suggest that there may exist other environmental factors (or potential confounding factors) involved in Hg-induced neurotoxicity – e.g. timing of exposure to methylmercury, genetic factors (relevant genetic polymorphisms) and combinatorial effect of protective confounders (PUFAs) and latitude/ vitamin D deficiency or sufficiency - warranting future research.

(V) This first-ever population study of vitamin D levels in ASD showed significantly lower vitamin D levels in participants with ASD (aged 15-24 years) living in the Faroe Islands, as compared to their siblings, parents, and typically developing comparisons. There was a trend for males having lower 25(OH)D<sub>3</sub> levels than females. Effects of age, month/season of birth, IQ, various subcategories of ASD and ADOS score were also investigated; however, no association was found. The very low 25(OH)D<sub>3</sub> levels in the ASD group suggests some underlying pathogenic mechanism.

The findings could reflect the consequences of ASD per se impacting on a person's lifestyle and diet or the underlying biology of ASD impacting in some way directly on the metabolism of vitamin D. Alternatively, the low vitamin D levels could be an indication of life-long vitamin D deficiency in ASD, and this hormone deficiency could, at least theoretically, have been involved in early aberrant development of the brain in these individuals, leading to the development of ASD.

## H. REFERENCES

- Aasheim, E. T., Hofso, D., Hjelmsaeth, J., Birkeland, K.I., & Bohmer, T. (2008). Vitamin status in morbidly obese patients: a cross-sectional study. *American Journal of Clinical Nutrition*, *87*, 362–369.
- Adams, J. S. & Hewison, M. (2010). Update in Vitamin D. *Journal of Clinical Endocrinology and Metabolism*, *95*, 471-478.
- Adams, J. B. & Romdalvik, J. (2007). Mercury, lead and zinc in baby teeth of children with autism versus controls. *Journal of Toxicology and Environmental Health*, *70*, 1046-1051.
- Agborsangaya, C., Toriola, A. T., Grankvist, K., Surcel, H. M., Holl, K., Parkkila, S., et al. The effects of storage time and sampling season on the stability of serum 25-hydroxy vitamin D and androstenedione. (2010). *Nutrition and Cancer*, *62*, 51–57.
- Ahn, J., Yu, K., Stolzenberg-Solomon, R., Simon, K. C., McCullough, M. L., Gallicchio, L., et al. (2010). Genome-wide association study of circulating vitamin D levels. *Human Molecular Genetics*, *19*, 2739-2745.
- Alexianu, M. E., Robbins, E., Carswell, S., & Appel, S. H. (1998). 1a,25-Dihydroxyvitamin D<sub>3</sub>-dependent up-regulation of calcium-binding proteins in motoneuron cells. *Journal of Neuroscience Research*, *51*, 58-66.
- Almeras, L., Eyles, D., Benech, P., Laffite, D., Villard, C., Patatian, A., et al. (2007). Developmental vitamin D deficiency alters brain protein expression in the adult rat: Implications for neuropsychiatric disorders. *Proteomics*, *7*, 769-780.
- Als, T. D., Jørgensen, T. H., Børglum, A. D., Petersen, P. A., Mors, O. & Wang, A. G. (2006). Highly discrepant proportions of female and male Scandinavian and British Isles ancestry within the isolated population of the Faroe Islands. *European Journal of Human Genetics*, *14*, 497-504.
- Anello, A., Reichenberg, A., Luo, X., Schmeidler, J., Hollander, E., Smith, C. J., et al. (2009). Brief report: Parental age and the sex ratio in autism. *Journal of Autism and Developmental Disorders*, *39*, 1487–1492.
- Altbäcker, A., Plózer, E., Darnai, G., Perlaki, G., Orsi, G., Nagy, A. A., Lucza, T., Schwarcz, A., Köszegi, T., Kovács, N., Komoly, S., Janszky, J., & Clemens, Z. (2014). Alexithymia is associated with low level of vitamin D in young healthy adults. *Nutritional Neuroscience* DOI: 10.1179/1476830514Y.0000000114.
- American Psychiatric Association. (1994). *Diagnostic and Statistical Manual of Mental Disorders*. 4<sup>th</sup> edition. American Psychiatric Association, Washington, DC.

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual of Mental Disorders*. 5<sup>th</sup> edition. American Psychiatric Association, Washington, DC.

Aschner, M., Syversen, T., Souza, D. O., Rocha, J. B. T., & Farina, M. (2007). Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. *Brazilian Journal of Medical and Biological Research*, *40*, 285-291.

Aschner, J. L. & Aschner, M. (2007). Methylmercury Neurotoxicity: Exploring Potential Novel Targets. *Open Toxicology Journal*, *1*, 1-9.

Aschner, M., Yao, C. P., Allen, J. W., & Tan, K. H. (2000). Methylmercury alters glutamate transport in astrocytes. *Neurochemistry International*, *37*, 199-206.

Atladóttir, H. O., Henriksen, T. B., Schendel, D. E., & Parner, E. T. (2012). Autism after infection, febrile episodes and antibiotic use during pregnancy: an exploratory study. *Pediatrics*, *130*, 1447-1454.

Atladóttir, H. O., Pedersen, M. G., Thorsen, P., Mortense, P. B., Deleuran, B., Eaton, W. W., & Parner, E. T. (2009). Association of family history of autoimmune disease and autism spectrum disorders. *Pediatrics*, *124*, 687-694.

Attwood, T. (2007). *The Complete Guide to Asperger's Syndrome*. London: Jessica Kingsley Publishers.

Auyeung, B., Taylor, K., Hackett, G., & Baron-Cohen, S. (2010). Foetal testosterone and autistic traits in 18 to 24-month-old children. *Molecular Autism*, *1*, 11.

Baird, G., Simonoff, E., Pickles, A., Chandler, S., Loucas, T., Meldrum, D, et al. (2006). Prevalence of disorders of the autism spectrum in a population cohort of children in South Thames: The Special Needs and Autism Project (SNAP). *The Lancet*, *368*, 210-215.

Bakare, M. O., Munir, K. M., & Kinney, D. K. (2011). Association of hypomelanotic skin disorders with autism: links to possible etiologic role of vitamin-D levels in autism? *Hypothesis*, *9*, 2-12.

Barbaresi, W. J., Colligan, R. C., Weaver, A. L., & Katusis, S. K. (2009). The incidence of clinically diagnosed versus research-identified autism in Olmstead County, Minnesota 1976-1997: Results from a retrospective, population-based study. *Journal of Autism and Developmental Disorders*, *39*, 464-470.

Barnevik-Olsson, M., Gillberg, C., & Fernell, E. (2008). Pervallence of autism in children born to Somali parents living in Sweden: a brief report. *Developmental Medicine and Child Neurology*, *50*, 598-601.

Baron-Cohen, S., Auyeung, B., Nørgaard-Pedersen, B., Hougaard, D. M., Abdallah, M. W., Melgaard, L., Cohen, A. S., Chakrabarti, B., Ruta, L. & Lombardo, M. V. (2014).

Elevated fetal steroidogenic activity in autism. *Molecular Psychiatry* 1–8 advance online publication, 3 June 2014; doi:10.1038/mp.2014.48

Baron-Cohen, S., Scott, F. J., Allison, C., Williams, J., Bolton, P., Matthews, F. E., & Brayne, C. (2009). Prevalence of autism-spectrum conditions: UK school-based population study. *British Journal of Psychiatry*, 194, 500-9.

Baron-Cohen, S., Knickmeyer, R. C., & Belmonte, M. K. (2005). Sex differences in the brain: Implications for explaining autism. *Science*, 310, 819–823.

Baron-Cohen, S., & Wheelwright, S. (2003). The friendship questionnaire: an investigation of adults with asperger syndrome or high-functioning autism, and normal sex differences. *Journal of Autism and Developmental Disorders*, 33, 509–517.

Baron-Cohen, S. (2002). The extreme male brain theory of autism. *Trends in Cognitive Sciences*, 6, 248-254.

Baron-Cohen, S. (1995). *Mindblindness: An Essay on Autism and Theory of Mind*. MIT Press/Bradford Books. Cambridge, MA.

Baron-Cohen, S., Allen, J., & Gillberg, C. (1992). Can autism be detected at 18 months? The needle, the haystack and the CHAT. *British Journal of Psychiatry*, 161, 839-843.

Baudo, R., Giesy, J., and Muntau, H. (1990). *Sediments: Chemistry and Toxicity of In-Place Pollutants*, Lewis Publishers, Michigan, USA.

Berk, M., Williams, L. J., Jacka, F. N., O’Neil, A., Pasco, J. A., Moylan, S., Allen, N. B., Stuart, A. L., Hayley, A. C., Byrne, M. L., & Maes, M. (2014). So depression is an inflammatory disease, but where does the inflammation come from? *BMC Medicine*, 11, 200.

Berquist, A. G., Schall, J. I., & Stallings, V. A. (2007). Vitamin D status in children with intractable epilepsy, and impact of the ketogenic diet. *Epilepsia*, 48, 66-71.

Berument, S. K., Rutter, M., Lord, C., Pickles, A., & Bailey, A. (1999). Autism screening questionnaire: diagnostic validity. *The British Journal of Psychiatry*, 175, 444-451.

Bilder, D., Botts, E. L., Smith, K. R., et al (2012). Excess mortality and causes of death in autism spectrum disorders: A Follow up to the 1980s Utah/UCLA autism epidemiology study. *Journal of Autism and Developmental Disorders*, Sept. 25, 1-9.

Billstedt, E., Gillberg, I. C., & Gillberg, C. (2005). Autism after adolescence: Population-based 13-22-year follow-up study of 120 individuals with autism diagnosed in childhood. *Journal of Autism and Development Disorders*, 35, 351-360.

Bischoff-Ferrari, H. A., Dawson-Hughes, B., Staehelin, H. B., Orav, J. E., Stuck, A. A., Theiler, R., Wong, J. B., Egli, A., Kiel, D. P., & Henschkowski, J. (2009). Fall

prevention with supplemental and active forms of vitamin D: A meta-analysis of randomised controlled trials. *British Medical Journal*, 339, b3692.

Blasi, F., Bacchelli, E., Pesaresi, G., Carone, S., Bailey, A. J., & Maestrini, E. (2006). Absence of coding mutations in the X-linked genes neuroligin 3 and neuroligin 4 in individuals with autism from the IMGSAC collection. *American Journal of Medical Genetics (Neuropsychiatric Genetics)*, 141B, 220–221.

Blaylock, R. L. & Strunecká, A. (2009). Immune-Glutamatergic dysfunction as a central mechanism of the Autism Spectrum Disorder. *Current Medicinal Chemistry*, 16, 157-170.

Bloch D. (2007). *Pilot whales and the drive in the Faroes. H.N. Jacobsen's Indispensable: Guide to the Faroe Islands.* H. N. Jacobsens Bokahandil, Tórshavn, Faroe Islands.

Bodnar, L. M., Catov, J. M., Simhan, H. N., Holick, M. F., Powers, R. W., & Roberts, J. M. (2007). Maternal vitamin D deficiency increases the risk of preeclampsia. *The Journal of Clinical Endocrinology & Metabolism*, 92, 3517-3522.

Boland, R. (1986). Role of vitamin D in skeletal muscle function. *Endocrine Reviews*, 7, 434-438.

