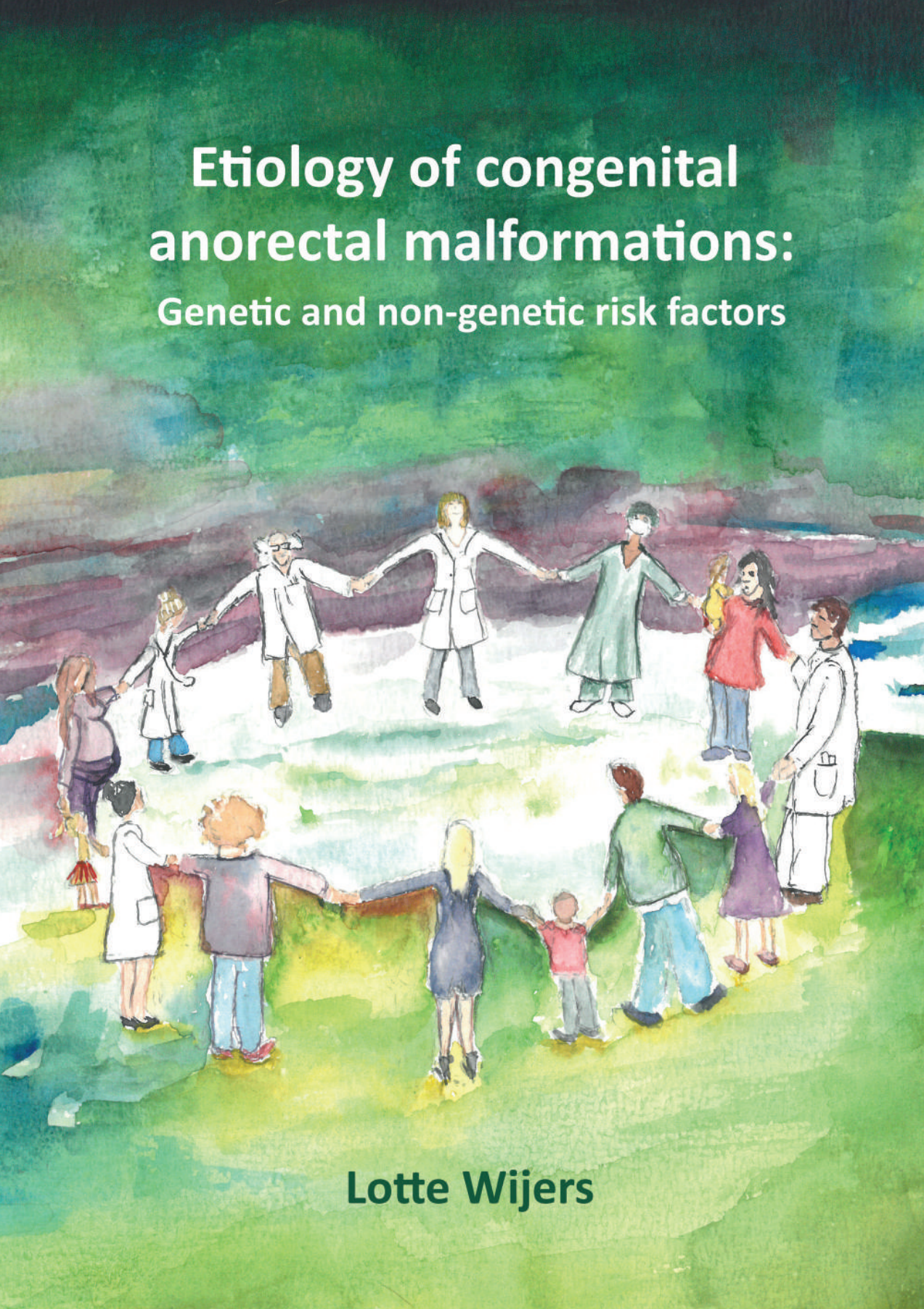
Etiology of congenital anorectal malformations:

Genetic and non-genetic risk factors



Lotte Wijers

Etiology of congenital anorectal malformations:

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Thesis Radboud University Nijmegen, with summary in Dutch

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Etiology of congenital anorectal malformations:

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Table of contents

Part 1: Introduction, prevalence, and definitions

Chapter 1	General introduction	11
Chapter 2	Genetic and non-genetic etiology of nonsyndromic anorectal malformations: a systematic review *Birth Defects Research Part C: Embryo Today: Reviews 2014;102: 382-400*	19
Chapter 3	Research perspectives in the etiology of congenital anorectal malformations using data of the International Consortium on Anorectal Malformations: evidence for risk factors across different populations *Pediatric Surgery International 2010;26:1093-1099*	85
Chapter 4	First results of a European multi-center registry of patients with anorectal malformations *Journal of Pediatric Surgery 2013;48:2530-2535*	97
Chapter 5	Bias in patient series with VACTERL association (Letter to the editor) *American Journal of Medical Genetics Part A 2011;155:2039-2041*	109

Part 2: Non-genetic factors

Chapter 6	Anorectal malformations and pregnancy-related disorders: a registry-based case-control study in 17 European regions *BJOG 2013;120:1066-1074*	119
Chapter 7	Maternal and paternal risk factors for anorectal malformations: a Dutch case-control study *Birth Defects Research Part A: Clinical and Molecular Teratology 2010;88:152-158*	139

Chapter 8 Parental subfertility, fertility treatment and the risk of congenital anorectal malformations 155
Epidemiology 2015;26:169-176

Part 3: Genetic factors and gene-environment interaction

Chapter 9 No major role for periconceptional folic acid use and its interaction with the *MTHFR C677T* polymorphism in the etiology of congenital anorectal malformations 179
Birth Defects Research Part A: Clinical and Molecular Teratology 2014;100:483-492

Chapter 10 Rare coding variants and the risk of congenital anorectal malformations: an exome chip association study 199

Part 4: General discussion and summaries

Chapter 11 General discussion 241

Chapter 12 Summary 261
Samenvatting 265
About the author 269
PhD portfolio 271
List of publications 273
Dankwoord 275

Part 1

Introduction, prevalence, and definitions

Chapter 1

General introduction

Anorectal malformations (ARM) are congenital malformations of the digestive system resulting from disturbed development of the hindgut during embryogenesis. ARM comprise a wide spectrum of phenotypes ranging from narrowing of the anorectal channel to anal atresia with or without fistulas to surrounding organs and even complex cloacal malformations. Table 1 shows the ARM phenotypes in both males and females, which are classified according to the international Krickenbeck criteria for ARM, developed in 2005.[1] Some of the common phenotypes are illustrated in Figure 1.

Table 1. Anorectal malformation phenotypes in male and female patients.

Males	**Females**
Perineal fistula	Perineal fistula
Rectourethral bulbar fistula	Vestibular fistula
Rectourethral prostatic fistula	Cloaca with common channel < 3 cm
Rectovesical (bladder neck) fistula	Cloaca with common channel > 3 cm
Atresia without fistula	Atresia without fistula
Anal stenosis	Anal stenosis
Rare complex malformations[a]	Rare complex malformations[a]

[a]Rare complex malformations include pouch colon, rectal atresia/stenosis, rectovaginal fistula, and H fistula.

Before 2005, several international classification systems for ARM were proposed. The classification has large impact on the choice for surgical approaches. The Wingspread classification, elaborated in 1984, distinguished between low, intermediate, and high atresias according to the level of termination of the rectum in relation to the levator ani muscle.[2] The Peña classification from 2000 was based on the presence and position of the fistula.[3] In 2005, the Krickenbeck classification was developed by consensus among a large group of medical professionals experienced in the management of patients with ARM. Nowadays, it is the most commonly used classification system for ARM worldwide. The Krickenbeck classification includes the above-mentioned diagnostic classification, but is also designed to enable adequate comparison of postoperative results.[1]

ARM occur in approximately 1 in 3,000 live born children worldwide.[5] In total, 40-70% of the ARM patients have one or more other major congenital malformations, mostly malformations that are part of the VACTERL (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal, and Limb defects) association.[6-8] Therefore, ARM can be classified into subgroups based on the presence or absence of additional congenital malformations, including isolated ARM, ARM with one or more other congenital malformations, or VACTERL.

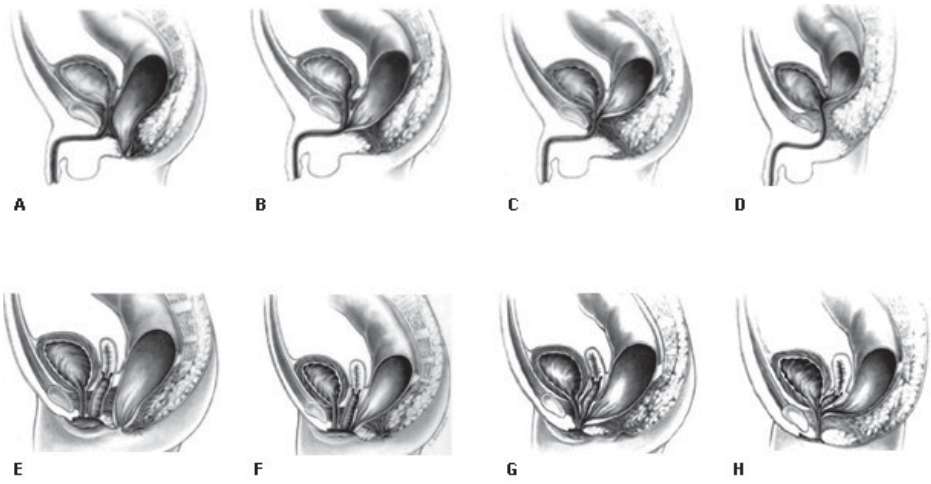

Figure 1. Common anorectal malformation phenotypes in male (A-D) and female patients (E-H)[4] (with permission)
A: perineal fistula, B: rectourethral bulbar fistula, C: rectourethral prostatic fistula, D: rectovesical fistula
E: perineal fistula, F: vestibular fistula, G: cloaca with common channel < 3 cm, H: cloaca with common channel > 3 cm

Treatment of ARM is considered an enormous challenge in reconstructive pediatric surgery. As the majority of patients need a colostomy prior to the definitive repair of the anomaly, multiple surgical procedures are often required in the first two years of a patient's life in order to restore bowel function.[9] Nowadays, the posterior sagittal approach, which was introduced in 1980, is the most commonly used and ideal method to repair ARM.[9] Postoperatively, intensive treatment with anal dilatations need to be performed by the parents, which can be painful for the child and have long-term impact on both the child and the parents as well.[10] The posterior sagittal approach clearly improved the anatomical restoration, but complete functional restoration is hardly ever achieved. As a result, ARM is often associated with lifelong psychosocial problems and physical impairments, such as constipation, fecal incontinence, and urological, sexual and gynecological problems.[11,12] Because of these problems, etiologic research is essential to obtain more insight in the development of ARM, with the ultimate aim to provide preventive strategies and improved genetic counseling.

In approximately 10% of all patients, a genetic cause is known as ARM develop as part of a chromosomal disorder or a syndrome,[7] such as 13q deletion syndrome, cat eye syndrome, Townes-Brocks syndrome, or Currarino syndrome.[13-15] In the remaining non-

syndromic ARM patients, however, the etiology is still unclear. Since various modes of inheritance have been shown in pedigrees with familial ARM and the majority of cases occur sporadically, a multifactorial etiology with involvement of both genetic and non-genetic factors seems most likely.[16-18]

Objectives and outline of this thesis

The main objective of this thesis is to increase knowledge on the involvement of genetic and non-genetic risk factors in the etiology of congenital anorectal malformations. To accomplish this objective, studies are performed on:

- **definitions and prevalence** of ARM phenotypes and associated congenital malformations (Part 1)
- a wide range of potential **non-genetic risk factors** for ARM (Part 2)
- the contribution of rare **genetic variants** and a potential **gene-environment interaction** to the development of ARM (Part 3)

Several databases and biobanks with ARM cases and control children were used for the studies performed, with the AGORA (Aetiologic research into Genetic and Occupational/ environmental Risk factors for Anomalies in children) data- and biobank as the main source. AGORA is a network organization of the departments for Health Evidence and Human Genetics and many clinical departments participating in the Amalia Children's Hospital of the Radboud university medical center (Radboudumc), which started with routine data collection to facilitate future research into childhood cancer and different congenital malformations, including ARM, in December 2004.[19] AGORA includes clinical data and DNA from the child and DNA and questionnaire data from their parents. As etiologic hetero-geneity may exist among different phenotypic subgroups of ARM, these subgroups were taken into account in the studies if case numbers were sufficient.

Part 1: Introduction, prevalence, and definitions

An extensive systematic review of the literature on the genetic and non-genetic etiology of ARM is presented in Chapter 2. Chapter 3 describes the ARM-Net Consortium that was initiated by clinicians, epidemiologists, geneticists, and patient organizations involved in ARM from the Netherlands, Germany, Italy, and France at the end of 2009 with the aim to exchange data and knowledge and to collaborate in research and clinical topics.[20] The first results on the prevalence of ARM phenotypes and associated congenital malformations and treatment of ARM within the ARM-Net Consortium are presented in Chapter 4. Chapter 5

describes a discussion of the definition of the VACTERL association using clinical data from AGORA and the German Network for Congenital Uro-REctal malformations (CURE-Net).[21]

Part 2: Non-genetic factors

In Chapter 6, associations between ARM and several pregnancy-related disorders are presented as assessed using the European Surveillance of Congenital Anomalies (EUROCAT) database,[22] a large European registry of congenital malformations. Several potential risk factors for ARM were also studied using parental questionnaires from 85 ARM cases collected within AGORA (Chapter 7). Thereafter, major efforts were done to enlarge the ARM case population with patients from the Radboudumc, Sophia Children's Hospital – Erasmus Medical Centre Rotterdam, and University Medical Center Groningen and to collect data from a population-based AGORA control group via municipalities throughout the Netherlands. These data are used to focus on associations between parental subfertility and a range of fertility treatments and the risk of ARM (Chapter 8).

Part 3: Genetic factors and gene-environment interaction

Chapter 9 concentrates on the assumed role of a gene-environment interaction between maternal periconceptional folic acid supplement use and the *MTHFR C677T* polymorphism in the occurrence of ARM. A genome-wide association study to identify rare genetic variants is described in Chapter 10. ARM cases were derived from AGORA and CURE-Net and controls from the Nijmegen Biomedical Study, a population-based survey of randomly selected inhabitants of the municipality of Nijmegen,[23] while ARM cases and controls from Hong-Kong in China were used for replication analyses.

Part 4: General discussion and summaries

A general discussion of the studies within this thesis and summaries in English and Dutch are included in Chapters 11 and 12.

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Chapter 2

Genetic and non-genetic etiology of nonsyndromic anorectal malformations: a systematic review

Charlotte HW Wijers, Iris ALM van Rooij, Carlo LM Marcelis, Han G Brunner, Ivo de Blaauw, Nel Roeleveld

Birth Defects Research Part C: Embryo Today: Reviews 2014;102:382-400

Abstract

Congenital anorectal malformations (ARM) are the most frequently observed birth defects of the digestive system, but their etiology remains elusive. Therefore, we aim to summarize and critically appraise all existing literature on the genetic and non-genetic etiology of nonsyndromic ARM and to conclude with unifying hypotheses and directions for future research. A structured literature search on English language human studies was conducted in PubMed and Embase up to October 1, 2013, resulting in 112 included articles. Research on the identification of genes underlying nonsyndromic ARM is remarkably scarce. Most studies were focused on screening of candidate genes for mutations or single nucleotide polymorphisms, which did not yield any substantial evidence. Non-genetic factors fairly consistently found to be associated with ARM are assisted reproductive techniques, multiple pregnancy, preterm delivery, low birth weight, maternal overweight or obesity, and pre-existing diabetes. This review provides indications for the involvement of both genes and non-genetic risk factors in the etiology of ARM. In future studies, large cohorts of ARM patients from national and international collaborations are needed to acquire new hypotheses and knowledge through hypothesis-generating approaches. Challenges for future studies may also lie in the investigation of gene-gene and gene-environment interactions.

Introduction

Phenotypic characterization

Anorectal malformations (ARM) are the most commonly observed congenital malformations of the digestive system, characterized by disturbed embryonic development of the hindgut 4-8 weeks after conception. The embryology of abnormal anorectal development is still partly unclear as discussed in a recent review.[1] The severity of ARM is variable, ranging from mild phenotypes with narrowing of the anorectal channel to anal atresia with or without fistulas and even more severe phenotypes, such as cloaca, as described in the international Krickenbeck classification for ARM developed in 2005.[2] Fistulas can be perineal, rectourethral (bulbar or prostatic urethra), or rectovesical in males and perineal or rectovestibular in females. In cloacas, the urethra, vagina, and rectum form one single common channel to the perineum. ARM is accompanied by one or more other congenital malformations in 40-70% of patients, mostly malformations involved in the VACTERL (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal, and Limb defects) association.[3-5] Of all patients with ARM, 10-15% are diagnosed with the VACTERL association and in approximately 10%, a syndrome or chromosomal anomaly is recognized.

Prevalence and prognosis

The prevalence of ARM ranges from 2 to 6 per 10,000 births worldwide.[6] Although no changes in prevalence rates have been observed over time, these rates vary considerably across countries. This may reflect true geographical and population variation or differences among registration systems, such as dissimilarities in identification, coding, or inclusion criteria.[7,8] Moreover, mild phenotypes of ARM without clinical symptoms may easily be missed, especially among female patients, which may partly explain male/female ratios being 1.2–1.6.[3,5,9-12] ARM is rarely diagnosed prenatally, but is usually detected during the early postnatal period.[13] Mortality rates vary between 3 and 16%, with the highest rates among children with ARM and other congenital malformations.[3,10,14,15] Surgical procedures are generally required during the first 2 years of life. Although surgical techniques have improved significantly in the last decades, complete anatomical and functional restoration cannot be achieved. This accounts for long-lasting problems in the majority of patients,[15] ranging from constipation to fecal soiling or even complete fecal incontinence, urological, gynecological and sexual difficulties, and psychosocial problems, which may decrease quality of life among patients and their families.[16]

Aim of the review

Primary prevention of ARM is needed, but this requires extensive knowledge about the etiology. In approximately 10% of patients, ARM develops as part of a chromosomal disorder or a syndrome caused by a single-gene mutation. Several chromosomal abnormalities described in patients with ARM, such as 13q deletion, cat eye syndrome, and trisomy 13, 18, and 21, were evaluated in a review by Marcelis et al.[17]. These loci predispose for ARM, but it remains unclear whether candidate genes in these regions cause nonsyndromic or syndromic ARM. Syndromes most frequently associated with ARM include Currarino syndrome, Townes-Brocks syndrome, and Pallister-Hall syndrome, which are caused by mutations in the *HLBX9*, *SALL1*, and *GLI3* genes, respectively.[18-20] The etiology of nonsyndromic ARM, however, remains unsolved but is suggested to be mainly multifactorial, involving both genetic and non-genetic risk factors. In this systematic review, we will summarize and critically appraise the existing literature about the genetic and non-genetic etiology of nonsyndromic ARM, involving many studies with small patient series and divergent results. Furthermore, we aim to conclude with unifying hypotheses regarding the etiology and to provide applicable directions for future research.

Methods

Literature search

We conducted a comprehensive systematic literature search on the etiology of nonsyndromic ARM in PubMed and Embase up to October 1, 2013. Relevant studies were identified using combinations of terms for ARM and etiology. As several researchers evaluated non-genetic risk factors for a range of congenital malformations and did not always mention a keyword for ARM in their titles or abstracts, these studies were not identified using the initial search. Therefore, we performed an additional search on all potential non-genetic risk factors for ARM that were evaluated in the studies included from the initial search, combined with terms for congenital malformations. Other relevant studies were identified by reviewing the reference lists of the selected articles. The complete search strategy is available from the authors.

Study selection

The full screening process and search results are shown in Figure 1. The initial search was restricted to English language human studies on ARM and provided 2,335 articles in PubMed and 1,175 in Embase, leaving a total of 2,688 unique potentially relevant articles of which titles and abstracts were screened. Forty articles turned out to be animal or *in vitro* studies and were excluded. As we focused our review on the etiology of nonsyndromic ARM, we

excluded all articles that were not about ARM or described ARM phenotypes, syndromic forms of ARM, surgery, management, long-term follow-up, or quality of life of patients with, and embryology or prevalence of ARM. Because we included peer-reviewed studies containing original data only, reviews, meta-analyses, commentaries, and conference abstracts were also excluded. In addition, case reports on non-genetic risk factors were excluded, whereas we included informative case reports on the involvement of genes in nonsyndromic isolated ARM. Full texts of the remaining 63 articles and those from the additional search and cross-reviewing were reviewed in detail. Studies that evaluated case populations consisting of patients with and without ARM, such as VACTERL cases or a heterogeneous group of all intestinal atresias, were excluded when we were not able to extract the results for patients with ARM separately. We included gene expression studies only if gene expression was investigated in tissues from patients with ARM. Epidemiological studies were included when a risk estimate was presented or sufficient information was provided to estimate the risk of ARM. This selection process resulted in 112 included articles, 19 on genetic and 93 on non-genetic risk factors for ARM.

Data extraction

A uniform data extraction sheet was used to extract the necessary information and to assess the quality of the research hypothesis, study design, study setting, study population (number and type of cases and controls), statistical analyses, and risk estimates (with 95% confidence interval [CI]), as well as potential biases. If a risk estimate was not presented, we calculated one based on numbers given. From the epidemiological studies, we extracted extra information about exposure assessment, such as method used and time window of exposure, response rates, and confounders and/or effect modifiers.

Results

Genetic factors

Although only 2–8% of patients with ARM have an affected first- or second degree relative,[10,12,21-24] ARM co-occurs more often among family members than would be expected by chance.[12,24-26] Familial occurrence seems to be most frequent when a perineal or vestibular fistula is present.[23] Nevertheless, research focused on the identification of genes involved in the etiology of nonsyndromic ARM has been limited, and therefore, only 19 genetic studies are included in this review (Table 1).

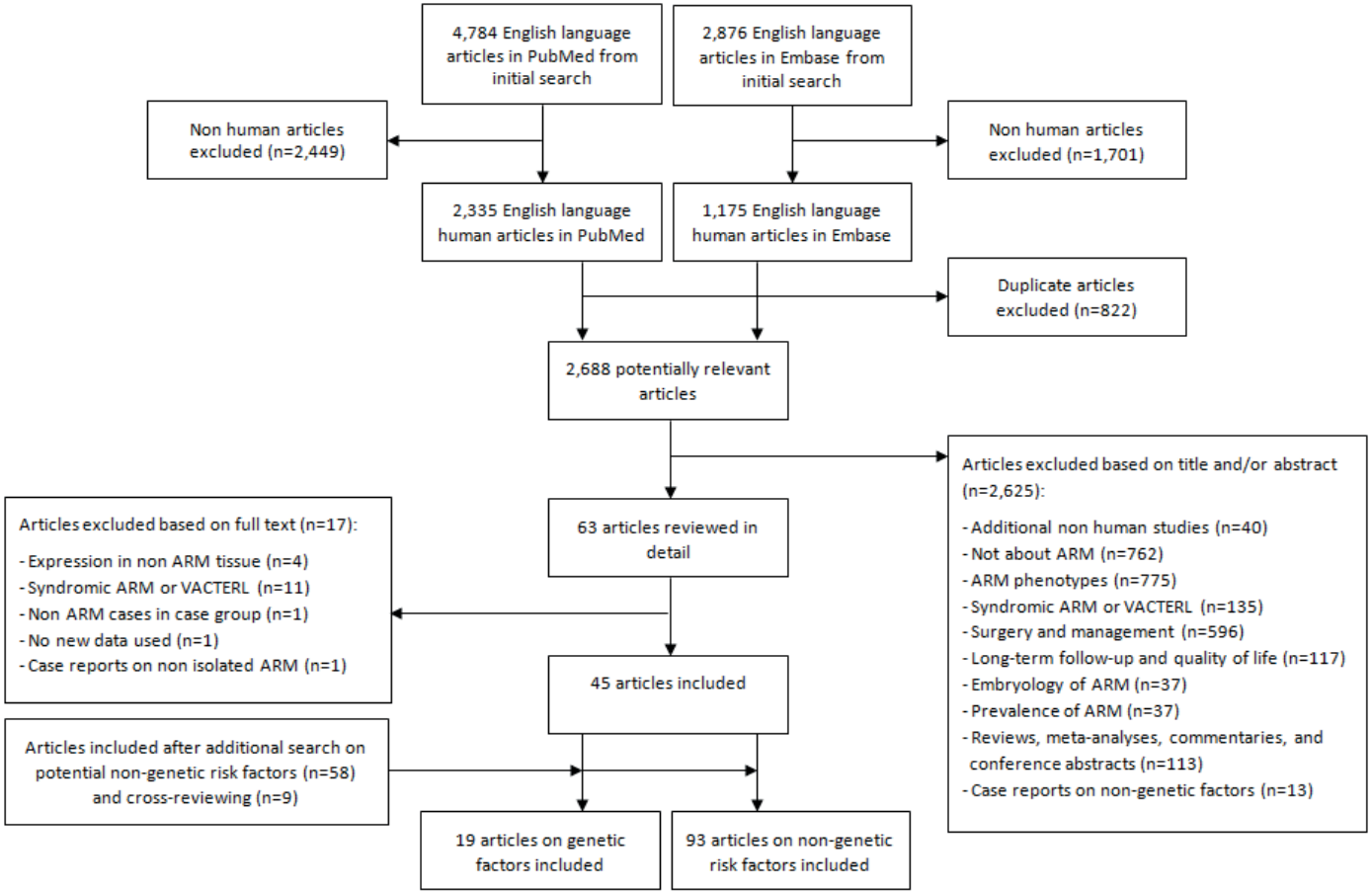

Figure 1. Flow chart of the screening process and search results for the systematic review on the etiology of ARM.

Most of these studies were focused on screening of specific genes in groups of ARM cases aiming to find causal mutations or genetic polymorphisms. The sonic hedgehog (SHH), wingless-type integration site (WNT), fibroblast growth factor (FGF), and bone morphogenetic protein (BMP) signaling pathways are essential in embryogenesis and are expected to be involved in hindgut development based on animal studies. Therefore, several genes (and downstream target) genes in these pathways were the subject of genetic studies on ARM. Significantly lower *SHH*, *GLI2*, and *BMP4* expression was noted in the posterior wall of the terminal rectum in patients with ARM when compared with controls.[27] Mutation screening, however, did not reveal relevant functional mutations in *SHH* or *GLI3* in two studies with 15 and 88 patients, respectively.[28,29] Single nucleotide polymorphisms (SNPs) at predicted regulatory sites in (downstream) targets of the SHH pathway, including GLI family zinc finger 2 (*GLI2*), homeobox D12 (*HOXD12*), *BMP4*, and proprotein convertase subtilisin/kexin type 5 (*PCSK5*), were suggested to be associated with isolated ARM in a study with patients of several ethnic populations. However, none of these associations remained after adjusting for multiple testing.[30] Other candidate SNPs did not reach statistical significance after Bonferroni adjustment in this study either.[30] The absence of any replicated findings may be explained by small sample sizes, inclusion of different phenotypes of ARM, or possible mutations in surrounding regulatory DNA elements or in upstream components of the same pathway. A few studies investigated the involvement of the WNT and FGF signaling pathways in the occurrence of ARM.[30-34] No causal mutations were observed by screening the *T Brachyury, FGF10*, *WNT3a*, *WNT5a*, *WNT11*, *DACT1*, and *FGFR2* genes,[31-33] except for one variant in the *T Brachyury* gene (C1013T),[31] but it remains unclear whether this variant has functional consequences. However, a *de novo* duplication of the dickkopf WNT signaling pathway inhibitor 4 (*DKK4*) gene was identified in one patient with ARM and a deletion in the inturned planar cell polarity effector homolog (*INTU*) gene in two patients with ARM, using a candidate gene approach after gene-based analyses of genome-wide copy number variation (CNV) data.[34] It is unclear whether the deletion in the *INTU* gene in the latter two patients was *de novo* or inherited from unaffected parents.

Candidate genes were also selected based on the knowledge from animal studies, syndromic forms of ARM or other congenital malformations of the colon. After screening the caudal type homeobox 1 (*CDX1*) gene, four heterozygous mutations (c.213-214InsGAA, c.6G>C, c.27G>T, c.18A>C) were identified and lower *CDX1* expression levels were found in cases when compared with controls.[35] Although the ret protooncogene (*RET*) is a major gene involved in Hirschsprung's disease, it was not associated with ARM.[36] In a family consisting of a son with ARM and Hirschsprung's disease and a mother and daughter having ARM, no causal mutations were identified in four major Hirschsprung's disease genes (*RET*, *EDNRB*, *EDN3*, and *GDNF*) either.[37] However, three SNPs in endothelin receptor type B (*EDNRB*)

seemed to be associated with specific ARM phenotypes in 14 sporadic South African patients with ARM.[38] The SNP 178G/A was more prevalent among female cases with a vestibular fistula when compared with controls, the 702C/T polymorphism among patients with a vesicorectal fistula or cloaca, and the SNP 831G/A among patients with bulbar or prostatic fistulas.[38] As these findings were based on a small study sample, replication is essential.

Few studies have been conducted using hypothesis-generating methods to identify disease-causing variants for ARM, such as genome-wide association studies (GWAS). To date, only one small GWAS was performed in 175 patients with ARM, which did not reveal any common SNPs associated with ARM.[34] However, a few recent genome-wide CNV analyses provided potential candidate regions. Wong et al. found an increase in rare deletions and duplications, which applied both to CNVs smaller and larger than 100 kb in size.[34] How many of these rare CNVs were *de novo* or were inherited from unaffected parents is unclear. Nevertheless, these findings provide strong support for a role of genetic factors in the etiology of ARM. The data seem to implicate a large number of different genes and loci, each with only limited support from other sources. Wong et al. also found 12 CNVs larger than 1 Mb in ostensibly isolated ARM patients, including patients who were diagnosed with autism or who had CNVs that had previously been reported in patients with clear syndromic features. These CNVs had not been associated with ARM previously. The data suggest that the distinction between syndromic and nonsyndromic ARM in this study was likely based on limited clinical information and follow-up. Genome-wide CNV screening also revealed a *de novo* duplication of 18p11.21-18q12.1 in a female nonsyndromic patient with,[39] and *de novo* microduplications at 1q41, 2q37.3, and 8q24.3 (involving the *SPATA17*, *GPR35*, *CAPN10*, *EPPK1*, *PLEC*, *PARP10* genes),[40] a *de novo* microduplication at 22q11.21,[41] and *de novo* 13q deletions including the *EFNB2* gene[42] in ARM cases with VACTERL or a VACTERL-like phenotype. Previously, 13q deletions have been described in seemingly syndromic patients with ARM and penoscrotal transposition, which were reviewed by Marcelis et al.[17]. Mutation screening of the *GPR35* and *EFNB2* genes among 192 and 331 patients with ARM, respectively, did not yield any causal mutations.[40,42] No causative CNVs were found among three monozygotic twins discordant for ARM and one or more other congenital malformations.[43]

Table 1. Studies screening genes in patients with anorectal malformations.

First author (year), ethnicity	Type of genetic analyses	No. cases	No. controls	Gene	Locus	Results	Population
Exonic mutation analyses							
Papapetrou et al.[31] (1999), white UK origin	Mutation analyses	28	60	*T Brachyury*	6p21.3	C1013T variant[a]	ARM case with sacral agenesis
Seri et al.[28] (1999), unknown	Mutation analyses	15	No controls	*SHH*	7q36	No mutations	Sporadic ARM cases with sacral hypodevelopment
Garcia-Barcélo et al.[29] (2008), Chinese	Mutation analyses	88	96	*SHH* *GLI3*	7q36 7p13	No mutations[b]	ARM cases (including cases with Down syndrome)
Krüger et al.[32] (2008), Caucasian	Mutation analyses	10	No controls	*FGF10*	5p13-p12	No mutations	Nonsyndromic sporadic ARM cases
Schramm et al.[37] (2011), Caucasian	Mutation analyses[c]	3	No controls	*RET* *EDNRB* *EDN3* *GDNF* *HLXB9*	10q11.2 13q22 20q13.2-q13.3 5p13.1-p12 7q36	No mutations	Familial cases of nonsyndromic ARM (mother and two children)
Draaken et al.[33] (2012), Caucasian	Mutation analyses	78	No controls	*WNT3A* *WNT5A* *WNT11* *DACT1* *FGF10*	1q42 3p21-p14 11q13.5 14q23.1 5p13-p12	No mutations[d]	ARM cases with one or more other congenital anomalies

First author (year), ethnicity	Type of genetic analyses	No. cases	No. controls	Gene	Locus	Results	Population
				FGFR2	10q26		
				T Brachyury	6p21.3		
Zhang et al.[35] (2013), Chinese	Mutation analyses	108	120	*CDX1*	5q32	c.213-214InsGAA, c.6G>C, c.27G>T, and c.18A>C variants[e]	ARM cases
Genetic association analyses							
Moore and Zaahl[38] (2007), South Africans	Single-strand confirmation polymorphism analyses	14	20	*EDNRB*	13q22	178G>A: ρ<0.05	ARM cases with vestibular fistula
						702C>T: ρ<0.05	ARM cases with vesicorectal fistula or cloaca
						831G>A: ρ<0.05	ARM cases with bulbar or prostatic fistula
Wu et al.[36] (2010), Taiwanese	SNP analyses	23	Number unknown	*RET*	10q11.2	c135G>A: ρ>0.05 c1296G>A: ρ>0.05 c2071G>A: ρ>0.05 c2307T>G: ρ>0.05 c2712C>G: ρ>0.05	ARM cases (including cases with Down syndrome)
Carter et al.[30] (2013), several ethnicities	SNP analyses[f]	150	623	*WNT3A*	1q42	rs12401893 G>A: ρ<0.05[g,h]	Isolated ARM cases
				GLI2	2q14	rs3738880 A>C: ρ<0.05[g,h]	
				HOXD12	2q31.1	rs35817516 G>A: ρ<0.05[h,i]	
				PCSK5	9q21.3	rs7040769 T>C: ρ<0.05[h,j] rs872189 C>T: ρ<0.05[g,h] rs2279659 C>T: ρ<0.05[g,h]	

First author (year), ethnicity	Type of genetic analyses	No. cases	No. controls	Gene	Locus	Results	Population
				BMP4	14q22-q23	rs17563 T>C: $\rho<0.05$[h,k]	
				TCF4	18q21.1	rs8766 A>G: $\rho<0.05$[h,j]	
				MKKS	20p12	rs2013178 T>A: $\rho<0.05$[h,l]	
						rs1003994 G>A: $\rho<0.05$[h,j]	
Genome-wide analyses							
Schramm et al.[41] (2011), Caucasian	CNV analyses	12	Parents		22q11.21	22q11.21 microduplication	ARM cases with the VACTERL association
Schramm et al.[39] (2011), Caucasian	CNV analyses	13	Parents		18p11.21-18q12.1	*de novo* duplication 18p11.21-18q12.1	Nonsyndromic ARM cases with other congenital anomalies
Wong et al.[34]	GWAS	175	2,971			No SNPs found	ARM cases (including
(2013), Chinese	CNV analyses	363	4,006	*INTU*	4q28.1	4q28.1 deletion[m]	syndromic ARM cases)
				DKK4	8p11.21	*de novo* 8p11.21 duplication	
						Other results see footnote[n]	
Dworschak et al.[42] (2013), Caucasian	CNV analyses	2	Parents	*EFNB2*	13q31. 2q34 and 13q33. 2q34	*de novo* 13q deletions	ARM cases
	Mutation analyses[o]	331	No controls	*EFNB2*	13q33	No mutations	
Baudisch et al.[43] (2013), Caucasian	CNV analyses	3	Discordant monozygotic twins			No causative CNVs found	ARM cases with one or more other congenital anomalies

First author (year), ethnicity	Type of genetic analyses	No. cases	No. controls	Gene	Locus	Results	Population
Hilger et al.[40] (2013), Caucasian	CNV analyses	47	Parents	*SPATA17* *GPR35, CAPN10* *EPPK1, PLEC, PARP10*	1q41 2q37.3 8q24.3	*de novo* microduplications	ARM cases with the VACTERL association or VACTERL-like phenotype
	Mutation analyses[o]	192	No controls	*GPR35*	2q37.3	No mutations	
Gene expression analyses							
Zhang et al.[27] (2009), unknown	Expression analyses	40	10	*SHH*	7q36	Lower expression in high-located ARM vs. controls: $\rho<0.05$	ARM cases with high or low location of the defect according to the Wingspread classification[145]
				GLI2	2q14	Lower expression in high-located ARM and low-located vs. controls: $\rho<0.05$	
				BMP4	14q22-q23	Lower expression in high-located ARM vs. controls: $\rho<0.05$	

ARM, anorectal malformation; SNP, single nucleotide polymorphism; CNV, copy number variation.

[a]Inherited from mother with possible same mild phenotype, but functional significance unclear.

[b]Four *SHH* and 10 *GLI3* variants were found, but the allele frequencies were not different between cases and controls and/or functional support was lacking.

[c]Previously performed CNV analyses did not reveal abnormalities.

[d]Three nucleotide substitutions were found in the *FGFR2* gene, but further investigations revealed that these variants were not causally related to ARM.

[e]These variants were not found in 120 controls and significantly lower *CDX1* expression levels were found in cases than controls, with lowest expression in high-located ARM.

[f]As 71 SNPs in 25 candidate genes were tested, we only presented the SNPs in 7 genes for which an association with ARM was found only. The other 18 genes for which at least one SNP was tested and no associations were found, included *EPHB2*, *WNT5A*, *EPHB3*, *FGF10*, *GLI3*, *HOXA13*, *SHH*, *MNX1*, *FGFR2*, *GDF11*, *HNF1A*, *EFNB2*, *SALL1*, *SALL4*, *PQBP1*, and *ZIC3*.

[g]Association found in African-Americans only.

[h]When the results were adjusted for multiple testing, no associations remained.

[i]Association found in Hispanics only.

[j]Association found in non-Hispanic whites only.

[k]Association found in Asians only.

[l]Association found in non-Hispanic whites and Hispanics.

[m]It is unclear whether this deletion was *de novo* or inherited from normal parents.

[n]An increase in rare CNVs was found ($p<0.05$), but it is unclear whether these were *de novo* or inherited from unaffected parents. Twelve chromosomal aberrations and 114 rare CNVs in 79 genes were found in cases but not in controls.

[o]These analyses were performed based on their results in CNV analyses.

Non-genetic factors

More scientific attention has been paid to involvement of non-genetic factors in the etiology of ARM. These findings are summarized in Table 2. Table 3 and Supplemental Tables 1-3 in Online Resource show all existing literature on associations between non-genetic factors and ARM in more detail.

Table 2. Summary of the existing literature on the involvement of non-genetic factors in the etiology of anorectal malformations.

Factors (fairly) consistently associated with ARM	**Factors (fairly) consistently not associated with ARM**
Assisted reproductive techniques (IVF and ICSI)	Maternal age at time of conception
Multiple birth	Paternal age at time of conception
Preterm delivery	Maternal alcohol use
Low birth weight	
Maternal overweight or obesity	
Maternal pre-existing diabetes mellitus	
Factors that seem to be associated with ARM	**Factors that do not seem to be associated with ARM**
Benzodiazepines	Contraceptives
Maternal job exposure to industrial cleaning agents and solvents	Maternal underweight
	Gestational diabetes
	Chronic hypertension
	Antimicrobial and antibiotic medication use
	Antidepressant medication use
	Maternal smoking
Factors showing inconsistent results	
Hormonal treatment for ovulation induction	Anti-asthmatics and anti-inflammatory medication use
Nulliparity	Paternal job exposure to exhaust fumes
Fever during early pregnancy	Paternal smoking
Antifungal medication use	Folic acid or multivitamin use
Factors investigated in one study only	
Maternal subfertility	Pandemic H1N1 influenza vaccination
Paternal subfertility	Maternal use of most other medications
Prolonged time to pregnancy	Most maternal and paternal job exposures
Preeclampsia	Paternal alcohol use
Influenza	Maternal illicit drug use
Common cold	Maternal caffeine intake
Acute respiratory infections	Living in urban or rural areas

Any chronic thyroid disorder	High altitude
Severe chronic dyspepsia	X-rays during pregnancy
Primary peptic ulcer disease	Paternal overweight or obesity
Epilepsy	Use of hot tubs
Bronchial asthma	Use of swimming pool
Glomerulonephritis during early pregnancy	Maternal injury

Fertility issues

In three studies, associations were investigated between ARM and common methods of contraception, including oral contraceptives, spermicides, male condoms and intrauterine devices, but no associations were found (Table 3).[22,44,45] However, assisted reproduction seems to attribute to the occurrence of ARM in several studies showing highly increased risk estimates ranging from 2.4 to 13.3.[46-50] The highest risks were observed in studies comparing personal interviews among case parents with register-based control data.[48,50] The size of the effect was lower in multiple births than in singletons,[49,50] but seemed to be similar for different manifestations of ARM, including isolated ARM, ARM with other defects and ARM-VACTERL.[50] In other studies no or only small size associations between ARM and assisted reproduction seemed to exist.[12,51-53] The study by Källén et al. published in 2010[51] was a repetition of their previous positive study,[47] using datasets from different time periods. The authors hypothesized that the risk may have been randomly low or declined over time. The second negative study suffered from aspecific exposure assessment,[12] whereas the third one focused specifically on the effect of clomiphene citrate, the most commonly used hormonal treatment for ovulation induction.[52] Two other studies, however, did find an association between ovulation stimulation and ARM.[46,53] Only one study looked at parental subfertility and reported an association between ARM and paternal but not maternal subfertility.[12] This association was mainly found among fathers of ARM-VACTERL cases, for whom a five-fold increased risk of ARM was shown.[12] This study also showed a prolonged time-to-pregnancy for parents of patients with ARM when compared with controls.[12]

Table 3. Studies on associations between anorectal malformations and fertility issues.