Borowicz, K. K., Morawska, M., Furmanek-Karwowska, K., Luszczki, J., & Czuczwar, S. J. (2007). Cholecalciferol enhances the anticonvulsant effect of conventional antiepileptic drugs in the mouse model of maximal electroshock. *European Journal of Pharmacology*, 573, 111-115.

Bosomworth, N. J. (2011). Mitigating epidemic vitamin D deficiency: The agony of evidence. *Canadian Family Physician*, 57, 16-20.

Bradley, J. V. (1968). *Distribution-Free Statistical Tests.* Prentice-Hall, Engelwood Cliffs, NJ.

Bromley, R. L., Mawer, G., Clayton-Smith, J., & Baker, G. A. (2008). Autism Spectrum Disorder following in utero exposure to antiepileptic drugs. *American Academy of Neurology*, 71, 997-1024.

Budtz-Jørgensen, E., Grandjean, P., & Weihe P. (2007). Separation of risks and benefits of seafood intake. *Environmental Health Perspectives*, 115, 323-327.

Burne, T. H., Feron, F., Eyles, D. W., McGarth, J. J., & Mackay-Sim, A. (2004). Combined prenatal and chronic postnatal vitamin D deficiency in rats impairs prepulse inhibition of acoustic startle. *Physiology and Behavior*, 81, 651-655.

Campbell, M., Reynolds, L., Cunningham, J., Minnis, H., & Gillberg, C. (2013). Autism in Glasgow: cumulative incidences and the effect of referral age, deprivation and geographical location. *Child: Care, Health and Development*, 39, 688-694.

- Cannell, J. J. (2013). Autism, will vitamin D treat core symptoms? *Medical Hypotheses*, *81*, 195-198.
- Cannell, J. J., & Grant, W. B. (2013). What is the role of vitamin D in autism? *Dermato-Endocrinology*, *5*, 1-6.
- Cannell, J. J. (2010). On the aetiology of autism. *Acta Paediatrica*, *99*, 1128-1130.
- Cannell, J. J. (2008). Autism and vitamin D. *Medical Hypotheses*, *70*, 750-759.
- Cannell, J. J. & Hollis, B. W. (2008a). Use of vitamin D in clinical practice. *Alternative Medicine Review*, *13*, 6-20.
- Cannell, J. J., Vieth, R., Willett, W., Zasloff, M., Hathcock, J. N., White, J. H., et al. (2008b). Cod liver oil, vitamin A toxicity, frequent respiratory infections, and the vitamin D deficiency epidemic. *Annals of Otolaryngology, Rhinology & Laryngology*, *117*, 864-870.
- Cantorna, M. T., Hayes, C. E., & DeLuca, H. F. (1996). 1,25-Dihydroxyvitamin D<sub>3</sub> reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis. *Immunology*, *93*, 7861-7864.
- Carlberg, C., Seuter, S., Heikkinen, S. (2012). The first genome-wide view of vitamin D receptor locations and their implications. *Anticancer Research*, *32*, 271-281.
- Carter, A. S., Black, D.O., Tewani, S., Connolly, C. E., Kadlec, M. B., & Tager-Flusberg, H. (2007). Sex differences in toddlers with autism spectrum disorders. *Journal of Autism and Developmental Disorders*, *37*, 86-97.
- Centers for Disease Control Prevention (2012, March 30). Prevalence of autism spectrum disorders – Autism and Developmental Disabilities Monitoring Network, 14 sites, United States 2008. *MMWR Surveillance Summary*, *61*, 1-22.
- Chaplin, G. & Jablonski, N. G. (2013). The human environment and the vitamin D compromise: Scotland as a case study in human biocultural adaptation and disease susceptibility. *Human Biology*, *85*, 529-552.
- Chawarska, K., Klin, A., Paul, R., Macari, S. Volkmar, F. (2009). A prospective study of toddlers with ASD: Short-term diagnostic and cognitive outcome. *Journal of Child Psychology and Psychiatry*, *50*, 1235-1245.
- Chawarska, K., Klin, A., Paul, R., & Volkmar, F. (2007). Autism spectrum disorder in the second year: stability and change in syndrome expression. *Journal of Child Psychology and Psychiatry*, *48*, 128-38.
- Chen, T. C., Chimeh, F., Lu, Z., et al. (2007). Factors that influence the cutaneous synthesis and dietary sources of vitamin D. *Archives of Biochemistry and Biophysics*, *460*, 213-217.

- Cheng, J. B., Levine, M. A., Bell, N. H., Mangelsdorf, D. J., & Russell, D. W. (2004). Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proceedings of the National Academy of Sciences*, *101*, 7711-7715.
- Choi, A. L., Cordier, S., Weihe, P., & Grandjean, P. (2008). Negative confounding in the evaluation of toxicity: The case of methylmercury in fish and seafood. *Critical Reviews in Toxicology*, *38*, 877-893; Erratum in 2009, *39*, 95.
- Choi, B. H., Lapham, L. W., Amin-Zaki, L., & Saleem, T. (1978). Abnormal neuronal migration, deranged cerebral cortical organization and diffuse white matter astrocytosis of human fetal brain ; a major effect of methylmercury poisoning in utero. *Journal of Neuropathology and Experimental Neurology*, *87*; 719-733.
- Clark, J. H., Rhoden, D. K., & Turner, D. S. (1993). Symptomatic vitamin A and D deficiencies in an eight year old with autism. *Journal of Parenteral and Enteral Nutrition*, *17*, 284-286.
- Clemens, T. L., Adams, J. S., Henderson, S. L., & Holick, M. F. (1982). Increased skin pigment reduces the capacity of skin to synthesize vitamin D3. *The Lancet*, *1*, 74-76.
- Coleman, M., & Gillberg, C. (2012). *The Autisms*, 4<sup>th</sup> ed. Oxford, UK: Oxford University Press, Oxford, p. 286-302.
- Constantino, J. N. & Charman, T. (2012). Gender bias, female resilience, and the sex ratio in autism. *Journal of the American Academy of Child and Adolescent Psychiatry*, *51*, 756-758.
- Constantino, J. N. & Todd, R. D. (2005). Intergenerational transmission of subthreshold autistic traits in the general population. *Biological Psychiatry*, *57*, 655-660.
- Crockett, M. J. (2009). The neurochemistry of fairness: clarifying the link between serotonin and prosocial behavior. *Annals of the New York Academy of Sciences*, *1167*, 76-86.
- Cui, X., McGrath, J. J., Burne, T. H. J., Mackay-Sim, A., & Eyles, D. W. (2007). Maternal vitamin D depletion alters neurogenesis in the developing rat brain. *International Journal of Developmental Neuroscience*, *25*, 227-232.
- Currenti, S. A. (2010). Understanding and determining the etiology of autism. *Cellular and Molecular Neurobiology*, *30*, 161-171.
- Czeisler, C. A., et al. (1995). Use of bright light to treat maladaptation to night shift work and circadian rhythm sleep disorders. *Journal of Sleep Research*, *4*, 70-73.
- Dalgård C., Petersen, M. S., Schmedes, A.V., Brandslund, I., Weihe, P., & Grandjean, P. (2010). High latitude and marine diet: vitamin D status in elderly Faroese. *British Journal of Nutrition*, *104*, 914-918.

- Daniels, A. M., Rosenberg, R. E., Law, J. K., Lord, C., Kaufmann, W. E., & Law, P. A. (2011). Stability of initial autism spectrum disorder diagnoses in community settings. *Journal of Autism and Developmental Disorders, 41*, 110-21.
- Davidson, P. W., Myers, G. J., Cox, C., Axtell, C., Shamlaye, C. F., Sloane-Reeves, J., Cernichiari, E., Needham, L., Choi, A., Yang, Y., Berlin, M., & Clarkson, T. W. (1998). Effects of prenatal and postnatal methylmercury exposure from fish consumption on neurodevelopment: Outcome at 66 months of age in the Seychelles child development study. *Journal of the American Medical Association, 280*, 701-707.
- Dealberto, M. J. (2011). Prevalence of autism according to maternal immigrant status and ethnic origin. *Acta Psychiatrica Scandinavica, 123*, 339-348.
- Debes, F., Budtz-Jørgensen, E., Weihe, P., White, R. F., & Grandjean, P. (2006). Impact of prenatal mercury exposure on neurobehavioral function at age 14 years. *Neurotoxicology and Teratology, 28*, 363-375.
- De Luca, G. C., Kimball, S. M., Kolasinski, J., Ramagopalan, S. V., & Ebers, G. C. (2013). Review: The role of vitamin D in nervous system health and disease. *Neuropathology and Applied Neurobiology, 39*, 458-484.
- De Souza Tostes, M. H., Polonini, H. C., Gattaz, W. F., Raposo, N. R. B., & Baptista, E. B. (2012). Low serum levels of 25-hydroxyvitamin D (25-OHD) in children with autism. *Trends in Psychiatry and Psychotherapy, 34*, 161-163.
- Dewailly, É., & Knap, A. (2006). Food from the oceans and human health: Balancing risks and benefits. *Oceanography, 19*, 84-93.
- Dworzynski, K., Ronald, A., Bolton, P., & Happé, F. (2012). How different are girls and boys above and below the diagnostic threshold for autism spectrum disorders? *Journal of the American Academy of Child Adolescent Psychiatry, 51*, 788-797.
- d'Hellencourt, L. C., Montero-Menei, C. N., Bernard, R., & Couez, D. (2003). Vitamin D3 inhibits proinflammatory cytokines and nitric oxide production by the EOC13 microglial cell line. *Journal of Neuroscience Research, 71*, 575-582.
- Ehlers, S., Gillberg, C., & Wing, L. (1999). A screening questionnaire for Asperger syndrome and other high-functioning Autism Spectrum Disorders in school age children. *Journal of Autism and Developmental Disorder, 29*, 129-41.
- Ehlers, S. & Gillberg, C. (1993). The epidemiology of Asperger's syndrome. A total population study. *Journal of Child Psychology and Psychiatry, 34*, 1327-1350.
- Einfield, S. L., Ellis, L. A., & Emerson, E. (2011). Comorbidity of intellectual disability and mental disorder in children and adolescents: a systematic review. *Journal of Intellectuals and Developmental Disability, 36*, 137-143.

Ellefsen, A., Kampmann, H., Billstedt, E., Gillberg, I. C., & Gillberg, C. (2007). Autism in the Faroe Islands. An epidemiological study. *Journal of Autism and Developmental Disorders*, *37*, 437–444.

Elsabbagh, M., Divan, G., Koh, Y.-J., Kim, Y. S., Kauchali, S., Marcín, C., Montiel-Nava, C., Patel, V., Paula, C. S., Wang, C., Yasamy, M. T., & Fombonne E. (2012). Global Prevalence of Autism and Other Pervasive Developmental Disorders. *Autism Research*, *5*, 160-179.

Emerson, E. & Einfield, S. (2010). Emotional and behavioural difficulties in young children with and without developmental delay: A bi-national perspective. *Journal of Child Psychology and Psychiatry*, *51*, 583-593.

Ercal, N., Gurer-Orhan, H., & Aykin-Burns, N. (2001). Toxic Metals and Oxidative Stress Part I: Mechanisms Involved in Metal induced Oxidative Damage. *Current Topics in Medicinal Chemistry*, *1*, 529-539.

Eyles, D. W., Burne, T. H., & McGrath, J. J. (2013). Vitamin D, effects on brain development, adult brain function and the links between low levels of vitamin D and neuropsychiatric disease. *Frontiers in Neuroendocrinology*, *34*, 47–64.