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Contraceptives							
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Oral contraceptives in 3 months before pregnancy Intra-uterine devices in 3 months before pregnancy	7.4% vs. 8.2%, NS 2.7% vs. 2.4%, NS	No
Gallaway et al.[45] (2009), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	444	4,723	Spermicides in first 3 months of pregnancy Male condoms in first 3 months of pregnancy	OR 2.0 (0.7-5.0) OR 0.7 (0.4-1.2)	No confounders identified
Waller et al.[44] (2010), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	382	4,000	Oral contraceptives: Last use 2-3 months before pregnancy Last use 1 month before pregnancy Use during pregnancy	 OR 0.8 (0.5-1.2) OR 0.6 (0.3-1.0) OR 0.7 (0.4-1.3)	No confounders identified
Assisted reproduction							
Robert and Francannet[46] (1996), France	ARM cases without chromosomal disorders or neural	Register data	224	7,873	Use of hormones for ovulation stimulation before conception	OR 2.4 (1.4-4.1)[b]	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
	tube defects Malformed controls		146	5,543	Progestin use during first trimester of pregnancy	OR 1.2 (0.5-2.7)[b]	
Källén et al.[47] (2005), Sweden	Nonchromosomal ARM cases Register-based controls	Register data	533	2,039,943	ART[c]	OR 4.7 (3.2-6.9)	Birth year
Midrio et al.[48] (2006), Italy	ARM cases Register-based controls	Personal interviews and register data	28	110,799	ART[c]	OR 13.3 (4.0-39.6)	No
Reefhuis et al.[49] (2009), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	435	4,792	Singleton birth after ART[c]	OR 3.7 (1.5-9.1)	Maternal age, study center, family income, parity
					Multiple birth after ART[c]	OR 1.5 (0.4-5.2)	Maternal age, family income, parity, use of multivitamins or folic acid, alcohol use
Källén et al.[51] (2010), Sweden	Nonchromosomal ARM cases Register-based controls	Register data	245	689,157	ART[c]	OR 0.9 (0.3-2.2)	Maternal age, parity, smoking, BMI, birth year

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Conception with medical treatment	OR 0.7 (0.2-2.0)	No confounders identified
Reefhuis et al.[52] (2011), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	592	6,500	Use of clomiphene citrate 2 months before conception through first month of pregnancy	OR 1.2 (0.6-2.3)	Maternal age, race, parity, BMI, previous miscarriages, education, smoking, alcohol, use of multi-vitamins or folic acid
Zwink et al.[50] (2012), Germany, CURE-Net	ARM cases Register-based controls	Personal interviews and register data	295	10,069, 986	ART[c] IVF ICSI	OR 8.7 (5.9-12.6)[d] OR 10.9 (6.2-19.0) OR 7.5 (4.6-12.2)	No
Wijers et al.[53] (2013), Europe	Nonsyndromic ARM cases[a] Malformed controls	Register data	1,417	13,371	Any type of fertility treatment ART[c] Hormonal treatment	OR 1.3 (0.9-1.8)[e] OR 0.8 (0.4-1.5) OR 2.1 (1.3-3.4)	No confounders identified, except for registry
Parental subfertility							
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Maternal subfertility Paternal subfertility Time to pregnancy > 6 months	OR 0.7 (0.3-1.7) OR 2.2 (0.8-6.1)[f] OR 1.8 (1.1-3.0)[b]	No confounders identified

ARM, anorectal malformations; NS, not statistically significant; NBDPS, National Birth Defects and Prevention Study; ART, assisted reproductive techniques; AGORA, Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children; CURE-Net, The German Network for Congenital Uro-REctal Malformations; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection.

[a]ARM cases without chromosomal or single gene disorders.

[b]Odds ratios and/or confidence intervals were calculated based on data provided in the article or by the authors on request.

[c]Assisted reproductive techniques (ART) includes IVF and ICSI treatments only.

[d]Similar results were found for subgroups of isolated ARM cases, ARM cases with other anomalies, and ARM cases with the VACTERL association.

[e]For isolated ARM cases: OR= 0.8 (0.4-1.5); for ARM cases with other defects: OR= 1.6 (1.0-2.4); and for ARM cases with the VACTERL association: OR= 1.6 (0.8-3.3).

[f]For ARM cases with the VACTERL association only: OR= 5.7 (1.2-27.5).

Parental, pregnancy and infant characteristics

Parental characteristics, including maternal and paternal age, may attribute to the occurrence of congenital malformations due to biological factors, such as ovarian age or mutations in sperm, or non-biological factors related to parental age, such as changing lifestyle patterns. However, neither maternal nor paternal age at time of conception seemed to increase the risk of ARM in most studies,[9,10,12,22,30,54-57] although two studies showed associations between ARM and higher maternal age (Supplemental Table 1).[58,59] In contrast, five studies showed an increased risk of having a child with ARM in the first pregnancy,[9,12,30,53,60] whereas other studies did not show associations between ARM and parity,[10,22,58,61] or showed the opposite effect.[59] The majority of these studies did not adjust for confounding, except three studies showing elevated risks of ARM in the first pregnancy.[9,53,60] ARM was almost consistently found to occur more frequently in multiple births than in singletons,[10,50,53,61-63] but these results were not adjusted for confounders, such as assisted reproduction, in any of the studies. Two studies showed a higher rate of isolated ARM, ARM with other congenital malformations, and ARM-VACTERL among multiple births.[50,53] Associations between ARM and preterm delivery and low birth weight were also fairly consistently reported,[10,12,22,61,64,65] although these factors are unlikely to be causal. They may, however, share a common pathogenic pathway with ARM.

Pregnancy complications and medical drug use

Only a few maternal health issues complicating pregnancy have been investigated extensively in relation to the risk of ARM. For mothers being overweight (25 ≤ body mass index [BMI] < 30 kg/m^2) or obese (BMI ≥ 30 kg/m^2) before pregnancy, increased risks of ARM were observed in most studies,[12,61,66,67] one of which also showed an elevated risk of isolated ARM separately among obese mothers (Supplemental Table 2).[66] Carter et al. hypothesized that interaction between maternal obesity and variants in the McKusick-Kaufman syndrome (*MKKS*) gene may be involved in ARM, but did not find evidence in their study, probably due to unreliable data on obesity.[30] No associations were observed between ARM and maternal underweight either. Several researchers found increased risks of ARM for pre-existing diabetes mellitus.[22,68-71] Others did not confirm these results,[53,72-74] probably due to lack of power or to using children with other congenital malformations as controls. The latter may have led to underestimation of the effect, because pre-existing diabetes is a strong risk factor for many congenital malformations. Most studies did not show clear associations between ARM and gestational diabetes,[30,69,73,75] except for the study by Correa et al. when looking at isolated ARM only.[70] Because gestational diabetes usually develops after the critical developmental period for ARM, a positive association may refer to undiagnosed pre-

existing diabetes. It has been argued that overweight or obesity and diabetes mellitus may be factors in the same causal pathway, whereby alternations between glycemic control and hyperglycemia may increase the risk of having a child with ARM.[66] Associations found between ARM and high dietary glycemic intake among non-diabetic mothers strengthen this hypothesis.[76,77] Some studies on pre-pregnancy BMI excluded mothers with pre-existing diabetes and still showed associations between ARM and overweight and/or obesity.[66,67] This may be explained by inclusion of mothers with undetected or subclinical diabetes mellitus in early pregnancy. Correa et al. found that the risk of ARM increased enormously (odds ratio [OR] 28.7; 95% CI: 5.5-151.0) when mothers with diabetes mellitus did not use multivitamins containing folic acid during the critical time period.[71]

The role of other pregnancy complications in the occurrence of ARM was less often investigated. Chronic hypertension did not seem to be associated with ARM in three studies.[22,61,78] In two other studies, preeclampsia was not associated with isolated ARM either,[53,79] but it was associated with ARM with other congenital malformations.[53] In addition, preeclampsia superimposed on chronic hypertension was associated with isolated ARM.[79] Maternal influenza, common colds, and acute respiratory infections during the second or third month of pregnancy did not increase the risk of ARM, but these results were mostly derived from single studies.[22,80-82] One rather small study did not find an association between ARM and maternal fever during pregnancy either,[22] whereas three other studies showed increased risks of ARM for mothers who had a fever during the first trimester of pregnancy,[12,24,53] mainly in ARM with other congenital malformations.[53] Chronic thyroid disorders and two gastro-intestinal disorders (severe chronic dyspepsia and primary peptic ulcer disease) seemed to be associated with ARM as well, especially with isolated ARM.[83-85] Strong associations were found between phenotypic subgroups of ARM and maternal epilepsy in a large European study,[53] but not in another small study.[22] Associations with ARM were not found for bronchial asthma, glomerulonephritis, mental disorders, and pandemic H1N1 influenza vaccination in single studies that were probably underpowered for these maternal disorders.[53,86-88]

Maternal use of therapeutic drugs, such as analgesics and antipyretics,[89-92] antimicrobials or antibiotics,[93-95] antidepressants,[96-98] antihistamines,[99] hypotensive medication,[100] barbiturates,[101] and decongestants[102] did not seem to be associated with increased risks of ARM, although separate medical drugs were mostly evaluated in one study only. Maternal use of opioid analgesics during early pregnancy seemed to increase the risk, mainly for isolated ARM.[91] Inconsistent results were found for the use of antifungal medication, with metronidazole and miconazole being associated with ARM,[103,104] whereas other antifungal drugs were not.[105,106] Benzodiazepines and other psychotropic drugs were

investigated the most, especially using data from the Hungarian Case-Control Surveillance of Congenital Anomalies. Increased risks were observed after maternal use of different kinds or combinations of benzodiazepines during the critical period of embryogenesis in three studies,[107-109] but not in two others.[110,111] However, one of the latter studies combined benzodiazepines and other psychotropic drugs in one exposure group.[111] One study showed anti-asthmatic drug use to be associated with ARM,[112] whereas another did not.[113] The above-mentioned study on thyroid disorders also showed an increased risk of ARM for thyreostatic medication, but not for thyroxine use.[83] An association between ARM and drugs for preterm labor (2-amino-1-phenylethanol) was observed in a study from the International Clearinghouse for Birth Defects.[114] Finally, for anti-inflammatory drugs, no associations with ARM were reported except for isolated ARM only.[113] However, we doubt the reliability of several of these findings as information was mostly based on hospital records or was retrospectively collected through interviews or questionnaires that were prone to underreporting and recall problems.[115] In general, it is difficult to evaluate whether the medication used or the underlying disease is involved in the etiology of ARM.

Occupational and lifestyle factors

Parental occupational exposures were not extensively studied in relation to ARM. However, mothers prenatally exposed to industrial cleaning agents and solvents while working as cleaners or janitors seemed to have an increased risk of giving birth to a child with ARM (Supplemental Table 3).[12,24,116,117] In one study, the risk was also elevated for mothers employed as scientists during pregnancy.[116] Other maternal occupational exposures did not seem to be associated with ARM, except for cytostatics with ARM-VACTERL only.[12] Indications for an elevated risk of ARM among fathers occupationally exposed to exhaust fumes were found in two studies,[12,118] but these results were not confirmed in a replication study.[24] Increased ORs observed for fathers working as printers, policemen, guards, vehicle manufacturers, and artists were mostly not statistically significant and found in single studies only.[118,119] For scientists and shippers, decreased risks were observed,[119] whereas for many other jobs and exposures among fathers no clear associations were seen.[12,118,119]

Maternal smoking was not associated with ARM in most studies[12,22,120-123] and only slightly in three.[30,61,124] However, one of the latter studies showed that this association was not present for isolated ARM only.[61] Paternal smoking or exposure to environmental tobacco smoke during embryogenesis was associated with ARM in two studies,[12,61] but not in two other studies.[24,122] Neither parental alcohol use nor maternal illicit drug use, including cannabis, cocaine or stimulant use during pregnancy, was found to be involved in the etiology of ARM.[12,22,61,122,125] Maternal caffeine intake, on the other hand, was associated

with an increased risk of ARM with the highest OR among mothers who consumed more than 300 mg of caffeine per day.[61] Although three of the five studies that investigated the effect of folic acid use on the etiology of ARM showed reduced ORs, these were not or only marginally statistically significant.[12,59,71,126,127] The results for maternal multivitamin use showed either no effect[68] or an adverse effect.[12] Living in urban or rural areas did not seem to be associated with ARM,[22] but a study in South America found an increased birth prevalence of infants with ARM at high altitudes.[128] Some other parental factors evaluated in single studies, such as undergoing X-rays during pregnancy, paternal overweight or obesity, use of hot tubs, and swimming pool use were not associated with ARM,[12,22,129,130] whereas mothers who reported being injured during early pregnancy seemed to have an increased risk of having a child with ARM.[131]

Conclusions

The etiology of ARM remains elusive for the majority of nonsyndromic ARM cases, but this review provides indications that both genetic and non-genetic factors are involved. ARM may include monogenic as well as multifactorial forms, but we can only speculate about the proportion of each as genetic research on ARM is remarkably scarce.

Most genetic studies on ARM were based on a candidate gene approach, which did not yield any substantial evidence. Candidate genes were mainly genes that are involved in the SHH, WNT and FGF signaling pathways, which control multiple processes, including embryonic patterning, proliferation, migration, and differentiation. Preliminary evidence for a contribution of genes and (downstream) targets in these pathways (*Shh, Gli2, Gli3, BMP4, Fgf10,* and *Wnt5a*) was derived from animal studies not reviewed in this article.[132-137] *SHH*, *GLI2*, and *BMP4* were also identified as candidate genes in a human expression study.[27] Only one candidate gene study identified functional mutations in the *CDX1* gene, as well as lower *CDX1* expression levels in patients with ARM.[35] *CDX1* is a transcription factor, which is involved in the regulation of proliferation and differentiation of embryonic intestinal tissue and is regulated by the WNT/β-catenin signaling pathway.[138] These studies may suggest that abnormal functioning of the SHH and WNT signaling pathways plays a role in the occurrence of nonsyndromic ARM. However, other candidate gene studies did not reveal any functional mutations or polymorphisms in genes involved in these pathways. This may be due to small sample sizes, which emphasizes that larger patient series are essential. On the other hand, it probably implies that other genetic regions contribute to the development of ARM. Candidate gene studies are driven by researchers' *a priori* presumptions and hypotheses, but do not necessarily generate new knowledge. Therefore, it seems highly valuable to acquire new hypotheses through hypothesis-generating approaches, such as GWAS to elucidate the

role of common variants, and exome or whole-genome sequencing to study rare variants. Application of the latter techniques in large cohorts of patients with ARM is currently still unfeasible, but may be useful to identify genes in large families with multiple affected individuals or *de novo* mutations in sporadic patients with severe phenotypes of ARM who frequently encounter reproductive problems.

Many rare CNVs that provide potential candidate regions for ARM were identified in a genome-wide CNV study.[34] However, information on *de novo* occurrence was lacking and no specific CNV was discovered in multiple patients with ARM, which may imply genetic heterogeneity. The authors also suggested a link between these rare CNVs and the WNT signaling pathway and cytoskeletal network using gene network analyses.[139] The cytoskeletal network has a role in, for example, ciliogenesis and cell movement and is regulated by WNT signaling. Furthermore, a few studies showed the importance of thorough phenotypic characterization and analyses in phenotypic subgroups of ARM. SNPs in the *EDNRB* gene, which induces smooth muscle differentiation, seemed to increase the risks of specific phenotypes of ARM only. Moreover, increased heritability of ARM was found among patients with perineal or vestibular fistulas,[23] and *SHH*, *GLI2*, *BMP4,* and *CDX1* were identified as candidate genes for mainly high-located ARM.[27,35]

Several non-genetic risk factors for ARM have been studied as well, but only a few factors were consistently found to be associated with ARM: assisted reproduction, multiple pregnancy, preterm delivery, low birth weight, maternal overweight or obesity and pre-existing diabetes. Regarding assisted reproduction, the true causal factor remains unclear. Is it hormonal treatment, the handling of oocytes and sperm, and/or the underlying parental subfertility? Low birth weight may be a consequence of fetal hypoxemia and decreased nutrient supply, which may in turn be due to alterations in placental development.[140] Similar circumstances may occur in multiple and nulliparous pregnancies[141] and may contribute to preeclampsia. Although associations found between ARM and nulliparity and preeclampsia still need confirmation, we hypothesize that placental insufficiency in early pregnancy may be involved in the etiology of ARM. Shared metabolic alternations, such as hyperglycemia, in overweight, obese, and diabetic mothers may clarify the associations with ARM for these factors.[66] A previous study showed that adverse pregnancy outcomes mainly occurred in mothers with poor glycemic control during the critical phase of embryogenesis,[142] which is the case in obese women with undiagnosed diabetes in particular. These studies consistently found that non-genetic risk factors are not unique for ARM, as assisted reproduction, multiple pregnancy, preterm delivery, low birth weight, preeclampsia, overweight or obesity, and pre-existing diabetes were also found to be associated with other congenital malformations, such as cardiac defects, neural tube defects, and hypospadias.[49,63-

[65,67,70,143,144] This may point to nonspecificity or the relevance of timing of exposure during embryonic development, although the latter seems to be less applicable to these specific risk factors. It is impossible to draw conclusions about the involvement of most other maternal illnesses, medical drug use, and parental occupational or lifestyle factors in the etiology of ARM as results were inconsistent or derived from single studies only.

Overall, most studies were limited by small samples sizes due to the relatively rare occurrence of ARM. Therefore, future studies should focus on the collection of data in large cohorts of patients with ARM, for which national and international collaborations seem essential.[24] This will also enable analyses on specific phenotypes of ARM to elucidate the potentially heterogeneous etiology. Additional challenges for future research may lie in studying the role of complex gene-gene and gene-environment interactions in the etiology of ARM. Ideally, increased knowledge on the pathogenesis of ARM will lead to better guidance of patients and their families, more precise estimation of recurrence risks, identification of high-risk groups, and tailored preventive strategies.

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Supplemental Tables

Supplemental Table 1. Studies on associations between anorectal malformations and parental, pregnancy and infant characteristics.

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Maternal age							
Hay and Barbano[58] (1972), USA	ARM cases Register-based controls	Register data	2,044	8,475,600	< 20 years 20-24 years 25-29 years 30-34 years 35-39 years ≥ 40 years	RR 1.0 (0.9-1.1)[a] RR 0.9 (0.8-1.0)[a] RR 1.0 (0.9-1.1)[a] RR 1.0 (0.9-1.1)[a] RR 1.2 (1.0-1.4)[a] RR 1.6 (1.3-2.0)[a]	No
Harris et al.[9] (1995), Sweden, France and USA	Nonsyndromic ARM cases[b] Register-based controls	Medical records and register data	1,310	4,684,204	< 20 years 20-24 years 25-29 years 30-34 years 35-39 years ≥ 40 years	OR 1.1 (0.9-1.4) OR 1.2 (1.1-1.5) OR 0.8 (0.7-0.9) OR 0.9 (0.7-1.0) OR 1.1 (0.9-1.4) OR 1.4 (0.8-2.3)	Program/race, birth year, parity
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Mean maternal age	26.9 vs. 26.1 years, NS	No
Myers et al.[59] (2001), China	ARM cases without anal stenosis and anterior displaced anus	Recorded during home visit	50	222,314	15-24 years 25-29 years ≥ 30 years	RR 1.0 (ref.) RR 1.3 (0.7-2.5) RR 2.9 (1.5-5.7)	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
	Population-based controls						
Forrester and Merz[10] (2002), Hawaii	ARM cases Register-based controls	Register data	124	282,900	< 20 years 20-24 years 25-29 years 30-34 years >= 35 years	RR 0.9 (0.5-1.5) RR 0.7 (0.5-1.1) RR 1.0 (ref.) RR 0.7 (0.4-1.0) RR 0.7 (0.4-1.1)	No
Materna-Kiryluk et al.[54] (2009), Poland	Isolated ARM cases Register-based controls	Questionnaire for physicians	99	902,452	Maternal age (continuous)	OR 0.9 (0.7-1.2)	Paternal age
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior displaced anus[b] Healthy controls	Telephone interviews	464	4,940	≤ 19 years 20-24 years 25-29 years 30-34 years ≥ 35 years	9.7% vs. 11.0% 26.9% vs. 22.3% 20.7% vs. 26.0% 27.4% vs. 26.8% 15.3% vs. 14.0%, overall ρ=0.042	No
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[b] Hospital-based controls	Parental questionnaires	85	650	≤ 34 years ≥ 35 years	OR 0.9 (0.5-1.6)[a]	No
Gill et al.[55] (2012), USA,	Nonsyndromic ARM cases[b]	Telephone interviews	661	8,169	< 20 years 20-24 years	OR 0.9 (0.6-1.2) OR 1.2 (0.9-1.5)	Maternal race or ethnicity, BMI, folic

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
NBDPS	Healthy controls				25-29 years	OR 1.0 (ref.)	acid use, gravidity,
					30-34 years	OR 1.1 (0.9-1.4)	education, smoking,
					35-39 years	OR 1.2 (0.9-1.6)	parental age
					≥ 40 years	OR 1.1 (0.6-2.0)	difference
Carter et al.[30] (2013), USA	Isolated ARM cases Register-based controls	Register data	150	623	< 20 years	OR 0.7 (0.3-1.5)[a]	No
					20-34 years	OR 1.0 (ref.)[a]	
					≥ 35 years	OR 1.2 (0.8-1.8)	
Paternal age							
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Mean paternal age	29.9 vs. 29.2 years, NS	No
Kazaura et al.[56] (2004), Norway	ARM cases Register-based controls	Register data	822	1,738,852	< 20 years	OR 1.4 (0.7-2.6)	Maternal age, parity,
					20-24 years	OR 0.9 (0.7-1.1)	institution, birth year
					25-29 years	OR 1.0 (ref.)	
					30-34 years	OR 0.8 (0.7-1.0)	
					35-39 years	OR 0.8 (0.6-1.1)	
					40-44 years	OR 1.0 (0.7-1.4)	
					45-49 years	OR 0.6 (0.2-1.2)	
					≥ 50 years	OR 0.6 (0.2-1.6)	
Yang et al.[57] (2007), USA	ARM cases Register-based controls	Register data	470	5,213,248	< 20 years	OR 1.2 (0.7-1.8)	Maternal age, race,
					20-24 years	OR 1.0 (0.8-1.3)	education, marital
					25-29 years	OR 1.0 (ref.)	status, prenatal care,
					30-34 years	OR 1.1 (0.8-1.4)	smoking, drinking

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
					35-39 years	OR 1.1 (0.8-1.5)	
					40-44 years	OR 1.2 (0.8-1.8)	
					45-49 years	OR 0.8 (0.3-1.7)	
					≥50 years	OR 1.2 (0.4-2.8)	
Materna-Kiryluk et al.[54] (2009), Poland	Isolated ARM cases Register-based controls	Questionnaire for physicians	99	902,452	Paternal age (continuous)	OR 1.1 (0.9-1.4)	Maternal age
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[b] Hospital-based controls	Parental questionnaires	85	650	≤ 34 years ≥ 35 years	OR 1.0 (0.6-1.6)[a]	No
Parity							
Hay and Barbano[58] (1972), USA	ARM cases Register-based controls	Register data	2,044	8,475,600	Nulliparity	RR 1.0 (1.0-1.2)[a]	No
Harris et al.[9] (1995), Sweden, France, and USA	Nonsyndromic ARM cases[b] Register-based controls	Medical records and register data	1,310	4,684,204	Parity ≥1	OR 0.7 (0.6-1.0)	Program/race, birth year, maternal age
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Nulliparity	46.3% vs. 46.1%, NS	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Myers et al.[59] (2001), China	ARM cases without anal stenosis and anterior displaced anus Population-based controls	Recorded during home visit	50	222,314	Parity ≥1	RR 2.2 (1.2-4.0)	No
Forrester and Merz[10] (2002), Hawaii	ARM cases Register-based controls	Hospital records	124	282,900	Nulliparity	RR 0.9 (0.6-1.2)	No
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior displaced anus[b] Healthy controls	Telephone interviews	464	4,940	Nulliparity	29.7% vs. 29.2%, NS	No
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[b] Hospital-based controls	Parental questionnaires	85	650	Nulliparity	OR 1.6 (1.0-2.6)[a]	No
Duong et al.[60] (2012), USA, NBDPS	Nonsyndromic ARM cases[b] Healthy controls	Telephone interviews	626	7,173	Nulliparity Primiparity Multiparity	OR 1.2 (1.0-1.4) OR 1.0 (ref.) OR 1.0 (0.8-1.3)	Maternal age, race and ethnicity, BMI, education, use of folic acid, smoking,

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
							alcohol use, pregnancy intention, previous fetal loss, gestational hypertension, fertility treatment, study site
Carter et al.[30] (2013), USA	Isolated ARM cases Register-based controls	Register data	150	623	Nulliparity	OR 1.4 (1.0-2.0)[a]	No
Wijers et al.[53] (2013), Europe	Nonsyndromic ARM cases[a] Malformed controls	Register data	1,417	13,371	Nulliparity	OR 1.6 (1.4-1.8)[c]	No confounders identified, except for registry
Multiple birth							
Källén[62] (1986), Sweden	ARM cases Register-based controls	Hospital records	unknown	896,953	Twin pregnancy	RR 1.9 (0.9-3.4)[a]	No
Forrester and Merz[10] (2002), Hawaii	ARM cases Register-based controls	Hospital records	124	282,900	Multiple birth	RR 3.3 (1.4-6.6)	No
Miller et al.[61] (2009), USA,	Nonsyndromic ARM cases without anal	Telephone interviews	464	4,940	Multiple birth	7.6% vs. 3.1%, ρ<0.001	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
NBDPS	stenosis and anterior displaced anus[b] Healthy controls						
Zhang et al.[63] (2011), China	ARM cases Register-based controls	Hospital records	218	545,018	Multiple birth	OR 2.1 (1.2-3.8)	No
Zwink et al.[50] (2012), Germany, CURE-Net	ARM cases Register-based controls	Personal interviews and register data	295	10,069,986	Multiple birth	OR 3.4 (2.3-5.0)[c]	No
Carter et al.[30] (2013), USA	Isolated ARM cases Register-based controls	Register data	150	623	Multiple birth	OR 1.4 (0.5-3.9)[a]	No
Wijers et al.[53] (2013), Europe	Nonsyndromic ARM cases[a] Malformed controls	Register data	1,417	13,371	Multiple birth	OR 1.6 (1.2-2.1)[d]	No confounders identified, except for registry
Preterm delivery							
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Length of gestation <37 weeks	19.4% vs. 21.6%, NS	No
Rasmussen et al.[64] (2001), USA	ARM cases Population-based controls	Hospital records	unknown	264,392	Length of gestation <37 weeks	RR 4.0 (2.5-6.5)[e]	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Forrester and Merz[10] (2002), Hawaii	ARM cases Register-based controls	Hospital records	124	282,900	Length of gestation <38 weeks	RR 3.9 (2.9-5.2)	No
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[b] Hospital-based controls	Parental questionnaires	85	650	Length of gestation <37 weeks	OR 1.6 (0.8-3.2)[a]	No
Low birth weight							
Mili et al.[65] (1991), USA	ARM cases Register-based controls	Register data	111	317,499	Birth weight ≤1499 grams 1500-1999 grams 2000-2499 grams 2500-3999 grams ≥4000 grams	 RR 11.0, ρ<0.05 RR 12.0, ρ<0.05 RR 6.2, ρ<0.05 RR 1.0 (ref.) RR 1.1, NS	No
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Mean birth weight	3.020 vs. 3.306 grams, ρ<0.01	No
Forrester and Merz[10] (2002), Hawaii	ARM cases Register-based controls	Hospital records	124	282,900	Birth weight <3000 grams	RR 2.8 (2.1-3.6)	No
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior	Telephone interviews	464	4,940	Birth weight <2500 grams	25.6% vs. 5.9%, ρ<0.001	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
	displaced anus[b] Healthy controls						
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[b] Hospital-based controls	Parental questionnaires	85	650	Birth weight ≤2500 grams	OR 1.8 (0.9-3.7)[a]	No

ARM, anorectal malformations; NS, not statiscally significant; RR, relative risk; NBDPS, National Birth Defects and Prevention Study; AGORA, Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children; CURE-Net, The German Network for Congenital Uro-REctal Malformations.

[a]Odds ratios and/or confidence intervals were calculated based on data provided in the article or by the authors on request.

[b]ARM cases without chromosomal or single gene disorders.

[c]Similar results were found for subgroups of isolated ARM cases, ARM cases with other anomalies, and ARM cases with the VACTERL association.

[d]For isolated ARM cases: OR= 1.1 (0.7-1.8); for ARM cases with other anomalies: OR= 1.8 (1.2-2.8); for ARM cases with the VACTERL association: OR= 2.5 (1.3-4.7).

[e]Similar results were found for isolated ARM cases only.

Supplemental Table 2. Studies on associations between anorectal malformations and pregnancy complications and medical drug use.

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Pre-pregnancy BMI (in kg/m^2)							
Waller et al.[66] (2007), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	380	3,904	Underweight (BMI <18.5) Normal (18.5≤ BMI <25.0) Overweight (25.0≤ BMI <30.0) Obese (BMI ≥30.0)	OR 0.8 (0.5-1.4) OR 1.0 (ref.) OR 1.2 (0.9-1.6)[b] OR 1.5 (1.1-2.0)[b]	Maternal age, race and ethnicity, educational level, parity, smoking, use of multivitamins containing folic acid
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior displaced anus[a] Healthy controls	Telephone interviews	464	4,940	Underweight (BMI <18.5) Normal (BMI 18.5-24.9) Overweight (BMI 25-29.9) Obese (BMI ≥ 30)	5.2% vs. 6.0% 50.7% vs. 56.6% 23.6% vs. 22.2% 20.5% vs. 15.3%, overall ρ=0.016	No
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Overweight and obesity (BMI ≥25)	OR 1.8 (1.1-2.8)	No confounders identified
Blomberg and Källén[67] (2010),	Nonchromosomal ARM cases	Register data	401	1,049,582	Underweight (BMI <18.5)	OR 1.2 (0.6-2.1)	Maternal age, parity, smoking, birth year

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Sweden	Register-based controls				Normal (BMI 18.5-24.9)	OR 1.0 (ref.)	
					Overweight (BMI 25-29.9)	OR 1.2 (1.0-1.5)	
					Obese class I (BMI 30-34.9)	OR 1.8 (1.3-2.4)	
					Obese class II (BMI 35-39.9)	OR 1.5 (0.7-2.6)	
					Obese class III (BMI ≥40)	OR 3.7 (1.7-7.0)	
Wijers et al.[24] (2010), Germany, CURE-Net[c]	Nonsyndromic ARM cases[a] Hospital-based controls	Parental interviews and questionnaires	79	650	Overweight and obesity (BMI ≥25)	OR 1.0 (0.6-1.7)	No
Diabetes mellitus							
Martínez-Frías[72] (1994), Spain, ECEMC	ARM cases Malformed controls	Personal interviews	196	18,759	Pre-existing diabetes	OR 2.6 (0.6-10.6)[d]	No
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Maternal diabetes	5.5% vs. 2.1%, ρ<0.01	No
Martínez-Frías et al.[75] (1998), Spain, ECEMC	ARM cases Malformed controls	Personal interviews	227	19,577	Gestational diabetes	OR 1.5 (0.6-3.6)	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Correa et al.[68] (2003), USA, Atlanta Birth Defects Case-Control study	Nonsyndromic ARM cases[a] Healthy controls	Telephone interview	88	3,029	Pre-existing diabetes	OR 4.3 (1.5-12.5)	No confounders identified
Frías et al.[69] (2007), Spain, ECEMC	Nonsyndromic ARM cases[a] Malformed controls	Hospital records	432	31,066	Pre-existing diabetes Gestational diabetes	FR 2.8 (1.2-6.7)[d] FR 1.2 (0.7-2.0)[d]	No
Correa et al.[70] (2008), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	430	4,895	Pre-existing diabetes Isolated ARM cases ARM cases with other congenital anomalies Gestational diabetes Isolated ARM cases ARM cases with other congenital anomalies	 OR 4.7 (1.6-14.3) OR 8.2 (3.6-18.7) OR 1.9 (1.0-3.6) OR 1.4 (0.7-2.7)	Maternal age, BMI, education, race/ethnicity, household income, entry into prenatal care, study center
Bánhidy et al.[73] (2010), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal care logbook, medical records, questionnaire	231	38,151	Pre-existing diabetes mellitus type 1 Gestational diabetes	OR 1.9 (0.3-13.6) OR 2.2 (0.7-6.8)	Maternal age, employment status, birth order, maternal hypertension

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Yazdy et al.[76] (2011), USA, BDS	Nonsyndromic ARM cases without sirenomelia or caudal regression[a] Healthy controls	Personal interviews with 99-item Willett Food Frequency Questionnaires	160	704	Dietary glycemic index ≥ 60 Dietary glycemic load ≥ 192	OR 1.5 (1.0-2.5)[b] OR 2.4 (1.1-4.9)[b]	Maternal age, race/ethnicity, income in 2005 US dollars, study center
Parker et al.[77] (2012), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews and Willett food frequency questionnaire	661	6,516	Dietary glycemic index < 49.1 49.1-52.3 52.4-55.6 >55.6	 OR 1.0 (ref.) OR 1.1 (0.8-1.3) OR 1.2 (0.9-1.5) OR 1.4 (1.1-1.8)	Maternal age, race, education, folic acid use, study center
Garne et al.[74] (2012), European countries, EUROCAT	Nonsyndromic ARM cases[a] Nonsyndromic malformed controls	Hospital records	1,093	81,303	Pre-existing diabetes	OR 1.3 (0.7-2.4)	No
Correa et al.[71] (2012), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	511	5,437	Pre-existing diabetes	OR 6.4 (3.5-11.8)	Maternal age, race/ethnicity, BMI, timing of entry into prenatal care, parity, annual household income
Carter et al.[30] (2013), USA	Isolated ARM cases Register-based	Register data	150	623	Gestational diabetes	OR 1.2 (0.5-2.9)[d]	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
	controls						
Wijers et al.[53] (2013), Europe	Nonsyndromic ARM cases[a] Malformed controls	Register data	1,417	13,371	Pre-existing diabetes	OR 0.8 (0.4-1.7)[e]	No confounders identified, except for registry
High blood pressure and preeclampsia							
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Arterial hypertension	10.2% vs. 3.4%, NS	No
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior displaced anus[a] Healthy controls	Telephone interviews	464	4,940	Pre-existing high blood pressure	14.2% vs. 12.7%, NS	No
Bánhidy et al.[78] (2011) / Bánhidy et al.[79] (2012), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	231	38,151	Chronic hypertension without preeclampsia Preeclampsia Preeclampsia with superimposed chronic hypertension	OR 1.6 (0.7-3.6) OR 1.0 (0.4-2.2) OR 3.7 (1.6-8.5)	Maternal age, birth order, employment status, related drug treatments, use of folic acid
Wijers et al.[53] (2013), Europe	Nonsyndromic ARM cases[a] Malformed controls	Register data	1,417	13,371	Preeclampsia	OR 2.2 (1.2-4.0)	No confounders identified, except for registry

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Fever, colds and infections							
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Fever during pregnancy Flu during pregnancy	7.4% vs. 8.1%, NS 6.5% vs. 6.9%, NS	No
Ács et al.[80] (2005) / Ács et al.[81] (2006) / Ács et al.[82] (2006), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Influenza[f] Common cold[f] Acute respiratory infections[f]	OR 0.7 (0.2-3.0) OR 1.4 (0.4-4.5)[g] OR 1.8 (0.8-4.1)	Maternal age, birth order, employment status, influenza or common cold, other acute diseases, use of supplements, use of antifever drugs
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Fever during first trimester	OR 5.1 (0.9-28.1)	Maternal multivitamin use
Wijers et al.[24] (2010), Germany, CURE-Net[c]	Nonsyndromic ARM cases[a] Hospital-based controls	Parental interviews and questionnaires	79	650	Fever during first trimester	OR 10.6 (2.8-40.4)	No
Wijers et al.[53] (2013), Europe	Nonsyndromic ARM cases[a] Malformed controls	Register data	1,417	13,371	Fever during the first 4 months of pregnancy	OR 2.2 (0.8-5.7)	No confounders identified, except for registry and year of birth

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Other pregnancy complications							
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Epilepsy	1.8% vs. 0.4%, NS	No
Tamási et al.[86] (2006), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Bronchial asthma	OR 1.0 (0.3-3.4)	Maternal age, birth order, employment status
Ács et al.[87] (2007), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Glomerulonephritis during first trimester of pregnancy	OR 0.7 (0.2-2.9)[d]	No
Browne et al.[83] (2009), USA, NBDPS	Nonsyndromic ARM cases Healthy controls	Telephone interviews	534	5,875	Any chronic thyroid disorder	OR 1.6 (0.9-2.7)[b]	Maternal age, state of residence, race/ethnicity
Ács et al.[84] (2009) / Bánhidy et al.[85] (2011), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Severe chronic dyspepsia Primary peptic ulcer disease[f]	OR 4.3 (1.7-10.5) OR 5.9 (1.5-28.4)	Maternal age, birth order, drug use for severe chronic dyspepsia, drug use for peptic ulcer disease, folic acid/multivitamin supplementation

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Wijers et al.[53] (2013), Europe	Nonsyndromic ARM cases[a] Malformed controls	Register data	1,417	13,371	Chronic lower obstructive pulmonary disease Epilepsy Mental disorder	OR 1.4 (0.8-2.3)[e] OR 5.1 (1.7-15.6) OR 1.3 (0.4-4.3)	No confounders identified, except for registry
Louik et al.[88] (2013), USA, BDS	ARM cases Healthy controls	Telephone interviews and vaccine records	47	951	Pandemic H1N1 influenza vaccination	OR 0.7 (0.3-2.0)	Propensity score using several parental factors
Analgesic and antipyretic medication use							
Czeizel et al.[89] (2000), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Kalmopyrin, istopyrin or aspirin (500 mg)[f]	OR 1.2 (0.7-2.8)[g]	Maternal age, birth order, disorders, other drug use
Feldkamp et al.[90] (2010), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	un-known	4,500	Acetaminophen[h]	OR ~ 1.0, NS	Maternal age, race or ethnicity, education, BMI, gestational diabetes, fever, smoking, use of folic acid, parity
Broussard et al.[91] (2011), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	623	6,701	Opioid analgesics[i] Codeine[i] Hydrocodone[i]	OR 1.5 (0.9-2.4)[b] OR 1.3 (0.5-3.2) OR 1.8 (0.8-4.0)	Maternal age, race/ethnicity, education, BMI,

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
							smoking, study center
van Gelder et al.[92] (2011), Norway, MoBa	Nonsyndromic ARM cases[a] Population-based controls	Maternal questionnaires	16	65,287	Prescribed and over-the-counter non-steroidal anti-inflammatory drugs during first 12 weeks of gestation	OR 1.4 (0.2-10.7)	No
Antimicrobial and antibiotic medication use							
Czeizel et al.[93] (2001), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Nitrofurantoin[f]	OR 2.3 (0.6-9.3)	Maternal age, birth order, disorders, other drug use
Czeizel et al.[94] (2001), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Matched healthy controls	Antenatal logbook, medical records, questionnaire	220	220	Ampicillin[f]	OR 1.2 (0.2-7.4)	No
Crider et al.[95] (2009), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	470	4,941	Any antibacterial[i] Penicillins[i] Erythromycins[i] Nitrofurantoins[i] Sulfonamides[i]	OR 1.0 (0.7-1.3) OR 0.8 (0.5-1.2) OR 1.0 (0.4-2.1) OR 1.1 (0.4-3.0) OR 1.0 (0.4-2.9) OR 1.6	Maternal age, race, education, BMI, time from date of delivery to interview, use of folic acid, smoking,

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
					Cephalosporins[i]	(0.7-3.5)	alcohol use
Antifungal medication use							
Czeizel et al.[103] (1998), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	160	30,663	Metronidazole[f]	OR 3.4 (1.1-10.6)	Maternal age, birth order, threatened abortion and preterm birth, disorders
Czeizel et al.[105] (1999), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	173	32,804	Clotrimazole[f]	OR 1.8 (0.3-11.6)	Maternal age, birth order, diseases, use of other drugs
Kazy et al.[104] (2005), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Combination of metronidazole and miconazole[f]	OR 8.8 (0.9-82.5)	Maternal age, birth order, employment status
Carter et al.[106] (2008), USA, NBDPS	Nonsyndromic isolated ARM cases[a] Healthy controls	Telephone interviews	199	4,565	Antifungal drugs[h]	OR 1.4 (0.7-3.1)	Maternal education, BMI
Psychotropic medication use							
Bonnot et al.[107] (2001), France, Italy, Japan,	ARM cases Malformed controls	Register data	un-known	13,703	Lorazepam[h]	OR 6.2 (2.4-15.7)	Maternal age, parity

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Brazil, Australia, Israel							
Eros et al.[108] (2002) / Czeizel et al.[109] (2003) / Czeizel et al.[110] (2004), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Five benzodiazepines combined[f] Diazepam[f] Chlordiazepoxide[f]	OR 10.4 (1.3-79.8) OR 5.2 (1.4-19.7) OR 3.0 (0.1-73.7)	Maternal age, birth order, disorders, use of other drugs
Kjær et al.[111] (2007), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	220	38,151	Phenytoin, phenobarbital, or diazepam[f]	OR 1.6 (0.7-3.6)	Exposure time trend[k]
Källén and Otterblad Olausson[96] (2007), Sweden[l]	ARM cases Register-based controls	Register data	495	860,215	Selective serotonin re-uptake inhibitors in early pregnancy	RR 0.6 (0.1-2.0)	No
Alwan et al.[97] (2007), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	418	4,092	Selective serotonin re-uptake inhibitors[i]	OR 0.7 (0.3-1.8)	Maternal race or ethnicity, obesity, smoking, family income
Reis and Källén[98] (2010), Sweden[l]	Nonchromosomal ARM cases	Register data	428	1,236,053	Any antidepressant[h]	RR 1.1 (0.4-2.4)	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
	Register-based controls						
Browne et al.[101] (2013), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	752	8,373	Any butalbital use	OR 3.0 (0.7-9.4)[m]	No
Other medication use							
Källén and Otterblad Olausson[112] (2007), Sweden	ARM cases Register-based controls	Register data	495	873,876	Anti-asthmatics in early pregnancy	OR 1.7 (1.1-2.6)	Birth year, maternal age, parity, smoking, number of previous miscarriages
Gilboa et al.[99] (2009), USA, NBDPS	Nonsyndromic isolated ARM cases[a] Healthy controls	Telephone interviews	223	4,982	Any antihistamines[i] Diphenhydramine[i] Doxylamine[i] Fexofenadine[i] Loratadine[i] Pheniramine[i] Promethazine[i]	OR 0.7 (0.4-1.2) OR 0.7 (0.2-2.1) OR 0.8 (0.3-2.5) OR 1.4 (0.4-4.8) OR 1.8 (0.8-3.8) OR 0.4 (0.1-1.5) OR 0.5 (0.2-1.4)	Maternal age, race or ethnicity, education, entry into prenatal care, parity, household income, study center
Browne et al.[83] (2009), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	534	5,875	Thyroxine[i] Thyreostatic medication[i]	OR 1.4 (0.7-2.6)[n] OR 8.6 (1.7-40.2)	Maternal age, state of residence, race/ethnicity No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Lisi et al.[114] (2010), Europe, Japan, and South America	Isolated ARM cases Malformed controls[o]	Maternal interview, pharmacy and/or prenatal records	155	18,131	2-amino-1-phenylethanol derivatives[h]	OR 2.0 (1.1-3.9)[p]	No
Bánhidy et al.[100] (2010), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Healthy controls	Antenatal logbook, medical records, questionnaire	231	38,151	Pholedrine[f]	OR 1.8 (0.3-9.7)	Maternal age, birth order, socioeconomic status, hypotension, folic acid use
Lin et al.[113] (2012), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	642	6,726	Anti-inflammatory[i] Bronchodilator[i] Both anti-inflammatory and bronchodilator[i]	OR 1.3 (0.7-2.3)[j] OR 0.9 (0.5-1.6)[b] OR 1.1 (0.4-3.0)	Maternal age, race/ethnicity, education, alcohol use, smoking, folic acid use, fever in first trimester, gender
Yau et al.[102] (2013), USA, BDS	Nonsyndromic ARM cases[a] Healthy controls	Personal and telephone interviews	274	7,606	Pseudoephedrine only[h]	OR 1.3 (0.8-2.3)	No confounders identified

ARM, anorectal malformations; BMI, body mass index; NBDPS, National Birth Defects and Prevention Study; OR, odds ratio; AGORA, Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children; CURE-Net, The German Network for Congenital Uro-REctal Malformations; ECEMC,

Spanish Collaborative Study of Congenital Malformations; FR, frequency ratio; HCCSCA, Hungarian Case-Control Surveillance of Congenital Malformations; BDS, Slone Epidemiology Center Birth Defects Study; NS, not statistically significant; RR, relative risk; MoBa, Norwegian Mother and Child Cohort Study.