Eyles, D., Burne, T., & McGrath, J. (2011). Vitamin D in fetal brain development. *Seminars in Cell & Developmental Biology*, *22*, 629-636.

Eyles, D. W. (2010). Vitamin D and autism: does skin colour modify risk? *Acta Paediatrica*, *99*, 645-647.

Eyles, D. W., Rogers, F., Buller, K., McGrath, J. J., Ko, P., French, K., et al. (2006). Developmental vitamin D (DVD) deficiency in the rat alters adult behaviour independently of HPA function. *Psychoneuroendocrinology*, *31*, 959-964.

Eyles, D. W., Smith, S., Kinobe, R., Hewison, M., & McGrath, J. J. (2005). Distribution of the Vitamin D receptor and 1 $\alpha$ -hydroxylase in human brain. *Journal of Chemical Neuroanatomy*, *29*, 21-30.

Eyles, D. W., Smith, S., Kinobe, R., Hewison, M., & McGrath, J. J. (2003). Distribution of the vitamin D receptor and 1 Alpha-hydroxylase in human brain. *Journal of Chemical Neuroanatomy*, *29*, 21-30.

Feldman, D., Pike, W. J., & Adams, J. S. (2011). *Vitamin D*. Elsevier, Amsterdam.

Fernell, E., Barnevik-Olsson, M., Bagenholm, G., Gillberg, C., Gustafsson, S., & Saaf, M. (2010a). Serum levels of 25-hydroxyvitamin D in mothers of Swedish and of Somali origin who have children with and without autism. *Acta Paediatrica*, *99*, 743-747.

Fernell, E., & Gillberg, C. (2010b). ASD diagnoses in Stockholm preschoolers. *Research in Developmental Disabilities*, *31*, 680-685.

- Feron, F., Burne, T. H., Brown, J., Smith, E., McGrath, J. J., Mackay-Sim, A. et al. (2005). Developmental Vitamin D3 deficiency alters the adult rat brain. *Brain Research Bulletin*, *15*, 141-148.
- Fieser, L. F. & Fieser, M. (1959). *Steroids*. Reinhold, New York.
- Freitag, C. M., Staal, W., Klauck, S. M., Duketis, E., & Waltes, R. (2010). Genetics of autistic disorders: review and clinical implications. *European Child & Adolescent Psychiatry*, *19*, 169-178.
- Fombonne, E. (2009). Epidemiology of pervasive developmental disorders. *Pediatric Research*, *65*, 591-598.
- Fombonne, E. (2008). Is autism getting commoner? *British Journal of Psychiatry*, *193*, 59.
- Fombonne, E. (1999). The epidemiology of autism: a review. *Psychological Medicine*, *29*, 769-786.
- Frith, U. (2012). Why we need cognitive explanation of autism. *Quarterly Journal of Experimental Psychology*, *65*, 2073-2092.
- Frith, U. (1996). Social communication and its disorder in autism and Asperger syndrome. *Journal of Psychopharmacology*, *10*, 48-53.
- Frith, U. & Happe, F. (1994). Autism: Beyond “theory of mind”. *Cognition*, *50*, 115-132.
- Frith, U. (1989). A look at language and communication in autism. *British Journal of Disorders of Communication*, *24*, 123-150.
- Fu, G. K., Lin, D., Zhang, M. Y., Bikle, D. D., Shackleton, C. H., Miller, W. L., & Portale, A. A. (1997). Cloning of human 25-hydroxyvitamin D-1 alpha-hydroxylase and mutations causing vitamin d-dependent rickets type 1. *Molecular Endocrinology*, *11*, 1961-1970.
- Fu, L., Yun, F., Oczak, M., Wong, B. Y. L., Vieth, R., & Cole, D. E. C. (2009). Common genetic variants of the vitamin D binding protein (DBP) predict differences in response of serum 25-hydroxyvitamin D [25(OH)D] to vitamin D supplementation. *Clinical Biochemistry*, *42*, 1174-1177.
- Garcion, E., Wion-Barbot, N., Montero-Menei, C. N., Berger, F., & Wion, D. (2002). New clues about vitamin D functions in the nervous system. *Trends in Endocrinology & Metabolism*, *13*, 100-105.
- Garrecht, M. & Austin D. W. (2011). The plausibility of a role for mercury in the etiology of autism: a cellular perspective. *Toxicological & Environmental Chemistry*, *93*, 1251-1273.

- Gardener, H., Spiegelman, D., & Buka, S. L. (2009). Prenatal risk factors for autism: comprehensive meta-analysis. *The British Journal of Psychiatry*, *195*, 7-14.
- Geier, G. A., Keru, J. K., & Geier, M. R. (2013). A comparison of the Autism Treatment Evaluation Checklist (ATEC) and the Childhood Autism Rating Scale (CARS) for the quantitative evaluation of autism. *Journal of Mental Health Research in Intellectual Disabilities*, *6*, 255-267.
- Gentile, I., Zappulo, E., Militerni, R., Pascotto, A., Borgia, G., & Bravaccio, C. (2013). Etiopathogenesis of autism spectrum disorders: Fitting the pieces of the puzzle together. *Medical Hypotheses*, *81*, 26–35.
- Giarelli, E., Wiggins, L. D., Rice, C. E., Levy, S. E., Kirby, R. S., Pinto-Martin, J. & Mandell, D. (2010). Sex differences in the evaluation and diagnosis of autism spectrum disorders among children. *Disability and Health Journal*, *3*, 107-116.
- Gillberg, C. (2010). The ESSENCE in child psychiatry: Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations. *Research in Developmental Disabilities*, *31*, 1543–1551.
- Gillberg, C. & Neville, B. (2010). Autism and epilepsy: Comorbidity coexistence or coincidence? *Clinics in Developmental Medicine*, *187*, 89.
- Gillberg, C., Cederlund, M., Lamberg, K., & Zeijlon, L. (2006). Brief report: "The autism epidemic". The registered prevalence of autism in a Swedish urban area. *Journal of Autism and Developmental Disorders*, *36*, 429-35.
- Gillberg, C. & Coleman, M. (2000). *The Biology of the Autistic Syndromes*. London: Cambridge University Press.
- Gillberg, C. & Wing, L. (1999) Autism: not an extremely rare disorder. *Acta Psychiatrica Scandinavica*, *99*, 399-406.
- Gillberg, C. (1998). Chromosomal disorders and autism. *Journal of Autism and Developmental Disorders*, *28*, 415-425.
- Gillberg, C., Schaumann, H., & Gillberg, C. (1995). Autism in immigrants: children born in Sweden to mothers born in Uganda. *Journal of Intellectual Disability Research*, *39*, 141-144.
- Gillberg, C. (1992). The Emanuel Miller Memorial Lecture 1991. Autism and autistic-like conditions: subclasses of disorders of empathy. *Journal of Child Psychology and Psychiatry*, *33*, 813-42.
- Gillberg, C. (1991). Clinical and neurobiological aspects of Asperger syndrome in six family studies. In *Autism and Asperger Syndrome* (U. Frith, Ed.), pp 122-146, Cambridge University Press, Cambridge.

Gillberg, C. (1990). Do children with autism have March birthdays? *Acta Psychiatrica Scandinavica*, 82, 152-156.

Gillberg, C. (1989). *Diagnosis and Treatment of Autism*, pp. 23-32, Plenum Press, New York.

Gillberg, I. C. & Gillberg, C. (1989). Asperger's syndrome: some epidemiological considerations: a research note. *Journal of Child Psychology and Psychiatry*, 30, 631-638.

Gillberg, C, Steffenburg S., Borjesson, B., & Andersson, L. (1987). Infantile autism in children of immigrant parents. A population-based study from Göteborg, Sweden. *British Journal of Psychiatry*, 150, 856-858.

Gillberg, C., & Steffenburg, S. (1987). Outcome and prognostic factors in infantile autism and similar conditions: A population-based study of 46 cases followed through puberty. *Journal of Autism and Developmental Disorders*, 17, 273-287.

Gong, Z. L., Luo, C. M., Wang, L., Shen, L., Wei, F., Tong, R. J., & Liu, Y. (2014). Serum 25-hydroxyvitamin D levels in Chinese children with autism spectrum disorders. *NeuroReport*, 25, 23-27.

Goodman, R. & Richards, H. (1995). Child and adolescent psychiatric presentations of second-generation Afro-Caribbeans in Britain. *British Journal of Psychiatry*, 167, 362-369.

Gotham, K., Risi, S., Dawson, G., *et al.* (2008). A replication of the Autism Diagnostic Observation Schedule (ADOS) revised algorithms. *Journal of the American Academy of Child Adolescent Psychiatry*, 47, 642-651.

Grandjean, P. & Landrigan, P. J. (2014). Neurobehavioural effects of developmental toxicity. *Lancet Neurology*, 13, 330-338.

Grandjean, P., Henriksen, J. E., Choi, A. L., Petersen, M. S., Dalgård, C., Nielsen, F., & Weihe, P. (2011). Marine food pollutants as a risk factor for hypoinsulinemia and type 2 diabetes. *Epidemiology*, 22, 410-7.

Grandjean, P. & Landrigan, P. (2006). Developmental neurotoxicity of industrial chemicals. *The Lancet*, 368, 2167-2178.

Grandjean, P., Weihe, P., White, R. F., Debes, F., Araki, S., Yokoyama, K., Murata, K., Sørensen, N., Dahl, R., & Jørgensen, P. J. (1997). Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. *Neurotoxicology and Teratology*, 19, 417-428.

Grandjean, P. & Weihe, P. (1993). Neurobehavioral effects of intrauterine mercury exposure: potential sources of bias. *Environmental Research*, 61, 176-183.

- Grant, W. B. & Soles, C. M. (2009). Epidemiologic evidence supporting the role of maternal vitamin D deficiency as a risk factor for the development of infantile autism. *Dermato-Endocrinology*, *1*, 223-228.
- Gundacker, C., Gencik, M., Hengstschläger, M. (2010). The relevance of the individual genetic background for the toxicokinetics of two significant neurodevelopmental toxicants: mercury and lead. *Mutation Research*, *705*, 130-140.
- Hallerbäck, M. U., Lugnegård, T., & Gillberg, C. (2012). ADHD and nicotine use in schizophrenia and Asperger syndrome: A controlled study. *Journal of Attention Disorders*, DOI: 10.1177/1087054712439099.
- Hallerbäck, M., Billstedt, E., Johansson, M, Gillberg, I. C., & Gillberg, C. (2010). Diagnostic Interview for Social and COmmunication Disorders – DISCO-11: the correspondence between the DISCO diagnosis and DSM-5. Unpublished manuscript.
- Hallmayer, J., Cleveland, S., Torres, A., Phillips, J., Cohen, B., Torigoe, T., Miler, J., Fedele, A., Collins, J., Smith, K., Lotspeich, L., Croen, L. A., Ozonoff, S., Lajonchere, C., Grether, J. K., Risch, N. (2011). Genetic heritability and shared environmental factors among twin pairs with autism. *Archives of General Psychiatry*, *68*, 1095-1102.
- Harada, M. (1995). Minamata disease: Methylmercury poisoning in Japan caused by environmental pollution. *Critical Reviews in Toxicology*, *25(1)*, 1-24.
- Harada, Y. Congenital (or fetal) Minamata disease. In: Study group of Minamata Disease eds. Minamata Disease. Japan; Kumamoto University, 1968: 93-18.
- Harms, L. R., Burne, T. H. J., Eyles, D. W., & McGrath, J. J. (2011). Vitamin D and the brain. *Best Practice and Research Clinical Endocrinology and Metabolism*, *25*, 657-669.
- Hayes, C. E., Nashold, F. E., Spach, K. M., & Pedersen, L. B. (2003). The immunological functions of the vitamin D endocrine system. *Cellular and Molecular Biology (Noisy-le-Grand)*, *49*, 277–300.
- Head, A. M., McGillivray, J. A., & Stokes, M. A. (2014). Gender differences in emotionality and sociability in children with autism spectrum disorders. *Molecular Autism*, *5*, 19-27.
- Hebert, K. J., Miller, L. L., & Joinson, C. J. (2010). Association of autistic spectrum disorder with season of birth and conception in a UK cohort. *Autism Research*, *3*, 185-190.
- Helles, A., Gillberg, I. C., Gillberg, C., & Billstedt, E. (2014) Asperger syndrome in males over two decades: stability and predictors of diagnosis. Submitted.
- Herndon, A. C., DiGuseppi, C., Johnson, S. L., Leiferman, J., & Reynolds, A. (2009). Does nutritional intake differ between children and Autism Spectrum Disorders and

children with typical development? *Journal of Autism & Developmental Disorders*, 39, 212-222.