[a] ARM cases without chromosomal or single gene disorders.

[b] Similar results were found for isolated ARM cases only.

[c] Replication study of results found by van Rooij *et al.* (2010), using Dutch controls.

[d] Odds ratios and/or confidence intervals were calculated based on data provided in the article or by the authors on request.

[e] Similar results were found for subgroups of isolated ARM cases, ARM cases with other anomalies, and ARM cases with the VACTERL association.

[f] During the second and/or third month of pregnancy.

[g] Results shown for medically recorded data only.

[h] In first trimester of pregnancy.

[i] In 1 month before conception through the end of the first trimester of pregnancy.

[j] For isolated ARM cases only: OR=2.1 (1.1-4.1).

[k] Case-time-control design was used.

[l] Both studies used the same data from 1995-2004. Reis and Källén (2010) also used additional data from 2005-2007, but excluded chromosomal anomalies.

[m] If other medications used to treat migraines were excluded OR=4.1 (1.0-13.8).

[n] For isolated ARM cases only: OR=2.5 (1.2-5.1).

[o] Controls were all exposed to any medication during pregnancy.

[p] OR with 99% confidence interval.

Supplemental Table 3. Studies on associations between anorectal malformations and parental occupational and lifestyle factors.

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Parental occupational exposures							
Schnitzer et al.[118] (1995), USA, Atlanta Birth Defects Case-Control study	ARM cases Healthy controls	Telephone interview	70	2,279	Paternal carpenters and woodworkers	OR 2.4 (0.7-8.5)	No confounders identified
					Paternal electricians and electrical workers	OR 1.7 (0.6-5.0)	
					Paternal printers	OR 2.9 (0.8-10.2)	Maternal age, education
					Paternal policemen and guards	OR 2.9 (0.8-9.9)	
					Paternal vehicle manufacturers	OR 5.1 (1.3-19.2)	
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Maternal job exposure to:		Family history of ARM, maternal BMI, multivitamin use, paternal smoking, paternal job exposure to exhaust fumes[c]
					X-rays	OR 0.6 (0.1-2.6)	
					Cytostatics	OR 1.5 (0.3-6.9)[b]	
					Industrial cleaning agents and solvents	OR 2.9 (0.9-9.3)	
					Paternal job exposure to:		
					Exhaust fumes	OR 1.9 (1.0-3.6)	
					Industrial cleaning agents and solvents	OR 0.6 (0.2-1.7)	

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
					Paint, varnish, adhesives, ink, thinner	OR 1.4 (0.6-3.7)	
					Welding fumes	OR 1.3 (0.5-3.3)	
Wijers et al.[24] (2010), Germany, CURE-Net[d]	Nonsyndromic ARM cases[a] Hospital-based controls	Parental interviews and questionnaires	79	650	Maternal job exposure to industrial cleaning agents and solvents	OR 7.4 (3.1-17.7)	No
					Paternal job exposure to exhaust fumes	OR 0.5 (0.2-1.4)	
Herdt-Losavio et al.[116] (2010), USA, NBDPS[e]	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	328	3,383	Maternal janitors or cleaners	OR 1.8 (1.1-3.1)	Maternal age, BMI, parity, folic acid use, smoking, alcohol use, study center
					Maternal scientists	OR 2.4 (1.2-4.6)	
Lin et al.[117] (2012), USA, NBDPS[e]	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	>328	>3,383	Maternal janitors	OR 3.8 (1.5-9.3)	Maternal age, education, BMI, race or ethnicity, pre-gestational diabetes, folic acid intake, smoking, alcohol use, parity, study center
Desrosiers et al.[119] (2012), USA,	Nonsyndromic ARM cases[a]	Telephone interviews	366	4,066	Paternal scientists	OR 0.7 (0.5-0.9)	Maternal age, education, race or
					Paternal artists	OR 8.8 (0.9-660)	

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
NBDPS	Healthy controls				Paternal shippers	OR 0.6 (0.4-0.9)	ethnicity, use of folic acid or vitamins, smoking, alcohol use, study center
Parental smoking							
Shiono et al.[120] (1986), USA, Kaiser Birth Defects Study	ARM cases Population-based controls	Maternal questionnaire	14	33,434	Maternal smoking in first 3 months of pregnancy	OR 0.4 (0.1-1.9)	No
van den Eeden[121] (1990), USA	ARM cases Healthy controls	Register data	35	4,500	Maternal smoking[f]	OR 0.9 (0.4-2.2)	Maternal age, parity
Yuan et al.[122] (1995), Japan	ARM cases Healthy controls	Register data	84	174	Maternal smoking[f] Paternal smoking[f]	OR 1.3 (0.6-2.9)[g,h] OR 1.2 (0.7-2.0)[g,h]	No
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Maternal smoking[f]	26.8 vs. 21.6%, NS	No
Källén[124] (2000), Sweden	Nonchromosomal ARM cases Register-based controls	Register data	410	1,413,811	Maternal smoking[f]	OR 1.3 (1.0-1.6)	Birth year, maternal age, parity, education
Honein et al.[123] (2001), USA	ARM cases Register-based controls	Register data	564	6,161,506	Maternal smoking[f]	PR 1.2 (0.9-1.5)	Maternal age, education, race/ethnicity

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior displaced anus[a] Healthy controls	Telephone interviews	464	4,940	Maternal smoking[j] Exposure to environmental tobacco smoke (at home or work)[i]	OR 1.2 (1.0-1.5)[j] OR 1.3 (1.0-1.6)[j]	No confounders identified
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Maternal smoking[f] Paternal smoking[k]	OR 0.8 (0.5-1.3) OR 1.8 (1.1-2.9)	No confounders identified
Wijers et al.[24] (2010), Germany, CURE-Net[d]	Nonsyndromic ARM cases[a] Hospital-based controls	Parental interviews and questionnaires	79	650	Paternal smoking[k]	OR 1.0 (0.6-1.6)	No
Carter et al.[30] (2013), USA	Isolated ARM cases Register-based controls	Register data	150	623	Maternal smoking[f]	OR 1.5 (0.9-2.5)[g]	No
Parental alcohol use							
Yuan et al.[122] (1995), Japan	ARM cases Healthy controls	Register data	84	174	Maternal alcohol use[f]	OR 1.5 (0.7-3.2)[g,h]	No
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Maternal alcohol use	5.5% vs. 4.9%, NS	No

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior displaced anus[a] Healthy controls	Telephone interviews	464	4,940	Maternal alcohol use[i]	OR 0.9 (0.8-1.1)[h]	No confounders identified
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Maternal alcohol use[f] Paternal alcohol use[k]	OR 1.0 (0.6-1.5) OR 1.3 (0.7-2.5)	No confounders identified
Maternal illicit drug use							
van Gelder et al.[125] (2009), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	468	4,967	Cannabis use[i] Cocaine use[i] Stimulant use[i]	OR 0.7 (0.4-1.2) OR 0.4 (0.1-2.7) OR 1.1 (0.3-3.8)	Maternal age, race or ethnicity, educational level, smoking, binge drinking, BMI, folic acid use[c]
Maternal caffeine intake							
Miller et al.[61] (2009), USA, NBDPS	Nonsyndromic ARM cases without anal stenosis and anterior displaced anus[a] Healthy controls	Telephone interviews	464	4,940	Caffeine intake per day in year before pregnancy <10 mg 10-99 mg	 OR 1.0 (ref.)[h] OR 1.4 (1.0-1.9)[h] OR 1.3 (1.0-1.8)[h]	No confounders identified

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
					100-299 mg ≥300 mg	OR 1.5 (1.0-2.2)[h]	
Folic acid or multivitamin use							
Czeizel et al.[126] (1996), Hungary, HCCSCA	Nonsyndromic isolated ARM cases[a] Matched healthy controls	Antenatal logbook, medical records, questionnaire	149	30,663	Use of folic acid supplements[f]	OR 0.8 (0.5-1.2)	No
Myers et al.[59] (2001), China	ARM cases without anal stenosis and anterior displaced anus Population-based controls	Recorded during home visit	50	222,314	Any folic acid use of 400 ug[l]	RR 0.6 (0.3-1.1)	Maternal age
Correa et al.[68] (2003), USA, Atlanta Birth Defects Case-Control Study	Nonsyndromic ARM cases[a] Healthy controls	Telephone interview	88	3,029	Regular multivitamin use (at least 3 times a week)[m]	OR 0.9 (0.5-1.7)	No confounders identified
Källén[127] (2007), Sweden	Nonchromosomal ARM cases Register-based controls	Register data	493	873,876	Use of folic acid in early pregnancy	OR 1.0 (0.6-1.8)	Birth year, maternal age, parity, smoking, number of previous miscarriages

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Folic acid use before and during pregnancy	OR 1.0 (0.6-1.7)[n]	No confounders identified
					Multivitamin use before and during pregnancy	OR 1.6 (1.0-2.7)[n]	
Correa et al.[71] (2012), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	511	5,437	Any use of vitamins or supplements containing folic acid[h]	OR 0.8 (0.6-1.0)[g]	No
Other parental lifestyle factors							
Stoll et al.[22] (1997), France	ARM cases Healthy controls	Hospital records	108	unknown	Living in urban area	38.9% vs. 45.0%, NS	No
					Living in rural area	61.1% vs. 55.0%, NS	
					X-rays during pregnancy	2.8% vs. 3.3%, NS	
Castilla et al.[128] (1999), South America	Isolated ARM cases Healthy controls	Register data	335	52,379	Altitude higher than 2,000 m above sea level	OR 1.6 (1.0-2.6)	Ethnicity, type of public health system, low birth weight
van Rooij et al.[12] (2010), The Netherlands, AGORA	Nonsyndromic ARM cases[a] Hospital-based controls	Parental questionnaires	85	650	Paternal overweight or obesity (BMI ≥25 kg/m^2)	OR 0.8 (0.5-1.3)	No confounders identified
Duong et al.[129] (2011), USA,	Nonsyndromic ARM cases[a]	Telephone interviews	626	6,795	Any use of hot tub during first trimester	OR 0.8 (0.5-1.2)	Race or ethnicity, education

First author (year), country, study name	Population	Method of data collection	No. cases	No. controls	Exposure	Results	Confounder adjustment
NBDPS	Healthy controls						
Tinker et al.[131] (2011), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	579	6,328	Maternal injury[i]	OR 1.7 (1.0-2.7)[h]	Maternal age, race or ethnicity, educational attainment, BMI, household income, alcohol use, smoking, working outside of the home, study site
Agopian et al.[130] (2013), USA, NBDPS	Nonsyndromic ARM cases[a] Healthy controls	Telephone interviews	659	6,826	Swimming pool use[i]	OR 1.1 (0.8-1.3)	Maternal age, race or ethnicity, education, parity, BMI, household income, smoking, folic acid use, surveillance site, season of conception

ARM, anorectal malformations; OR, odds ratio; AGORA, Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children; CURE-Net, The German Network for Congenital Uro-REctal Malformations; NBDPS, National Birth Defects and Prevention Study; NS, not statistically significant; PR, prevalence ratio; HCCSCA, Hungarian Case-Control Surveillance of Congenital Malformations; RR, relative risk.

[a] ARM cases without chromosomal or single gene disorders.

[b] For ARM cases with the VACTERL-association only: OR=10.2 (2.0-51.8).

[c] These factors were not identified as confounder for every association (based on 10% change of the OR and a minimum number of exposed cases).

[d] Replication study of results found by van Rooij *et al.* (2010), using Dutch controls.

[e] Both studies used the same data from 1997-2003. Lin *et al.* (2012) also used additional data from 2004 and divided cleaners in subgroups.

[f] Exposure during pregnancy.

[g] Odds ratios and/or confidence intervals were calculated based on data provided in the article or by the authors on request.

[h] Similar results were found for isolated ARM cases only.

[i] Exposure in 1 month before conception through the end of the first trimester of pregnancy.

[j] For isolated ARM cases only, the associations disappeared.

[k] Exposure in 3 months before conception.

[l] Use from premarital examination through the end of the first trimester of pregnancy.

[m] Use in 3 months before conception through the end of the first trimester of pregnancy.

[n] Similar results were found for ARM cases with the VACTERL association only.

Chapter 3

Research perspectives in the etiology of congenital anorectal malformations using data of the International Consortium on Anorectal Malformations: Evidence for risk factors across different populations

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Abstract

Purpose The recently established International Consortium on Anorectal Malformations aims to identify genetic and environmental risk factors in the etiology of syndromic and nonsyndromic anorectal malformations (ARM) by promoting collaboration through data sharing and combined research activities.

Methods The consortium attempts to recruit at least 1,000 ARM cases. DNA samples are collected from case-parent triads to identify genetic factors involved in ARM. Several genetic techniques will be applied, including SNP arrays, gene and whole exome sequencing, and a genome-wide association study. Questionnaires inquiring about circumstances before and during pregnancy will be used to obtain environmental risk factor data.

Results Currently, 701 ARM cases have been recruited throughout Europe. Clinical data are available from all cases, and DNA samples and questionnaire data mainly from the Dutch and German cases. Preliminary analyses on environmental risk factors in the Dutch and German cohort found associations between ARM and family history of ARM, fever during first trimester of pregnancy, and maternal job exposure to cleaning agents and solvents.

Conclusion First results show that both genetic and environmental factors may contribute to the multifactorial etiology of ARM. The International Consortium on Anorectal Malformations will provide possibilities to study and detect important genes and environmental risk factors for ARM, ultimately resulting in better genetic counseling, improved therapies, and primary prevention.

Introduction

Congenital anorectal malformations (ARM) are rare disorders, occurring in approximately 1 in 5000 to 1 in 1500 live births worldwide.[1] These disorders usually require surgical interventions in the neonatal period and postoperative follow-up and treatment to obtain and maintain fecal and urinary continence. Sequelae of ARM continue into adulthood and may include fecal incontinence and sexual dysfunction. All these issues cause major concerns for parents and children at school age and in later life, and pose a large social problem connected with acceptance of the population suffering from these problems. Clinical outcomes of ARM, such as functional stooling problems, are found to be directly associated with poor quality of life in ARM patients.[2,3] These outcomes often deteriorate with age, which emphasizes the need for long-term follow-up and psychosocial treatment.[2,3] As experience concerning ARM is scattered in many different hospitals in most European countries,[4] regional differences in clinical outcome and quality of life have been reported. Moreover, case comparison is difficult, as a detailed diagnosis according to Krickenbeck is often not stated in discharge letters.[5]

Despite the great physical and social burden on ARM patients and their parents, the etiology is largely unknown. In rare cases, ARM can be explained by chromosomal abnormalities or is part of a well-known syndrome. Townes-Brocks syndrome, comprising ear, limb, anal, renal, and heart anomalies, is caused by mutations in the *SALL1* gene,[6,7] whereas mutations in the homeobox gene *HLXB9* lead to Currarino syndrome, a combination of sacral agenesis, presacral mass, and ARM.[8-10] However, clinical manifestations of syndromic forms of ARM may vary and genetics and underlying mechanisms remain elusive. The majority of ARM cases are not part of a syndrome and their etiology is assumed to be multifactorial with a causal interplay of genetic and environmental factors. Evidence for genetic factors involved in the anorectal development is mainly derived from syndromic ARM and animal studies rather than from studies on nonsyndromic ARM. In addition to genetic factors, an essential role is expected for environmental factors as ARM rarely aggregates in families.[11] Some researchers studied environmental hazards and found associations between ARM and maternal alcohol intake,[12] tobacco smoke and caffeine,[13] the benzodiazepine lorazepam,[14] paternal exposure to occupational hazards,[15,16] and folic acid supplementation.[17] In addition to these factors, increased risks were found after in vitro fertilization.[18-20] However, strong evidence is still scarce as most potential risk factors were found in only one study and the majority of the studies had low power.

To summarize, large scale studies on genetic and environmental risk factors with human ARM cases have not yet been performed, but could provide substantial clues on the pathogenesis of ARM. ARM would benefit from prevention, accurate diagnosis, and outlined

clinical treatment. Clinical research on regional differences in clinical outcome and quality of life may contribute to improvements in patient care. Recently, the International Consortium on Anorectal Malformations, consisting of the Dutch AGORA-project (Aetiologic research into Genetic, Occupational and environmental Risk factors for Anomalies in children), the German CURE-NET (German Network for Congenital Uro-REctal Malformations), the Italian MUGAR-Net (Network for Uro-Genito-Ano-Rectal malformations in Italy), and the French MAREP (National Center for AnoRectal Malformations and rare Pelvic Anomalies), has been established for etiologic and clinical research. Our multidisciplinary consortium of geneticists, epidemiologists, and clinician-scientists strongly believes that a fundamental prerequisite for progress in the field of rare developmental disorders, such as ARM, will be to strengthen the ties between clinical and basic research activities. In this report, our mission and specific research goals regarding the pathobiology of ARM will be addressed. In addition, the first results on environmental risk factors from the Dutch and German ARM cohort will be presented.

Methods

Consortium on Anorectal Malformations

Researchers from the participating countries initiated the establishment of an International Consortium on Anorectal Malformations. Our common goal is to collaborate and exchange knowledge on a research topic that is still in its infancy. We aim to perform high quality etiologic research on ARM and to standardize diagnostic classifications and clinical outcomes of ARM patients throughout Europe. The consortium will create a registry of ARM patients, which will serve as a base for all research on ARM. This registry is essential for etiologic research, which needs adequately registered diagnoses of ARM patients, as different phenotypes of ARM may originate from different pathogenic processes.

At the current stage of research, more and more emphasis is placed on large numbers of patients and development of biobanks for both rare and frequently occurring disorders. Therefore, the participants in this consortium consider it of utmost importance to collect a critical mass of clinical data and biomaterials from children affected by ARM and their parents in a collaborative effort. The international collaboration will create case series of patients with data and biomaterials that would be hard to achieve on a national scale in any country. We aim to recruit at least 1,000 ARM cases within the countries participating in the consortium. Blood or saliva samples are collected from case-parent triads for genetic analyses. Questionnaires inquiring about circumstances three months before and during pregnancy will be used to obtain environmental risk factor data from the parents. Potential environmental risk factors are those that are capable of affecting the formation of oocytes

and spermatozoa and/or may influence the development of the embryo. A special database for questionnaire and clinical data will be set up that can be used for data entry in all participating countries. Professional data management will facilitate ongoing clinical collaborative projects ensuring data quality and compatibility. The following paragraphs describe the existing projects of patient and data collection in the currently participating countries of the consortium.

AGORA - The Netherlands

AGORA (Aetiologic research on Genetic, Occupational and environmental Risk factors for Anomalies in children) started at the Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, The Netherlands in 2005. Several disciplines collaborate in AGORA, namely genetic, epidemiologic, and pediatric departments, with the common aim to study the role of genetic and environmental risk factors in the etiology of a variety of congenital malformations, including ARM. Blood or saliva samples and environmental data are routinely collected from case-parent-triads during diagnostic or surgical procedures. The ideal control group, which consists of a random sample of the Dutch population in the relevant age group, will also be recruited to perform research on all congenital malformation patient groups within AGORA.

CURE-NET - Germany

The German Network for Congenital Uro-REctal Malformations (CURE-Net) represents the first systematic approach in Germany to investigate the molecular causes of congenital uro-rectal malformations, employing an integrated network structure that involves basic research disciplines, and to establish the first prospective multicenter study into the clinical implications and psychosocial outcome of these disorders. CURE-NET was founded in 2008 with federal funding by the German Federal Ministry of Education and Research (BMBF) starting in 2009. To ensure efficient recruitment of patients and their families, central recruitment has been established with all participating families being assessed in person by one of four physicians specialized in clinical genetics and/or pediatric surgery. DNA samples of each participating individual are stored in a central DNA biomaterial bank (DNA-BMB). The phenotypic information is documented in a nationwide register.

MUGAR-Net - Italy

The MUGAR-Net project (Network for Uro-Genito-Ano-Rectal malformations in Italy) is an ongoing project that does not exist in Italy at the moment, which aims to set up an Italian registry for urogenital and anorectal malformations. The registry will allow us to perform

epidemiological studies, make the healthcare workers aware of the problem, collect blood and saliva samples from affected patients in a biobank, and finally establish cooperation with other international centers.

MAREP - France

The national center for AnoRectal Malformations and rare Pelvic anomalies (MAREP) was created in 2007 as part of the National French Program for Rare Disease (2005–2008). Its main objectives are to develop a French registry gathering anatomical and clinical data, to edit guidelines for the optimal management and follow-up of patients with ARM, and to diffuse information to patients, families, and medical actors to ensure equality for diagnostics, treatment, and care. Genetic diseases screening, research development and European collaborations form a large part of our missions as well. Data are collected and recorded online in a database called CEMARA (for CEntre MAladies RAres), which interacts with the Orphanet database. Blood samples are already collected in syndromic forms and collection will be extended to all enrolled patients. This center is also the national referent for Currarino syndrome molecular sequencing, and 90 index cases have been recorded. MAREP currently collects about 50–60 new ARM cases per year and is thus ready to participate in the consortium.

Research possibilities

The substantial amount of data that will be obtained within the consortium offers many possibilities for ARM research. Due to rapid developments in the genetic field, existing techniques in performing genetic research have also changed rapidly. SNP arrays and new promising gene sequencing techniques are appropriate in performing research regarding syndromic forms of ARM. Identification of genes mediating syndromic forms of ARM may also provide suggestions for the role of genetic factors in the etiology of more isolated forms of ARM. At the current stage, however, indications for specific candidate genes involved in the more isolated forms of ARM are nearly absent. Therefore, hypothesis-free research on nonsyndromic ARM seems to be most appropriate. We intend to perform a genome-wide association study (GWAS) in a large cohort of more isolated nonsyndromic ARM cases. In this hypothesis-generating approach, the whole genome will be scanned to identify potential SNPs involved in nonsyndromic ARM. To identify specific environmental risk factors, questionnaire data will be investigated. Since this will be a unique and relatively large cohort of ARM cases, identification of environmental risk factors seems likely. In a GWAS with 1,000 cases and controls and assuming 80% power, main genetic effects ≥ 1.5 can be identified for

genes with an allele frequency > 0.05. For environmental factors with an exposure prevalence of 0.1, main effects ≥ 1.5 can also be detected.

Dutch and German studies on environmental risk factors

In our first Dutch case-control study among 85 nonsyndromic ARM cases and 650 controls, potential environmental risk factors for ARM were studied.[21] ARM cases with chromosomal anomalies, known syndromes, cloaca, and cloacal exstrophies were excluded. Parents of these cases and controls filled out questionnaires, concerning exposures just before and during pregnancy. Recently, we tried to replicate the findings of the Dutch case-control study in 79 nonsyndromic ARM cases from the German CURE-Net. Due to the close geographic proximity and the assumed analogy in ethnicity, controls were used from the Dutch case-control study. Dutch and German questionnaires were nearly similar with regard to the environmental risk factors studied, apart from time to pregnancy (TTP), which was only included in the Dutch questionnaire.

Results

Data collection within the International Consortium on Anorectal Malformations

A relatively large amount of data has already been collected in Europe during the past few years. Currently, approximately 700 ARM cases have been recruited: 275 cases in the Netherlands, 172 cases in Germany, 140 cases in Italy, and 114 cases in France. Clinical data are available for all these cases. The members of the consortium are currently harmonizing their case reports to combine these data. As Italy and France had just started data collection, DNA samples and questionnaire data were mainly collected from ARM cases in the Netherlands and Germany (Table 1). Some other potential (non-)European collaborators may also contribute data to increase the total number of ARM cases in the future.

Table 1. Collected data of ARM cases per country.

	Number of collected data of ARM cases			Intend to collect in total[b]
	Clinical data	DNA samples	Environmental data	
Netherlands (Nijmegen)	275	275	220	300
Germany (Bonn and Heidelberg)	172	131	156	300
Italy (Padua)	140	20	10	200
France (Paris)	114	50	114[a]	200
Total	**701**	**476**	**500**	**1,000**

[a]Partial questionnaire data.

[b]Minimum.

Results of Dutch case-control study

In our Dutch case-control study among 85 nonsyndromic ARM cases, we found associations between ARM and family history of ARM, time to pregnancy > 6 months, pre-pregnancy BMI ≥ 25 kg/m^2, fever during first trimester of pregnancy, maternal job exposure to industrial cleaning agents and solvents, paternal smoking, and paternal job exposures to exhaust fumes (Table 2).[21]

Table 2. Summary of results of our Dutch case-control study on risk factors of ARM.[21]

Potential risk factors	**ARM (n=85) No. (%)**	**Controls (n=650) No. (%)**	**Adjusted OR (95% CI)**
Family history of ARM 1st and 2nd degree	6 (7.2)	1 (0.2)	33.9 (2.9–398)
Time to pregnancy > 6 months	26 (32.9)	135 (21.4)	1.8 (1.1–3.0)
Maternal risk factors			
BMI before pregnancy ≥ 25 kg/m^2	32 (38.1)	160 (26.0)	1.8 (1.1–2.8)
Fever 1st trimester pregnancy (> 38°C)	3 (3.7)	4 (0.6)	5.1 (0.9–28.1)
Maternal job exposure to industrial cleaning agents and solvents	5 6.0)	12 (1.9)	2.9 (0.9–9.3)
Paternal risk factors (3 months prior to conception)			
Paternal smoking of cigarettes	41 (50.0)	210 (35.7)	1.8 (1.1–2.9)
Paternal job exposure to exhaust fumes	14 (17.3)	59 (10.0)	1.9 (1.0–3.6)

[a]Results derived from the article in Birth Defects Res A Clin Mol Teratol.[21]

Preliminary results of the German CURE-Net

The Dutch results were reanalyzed with 79 ARM cases of the German CURE-Net study. These cases were classified according to Krickenbeck.[5] The most frequently occurring diagnoses were perineal fistula (total 19.0%; 9.3% in boys and 30.5% in girls), vestibular fistula in girls (36.1%), and rectourethral fistula in boys (51.1%). The preliminary results showed associations between ARM and family history of ARM, fever during first trimester of pregnancy, and maternal job exposure to industrial cleaning agents and solvents (Table 3). No associations were found between ARM and BMI before pregnancy ≥ 25 kg/m^2, paternal smoking, and paternal job exposure to exhaust fumes. These results were not adjusted for confounders.

Table 3. Replication of results on environmental risk factors for ARM using German ARM cases.

Potential risk factors	German ARM (n=79) No.(%)	Dutch Controls (n=650) No. (%)	Crude OR (95% CI)
Family history of ARM 1st and 2nd degree	6 (7.6)	1 (0.2)	49.0 (5.8–412.6)
Maternal risk factors			
BMI before pregnancy ≥ 25 kg/m^2	20 (25.3)	160 (26.0)	1.0 (0.6–1.7)
Fever 1st trimester pregnancy (> 38°C)	5 (6.3)	4 (0.6)	10.6 (2.8–40.4)
Maternal job exposure to industrial cleaning agents and solvents	10 (12.7)	12 (1.9)	7.4 (3.1–17.7)
Paternal risk factors (3 months prior to conception)			
Paternal smoking of cigarettes	28 (35.4)	210 (35.7)	1.0 (0.6–1.6)
Paternal job exposure to exhaust fumes	4 (5.1)	59 (10.0)	0.5 (0.2–1.4)

Discussion

The replication of the Dutch results using the German CURE-Net data shows the added value of our international consortium. Interestingly, associations between ARM and family history of ARM, fever during first trimester of pregnancy, and maternal job exposure to industrial cleaning agents and solvents could be replicated in the German cohort of ARM cases. Family history of ARM has consistently been identified as a risk factor for ARM,[22] which argues for the importance of genetic factors involved in ARM. Maternal fever during first trimester of pregnancy was indicated as a potential risk factor for ARM in the Dutch case-control study for the first time. As this association was replicated in the German cohort, it seems extremely worthwhile to further study this potential risk factor in detail. It may be essential to incorporate maternal fever, maternal infections, and antifebrile treatment in the consortium questionnaire on environmental risk factors to explore the underlying mechanism of the association between ARM and fever. In previous studies and in the Dutch and German cohorts, some job exposures were found to be associated with ARM, such as organic solvents and metals.[15,16] As it remains questionable whether the rather crude assessment of parental exposure to occupational agents reflects real exposure, we intend to analyze these occupational exposures in a more accurate way and in a larger cohort of ARM patients. Although the Dutch case-control study[21] and previous studies[23,24] found an association between ARM and BMI before pregnancy ≥ 25 kg/m^2, it could not be confirmed in the German study. Associations between ARM and paternal smoking and paternal job exposure to exhaust fumes were not replicated in the German cohort either. These preliminary results have some limitations, which should also be considered. In the German

analyses, the control group of the Dutch case-control study was used. Although we expect that the Dutch and German population correspond for the most part with regard to social standards and lifestyle factors, some geographical differences may exist. In the future, we intend to recruit controls in all participating countries to achieve true geographical similarity between cases and controls. In addition, the small sample sizes of the two case series made it difficult to provide reliable findings for less frequently occurring risk factors. However, these findings encourage us to reanalyze the current findings in a larger international study population, in which other interesting environmental risk factors may be found as well.

Conclusion

The recently established International Consortium on Anorectal Malformations, uniting researchers from the Netherlands, Germany, Italy, and France, will collect a large amount of data available for ARM research. The ensuing large cohort of ARM cases will provide possibilities to study and detect important genes and environmental risk factors involved in the etiology of syndromic and nonsyndromic forms of ARM, finally combining these to identify gene-environment interaction as well. Consequently, it is expected that the etiology of ARM will gradually be unraveled in the future, which will also provide a substantial amount of information on the pathogenesis of ARM. This knowledge may result in better genetic counseling, perinatal care, and ultimately primary prevention of ARM. While the current research focus is on finding causative factors, clinical issues will also become important. The consortium brings together pediatric surgeons to harmonize diagnostic classifications, interventions, and follow-up and to improve quality of life of ARM patients. In the future, other international pediatric surgery, genetic or epidemiological research groups may be involved in the consortium.

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Chapter 4

First results of a European multi-center registry of patients with anorectal malformations

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Abstract

Background The European consortium on anorectal malformations (ARM-NET) was established to improve the health care of patients and to identify genetic and environmental risk factors. The aim of the present study was to present the first results on clinical data of a large European cohort of ARM patients based on our registry.

Methods In 2010, the registry was established including patient characteristics and data on diagnosis, surgical therapy, and outcome regarding complications. Patients born between 2007 and 2012 were retrospectively added. A descriptive analysis of this cohort was performed.

Results Two hundred and three ARM patients were included. Syndromes or chromosomal abnormalities were present in 9%. Perineal fistulas were seen most in boys (42%) and girls (29%). Rare forms of ARM were found in 4% of the male and in 14% of the female patients. Forty-five percent of the patients had additional urogenital abnormalities. However, 32% of the patients were never screened for bladder abnormalities. Eight percent were never screened for renal malformations. In the majority of patients (79%), a PSARP was performed for the definitive reconstruction.

Conclusion This collaborative effort provides a representative basis to estimate incidence of ARM types, to discuss differences and similarities in treatment, and health consequences throughout Europe.

Introduction

Congenital anorectal malformations (ARMs) are rare birth defects of the digestive system affecting 2–6 per 10,000 births worldwide with an estimated prevalence rate of 3.0 per 10,000 births in Europe.[1,2] ARMs are found as isolated congenital birth defects, as part of a syndrome or associated with other anomalies.[3] Associated anomalies have been reported to occur in approximately 45%–65% of the patients, mostly of the urogenital tract, central nervous system, skeletal system (vertebrae) or the remaining gastrointestinal tract.[4-6] The exact frequency of associated congenital anomalies is still unclear and often based on single center studies showing contradicting results. The largest series is a single center study by Levitt and Peña and may be biased because of its nationwide referral function as Colorectal Center in the US.[7]

Almost all ARMs require surgery early in life.[4] Several surgical procedures are performed to correct anorectal malformation. The spectrum of malformations sometimes mandates different techniques for different malformations but the preferred technique is also influenced by surgeon's preference and surgical education. Although these surgical procedures generally take place in specialized surgical centers to provide optimal care, the surgical care does not always restore functionality completely accounting for substantial morbidity among ARM patients. Outcome parameters thus vary accordingly. These parameters also vary due to the use of different scoring systems for evaluation of functional outcome and the retrospective character of most studies.

Therefore, in 2010 the ARM-NET Consortium was founded and withholds three main goals. First, the consortium collects epidemiological data and DNA specimens to elucidate the etiology of anorectal malformations. It further aims to represent and independently monitor frequencies of types of ARM, additional malformations, surgical procedures, complications and outcome, especially because presently the Krickenbeck classification for ARM is not established in the WHO-ICD-10 or EUROCAT registry. A third goal is to harmonize diagnostic classification, possible interventions and follow up to improve quality of life of ARM patients. Here, we present our first results on co-morbidity and surgical procedures of a comprehensive multicenter European cohort of ARM patients.

Methods

ARM-NET was founded in 2010 as a consortium to collaborate in genetic and epidemiological research as well as to set up an anonymized registry on all new ARM patients in the participating centers in Europe (http://www.arm-net.eu).[8] The initiative was a joint approach of four national research projects and their collaborating national patient organizations: The German CURE-Net (German Network for Congenital Uro-REctal

Malformations), the Dutch AGORA project (Aetiologic research into Genetic, Occupational and environmental Risk factors for Anomalies in children), the Italian MUGAR-Net (Network for Uro-Genito-Ano-Rectal malformations in Italy), and the French MAREP project (National centre for AnoRectal Malformations and rare Pelvic anomalies).

The studies were approved by all local Ethics Committees and informed consent has been obtained from all patients and/or their parents as legal representatives. The first participating centers were from the Netherlands (Nijmegen, Groningen), Germany (Wurzburg, Heidelberg, Bremen), France (Paris) and Italy (Padua). The consortium has now expanded with further centers from Italy (Milan and Rome), Spain (Madrid), Turkey (Ankara), Germany (Hannover), and Ukraine (Dnepropetrovsk). In every center one pediatric surgeon is responsible for data management, except for Padua and Paris in which there are two.

A web-based registry was constructed to monitor anonymized background data (maternal age at birth, gender, twin pregnancy, family history), diagnostic data such as type of malformation, following the Krickenbeck classification,[9] associated anomalies, surgical procedures, complications and outcome. The VACTERL association was defined as having at least three of the following congenital malformations: vertebral anomalies, anorectal malformation, cardiac anomalies, tracheo-esophageal fistula, renal anomalies and limb anomalies.[10] For the current study newborns were also retrospectively included, to survey all ARM patients born between 2007 and 2012. All data were anonymized, filled in and confirmed by pediatric surgeons from the collaborating centers.

Descriptive analyses of absolute and relative frequencies have been performed on patient characteristics, diagnostic classification of the anorectal malformation and associated malformations, and surgery characteristics. Statistical analyses were performed using the statistical package SPSS 20.0 for Windows (SPSS Inc., Chicago, IL).

Results

A total of 203 patients were eligible for this study, as centers with incomplete patient data were excluded. ARM occurred equally in boys and girls (Table 1). Of these patients, 9% were syndromic or part of a known chromosomal defect such as trisomy 21, Currarino syndrome, Casamassima syndrome, Cri-du-Chat, Cat Eye syndrome or 22q11 microdeletion syndrome.In male patients the largest proportion had perineal fistulas (43%) followed by urethral fistulas (29%) (Table 2). In females the largest group had perineal fistulas as well (41%) followed by vestibular fistulas (28%). No fistulas were found in 15% of the males and only in 4% of females. Anal stenosis was observed similarly in males (3%) as in females (5%). Rare types of anorectal malformations were found in 9% of cases: 4% in males and 14% in females.

Table 1. Basic characteristics of a representative European cohort of 203 newborns with an anorectal malformation.

Age mother at childbirth, mean (SD)	32 (5.5)
Twins, no. (%)	11 (5.4)
Male : Female ratio	1:1
Isolated ARM patients, no. (%)	57 (28)
Syndromic or chromosomal defect, no. (%)	18 (8.9)

Table 2. Incidence of type of anorectal malformation according to the Krickenbeck classification.

Male (n=98)[a]	**No.**	**%**	**Female (n=102)**	**No.**	**%**
Perineal	42	43	Perineal	42	41
Rectourethral	28	29	Vestibular	29	28
bulbar	14	15			
prostatic	12	12			
unspecified	2	2			
Bladder neck	6	6	Cloaca	8	8
			< 3cm common channel	2	2
			> 3 cm common channel	2	2
			unspecified	4	4
No fistula	15	15	No fistula	4	4
Anal stenosis	3	3	Anal stenosis	5	5
Rare type*	4	4	Rare type*	14	14

[a]For 3 male patients the type of malformation was unknown.

[b]Rare type: 1 anterior ectopic syndrome, 2 cloacal extrophy, 1 rectal atresia, 1 rectal stenosis, 2 recto-vaginal fistula, 2 H-type fistula, 3 pouch colon, 6 other.

The majority of patients (72%) had associated anomalies, most often renal, cardiac, or skeletal anomalies (Table 3). Nine percent of the patients had an esophageal atresia. The majority of these patients (89%) were classified as Vogt 3b/Gross C. Other gastrointestinal anomalies were seen in 6% of the ARM patients including duodenal atresia, malrotation, omphalocele and one had a hepatoblastoma. Cardiac anomalies were observed in 31% of the patients, mostly atrial septal or ventricular septal defects. Almost the same proportion of patients were found to have renal abnormalities (29%): hydronephrosis in 6%, single kidney in 6%, dysplastic kidney in 3%, double system 5%, horseshoe kidney in 2%, ectopic kidney in 1% and other anomalies in 9%. Only 68% of the patients were screened for bladder anomalies. Excluding bladder exstrophy, in 8% of these patients abnormalities were found. Six patients had a neurogenic bladder, one had megacystis, and another had bladder

agenesis with trigonal hypoplasia. Regarding all neurogenic bladders it was unknown whether these were congenital or acquired.

Forty-five percent of the patients were screened for vesicourethral reflux (gr I-V) and 17% of those patients presented some grade of vesicourethral reflux (63% grade I or II). Skeletal abnormalities of the upper limb were seen in 7% of the patients and abnormalities of the lower limb in 11% of the patients. Vertebral anomalies (other than sacral and coccyx region) were seen in 18% whereas sacral anomalies were observed in 17% of the patients. However, the sacral ratio was recorded (25%) in only one center. Spinal cord anomalies were seen in 18% of those patients who were screened for this (78%), including thickened filum, tethered cord, syrinx conus, intraspinal lipoma's or masses and meningoceles.

Table 3. Absolute and relative frequency of associated anomalies seen in newborns with anorectal malformations.

Type of associated anomalies	$n_{anomaly}/n_{total\ group}$ [a]	%
Esophageal atresia	18/195	9
Other gastrointestinal anomalies	10/170	6
Cardiac anomalies	57/185	31
VSD[b]	16/185	9
ASD[b]	18/185	10
Renal anomalies	53/186	29
Hydronephrosis[c]	12/186	6
Single kidney	11/186	6
Dysplastic kidney	6/186	3
Double system	9/186	5
Horse shoe kidney	4/186	2
Ectopic kidney	2/186	1
Other	17/186	9
Skeletal anomalies	73/203	36
Upper Limb	13/191	7
Lower Limb	21/192	11
Vertebra (not sacral and coccyx region)	29/164	18
Sacral anomalies	28/162	17
Coccyx anomalies	31/140	22
Spinal canal/cord anomaly	29/158	18

[a]Total group of patients without missing data.

[b]With and without other cardiac anomalies.

[c]With and without other renal anomalies.

Forty percent of the patients were given an enterostomy of which 84% were divided colostomies (Table 4). In most cases (77%) stomas were placed at the end of the descending colon or at the beginning of sigmoid colon. In the other 23% of the patients the stoma was mostly placed at the transverse colon. In males, all bladder neck, recto-bulbar and recto-prostatic fistulas were given a colostomy. In females, all patients with cloacas received a colostomy. Females with a vestibular fistula were given a colostomy in 27%. Male and female patients with a perineal fistula were given an enterostomy in only 4%. In 92% a definitive anorectal reconstruction was done. For the final surgery a posterior sagittal anorectoplasty (PSARP) was performed in 79% of the patients. Four percent of the patients were treated with an anoplasty or cutback procedure (perineal patients only) (Table 5). An anterior sagittal anorectoplasty (ASARP) was performed in 6% of the patients; four percent of the patients received a laparascopic anorectoplasty (mainly for bladder-neck fistulas).