Hiraki, L. T., Major, J. M., Chen, C., Cornelis, M. C., Hunter, D. J., Rimm, E. B., et al. (2012). Exploring the genetic architecture of circulating 25-hydroxyvitamin D. *Genetic Epidemiology* 37, 92-98.

Holick, M. F., Binkley, N. C., Bischoff-Ferrari, H. A., Gordon, C. M., Hanley, D. A., Heaney, R. P., et al. (2011). Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society Clinical Practice Guideline. *Journal of Clinical Endocrinology & Metabolism*, 96, 1911-1930.

Holick, M. F. (2010). *The Vitamin D Solution*. Penguin Book (USA) Inc, New York, p. 44.

Holick, M. F. (2007). Vitamin D Deficiency. *New England Journal of Medicine*, 357, 266-281.

Holick, M. F. (2005). The Influence of Vitamin D on Bone Health Across the Life Cycle. The vitamin D epidemic and its health consequences. *The Journal of Nutrition*, 135, 2739S-2748S.

Holick, M. F., Tian, X. Q., & Allen, M. (1995). Evolutionary importance for the membrane enhancement of the production of vitamin D<sub>3</sub> in the skin of poikilothermic animals. *Proceedings of the National Academy of Sciences, USA*, 92, 3124-3126.

Hollis, B. W. (2007). Vitamin D requirement during pregnancy and lactation. *Journal of Bone and Mineral Research*, 22, V39-V44.

Howlin, P., Goode, S., Hutton, J., & Rutter, M. (2004). Adult outcome for children with autism. *Journal of Child Psychology and Psychiatry*, 45, 212-229.

Humble, M. B., Gustafsson, S., & Bejerot, S. (2010). Low serum levels of 25-hydroxyvitamin D (25-OHD) among psychiatric out-patients in Sweden: Relations with season, age, ethnic origin and psychiatric diagnosis. *Journal of Steroid Biochemistry & Molecular Biology*, 121, 467-470.

Jaffe, J. S., Melnyk S., Fuchs, G., et al (2009). Efficacy of methylcobalamin and folic acid treatment on glutathione redox status in children with autism. *The American Journal on Clinical Nutrition*, 89, 425-430.

Jaffe, J. S., Timmell, A. M., & Gulanski, B. J. (2001). Prevalence of low bone density in women with developmental disabilities. *Journal of Clinical Densitometry*, 4, 25-29.

Jamain, S., Quach, H., Betancur, C., Rastam, M., Colineaux, C., Gillberg, I. C., et al. (2003). Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nature of Genetics*, 34, 27-29.

- Johansson, C., Castoldi, A. F., Onishchenko, N., Manzo, L., Vahter, M., & Ceccatelli, S. (2007). Neurobehavioural and molecular changes induced by methylmercury exposure during development. *Neurotoxicity Research*, *11*, 241-260.
- Johnson, D. D., Wagner, C. L., Hulsey, T. C., McNeil, R. B., Ebeling, M., & Hollis, B. W. (2011). Vitamin D deficiency and insufficiency is common during pregnancy. *American Journal of Perinatology*, *28*, 7-12.
- Johnson, L. K., Hofsø, D., Aasheim, E. T., Tanbo, T., Holven, K. B., Andersen, L. F., Roislien, J., & Hjelmæsæth, J. (2012). Impact of gender on vitamin D deficiency in morbidly obese patients: a cross-sectional study. *European Journal of Clinical Nutrition* *66*, 83–90.
- Johnson, S. (2001). Micronutrient accumulation and depletion in schizophrenia, epilepsy, autism and Parkinson's disease? *Medical Hypotheses*, *56*, 641-645.
- Jørgensen, T. H., Buttenschön, H. N., Wang, A. G., Als, T. D., Børghlum, A. D., & Ewald, H. (2004). The origin of the isolated population of the Faroe Islands investigated using Y chromosomal markers. *Human Genetics*, *115*, 19-28.
- Juhlshamn, K., Andersen, A., Ringdal, O., & Mørkøre, J. (1987). Trace elements in the Faroe Islands. I. Element levels in edible parts of pilot whales (*Globicephalus meleanus*). *Science of the Total Environment*, *65*, 53-62.
- Julvez, J., Davey Smith, G., Golding, J., Ring, S., St Pourcain, B., Gonzales, J. R., & Grandjean, P. (2013). Prenatal methylmercury exposure and genetic predisposition of cognitive deficit at age 8 years. *Epidemiology*, *24*, 643-650.
- Kalueff, A. V., Keisala, T., Minasyan, A., Kuuslahti, M., Miettinen, S., & Tuohimaa, P. (2006). Behavioural anomalies in mice evoked by 'Tokyo' disruption of the vitamin D receptor gene. *Neuroscience Research*, *54*, 254-260.
- Kalueff, A. V., Minayan, A., & Tuohimaa, P. (2005). Anticonvulsant effects of 1,25-dihydroxyvitamin D in chemically induced seizures in mice. *Brain Research Bulletin*, *67*, 156-160.
- Kantzer, A.-K., Fernell, E., Gillberg, C., & Miniscalco, C. (2013). Autism in community pre-schoolers: Developmental profiles. *Research in Developmental Disabilities*, *34*, 2900-2908.
- Keen, D. V., Reid, F. D., & Arnone, D. (2010). Autism, ethnicity and maternal immigration. *British Journal of Psychiatry*, *196*, 274-281.
- Kennel, K. A., Drake, M. T., & Hurley, D. L. (2010). Vitamin D Deficiency in Adults: When to Test and How to Treat. Concise Review for Clinicians. *Mayo Clinic Proceedings*, *85*, 752-758.

Kern, J. K. & Jones, A. M. (2006). Evidence of toxicity, oxidative stress, and neuronal insult in autism. *Journal of Toxicology & Environmental Health, Part B: Critical Reviews*, 9, 485-499.

Kerper, L. E., Ballatori, N., & Clarkson, T. W. (1992). Methylmercury transport across the blood-brain barrier by an amino acid carrier. *American Journal of Physiological*, 262, 761-765.

Kim, B-M., Choi, A. L., Ha, E-H., Pedersen, L., Nielsen, F., Weihe, P., Hong, Y-C., Budtz-Jørgensen, B. & Grandjean, P. (2014). Effect of hemoglobin adjustment on the precision of mercury concentrations in maternal and cord blood. *Environmental Research*, 132, 407-412.

Kim, Y. S., Leventhal, B. L., Koh, Y. J., Fombonne, E., Laska, E., Lim, E. C., et al. (2011). Prevalence of autism spectrum disorders in a total population sample. *The American Journal of Psychiatry*, 168, 904-912.

Kjellstrom, T., Kennedy, P., Wallis, S., & Mantell, C. (1989). Physical and mental development of children with prenatal exposure to mercury from fish. Stage 2: Interviews and psychological tests at age 6. Report 3642. Solna, Sweden: national Swedish Environmental Protection Board.

Kleinman, J. M., Ventola, P. E., Pandey, J., Verbalis, A. D., Barton, M., Hodgson, S., Green, J., Dumont-Mathieu, T., Robins, D. L., & Fein, D. (2008). Diagnostic stability in very young children with autism spectrum disorders. *Journal of Autism and Developmental Disorders*, 38, 606-15.

Knapp, M., Romeo, R., & Beecham, J. (2009). Economic cost of autism in the UK. *Autism*, 13, 317-336.

Knapp, M., Romeo, R., & Beecham, J. (2007). The economic consequences of autism in the UK. London: Foundation for People with Learning Disabilities.

Kočovská, E., Andorsdóttir, G., Weihe, P., Halling, J., Fernell, E., Stórá, T., Biskupstø, R., Gillberg, I. C., Shea, R., Billstedt, E., Bourgeron, T., Minnis, H., & Gillberg, C. (2014). Vitamin D in the General Population of Young Adults with Autism in the Faroe Islands. *Journal of Autism and Developmental Disorders*. Submitted

Kočovská, E., Billstedt, E., Ellefsen, Á., Kampmann, H., Gillberg, I. C., Biskupstø, R., Andorsdóttir, G., Stórá, T., & Gillberg, C. (2013). Autism in the Faroe Islands: Diagnostic stability from childhood to early adult life. *The Scientific World Journal*, Special Issue on ESSENCE, Article ID 592371, <http://dx.doi.org/10.1155/2013/592371>.

Kočovská, E., Fernell, E., Billstedt, E., Minnis, H., & Gillberg, C. (2012a). Vitamin D and autism: Clinical review. *Research in Developmental Disabilities*, 33, 1541-1550.

Kočovská, E., Biskupstø, R., Gillberg, I. C., Ellefsen, Á., Kampmann, H., Stórá, T., Billstedt, E., & Gillberg, C. (2012b). The rising prevalence of autism: A prospective

longitudinal study in the Faroe Islands. *Journal of Autism and Developmental Disorders*, 42, 1959–1966.

Kohane, I. S., McMurry, A., Weber, G. et al. (2012). The co-morbidity burden of children and young adults with autism spectrum disorders. *PloS one*, 7, e33224.

Kolevzon, A., Gross, R., & Reichenberg, A. (2007). Prenatal and perinatal risk factors for autism. *Archives of Pediatrics & Adolescent Medicine*, 161, 326-333.

Kolevzon, A., Weiser, M., Gross, R., Lubin, G., Knobler, H. Y., Schmeidler, J., et al. (2006). Effects of season of birth on autism, spectrum disorders: fact or fiction? *American Journal of Psychiatry*, 163, 1288-1290.

Kopp, S., Kelly, K., & Gillberg, C. (2010). Girls with social and/or attention deficits: a descriptive study of 100 clinic attenders. *Journal of Attention Disorders*, 14, 167-181.

Kopp, S. & Gillberg, C. (1992). Girls with social deficits and learning problems: autism, atypical Asperger syndrome or a variant of these conditions. *European Child and Adolescent Psychiatry*, 1, 89-99.