Table 4. Type and placement of enterostomy for anorectal malformations.

	$n_{anomaly}/n_{total\ group}$[a]	%
Enterostomy	79/198	40
Type		
Loop	12/77	16
Divided colostomy	65/77	84
Place		
Ileum	3/75	4
Transverse colon	14/75	19
Sigmoid-Descending	57/75	77

[a]Total group of patients without missing data.

Table 5. Final reconstructive surgery for the anorectal malformation.

	$n_{anomaly}/n_{total\ group}$[a]	%
Cutback	3/168	2
Anoplasty	4/168	2
(mini)PSARP	42/168	25
PSARP	90/168	54
ASARP	10/168	6
LAARP	6/168	4
PSARVUP	1/168	0.6
TUM	3/168	2

PSARP: posterior sagittal anorectoplasty; ASARP: anterior sagittal anorectoplasty; LAARP: laparoscopic anorectoplasty; PSARVUP: posterior sagittal anorectal-vagino-urethroplasty; TUM: total urogenital mobilization.

[a]Total group of patients without missing data.

Discussion

In this study we collected and analyzed anonymized data on newborns with ARM in five European pediatric surgical centers, representative for six consecutive years (2007–2012). Members of the ARM-NET Consortium are dedicated pediatric colorectal surgeons and representatives of patient organizations and as well as epidemiologists and geneticists. Despite this, we have noticed that the classification of ARM is still difficult and debatable in specific patients, e.g. urethral fistulas (bulbar or prostatic) in males or perineal fistulas in females. We have noticed this through the remarks added to the registry and after a collective discussion. Urethral fistulas in males, either bulbar or prostatic, are part of a spectrum of anorectal malformations as described by Levitt and Peña.[4] In this spectrum there is a certain overlap in bulbar and prostatic fistulas and the diagnostic tool (high pressure colostogram) sometimes gives results in which some clinicians and radiologists see a bulbar fistula whereas others see a prostatic fistula. Although the importance of the difference may not appear to be so relevant, yet in some patients (prostatic fistula) the laparoscopic approach could be chosen whereas in other patients (bulbar fistula) the posterior sagittal approach would be appropriate. Furthermore, it is generally thought that the functional outcome of a bulbar fistula is far better than that of a prostatic fistula.[7]

In male patients the incidence of patients without a fistula was rather high (15%) in comparison to previous reports. Although this may be a true observation, it could be related to a possible misdiagnosis. A improperly performed colostogram may not reveal the fistula. This will not only overestimate the group of no-fistula patients but would also have the consequence of leaving a fistula after a PSARP. Further follow up on outcome will show this to be the case.

Another remarkable observation is the incidence of the different types of anorectal malformations. The majority of malformations in females were perineal fistulas. This is contradictory to one of the largest study published by Levitt and Peña that showed that vestibular fistulas were the most frequently seen type of anorectal malformations.[7] The difference may be explained by the fact that the center of that study also functions as a national referral center in the U.S. and thus may have a bias towards more severe patients. Another remarkable observation was that we found a relatively high percentage of rare anorectal malformations, particularly in females (14%). If this survey could be considered as representative, and our peer-review proved the correct classification, the so-called rare types may occur more often than previously expected. These patients often need thorough clinical judgment, decision-making or complex surgical care. The clinical relevance of our observation suggests that these patients may need to go to specific pediatric surgical care centers (specialized in colorectal care). In most European countries this is under debate and

rules are now being made by the European Committee to have centralized care for certain rare diseases.[11] Therefore the opportunity of an independent, voluntarily and anonymized comparison, which is offered by such a registry, is important.

The frequencies of associated anomalies were more or less in the range observed by others.[4-6] Although associated urogenital anomalies were seen most, a large group was not routinely screened for bladder anomalies or vesico-ureteral reflux. A large group of patients were only examined on the basis of clinical symptoms. Although 17% of the screened patients showed some sort of vesico-ureteral reflux, the significance of routine screening needs to be further examined particularly whether such a screening also leads to a better urogenital long-term outcome. For the present time we recommend routine ultrasound of the urinary tract and kidneys in every newborn with an ARM. Voiding cysto-urethrography can be reserved for patients with dilated upper urinary tracts, lumbosacral and spinal abnormalities, or in case of additional urinary tract infections. Similarly, urodynamic investigations should be reserved for patients with suspected neurogenic bladders, recurrent infections or urinary incontinence at an older age in the follow up.

Another remarkable finding concerned the screening of the sacral ratio.[6] In our group of patients a sacral X-ray, MRI or ultrasound was performed to find sacral and spinal anomalies. In the latter 2 investigations the sacrum is described as normal or abnormal. However, the sacral ratio was only measured in one center (25% of patients). The clinical importance has been shown,[12,13] but confirmation and validation by other centers may be helpful. In our registry, the measurement of the sacral ratio was reported to be difficult in very young children and newborns, thus making the measurement of the ratio quite variable. Ultrasound is also a validated method to investigate malformations both of the sacrum and of the spinal canal in newborns and infants. Therefore, the sacrum was described as either being normal or abnormal on ultrasound exams in most of the centers. We do, however, recognize the clinical importance of the sacral abnormalities and sacral ratio and hope to report better registration in the future and maintain the item in the registry despite these initial difficulties.

The posterior sagittal approach has become the standard of care for most patients regardless the center or country of care. Laparoscopy is sometimes used but this is mostly limited to (male) patients with bladder neck fistulas.[14] In females a small group of patients with perineal and vestibular fistulas were operated using an ASARP.[15] This procedure has the preference of some surgeons because it leaves the posterior part of the sphincter complex as one similar as the mini-PSARP. The difference with the mini-PSARP lies mainly in the positioning of the patient. The ASARP is in lithotomy position whereas the mini-PSARP is in prone position. Both ASARP and mini-PSARP are procedures with less exposure but also

reduced visibility during surgery. Whether these procedures result in differences in long term outcome remains to be elucidated. This will be one of the long-term outcome measurements of our registry.

In regards to the placement of a colostomy, vestibular fistulas in females are still the most controversial on whether to place one or not. In all bulbar-, prostatic, bladder-neck fistulas and cloacal malformations a colostomy was created soon after birth. In patients with a vestibular fistula approximately 27% were given an enterostomy whereas in the remaining 73% the reconstruction was done with a short or long period of parenteral nutrition. Evidence for either one of the protective procedures is still lacking and the choice is fully case individual decided and based on personal preferences. It will be a challenge for the ARM-NET Consortium to start a randomized controlled trial to give evidence for either one of the perioperative options.

The major limitation of this cross-sectional study lies in the fact that data were collected by several surgeons throughout different countries. Peer review or independent monitoring is needed to ensure that the same kind of diagnostic classification is used. The registration in different centers increases the variance and the need to collect more data of a larger group of patients before issues are proven. Simultaneously the multi-center and cross-country character of our anonymized registry is a great strength and the first step for an independent and representative comparison. This registry delivers more precise phenotypic and surgical treatment data than general malformation registries and multi-center data and in the future it provides the possibility to assess outcome evaluation of anorectal malformations in Europe.

Regarding this issue, it seems clear that an evaluation procedure independent of the surgeon treating the patient is best to be established, as was shown recently.[16] The ARM-Net Consortium furthermore brings geneticists and epidemiologists together for a virtual biobank and opens possibilities for etiologic and pathobiological research into this rare malformation. Finally, several patient organizations are co-founder and member of this network and they are not only able to give a quality control on the surgical care, but in certain sense also have the moral authority to do so.[16]

In conclusion, this collaborative effort of five European pediatric surgical centers brings national research projects together and gives a European, representative dataset of ARM patient. These first results show that basic screening of all patients entering our registry should improve and include e.g. sacral abnormalities and ultrasound screening of the urinary tract of every patient. The data provide a basis to discuss differences in treatment and health consequences in different types of ARM, but also shows the similarities in surgical treatment throughout different countries in Europe.

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Chapter 5

Bias in patient series with VACTERL association

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To the Editor

Strict delineation and clear limits to the VACTERL association (OMIM 192350) have been debated since the first description of the VATER association in 1972.[1] We welcome the presentation of a complete series of 60 patients presenting at least three component features of the VACTERL association by Solomon et al. in the September issue of the *American Journal of Medical Genetics Part A*.[2] They mention among their cardinal findings that vertebral defects, cardiac malformations, and renal anomalies build the strongest cluster, suggesting that these three VACTERL component features tend to appear together more often than do any other component features (anorectal malformation (ARM), tracheo-esophageal (TE) fistula with esophageal atresia, and limb dysplasia). However, we have serious doubts about the external validity of the study results and we are not convinced that these can be extrapolated to the overall group of VACTERL patients for several reasons.

First, the authors claim the advantage of recruiting their patients based on a predefined association definition as inclusion criterion and not focusing on a population-based registry. However, this may be the most important study limitation. We wonder about the effect of sampling bias on the results, as the authors already mentioned that more severely affected individuals are more likely to seek participation in their study. Moreover, without reliable genetic or epidemiological proof of concept regarding the classification of component features or inclusion criteria of clinical features for VACTERL, all further results may be arbitrary. Even the ostensible proof of inheritance of component features of the VACTERL association might be reduced to the inheritance of congenital anomalies of the VACTERL association spectrum among patients and families with TE.[3] The current broad definition and classification of component features for the VACTERL association has not been supported by independent epidemiological studies. While the first population based studies[4,5] used an extended definition of VACTERL according to Kaufman,[6] later and more comprehensive analyses could not support this extension.[7-9]

Secondly, to compare prevalence rates of the component features of the VACTERL association between independent studies, equal diagnostic criteria are desired, which was also stated by Solomon et al. However, vertebral, cardiac, renal, and limb defects are rather collective categories, which include a broad spectrum of anomalies differing in severity and timing of appearance, while ARM and TE are more specific defects, at least not differing in their timing of appearance. Here, we see certain shortcomings of the authors not providing a more detailed and specific description on their classification of component features. For example, the following defects might all occur during a certain phase of pre- or postnatal development but also tend to disappear again, without any need for intervention why most

of them are not considered real congenital defects, e.g., patent ductus arteriosus, patent foramen ovale, vesicouretral reflux, and secondary hydronephrosis, etc. Furthermore, it is not clear whether sacral dysplasia, a common component in ARM, is included in vertebral malformations. In this respect and in regards to limb defects, population based evidence exist only for upper preaxial (radial) defects.[7-9]

To our opinion, the study results may thus be weakened by sampling bias and an unclear definition or classification of classic component features among patients with VACTERL association. Solomon et al. found the highest prevalence rates (70–80%) for vertebral, cardiac, and renal defects in their study compared to prevalence rates of approximately 50% of the other three component features of the VACTERL association. Hence, it is not surprising that they found vertebral, cardiac, and renal defects cluster together in hierarchical clustering analyses. Nevertheless, previous population based studies do not support their findings: Rittler et al.[7] even found the lowest association rate (observed/expected) for the co-occurrence of vertebral, cardiac, and renal defects as multiple congenital anomalies (MCA). Even more, these population based studies[8,9] do not count cardiac defects as part of the VACTERL association, as these defects do not seem to be more common among VACTERL patients than in other MCAs.

To avoid sampling bias it is reasonable to focus on population based studies. In lack of such populations, another valid approach may be focusing on a reliable component feature like TE or ARM. The systematic screening for other component features of the VACTERL association in such cases excludes arbitrary feature patterns. Hence, we compared the presence of each component feature of the VACTERL association in the patients described by Solomon et al.[2] to our Dutch (14 Triads, 10 Tetrads, 5 Pentads, and 1 Hexad) and German (25 Triads, 13 Tetrads, and 6 Pentads) cohorts of patients with VACTERL association all presenting with ARM (Table 1), recruited through pediatric surgeons and clinical geneticists. Patients with associated features suggestive of an alternate diagnosis were excluded as well. We used a rigid and conservative classification system of component features of the VACTERL association for our own patients and compared these with the 34 patients with VACTERL association and ARM (33 patients plus patient #66) described by Solomon et al.[2] Solomon et al.[2] did not account patients #28 and #66 to have an ARM, which of course must be corrected according to standard classification system.[10,11] Looking at the prevalence rates of cardiac and limb defects among patients with VACTERL association who all present an ARM, the cohort of Solomon et al. shows higher prevalences than in our cohorts (Table 1), demonstrating differences in component feature prevalences among VACTERL cohorts.

Table 1. Anorectal malformation and at least two further major component features.

Component feature	Solomon et al.[2], (n=34) No. (%)	German cohort, (n=44) No. (%)	Dutch cohort, (n=30) No. (%)
V	25 (74)	26 (59)	22 (73)
A	34 (100)	44 (100)	30(100)
C	27 (79)	29 (66)	18 (60)
TE	15 (44)	18 (41)	14 (47)
R	22 (65)	30 (68)	21 (70)
L	14 (41)	13 (30)	8 (27)

V, vertebral malformations; A, anorectal malformations; C, cardiac malformations; TE, tracheo-esophageal fistula; R, renal malformations; L, limb malformations, for the German and Dutch cohort only radial defects.

Finally, we are confused with parts of the discussion of Figure 4. It is mentioned that Cluster 1 has the lowest rate of anorectal defects, but this should be Cluster 2 according to the Figure. Although patients in Clusters 2 and 3 are stated to have low probability of TE defects, the probability of having these defects is the highest for patients in Cluster 2 according to the Figure.

Overall accounting for the lack of nationwide active birth registries in most countries we want to stress the importance of multicenter and multidiscipline collaborations in the field of rare congenital anomalies comprising active and passive birth registries, pediatric surgeons, and geneticists to harmonize diagnostic classification systems and to avoid sampling bias.

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Part 2

Non-genetic factors

Chapter 6

Anorectal malformations and pregnancy-related disorders: a registry-based case-control study in 17 European regions

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Abstract

Objective To identify pregnancy-related risk factors for different manifestations of congenital anorectal malformations (ARM).

Design A population-based case-control study.

Setting Seventeen EUROCAT (European Surveillance of Congenital Anomalies) registries, 1980–2008.

Population The study population consisted of 1,417 cases with ARM, including 648 isolated ARM cases, 601 ARM cases with additional congenital anomalies and 168 ARM-VACTERL (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal, and Limb defects) cases, and 13,371 controls with recognized syndromes or chromosomal abnormalities.

Methods Multiple logistic regression analyses were used to calculate adjusted odds ratios (OR) for potential risk factors for ARM, such as fertility treatment, multiple pregnancy, primiparity, maternal illnesses during pregnancy, and pregnancy-related complications.

Main outcome measures Adjusted ORs for pregnancy-related risk factors for ARM.

Results ARM cases were more likely to be firstborn than controls (OR 1.6, 95% CI 1.4–1.8). Fertility treatment and being part of a twin or triplet seemed to increase the risk of ARM in cases with additional congenital anomalies or VACTERL (ORs ranging from 1.6 to 2.5). Maternal fever during pregnancy and preeclampsia were only associated with ARM when additional congenital anomalies were present (OR 3.9, 95% CI 1.3–11.6 and OR 3.4, 95% CI 1.6–7.1, respectively), whereas maternal epilepsy during pregnancy resulted in a fivefold elevated risk of all manifestations of ARM (OR 5.1, 95% CI 1.7–15.6).

Conclusions This large European study identified maternal epilepsy, fertility treatment, multiple pregnancy, primiparity, preeclampsia, and maternal fever during pregnancy as potential risk factors primarily for complex manifestations of ARM with additional congenital anomalies and VACTERL.

Introduction

Congenital anorectal malformations (ARM) are the most frequently occurring birth defects of the digestive system affecting 2–6 per 10,000 births worldwide.[1] ARM is characterized by a narrowing of the anorectal canal or anal atresia with or without fistula to neighboring organs. Surgical procedures are often required at a very early age, but may not restore functionality completely, accounting for substantial physical and social morbidity among patients and their families. ARM can occur in isolation, but additional anomalies are present in approximately 50% of cases, especially as components of the VACTERL (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal, and Limb) association.[2,3]

Although the diversity in phenotypes and the presence of associated malformations indicate causal heterogeneity, most etiological studies analyzed all ARM types collectively. These studies showed contradictory results for associations between ARM and parental lifestyle factors, such as alcohol intake and smoking.[4-7] Several studies have been undertaken to investigate other aetiological parameters for ARM, suggesting risk factors, such as maternal pre-pregnancy overweight or obesity [6,8,9], pre-existing and/or gestational diabetes,[10,11] prolonged time to pregnancy,[6] and use of assisted reproductive techniques.[12-14] In addition, indications exist for a role of maternal fever during pregnancy,[6,15] maternal drug use (anti-asthmatic drugs,[16] thyroid medication,[17] and the benzodiazepine lorazepam[18]), folic acid supplementation,[19] and parental occupational hazards.[6,20] Scientific evidence for the above-mentioned potential risk factors for ARM is still growing, but most studies lack power to perform subanalyses on different manifestations of ARM. For these analyses, large population-based case-control studies are most appropriate.

The European Surveillance of Congenital Anomalies (EUROCAT) is a network of population-based congenital anomalies registries in Europe covering more than 1.7 million births per year collecting data on congenital anomalies for public health, preventive, and epidemiologic purposes.[21] The EUROCAT database enabled us to conduct a case-control study for ARM in a large study sample, using information from multiple sources from 17 EUROCAT registries to study potential pregnancy-related risk factors for ARM and especially for subgroups with different manifestations of ARM.

Methods

EUROCAT

The EUROCAT database contains standardized data about congenital anomalies recorded by each registry using uniform definitions and coding, which are described elsewhere.[22-24] The data used in this study were routinely collected between 1980 and 2008 by 17 EUROCAT registries in 13 countries (Figure 1), including live births, stillbirths from 20 weeks of

gestation onwards, and terminations of pregnancy for fetal anomaly following prenatal diagnosis. Major malformations, syndromes, and chromosomal abnormalities were coded according to the *International Classification of Diseases (ICD)* version 9 or 10, whereas specified minor anomalies were excluded according to the EUROCAT classification.[23]

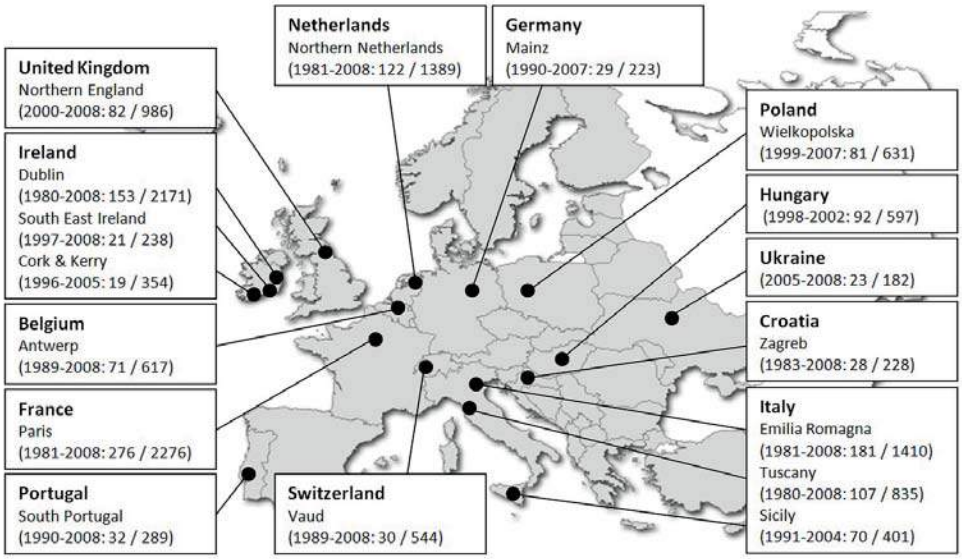

Figure 1. Map of 17 participating EUROCAT registries with the birth years in the registries and the total numbers of cases and controls.

Case and control definitions

The study population consisted of 21,606 fetuses or infants with ARM, syndromes, and/or chromosomal abnormalities. Cases were defined as live births, stillbirths, and prenatally diagnosed fetuses with subsequent terminations of pregnancy with all phenotypes of ARM. Fetuses or infants with ARM being part of a recognized syndrome or the result of a chromosomal abnormality, as well as with exstrophy of the cloaca or bladder were excluded (n=268). Excluded syndromes or chromosomal abnormalities (n=217; 13% of ARM cases) mainly comprised trisomy 21 (26%), sirenomelia (11%), and trisomy 18 (8%) as well as many others. We divided the case group into three different manifestations of ARM: isolated ARM (if ARM was the only malformation); ARM and one or more other major congenital anomalies; and ARM cases with the VACTERL association (ARM-VACTERL). Associated anomalies in the latter two manifestations of ARM are shown in Table 1. The VACTERL association includes vertebral defects (only thoracic and lumbar defects), anorectal malformations, cardiac defects (mainly atrial septal defect, ventricular septal defect, and

tetralogy of Fallot), tracheo-esophageal atresia, renal malformations, and radial limb defects. ARM-VACTERL was defined as ARM and at least two of the additional congenital anomalies included in the VACTERL association, which corresponds with the VACTERL definition of the presence of at least three of the associated malformations.[25] Diagnoses of ARM-VACTERL were assigned by the first author (C.W.) based on this definition and confirmed by a clinical geneticist (C.M.). Because no healthy controls are included in the EUROCAT registries, by convention the control group included live births, stillbirths, and prenatally diagnosed fetuses with subsequent terminations of pregnancy with recognized syndromes (mostly single gene disorders) or chromosomal anomalies, excluding ARM and VACTERL (n=45). Frequently occurring syndromes or chromosomal abnormalities among controls were trisomy 21 (54%), trisomy 18 (8%), and Turner syndrome (4%). As some pregnancy-related factors, such as preeclampsia and gestational diabetes, are normally not yet diagnosed before termination of pregnancy, the proportion of terminated pregnancies after prenatal diagnosis in the control group was reduced to the proportion in the case group for reasons of comparability, by randomly excluding part of these controls (n=6,505).