Kripke, D. F. (1998). Light treatment for nonseasonal depression: Speed, efficacy, and combined treatment. *Journal of Affective Disorders*, 49, 109-117.

Kurita, H. (2001). Current status of autism studies. *Seishin Shinkeigaku Zasshi*, 103, 64-75.

Lagunova, Z., Porojnicu, A. C., Lindberg, F., Hexeberg, S., & Moan, J. (2009). The dependency of vitamin D status on body mass index, gender, age and season. *Anticancer Research*, 29, 3713–3720.

Landau, E. C., Cicchetti, D. V., Klin, A., & Volkmar, F. R. (1999). Season of birth in autism: a fiction revisited. *Journal of Autism and Developmental Disorders*, 29, 385-393.

Landis, R. J. & Koch, G. G. (1977). The measurement of observer agreement for categorical data. *Biometrics*, 33, 159-174.

Landner, L. (1972). The biological alkylation of mercury. *The Biochemical Journal*, 130, 67-69.

Landrigan, P. J. (2010). What causes autism? Exploring the environmental contribution. *Current Opinion in Pediatrics*, 22, 219-225.

Lansdowne, A. T. & Provost, S. C. (1998). Vitamin D3 enhances mood in healthy subjects during winter. *Psychopharmacology (Berl)*, 135, 319-323.

Lappe, J. M. (2011). The role of vitamin D in human health: A paradigm shift. *Journal of Evidence-Based Complementary and Alternative Medicine*, 16, 58-72.

Lawrence, Y. A., Kemper, T. L., Bauman, M. L., & Blatt, G. J. (2010). Parvalbumin-, calbindin-, and calretinin-immunoreactive hippocampal interneuron density in autism. *Acta Neurologica Scandinavica*, *121*, 99-108.

Le Couteur, A., Lord, C., Rutter, M., *Autism Diagnostic Interview-Revised (ADI-R)* (2006) Western Psychological Services, Wilshire Blvd., Los Angeles, California.

Leekam, S. R. (2011). Diagnostic Interview for Social and Communication Disorders. Entry for *Encyclopedia of Autism Spectrum Disorders* (Volmar, F. R., Ed.). Springer Publishers, Berlin.

Leonard, H., Dixon, G., Whitehouse, A. J. O., Bourke, J., Aiberti, K., Nassar, N., & Glasson, E. J. (2010). Unpacking the complex nature of the autism epidemic. *Research in Autism Spectrum Disorders*, *4*, 548-554.

Levy, S. E., Mandell, D. S., Schultz, R.T. (2009). Autism. *The Lancet*, *374*, 1627-1638.

Lichtenstein, P., Carlström, E., Rastam, M., Gillberg, C., & Ancharater, H. (2010). The genetics of autism spectrum disorders and related neuropsychiatric disorders in childhood. *American Journal of Psychiatry*, *167*, 1357-1363.

Lindsay, R. L., Arnold, L. E., Aman, M. G., Vitiello, B., Posey, D. J., McDougle, C. J., et al. (2006). Dietary status and impact of risperidone on nutritional balance in children with autism: A pilot study. *Journal of Intellectual & Developmental Disability*, *31*, 204-209.

Liptak, G. S., Kennedy, J. A, & Dosa, N. P. (2011). Social participation in a nationally representative sample of older youth and young adults with autism. *Journal of Developmental Behavior Pediatrics*, Apr 15 2011 (<http://www.ncbi.nlm.nih.gov/pubmed/21285894>).

Lord, C., Risi, S., Lambrecht, L., Cook, E. H., Leventhal, B. L., DiLavore, P. C., Pickles, A., & Rutter, M. (2000). The autism diagnostic observation schedule-generic: a standard measure of social and communication deficits associated with the spectrum of autism. *Journal of Autism and Developmental Disorders*, *30*, 205-223.

Lord, C., Rutter, M., Goode, S., Heemsbergen, J., Jordan, H., Mawhood, L., et al. (1989). Autism diagnostic observation schedule: a standardized observation of communicative and social behavior. *Journal of Autism and Developmental Disorders*, *19*, 185-212.

Losh, M. & Piven, J. (2007). Social-cognition and the broad autism phenotype: identifying genetically meaningful phenotypes. *Journal of Child Psychology and Psychiatry*, *48*, 105-112.

Lundström, S., Chang, Z., Rastam, M., Gillberg, C., Larsson, H., Anckarsater, H., et al. (2012). Autism spectrum disorders and autistic-like traits: similar etiology in the extreme end and the normal variation. *Archives of General Psychiatry*, *69*, 46-52.

Lutchmaya, S. & Baron-Cohen, S. (2002). Human sex differences in social and non-social looking preferences, at 12 months of age. *Infant Behavior and Development*, *25*, 319-325.

Mahaffey, K. R., Sunderland, E. M., Chan, H. M., Choi, A. L., Grandjean, P., Mariën, K., Oken, E., Sakamoto, M., Shoeny, R., Weihe, P., Yan, C.-H., & Yasutake, A. (2011). Balancing the benefits of n-3 polyunsaturated fatty acids and the risk of methylmercury exposure from fish consumption. *Nutrition Reviews*, *69*, 493-508.

Mahaffey, K., Clickner, R., & Jeffries, R. (2009). Adult women's blood mercury concentrations vary regionally in USA: Association with patterns of fish consumption (NHANES 1999-2004). *Environmental Health Perspective*, *117*, 47-53.

Mandy, W., Chilvers, R., Chowdhury, U., Salter, G., Seigal, A., & Skuse, D. (2012). Sex differences in autism spectrum disorder: Evidence from a large sample of children and adolescents. *Journal of Autism and Developmental Disorders*, *42*, 1304-1313.

Matson, J. L. & Kozlowski, A. (2011). The increasing prevalence of autism spectrum disorders. *Research in Autism Spectrum Disorders*, *5*, 418-425.

Matson, J. L., Fodstad, J. C., & Dempsey, T. (2009). What symptoms predict the diagnosis of autism or PDD-NOS in infants and toddlers with developmental delays using the Baby and Infant Screen for autism traits. *Developmental Neurorehabilitation*, *12*, 381-388.

McGrath, J., Feron, D., & Mackay-Sim, A. (2001). Vitamin D: the neglected neurosteroid? *Trends in Neurosciences*, *24*, 570-572.

Meguid, N. A., Hashish, A. F., Amwar, M., & Sidhom, G. (2010). Reduced serum levels of 25-hydroxy and 1,25-dihydroxy vitamin D in Egyptian children with autism. *The Journal of Alternative and Complementary Medicine*, *16*, 641-645.

Mehrotra, P., Marwaha, R. K., Aneja, S., Seth, A., Singla, B. M., Ashraf, G., et al. (2010). Hypovitaminosis D and hypocalcemic seizures in infancy. *Indian Paediatrics*, *47*, 581-586.

Moher, D., Liberati, A., Tetzlaff, J., & Altman, D. G. (2009). Preferred reporting items for systematic reviews and meta-analyses. The PRISMA statement. *PLoS Med*, *6*, 11000097; DOI: 10.1371/journal.pmed1000097.

Molloy, C. A., Kalkwarf, H. J., Manning-Courtney, P., Mills, J. L., & Hediger, M. L. (2010). Plasma 25(OH)D concentration in children with autism spectrum disorder. *Developmental Medicine and Child Neurology*, *52*, 969-971.

- Morrissette, J., Takser, L., St-Armour, G., Smargiassi, A., Lafond, J., & Mergler, D. (2004). Temporal variation of blood and hair mercury levels in pregnancy in relation to fish consumption history in population living along the St. Lawrence River. *Environmental Research*, *95*, 363-364.
- Moss, J., Magiati, I., Charman, T. & Howlin, P. (2008). Stability of the Autism diagnostic interview-revised from pre-school to elementary school age in children with autism spectrum disorders (2008). *Journal of Autism and Developmental Disorders*, *38*, 1081-91.
- Mostafa, G. A. & AL-Ayadhi, L. Y. (2012). Reduced serum concentrations of 25-hydroxy vitamin D in children with autism: Relation to autoimmunity. *Journal of Neuroinflammation*, *9*, 201-207.
- Muhle, R., Trentacoste, S. V., & Rapin, I. (2004). The Genetics of Autism. *Pediatrics*, *113*, e472-e482.
- Murad, M. H., Elamin, K. B., Abu Elnour, N. O., Elamin, M. B., Alkatib, A. A., Fatourechi, M. M., Almandoz, J. P., Mullan, R. J., Lane, M. A., Liu, H., Erwin, P. J., Hensrud, D. D., & Montori, V. M. (2011). Interventions to raise vitamin D level and functional outcomes: A systematic review and meta-analysis. *The Journal of Clinical Endocrinology and Metabolism*, *96*, 2997-3006.
- Munger, K. L., Levin, L. I., Massa, J., Horst, R., Orban, T., & Ascherio, A. (2012). Preclinical serum 25-hydroxyvitamin D levels and risk of Type 1 Diabetes in a cohort of US military personnel. *American Journal of Epidemiology*, *177*, 411-419.
- National Research Council (NRC). (2000). Toxicological effects of methylmercury. Committee on the Toxicological Effects of Methylmercury, Board on Environmental Studies and Toxicology, Commission on Life Sciences, National Research Council. Washington, DC: National Academy press.
- Neumeyer, A. M., Gates, A., Ferrone, C., Lee, H., & Misra, M. (2012). *Journal of Autism and Developmental Disorders*, Nov. 4, PMID: 231243396.
- Neveu, I., Naveilhan, P., Menea, C., Wion, D., Brachet, P., & Garabedian, M. (1994). Synthesis of 1,25-dihydroxyvitamin D3 by rat brain macrophages in vitro. *Journal of Neuroscience Research*, *38*, 214-220.
- Nichols, S., Moravcik, G., & Tetenbaum, S. P. (2008). *Girls growing up on the autism spectrum: What parents and professionals should know about the pre-teen and teenage years*. London: Jessica Kingsley Publishers.
- Noble, J. M., Mandel, A., & Patterson, M. C. (2007). Scurvy and rickets masked by chronic neurologic illness: Revisiting "Psychological Malnutrition". *Pediatrics*, *119*, e783-e790.

- Noriega, D. B. & Savelkoul, H. F. J. (2014). Immune dysregulation in autism spectrum disorder. *European Journal of Pediatrics*, *173*, 33-43.
- Novakovic, B., Sibson, M., Ng, H. K., Manualpillai, U., Rakyan, V., Down, T., et al. (2009). Placenta-specific methylation of the vitamin D 24-hydroxylase gene. *The Journal of Biological Chemistry*, *284*, 14838-14848.
- Nseir, W., Mograbi, J., Abu-Rahmeh, Z., Mahamid, M., Abu-Elheja, O., & Shalata, A. (2012). The association between vitamin D levels and recurrent group A streptococcal tonsillopharyngitis in adults. *International Journal of Infectious Diseases*, *16*, e735-e738.
- Nygren, G., Cederlund, M., Sanderg, E., Gillstedt, F., Arvidsson, T., Gillberg, I. C., et al. (2010). The prevalence of Autism Spectrum Disorders in toddlers: A population study of 2-year-old Swedish children. *Journal of Autism and Developmental Disorders*, *42*, 1491-1497. Erratum: *Journal of Autism and Developmental Disorders*, 2012, *42*, 1498.
- Nygren, G., Hagberg, B., Billstedt, E., Skoglund, A., Gillberg, C., & Johansson, M. (2009). The Swedish version of the Diagnostic Interview for Social and Communication Disorders (DISCO-10). Psychometric properties. *Journal of Autism and Developmental Disorders*, *39*, 730-741.
- Ornitz, E. (1988). Autism: A disorder of directed attention. *Brain Dysfunction*, *1*, 309-322.
- Ornitz, E., Guthrie, & D. Farley, A. J. (1978). The early symptoms of childhood autism, in *Cognitive Defects in the Development of Mental Illness* (Serban, G. Ed.), pp. 24-42, Brunner/Mazel, New York.
- Orton, S.-M., Herrera, B. M., Yee, I. M., Valdar, W., Ramagopalan, S. V., Sadovnick, A. D., et al. (2006). Sex ratio of multiple sclerosis in Canada: a longitudinal study. *Lancet Neurology*, *5*, 932-936.
- Patrick, R. P. & Ames, B. N. (2014). Vitamin D hormone regulates serotonin synthesis. Part 1: relevance for autism. *The FASEB Journal*, *28*, DOI: 10.1096/fj.13-246546.
- Petersen, M. S., Halling, J., Bech, S., Wermuth, L., Weihe, P., Nielsen, F., Jørgensen, P. J., Budtz-Jørgensen, E., & Grandjean, P. (2008) Impact of dietary exposure to food contaminants on the risk of Parkinson's disease. *Neurotoxicology*. *29*, 584-90.
- Philippe, A., Martinez, M., Guilloud-Bataille, M., Gillberg, C., Råstam, M., Sponheim, E., Coleman, M., Zappella, M., Aschauer, H., Van Maldergem, L., Penet, C., Feingold, J., Brice, A., & Leboyer, M. (1999). Genome-wide scan for autism susceptibility genes. Paris Autism Research International Sibpair Study. *Human Molecular Genetics*, *8*, 805-812.

- Pickett, J. A., Paculdo, D. R., Shavelle, R. M., et al. (2006). 1998-2002 Update on "Causes of death in autism". *Journal of Autism and Developmental Disorders*, 36, 287.
- Pinkel, D. & Albertson, D. G. (2005). Comparative genomic hybridization. *Annual Review of Genomics and Human Genetics*, 6, 331-354.
- Plotnikoff, G. (2013). Vitamin D deficiency: Time for inaction? *Global Advances in Health and Medicine*, 2, 5-6.
- Posserud, M.-B., Lundervold, A. J., & Gillberg, C. (2009). Validation of the autism spectrum screening questionnaire in a total population sample. *Journal of Autism and Developmental Disorders*, 39, 126-134.
- Posserud, M.-B., Lundervold, A. J., & Gillberg, C. (2006). Autistic features in a total population of 7-9-year-old children assessed by the ASSQ (Autism Spectrum Screening Questionnaire). *Journal of Child Psychology and Psychiatry*, 47, 167-175.
- Priemel, M., von Domarus, C., Klatter, T. O., Kessler, S., Schlie, J., Meier, S., Proksch, N., Pastor, F., Netter, C., Streicher, T., Püschel, K., & Amling, M. (2010). Bone mineralization defects and vitamin D deficiency: Histomorphometric analysis of iliac crest bone biopsies and circulating 25-hydroxyvitamin D in 675 patients. *Journal of Bone and Mineral Research*, 25, 305-312.
- Ramagopalan, S. V., Heger, A., Berlanga, A. J., Maugeri, N. J., Lincoln, M. R., Burrell, A., et al. (2010). A ChIP-seq-defined genome-wide map of vitamin D receptor binding: Associations with disease and evolution. *Genome Research*, 20, 1352-1360.
- Rice, D. C., Schoeny, R., & Mahaffey, K. (2003). Methods and rationale for derivation of a reference dose for methylmercury by the U.S. EPA. *Risk Analysis*, 23, 107-115.
- Rice, D. & Barone, S. Jr. (2000). Critical periods of vulnerability for the developing nervous system: Evidence from humans and animal models. *Environmental Health Perspectives*, 108(suppl 3), 511-533.
- Rimland, B. & Edelson, M. (1999). Autism Treatment Evaluation Checklist. Autism Research Institute, 4812 Adams Avenue, San Diego, CA 92116. Retrieved from <https://www.autismeval.com/ari-atec/report1.html>
- Rivet, T. T. & Matson, J. L. (2011). Review of gender differences in core symptomatology in autism spectrum disorders. *Research in Autism Spectrum Disorders*, 5, 957-976.
- Rogers, S. J. (2009). What are infant siblings teaching us about autism in infancy? *Autism Research*, 2, 125-137.
- Ronald, A., Simonoff, E., Kuntsi, J., Asherson, P., & Plomin, R. (2008). Evidence for overlapping genetic influences on Autistic and ADHD behaviours in a community twin sample. *Journal of Child Psychology and Psychiatry*, 49, 535-542.

- Rondeau, E., Klein, L. S., Masse, A., Bodeau, N., Cohen, D., & Guilé, J. M. (2011). Is pervasive developmental disorder not otherwise specified less stable than autistic disorder? A meta-analysis. *Journal of Autism and Developmental Disorders*, *41*, 1267-76.
- Rutter, M. (2005). Incidence of autism spectrum disorders: changes over time and their meaning. *Acta Paediatrica*, *94*, 2-15.
- Rutter, M., Bailey, A., & Lord, C. (2003). *Social Communication Questionnaire (SCQ)*. Western Psychological Services, Los Angeles.
- Rutter, M., Greenfeld, D., & Lockyer, L. (1967). A five to fifteen years follow-up study of infantile psychosis: 1. Description of sample. *British journal of Psychiatry*, *113*, 1169-1182.
- Sadowska, J. & Cierebiej M. (2011). Evaluation of the nutrition manner and nutritional status of children with autism. *Pediatrics Wspolczesna*, *13*, 155-60.
- Saemundsen, E., Magnússon, P., Georgsdóttir, I., Egilsson, E., & Rafnsson, V. (2013). Prevalence of autism spectrum disorders in an Icelandic birth cohort. *British Medical Journal Open*, *3*, DOI: 10.1136/bmjopen-2013-002748.
- Sakamoto, M., Kakita, A., Wakabayashi, K., Takahashi, H., Nakano, A., & Akagi, H. (2002). Evaluation of changes in methylmercury accumulation in the developing rat brain and its effects: a study with consecutive and moderate dose exposure throughout gestation and lactation periods. *Brain Research* *949*, 51–59.
- Sakamoto, M. K., Murata, K., Kubota, M., Nakai, K., & Satoh, H. (2010). Mercury and heavy metal profiles of maternal and umbilical cord RBCs in Japanese population. *Exotoxicology and Environmental Safety*, *73*, 1-6.
- Sakamoto, M., Kubota, M., Liu, X.-J., Murata, K., Nakai, K., & Satoh, H. (2004). Maternal and fetal mercury and n-3 polyunsaturated fatty acids as a risk and benefit of fish consumption to fetus. *Environmental Science and Technology*, *38*, 3860-3863.
- Sasson, N. J., Lam, K. S., Childress, D., Parlier, M., Daniels, J. L., & Piven, J. (2013). The broad autism phenotype questionnaire: Prevalence and diagnostic classification. *Autism Research*, p. 20.
- Schaaf, C. P. & Zoghbi, H. Y. (2011). Solving the autism puzzle a few pieces at a time. *Neuron*, *70*, 806-808.
- Schoeman, K., Tanaka, T., Bend, J. R., & Koren, G. (2010). Hair mercury levels of women of reproductive age in Ontario, Canada: Implications to fetal safety and fish consumption. *Journal of Pediatrics*, *57*, 127-131.

- Schopler, E., Reichler, R. J., DeVellis, R. F., & Daly, K. (1980). Toward objective classification of childhood autism: Childhood Autism Rating Scale (CARS). *Journal of Autism and Developmental Disorders*, *10*, 91-103.
- Schöttker, B., Jansen, E. H., Haug, U., Schomburg, L., Köhrle, J., & Brenner, H. (2012). Standardization of misleading immunoassay based 25-hydroxy-vitamin D levels with liquid chromatography tandem-mass spectrometry in a large cohort study. *PLoS ONE*, *7*, e48774.
- Schreck, K. A., Williams, K., & Smith, A. F. (2004). A comparison of eating behaviours between children with and without autism. *Journal of Autism and Developmental Disorders*, *34*, 433-438.
- Schwalfenberg, G. (2007). Not enough vitamin D. Health consequences for Canadians. *Canadian Family Physician*, *53*, 841-854.
- Scott, F. J., Baron-Cohen, S., Bolton, P., & Brayne. (2002). Brief Report: Prevalence of Autism Spectrum Conditions in Children Aged 5–11 Years in Cambridgeshire, UK. *Autism* *6*, 231-237.
- Shagun, V. A., Shevchenko, S. G., Smirnov, V. I., & Frolov, Y. L. (2005). Quantum chemical study of the effects of interaction of polyhydrated Hg(II) with methylcobalamin - A derivative of vitamin B<sub>12</sub>. *Journal of Structural Chemistry*, *46*, 34-42.
- Shamberger, R. J. (2011). Autism rates associated with nutrition and the WIC Program. *Journal of the American College of Nutrition*, *30*, 348-353.
- Shavelle, R. M., Strauss, D. J., & Pickett, J. (2001). Causes of death in autism. *Journal of Autism and Developmental Disorders*, *31*, 569-576.
- Shea, R. L. & Berg, J. D. (2013). Measuring 25-hydroxy vitamin D in haemolysed whole blood samples. *Annals of Clinical Biochemistry*, *50* (supplement 1), 25–26.
- Siegel, A., Malkowitz, L., Moskovits, M. J., & Christakos, S. (1984). Administration of 1,25-dihydroxyvitamin D<sub>3</sub> results in the elevation of hippocampal seizure threshold levels in rats. *Brain Research*, *298*, 125-129.
- Sifneos, P. E. (1973). The prevalence of 'alexithymic' characteristics in psychosomatic patients. *Psychotherapy and psychosomatics*, *22*, 255-262.
- Sigmundsdóttir, H. (2011). New aspects on immunoregulation by vitamin D analogs. *Dermato-Endocrinology*, *3*, 187-192.
- Skuse, D. H. (2000). Imprinting, the X-chromosome, and the male brain: Explaining sex differences in the liability to autism. *Pediatric Research*, *47*, 9–16.

- Spach, K. M. & Hayes, C. E. (2005). Vitamin D<sub>3</sub> confers protection from autoimmune encephalomyelitis only in female mice. *The Journal of Immunology*, *175*, 4119-4126.
- Stamm, C., Friehs, I., Ho, S. Y., Moran, A. M., Jonas, R. A., & del Nido, P. J. (2001). Congenital supraaortic stenosis: a simple lesion? *European Journal of Cardio-Thoracic Surgery*, *19*, 195–202.
- Steffenburg, S. & Gillberg, C. (1986). Autism and autistic-like conditions in Swedish rural and urban areas: a population study. *The British Journal of Psychiatry*, *149*, 81-7.
- Stewart, C. & Latif, A. (2008). Symptomatic nutritional rickets in a teenager with autistic spectrum disorder. *Child: Care Health and Development*, *34*, 276-278.
- Strachan, T. & Read, A. P. (2010). *Human Molecular Genetics*: Garland Science.
- Swinkels, S. H., Dietz, C., Van Daalen, E., Kerkhof, I. H., van Engeland, H., & Buitelaar, J. K. (2006). Screening for autistic spectrum in children aged 14 to 15 months. I: the development of the Early Screening of Autistic Traits Questionnaire (ESAT). *Journal of Autism and Developmental Disorders*, *36*, 723–732.
- Taniura, H., Ito, M., Sanada, N., Kuramoto, N., Ohno, Y., Nakamichi, N., et al. (2006). Chronic vitamin D<sub>3</sub> treatment protects against neurotoxicity by glutamate in association with upregulation of vitamin D receptor mRNA expression in cultured rat cortical neurons. *Journal of Neuroscience Research*, *83*, 1179-1189.
- Teitelbaum, P., Teitelbaum, O., Nye, J., Fryman, J., & Maurer, R. G. (1998). Movement analysis in infancy may be useful for early diagnosis of autism. *Proceeding of the National Academy of Sciences*, *95*, 13982-13987.
- Torigoe, T., Miller, J. Fedele, A., Collins, J., Smith, K., Lotspeich, L., Croen, L. A., Ozonoff, S., Lajonchere, C., Grether, J. K., & Risch, N. (2011). Genetic heritability and shared environmental factors among twin pair with autism. *Archives of General Psychiatry*, *68*, 1095-1102.
- Tyler, C. V., Schramm, S. C., Karafa, M., et al. (2011). Chronic disease risk in young adults with autism spectrum disorder: Forewarned is forewarned. *American Journal on Intellectual and Developmental Disabilities*, *116*, 371-380.
- Van Daalen, E., Kemner, C., Dietz, C., Swinkels, S. H., Buitelaar, J. K., & van Engeland, H. (2009). Inter-rater reliability and stability of diagnoses of autism spectrum disorder in children identified through screening at a very young age. *European Child and Adolescent Psychiatry*, *18*, 663-74.
- Vincent, J. B., Kolozsvari, D., Roberts, W. S., Bolton, P. F., Gurling, H. M., & Scherer, S. W. (2004). Mutation screening of X-chromosomal neurologigin genes: No mutations in 196 autism probands. *American Journal of Medical Genetics (Neuropsychiatric Genetics)*, *129B*, 82–84.

- Walker, S. J., Fortunato, J., González, L. G., & Krigsman, A. (2013). Identification of unique gene expression profile in children with regressive autism spectrum disorder (ASD) in ileocolitis. *PLoS ONE* 8:e58058. DOI: 10.1371/journal.pone.0058058.
- Weaver, K. L., Ivester, P., Chilton, J. A., Wilson, M. D., Pandey, P., & Chilton, F. H. (2008). The content of favourable and unfavourable polyunsaturated fatty acids found in commonly eaten fish. *The Journal of American Dietetic Association*, 108, 1178-1185.
- Wechsler, D. (1992). *Wechsler Intelligence Scale for Children (3<sup>rd</sup> ed.)*. Psychological Corporation, London, UK.
- Wechsler, D. (1981). *Wechsler Adult Intelligence Scale-Revised*. Psychological Corporation. San Antonio, Texas.
- Wechsler, D. (1974). *Manual for the Wechsler Intelligence Scale for Children – Revised*. Psychological Corporation, New York.
- Weig, S. G. (2009). BM-7 A 6-year-old boy with autism and painful leg weakness. *Journal of Clinical Neuromuscular Disease*, 10, 160-161.
- Weihe, P. & Grandjean, P. (2013). The Faroese birth cohorts and methylmercury exposure from marine diet. *Proceedings of the 11<sup>th</sup> International Conference on Mercury as a Global Pollutant*, T4-0830.
- Weihe, P., Debes, F., White, R. F. Sorensen, N., Budtz-Jørgensen, E., Keiding, N., & Grandjean, P. (2003). Environmental epidemiology research leads to a decrease of the exposure limit for mercury. *Ugeskrift for Laeger*, 6, 107-111.
- Wermuth, L., von Weitzel-Muderbach, P., & Jeune, B. (2000). A twofold difference in the age-adjusted prevalences of Parkinson's disease between the island of Als and the Faroe Islands. *European Journal of Neurology*, 7, 655–660.
- Wermuth, L., Joensen, P., Bünger, N., & Jeune, B. (1997). High prevalence of Parkinson's disease in the Faroe Islands. *Neurology*, 49, 426.
- Whitehouse, A. J. O., Holt, B. J., Serralha, M., Holt, P. G., Kusel, M. M. H., & Hart, P. H. (2012). Maternal serum vitamin D levels during pregnancy and offspring neurocognitive development. *Pediatrics*, 129, 485-493.
- Wiener, J., Krabbenhoft, D. P., Heinz, G., & Sheuhammer, A. (2003). Exotoxicology of mercury. In *Handbook of Exotoxicology* (Hoffmann, D., Rattner, B., Burton, B. J., Cairns, J. J., Eds.). CRS Press, Boca Raton, FL, pp. 407-461.
- Wing, L., Leekam, S. R., Libby, S. J., Gould, J., & Larcombe M. (2002). The diagnostic interview for social and communication disorders: Background, inter-rater reliability and clinical use. *Journal of Child Psychology and Psychiatry*, 43, 307–325.
- Wing, L. (2001). *The Autistic Spectrum*, Ulysses Press, Berkeley, California.

Wing, L. (1996). Wing autistic disorder interview checklist (WADIC). In: *Preschool children with inadequate communication* (Rapin, I.; editor). Mac Keith Press, London, pp. 247–252.

Wing, L. (1981). Sex ratios in early childhood autism and related conditions. *Journal of Autism and Developmental Disorders*, 12, 89-99.

Wing, L. (1980). *Early Childhood Autism*. Oxford: Pergamon Press.

Witsch-Baumgartner, M., Schwenter, I., Gruber, M., Benlian, P., Bertranpetit, J., et al (2008). Age and origin of major Smith-Lemli-Opitz syndrome (SLOS) mutations in European population. *Journal of Medical Genetics*, 45, 200-209.

Woolfenden, S., Sarkozy, V., Ridley, G., et al. (2012). A systematic review of two outcomes in autism spectrum disorder-epilepsy and mortality. *Developmental Medicine and Child Neurology*, 54, 306-312.

World Health Organization. (1993). *The ICD-10 classification of mental and behavioural disorders. Diagnostic criteria for research*. Geneva: World Health Organisation. (<http://www.who.int/classifications/icd/en/GRNBOOK.pdf>; accessed 21<sup>st</sup> March 2014).

World Health Organization (1990). Methylmercury (Environmental Health Criteria 101). Geneva: World Health Organization.

Zahir, F., Rizwi, S. J., Haq, S. K., & Khan, R. H. (2005). Low dose mercury toxicity and human health. *Environmental Toxicology and Pharmacology*, 20, 351-360.

Zalups, R. K. & Bridges, C. C. (2012). Relationships between the Renal Handling of DMPS and DMSA and the Renal Handling of Mercury. *Chemical Research in Toxicology*, 25, 1825-1838.

Zerbo, O., Iosif, A. M., Delwiche, L., Walker, C., & Hertz-Picciotto, I. (2011). Month of conception and risk of autism. *Epidemiology*, 22, 469-475.

Zwaigenbaum, L., Bryson, S. E., Szatmari, P., Brian, J., Smith, I. M., Roberts, W., Vaillancourt, T., & Roncadin, C. (2012). Sex Differences in Children with Autism Spectrum Disorder Identified Within a High-Risk Infant Cohort. *Journal of Autism and Developmental Disorders*, 42, 2585–2596.

# **I. APPENDICES**

## Appendix 1

**Feasibility Study V:** Dietary/life-style trends among Faroese mothers during their pregnancy with a child from the target group later diagnosed with ASD or with a typically developing child from the control group of study IV

### Dietary Questionnaire 2011

Mother's name: .....

"MUM to" (ID number of the child): .....

- 1.0. What were your housing conditions during your pregnancy?
- 2.0. Did you live in a foreign country during your pregnancy?
  - 2.1. If yes, what country?
  - 2.2. If yes, what period of time?
- 3.0. Did you have any illness during your pregnancy?
  - 3.1. If yes, what kind?
- 4.0. Did you have any contact with doctor consultant during your pregnancy?
  - 4.1. If yes, for which reason?
5. Where you hospitalized during your pregnancy?
  - 5.1. If yes why?
  - 5.2. If yes, when?
  - 5.3. If yes, where?
6. Did you take any medication during your pregnancy, incl. vitamin supplement and/or any kind of OTC medication?
  - 6.1. If yes, describe it:
7. Do you have any kind of mental disorder?
  - 7.1. If yes, describe it:
8. In summary, how was your health condition during your pregnancy?
9. Did you work during your pregnancy?
  - 9.1. If yes, what kind of work was it?
  - 9.2. If yes, for how long time?

10. Did you smoke every day, sometimes, or not at all?
  - 10.1. How many cigarettes
11. How old were you when you started to smoke every day?
12. How many years have you been smoking before you got pregnant?
13. Have you been drinking alcohol while you were pregnant?
14. How many beers did you drink during your pregnancy (a bottle of 33 cl.)
  - 14.1. Quantity of alcohol?
15. How much wine did you drink during your pregnancy (a glass of 12 cl.)
  - 15.1. Quantity of alcohol?
16. How much hard liquor did you drink during your pregnancy (a glass of 2 cl.)
  - 16.1. Quantity of alcohol?
17. Did you use any drugs during your pregnancy?
  - 17.1. If yes, what kind?
18. Have you been eating pilot whales during your childhood and up to 12 years of age?
  - 18.1. If yes, what time(s) during the year?
  - 18.2. If yes, what time(s) during the month?
  - 18.3. If yes, what time(s) during the week?
19. Have you been eating pilot whale meat from 13 years of age and up to your pregnancy?
  - 19.1. If yes, what time(s) during the year?
  - 19.2. If yes, what time(s) during the month?
  - 19.3. If yes, what time(s) during the week?
- 20.0. Have you been eating pilot whale meat during your pregnancy?
  - 20.1. If yes, what time(s) during the year?
  - 20.2. If yes, what time(s) during the month?
  - 20.3. If yes, what time(s) during the week?
- 21.0. Have you been eating blubber of pilot whales during your childhood and up to 12 years of age?
  - 21.1. If yes, what time(s) during the year?
  - 21.2. If yes, what time(s) during the month?
  - 21.3. If yes, what time(s) during the week?

- 22.0. Have you been eating blubber of pilot whales from 13 years of age and up to your pregnancy?
- 22.1. If yes, what time(s) during the year?
- 22.2. If yes, what times during the month?
- 22.3. If yes, what times during the week
- 23.0. Have you been eating blubber of pilot whales during your pregnancy?
- 23.1. If yes, what time(s) during the year?
- 23.2. If yes, what time(s) during the month?
- 23.3. If yes, what time(s) during the week?
- 24.0. Have you been eating seabird during your childhood and up to 12 years of age?
- 24.1. If yes, what time(s) during the year?
- 24.2. If yes, what time(s) during the month?
- 24.3. If yes, what time(s) during the week?
- 25.0. Have you been eating seabird from 13 years of age and up to your pregnancy?
- 25.1. If yes, what time(s) during the year?
- 25.2. If yes, what time(s) during the month?
- 25.3. If yes, what times during the week?
- 26.0. Have you been eating seabird during your pregnancy?
- 26.1. If yes, what time(s) during the year?
- 26.2. If yes, what time(s) during the month?
- 26.3. If yes, what time(s) during the week?
- 27.0. Have you been eating fish during your childhood and up to 12 years of age?
- 27.1. If yes, what time(s) during the year?
- 27.2. If yes, what time(s) during the month?
- 27.3. If yes, what times during the week?
- 28.0. Have you been eating fish from 13 years of age and up to your pregnancy?
- 28.1. If yes, what time(s) during the year?
- 28.2. If yes, what times during the month?
- 28.3. If yes, what times during the week?
- 29.0. Have you been eating fish during your pregnancy?
- 29.1. If yes, what time(s) during the year?
- 29.2. If yes, what time(s) during the month?

- 29.3. If yes, what time(s) during the week?
- 30.0. Have you been eating fruit during your childhood and up to 12 years of age?
- 30.1. If yes, what time(s) during the year?
- 30.2. If yes, what time(s) during the month?
- 30.3. If yes, what time(s) during the week?
- 31.0. Have you been eating fruit from 13 years of age and up to your pregnancy?
- 31.1. If yes, what time(s) during the year?
- 31.2. If yes, what time(s) during the month?
- 31.3. If yes, what time(s) during the week?
- 32.0. Have you been eating fruit during your pregnancy?
- 32.1. If yes, what time(s) during the year?
- 32.2. If yes, what time(s) during the month?
- 32.3. If yes, what time(s) during the week?
- 33.0. Have you been eating vegetables during your childhood and up to 12 years of age?
- 33.1. If yes, what time(s) during the year?
- 33.2. If yes, what time(s) during the month?
- 33.3. If yes, what time(s) during the week?
- 34.0. Have you been eating vegetables from 13 years of age and up to your pregnancy?
- 34.1. If yes, what time(s) during the year?
- 34.2. If yes, what time(s) during the month?
- 34.3. If yes, what time(s) during the week?
- 35.0. Have you been eating vegetables during your pregnancy?
- 35.1. If yes, what time(s) during the year?
- 35.2. If yes, what time(s) during the month?
- 35.3. If yes, what time(s) during the week?
- 36.0. Have you been eating potatoes during your childhood and up to 12 years of age?
- 36.1. If yes, what time(s) during the year?
- 36.2. If yes, what time(s) during the month?
- 36.3. If yes, what time(s) during the week?
- 37.0. Have you been eating potatoes from 13 years of age and up to your pregnancy?
- 30.1. If yes, what time(s) during the year?
- 30.2. If yes, what time(s) during the month?

- 30.3. If yes, what time(s) during the week?
- 38.0. Have you been eating potatoes during your pregnancy?
- 38.1. If yes, what time(s) during the year?
- 38.2. If yes, what time(s) during the month?
- 38.3. If yes, what time(s) during the week?
- 39.0. Did you change your food habits when you confirmed your pregnancy?
- 39.1. If yes, describe it.
- 40.0. Did you always stock pilot whales and blubber at home?
- 41.0. Were you often in the sunlight during your pregnancy?
- 41.1. If yes, describe it?
- 42.0. Did you take any vitamin-supplements during your pregnancy?
- 42.1. If yes, what kind?
- 43. Have you ever experienced miscarriage or extra-uterine pregnancy?
- 44.1. If yes, describe it?
- 43.0. Did you take any vitamin-supplements before you became pregnant?
- 43.1. If yes, what kind?
- 44.0. Please write all your childbirths below.
- 45.0. When were you pregnant for the first time?
- 46.0. Was your pregnancy planned?
- 47.0. Did you and your partner use any kind of contraception?
- 47.1. If yes, what kind?
- 48.0. How many months did it take before you got pregnant for the first time?
- 49.0. Did you use this contraception regularly?
- 49.1. How long did you use contraception before you got pregnant?
- 50. Did you use “p-pills” during the last 12 months before you got pregnant for the first time?
- 51. How long did it take to get pregnant, after you stopped using contraception?
- 52. Medication in pregnancy yes/no
- 52.1. If yes, what kind
- 52.2. Medication in pregnancy notes
- 53. Number of childbirths
- 54. Did you have a caesarean section?

## Appendix 2

### *A personal experience of the climate in the Faroe Islands*

The only international airport on the Faroe Islands was built by the British on the island of Vagar during World War II. It is classified as one of the most dangerous airports in the world due to the landscape: It is situated between two mountain ranges and has a very short runway, only 1,200 m long. The harsh climate, namely high precipitation, extremely low visibility, and frequent strong winds add to the dangers.

To enable take-off from such a short runway, the British engineers came up with an ingenious exploitation of the Vagar terrain: the runway ends on a cliff and the plane, like a puffin, uses the height and glides...or ...luckily for me those 7 times did glide, anyway.

The Atlantic Airways and Icelandic Airways pilots are specially trained experts and their 'Fatal Event Score' since 1970 has been zero. One has to have a faith in the statistics and the advertised above-average skills of the pilots when doing PhD research in the Faroe Islands.

Travelling to the Faroe Islands, however, also requires some special practical arrangements. Due to the unpredictable weather conditions and travel difficulties, one has to always have a 'time reserve' of at least 24 hours at both ends of the trip and it is not advisable to schedule the first meeting for an early morning after the planned arrival the night before.

The only connection from Glasgow to the Faroe Islands is via London and Copenhagen (depending on the day of the week). Out of my seven trips to the Faroe Islands, 6 times the arrival flight was significantly delayed, shortening the sleep period to less than 4 hours as one has to travel around an hour from the airport on the island of Vagar to the capital Tórshavn on the Streymoy island through several under-sea or through-the-mountain tunnels; 6 times the flight back had been delayed or cancelled completely, resulting in missed connection flights to Britain. Once the flight from Copenhagen to Vagar was completely re-directed to Norway due to a snow storm and after three unsuccessful attempts to land at Vagar. The first morning of the next working day had thus been lost completely.

*One of my several 'Faroese Odysseys' - June 2011*

On the way from Copenhagen to the Faroe Islands on Tuesday, the flight was delayed by 3 hours which, as I noticed, had become the 'norm' during my journeys. These delays were always very tiring because one has to leave Glasgow at 7 am and therefore wake up at 4 am. It was already raining but amazingly there was still light in the Faroe Islands - at midnight it looked like 8 pm in Glasgow.

The first meeting on Wednesday was scheduled for 8 am and the weather was progressively worsening. I managed to walk in the rain and horrible wind with my umbrella permanently in the 'Walden's turnover' (a type of a chemical reaction when a molecule looks like a completely turned over umbrella in a wind) to Pál's office.

With the distance of any place in the Faroe Islands from the sea being less than 5 km, when clouds are extremely low, a hurricane is blowing and the rain becomes an uninterrupted wall of water, one has a feeling that it is still before the second day of Genesis – before 'the waters in the sky were separated from the waters of the Earth'. It is impossible to say where the land ends and where the ocean begins, and it feels like standing in the middle of the sea. Hurricanes are very frequent there at any time of the year, most of them have names – e.g., Oscar, Ofelia, Felicity, etc. - and they are ruling the islands with a mighty force. I always had to carry my laptop when outside and despite its weight I could hardly stay on the ground – flying like Mary Poppins - seemingly defying gravity.

While working in the Faroe Islands, I used to stay in a hospital flat without a phone or internet access; therefore, I would usually book my taxi for the departure on the arrival and then hope for the best. On the day of my departure on this particular trip on Friday morning at 5.55 am I was waiting for the taxi outside the hospital block of flats in an enormous gale and torrential rain (again my umbrella was useless) and the taxi did not appear! I tried to phone the taxi company several times but my foreign mobile did not work there. So, I dragged my suitcase to the hospital (500 m) where I was supposed to return the key from the flat but could not find anybody there, even in the Emergency Department, and there was not a single soul or a car to be seen anywhere around!

I started panicking, fearing that I would miss my flight. I could not see the building across the road – this is how bad it was. This scenario is usually a ‘bad omen’ (or rather a very practical one) for aviation purposes in the Faroe Islands. Once, during one of my earlier trips, a Danish consultant, who was sharing a taxi from the airport with me, educated me that when, at the airport waiting hall, one cannot see the runway (at a ~50 m distance), it means no plane would land in such conditions, and that consequently means no chance of getting back to Europe.

However, I still needed to get a taxi and get to the airport. I was hopelessly and helplessly standing in the middle of the road near the hospital, or rather in the middle of a ‘river’, hoping for a miracle – preferably in the form of a taxi - and eventually, one car emerged from the rainy cloud. The lady driver kindly stopped and phoned the taxi company from her mobile phone, explaining that the pre-booked taxi did not show up and that I was unable to phone them from my foreign mobile. I learned that they did not come because the flight was delayed to 10.30 am and they would come at 8 am. So, I dragged my suitcase up the hill, went back to the flat (thank God I still had the key!), wringed my completely soaked clothes, made a cup of hot tea and glued myself to a radiator of central heating.

At 8 am the taxi finally arrived but when we got to the airport the runway was obscured by a cloud and the flight meanwhile was delayed to 12.30 pm, then to 2 pm and then to 3.30 pm and by that time I knew I had missed all my connecting flights from Copenhagen to London - yet again. This hurricane was particularly persistent - all flights on the previous day (Thursday) were cancelled, not a single plane managed to land and the first one finally landed after 45 hours at 5 pm! And I must say I was highly impressed by how on earth the pilot managed to land because we, in the waiting hall, still could not see the runway. Clearly, the Atlantic Airways pilots really need to be praised for their skills. Eventually, we left after 6.30 pm. At midnight I was, at long last, able to take off the soaked cloths after having safely arrived in a Copenhagen hotel after 19 hours of travel adventures. The flight back home the next day was boringly uneventful.

## Appendix 3

**Kočovská, E.,** Biskupstø, R., Gillberg, I. C., Ellefsen, Á., Kampmann, H., Stóra, T., Billstedt, E., & Gillberg, C. (2012).

The rising prevalence of autism: A prospective longitudinal study in the Faroe Islands. *Journal of Autism and Developmental Disorders*, 42, 1959-1966.

## Appendix 4

**Kočovská, E.,** Fernell, E., Billstedt, E., Minnis, H., & Gillberg, C. (2012).  
Vitamin D and Autism: Clinical Review.  
*Journal of Research in Developmental Disabilities*, 33, 1541-1550.

## Appendix 5

Kočovská, E., Billstedt, E., Ellefsen, Á., Kampmann, H., Gillberg, I. C., Biskupstø, R., Andorsdóttir, G., Stóra, T., & Gillberg, C. (2013).  
Autism in the Faroe Islands: Diagnostic stability from childhood to early adult life.  
*The Scientific World Journal*, Special Issue on ESSENCE, Article ID 592371,  
<http://dx.doi.org/10.1155/2013/592371>.

## Appendix 6

Kočovská, E., Andorsdóttir, G., Weihe, P., Halling, J., Fernell, E., Stóra, T., Biskupstø, R., Gillberg, I. C., Shea, R., Billstedt, E., Bourgeon, T., Minnis, H., & Gillberg, C. (2014).

Vitamin D in the general population of young adults with autism in the Faroe Islands  
*Journal of Autism and Developmental Disorders*, DOI 10.1007/s10803-041-2155-1.



After the successful take-off from the Vagar cliff (picture by EK).