Determinant definitions

In the EUROCAT database, information was recorded after birth of the child using multiple sources, such as hospital records, birth and death certificates, and post mortem examinations using uniform definitions and coding.[23] A few registries also incorporated maternal interviews after birth. Several fetus/infant characteristics, including gender, survival (beyond one week of age), gestational age (in completed weeks) and birth weight (in grams) were recorded in the database. In addition, information was available on maternal age at delivery, fertility treatment (including hormonal treatment, artificial insemination, *in vitro* fertilization [IVF], intracytoplasmic sperm injection [ICSI], and gamete intra-fallopian transfer [GIFT]), multiple pregnancy (twin or triplet), parity (no vs one or more previous pregnancies), maternal chronic illnesses, maternal illnesses during first four months of pregnancy, and pregnancy complications. The latter three were coded according to the ICD-9 or ICD-10 and we included pre-existing and gestational diabetes mellitus, chronic obstructive lower pulmonary diseases (e.g. asthma and bronchitis), epilepsy, mental disorders (e.g. depressive and anxiety disorders), fever (>38°C) during first four months of pregnancy, and preeclampsia, also including eclampsia, as a proxy for placental dysfunction in this study.

Table 1. Anomalies among anorectal malformation cases with additional congenital anomalies and ARM-VACTERL cases (17 EUROCAT registries; 1980-2008).

	ARM and other defects (n= 601)		ARM-VACTERL (n= 168)		All ARM cases[a] (n= 1,417)	
	No.	**%**	**No.**	**%**	**No.**	**%**
Skeletal anomalies	125	20.8	87	51.8	212	15.0
Vertebral anomalies[b]	20	3.3	57	33.9	77	5.4
Other musculo-skeletal anomalies	113	18.8	48	28.6	161	11.4
Congenital heart defects	137	22.8	98	58.3	235	16.6
Cardiac defects[b]	97	16.1	87	51.8	184	13.0
Other cardiovascular anomalies	53	8.8	29	17.3	82	5.8
Tracheo-Esophageal atresia[b]	62	10.3	80	47.6	142	10.0
Urological anomalies	236	39.3	122	72.6	358	25.3
Renal anomalies[b]	126	21.0	107	63.7	233	16.4
Urinary tract anomalies	136	22.6	36	21.4	172	12.1
Limb defects	121	20.1	68	40.5	189	13.3
Radial Limb defects[b]	33	5.5	50	29.8	83	5.9
Other limb defects	101	16.8	28	16.7	129	9.1
Central nervous system anomalies	89	14.8	13	7.7	102	7.2
Respiratory anomalies	50	8.3	15	8.9	65	4.6
Orofacial clefts	35	5.8	4	2.4	39	2.8
Other facial anomalies	66	11.0	20	11.9	86	6.1
Gastro-intestinal anomalies	60	10.0	21	12.5	81	5.7
Abdominal wall defects	32	5.3	0	0.0	32	2.3
Genital anomalies	148	24.6	34	20.2	182	12.8

[a]Including isolated ARM cases, ARM cases with additional congenital anomalies, and ARM-VACTERL cases.

[b]Only including defects belonging to the VACTERL association.

Statistical analyses

Statistical analyses were performed using the statistical package SPSS 18.0 for Windows (SPSS Inc., Chicago, IL). We calculated crude odds ratios (ORs) with 95% confidence intervals (CIs) for potential risk factors for ARM, namely fertility treatment, multiple pregnancy, primiparity, and the above-mentioned maternal illnesses and pregnancy complications. Multiple logistic regression analyses were used to calculate adjusted ORs when at least three cases were exposed. We defined confounders as those factors that were either causally or accidentally associated with both ARM and the determinant. The reporting registry was selected as *a priori* confounder and was included in every model. In addition, maternal age at delivery (divided into ≤35 vs >35 years), year of birth, fertility treatment, multiple pregnancy, primiparity, preeclampsia, and maternal epilepsy were considered as potential confounding factors when they were not the primary factor of interest. All factors that changed the ORs in bivariable models were included in the full models, from which they were excluded if the OR did not change more than 10% upon removal. None of the potential confounders proved to be true confounders, except for maternal age at delivery, primiparity, and year of birth on only a few occasions. Subanalyses were performed for the different manifestations of ARM. Registries were excluded from specific analyses when data on certain determinants were not collected.

Results

A total of 1,417 cases and 13,371 controls were eligible for this case-control study. Of the cases, 648 had isolated ARM (46%), 601 had ARM with one or more major congenital anomalies (42%), and 168 were ARM-VACTERL cases (12%). Most ARM cases with associated anomalies had urological (25%), cardiac (17%), skeletal (15%), limb (13%), or genital (13%) anomalies (Table 1). Cases were more often boys than girls, whereas the proportions of control boys and girls were almost equal (Table 2). Approximately 87% of the cases were live-born, 3% were stillborn, and 10% of the pregnancies were terminated after prenatal diagnosis. Survival after one week of age was 90% among all live-born ARM cases, 99% among live-born isolated ARM cases, 84% among live-born ARM cases with other anomalies, and 73% among live-born ARM-VACTERL cases. The proportions of mothers whose children were delivered preterm and/or had low birth weight were almost the same for live-born cases and controls. Case mothers were slightly younger than control mother (28.9 years and 32.2 years, respectively), of which a larger proportion was over 35 years of age, because of the selection of controls, which included many infants or fetuses with Down syndrome.

Table 2. Characteristics of anorectal malformation cases and controls and their mothers (17 EUROCAT registries; 1980-2008).

	ARM cases (n= 1,417) No.[a] (%)	Controls (n= 13,371) No.[a] (%)
Gender		
Male	894 (63.5)	6,748 (50.8)
Female	490 (34.8)	6,487 (48.9)
Indeterminate	24 (1.7)	37 (0.3)
Birth type		
Live birth	1,228 (86.7)	11,210 (84.0)
Stillbirth	45 (3.2)	793 (5.9)
Termination of pregnancy	143 (10.1)	1,349 (10.1)[b]
Survival (>one week of age)[c]	827 (90.3)	8,551 (92.4)
Preterm delivery (<37 weeks)[c]	242 (21.3)	2,125 (20.3)
Low birth weight (<2500 gr)[c]	294 (24.9)	2,915 (26.9)
Maternal age at delivery (>35 years)	180 (13.7)	4,264 (33.2)

[a]Numbers do not add up to total number due to missing values.

[b]Artificially reduced to the same percentage as the cases.

[c]Only calculated for live births.

Tables 3 and 4 show the associations between ARM and fertility treatment, multiple pregnancy, primiparity, and maternal chronic illnesses, maternal illnesses during the first four months of pregnancy and pregnancy complications. ARM seemed to be weakly associated with fertility treatment (OR 1.3, 95% CI 0.9–1.8). No association was found between isolated ARM and fertility treatment (OR 0.8, 95% CI 0.4–1.5), but an increased risk was observed for ARM and other congenital anomalies (OR 1.6, 95% CI 1.0–2.4) and possibly for ARM-VACTERL (OR 1.6, 95% CI 0.8–3.3). Comparable odds ratios were found when multiple pregnancies were excluded from the analyses. Among the parents who received fertility treatment, 118 mothers were given hormonal treatment only (21 case and 97 control mothers), 87 mothers were artificially inseminated (ten case and 77 control mothers), 109 parents underwent IVF (seven case and 102 control parents), 14 parents conceived through ICSI (zero case and 14 control parents), 16 pregnancies resulted from GIFT (three cases and 13 controls), and other unspecified techniques were used 38 times. In fact, the only effect was observed for hormonal treatment (OR 2.1, 95% CI 1.3–3.4), whereas no association was seen between ARM and IVF, ICSI, and GIFT taken together (OR 0.8, 95% CI 0.4–1.5). In total, 398 multiple pregnancies occurred, of which 388 were twin pregnancies and ten were triplet pregnancies. Cases were more likely to be part of a twin or triplet than

controls (OR 1.6, 95% CI 1.2–2.1), but this was only seen among ARM cases with other congenital anomalies and ARM-VACTERL cases (OR 1.8, 95% CI 1.2–2.8 and OR 2.5, 95% CI 1.3–4.7, respectively). We found comparable results when the analyses were restricted to infants whose parents did not receive fertility treatment, but the association between ARM-VACTERL and multiple pregnancy disappeared (OR 0.5, 95% CI 0.1–3.6). Primiparity was associated with an approximately 1.5-fold elevated risk of all manifestations of ARM. We found preeclampsia to be associated with ARM (OR 2.2, 95% CI 1.2–4.0), but only among ARM cases with other congenital anomalies (OR 3.4, 95% CI 1.6–7.1). Similar risk estimates were seen for ARM patients whose mothers had fever during the first four months of pregnancy (OR 2.2, 95% CI 0.8–5.7 and OR 3.9, 95% CI 1.3–11.6, respectively). The risks of all manifestations of ARM were approximately five times increased when the mother suffered from epilepsy during pregnancy. In total, 14 mothers had epilepsy during pregnancy (five cases and nine controls), of whom at least ten mothers reported antiepileptic drug use during the first four months of pregnancy (five case and five control mothers). We did not have information on type and dose of the antiepileptic drugs. Finally, pre-existing and gestational diabetes, maternal chronic obstructive lower pulmonary diseases (e.g. asthma and bronchitis), and mental disorders (e.g. depressive and anxiety disorders) during the first four months of pregnancy were not associated with ARM. Subanalyses including only live births resulted in ORs which were very similar to those in Tables 3 and 4 for all pregnancy-related disorders (data not shown).

Table 3. Associations between anorectal malformations and pregnancy-related disorders (17 EUROCAT registries; 1980-2008).

	Cases (n= 1,417) No. (%)	Controls (n= 13,371) No. (%)	Crude OR (95% CI)	Adjusted OR (95% CI)
Fertility treatment[a]	45 (6.0)	337 (4.8)	1.3 (0.9–1.8)	1.3 (0.9–1.8)
Multiple pregnancy (twin or triplet)	56 (4.0)	342 (2.6)	1.6 (1.2–2.1)	1.6 (1.2–2.1)
Primiparity[b]	472 (43.2)	3,472 (32.5)	1.6 (1.4–1.8)	1.6 (1.4–1.8)
Preeclampsia[c]	14 (2.2)	71 (1.3)	1.7 (1.0–3.1)	2.2 (1.2–4.0)
Fever during the first 4 months of pregnancy[c]	5 (0.8)	30 (0.5)	1.5 (0.6–3.8)	2.2 (0.8–5.7)[d]
Diabetes mellitus[c]				
Pre-existing	8 (1.1)	85 (1.4)	0.8 (0.4–1.7)	0.8 (0.4–1.7)
Gestational	1 (0.1)	37 (0.6)	0.2 (0.0–1.7)	–
Chronic lower obstructive pulmonary disease[c]	17 (2.4)	128 (2.1)	1.2 (0.7–1.9)	1.4 (0.8–2.3)
Epilepsy during pregnancy[c]	5 (0.8)	9 (0.2)	4.9 (1.6–14.6)	5.1 (1.7–15.6)
Mental disorder during the first 4 months of pregnancy[c]	3 (0.5)	28 (0.5)	0.9 (0.3–3.1)	1.3 (0.4–4.3)

Maternal age at delivery, year of birth, fertility treatment, multiple pregnancy, primiparity, preeclampsia, and maternal epilepsy were included as potential confounders, but they were excluded from the models if their removal did not change the OR by more than 10%. All factors were adjusted for registry. Adjusted OR was calculated if >2 cases were exposed.

[a]Five registries were excluded as data were not available, leaving 1,071 cases and 10,518 controls.

[b]One registry was excluded as data were not available, leaving 1,325 cases and 12,774 controls.

[c]Six registries were excluded as data were not available, leaving 918 cases and 8,347 controls.

[d]Adjusted for registry and year of birth.

Table 4. Adjusted associations between different manifestations of anorectal malformations and pregnancy-related disorders (17 EUROCAT registries; 1980-2008).

	Isolated ARM cases (n= 648)		ARM and other defects (n= 601)		ARM-VACTERL (n= 168)	
	No.	Adjusted OR (95% CI)	No.	Adjusted OR (95% CI)	No.	Adjusted OR (95% CI)
Fertility treatment[a]	13	0.8 (0.4–1.5)[b]	23	1.6 (1.0–2.4)	9	1.6 (0.8–3.3)
Multiple pregnancy (twin or triplet)	18	1.1 (0.7–1.8)	27	1.8 (1.2–2.8)	11	2.5 (1.3–4.7)
Primiparity[c]	213	1.5 (1.3–1.8)	201	1.5 (1.3–1.9)	58	1.8 (1.3–2.6)
Preeclampsia[d]	3	1.3 (0.4–4.1)	9	3.4 (1.6–7.1)	2	1.8 (0.4–7.4)
Fever during the first 4 months of pregnancy[d]	0	–	4	3.9 (1.3–11.6)	1	2.1 (0.3–15.8)
Diabetes mellitus[d]						
Pre-existing	3	0.8 (0.3–2.5)	4	1.2 (0.4–3.2)[e]	1	0.8 (0.1–5.9)
Gestational	1	0.5 (0.1–3.9)	0	–	0	–
Chronic obstructive lower pulmonary disease[d]	7	1.4 (0.6–3.0)	7	1.3 (0.6–2.9)	3	1.4 (0.4–4.7)[f]
Epilepsy during pregnancy[d]	2	4.5 (1.0–20.8)	2	4.6 (1.0–21.2)	1	7.1 (0.9–56.5)
Mental disorder during the first 4 months of pregnancy[d]	2	1.4 (0.3–6.1)	1	0.7 (0.1–5.4)	0	–

The same controls were used as in Table 3. Maternal age at delivery, year of birth, fertility treatment, multiple pregnancy, primiparity, preeclampsia, and maternal epilepsy were included as potential confounders, but they were excluded from the models if their removal did not change the OR by more than 10%. All factors were adjusted for registry. Crude OR was presented if <3 cases were exposed.

[a]Five registries were excluded as data were not available, leaving 460 isolated ARM cases, 470 ARM cases with other defects, 141 ARM-VACTERL cases, and 10,518 controls.

[b]Adjusted for registry and primiparity.

[c]One registry was excluded as data were not available, leaving 595 isolated ARM cases, 569 ARM cases with other defects, 161 ARM-VACTERL cases, and 12,774 controls.

[d]Six registries were excluded as data were not available, leaving 390 isolated ARM cases, 406 ARM cases with other defects, 122 ARM-VACTERL cases, and 8,347 controls.

[e]Adjusted for registry and maternal age.

[f]Adjusted for registry and year of birth.

Discussion

In this large European registry-based case-control study, the risk of ARM appeared to be associated with fertility treatment, multiple pregnancy, primiparity, preeclampsia, maternal fever during the first four months of pregnancy, and epilepsy during pregnancy. These pregnancy-related disorders were mainly found to increase the risk of ARM when ARM occurred in combination with other anomalies, whether or not belonging to the VACTERL association.

The EUROCAT network provides many possibilities for large-scale epidemiologic studies on congenital anomalies. The study sample of 1,417 ARM cases was the largest sample ever studied, which made it possible to investigate different manifestations of ARM. The importance of performing these subanalyses was emphasized by risk estimates that varied between different manifestations of ARM. Causal heterogeneity may exist due to differences in the time windows of exposure or the underlying biological mechanisms. Despite the large study sample, the power may have been insufficient to detect moderate effects of specific risk factors for ARM, especially in ARM-VACTERL cases. As EUROCAT only collects data on pregnancies of affected infants/fetuses, we used controls that had malformations instead of controls without malformations. Teratogenicity non-specificity bias may occur when a certain exposure leads to malformations in both cases and controls. To reduce this type of bias, only controls with recognized syndromes or chromosomal abnormalities were included, under the assumption that these abnormalities originated due to genetic defects only. Because these syndromes and chromosomal abnormalities arise before or during conception, it is not likely that pregnancy-related factors influenced their occurrence. Furthermore, previous studies argued that these types of congenital anomalies are suitable and representative sources of controls.[26,27] A few potential risk factors, including gestational diabetes mellitus, chronic lower obstructive pulmonary disease and mental disorders, seemed to be underreported in mothers of both cases and controls, but the figures for most determinants in controls were similar to those found in the general pregnant population.[28,29] As post mortem examination after a stillbirth or for prenatally diagnosed fetuses with subsequent termination of pregnancy was not always performed, some controls may have had ARM. This could have resulted in dilution of the effects that were found. Differential misclassification of exposure status because of poorer recording of exposures for controls compared with cases is unlikely, as information on pregnancy-related factors was obtained from hospital records by the EUROCAT registry, with already existing data gathered prospectively during pregnancy for most infants in our study, regardless of the type of malformation. Only a few registries used maternal interviews after birth, but pregnancy-related factors, such as fertility treatment, multiple pregnancy, primiparity,

preeclampsia, and other maternal disorders, are not expected to be prone to recall bias. A limitation of this registry-based study was the lack of information on parental lifestyle factors and of detailed information on certain potential risk factors, such as zygosity in multiple pregnancies, the type of fertility problems of the parents, and specific information on illnesses and drug use. Therefore, residual confounding cannot be excluded. All associations were adjusted for registry, which was appropriate as small differences existed between the EUROCAT registries included. Although we described some potential biases that are usually associated with observational studies, we expect that these biases only influenced our findings to a minimal extent.

Approximately 54% of the ARM cases without syndromes, chromosomal abnormalities, or cloacal or bladder exstrophy had one or more additional congenital anomalies. Among all ARM cases up to 62% had additional congenital anomalies. This is in line with previous studies that reported percentages between 40% and 70%, usually including syndromes and chromosomal abnormalities.[2,30] Urological (25%) and cardiac (17%) malformations appeared to be the most prevalent additional malformations, which also confirms other studies.[2,30] The male/female ratio of 1.8 among ARM cases was higher than previously described ratios of 1.2–1.4.[6,30] Females more often suffer from mild and unrecognized phenotypes of ARM without clinical symptoms than males. In this registry-based study, diagnoses of these mild phenotypes of ARM may have been missed or delayed, which could have led to a relatively high male/female ratio.

To our knowledge, this is the first study to perform subanalyses on associations between fertility treatment and different manifestations of ARM. Even after adjustment for multiple pregnancy, we found fertility treatment to be associated with ARM in combination with other defects only and we found an effect for hormonal treatment without IVF or ICSI in general. In contrast, clomiphene citrate, a hormonal drug for subfertility treatment, was not found to be associated with ARM in a study on the effect of this drug on major birth defects.[31] Other studies showed that assisted reproductive techniques, including IVF and ICSI, increased the risk of several major birth defects, including ARM.[12-14] However, these studies did not perform subanalyses for different manifestations of ARM. Although contrasting, all of these findings seem to point towards an association between ARM and fertility treatment. This may be due to epigenetic mechanisms, which were also described for associations between fertility treatment and certain syndromes (e.g. Beckwith-Wiedemann syndrome).[32,33] However, it may also indicate that the underlying subfertility of the parents is the main risk factor in the etiology of ARM.

We found an over-representation of ARM with additional congenital anomalies and ARM-VACTERL among multiple pregnancies, whereas few other researchers found increased

numbers of heterogeneous groups of gastro-intestinal atresias, including ARM, in multiple pregnancies.[34,35] Doyle et al. showed the proportion of chromosomal abnormalities to be lower in multiple pregnancies,[34] which could correspond with a decreased number of multiple pregnancies among our controls. Since more recent studies did not confirm this result, it is debatable whether our ORs for multiple pregnancies were overestimated.[35,36] Primiparity was associated with all manifestations of ARM and preeclampsia with ARM and additional congenital anomalies only, although the risk estimates for primiparity may be slightly inflated as firstborns are relatively uncommon among controls with Down syndrome.[37]. Interestingly, multiple pregnancy, primiparity, and preeclampsia are all factors that may reflect situations in which the placenta supplies the fetus with nutrients and hormones insufficiently. Therefore, we hypothesize placental insufficiency in early pregnancy to partly explain the associations found, especially those for ARM occurring in combination with other congenital anomalies. Similar results have been found for hypospadias, a frequently occurring congenital malformation among boys.[38] Although the rates of low birth weight among cases and controls were almost equal, these were high compared with non-malformed controls.[28] Low birth weight may also be a consequence of placental insufficiency, which strengthens our placental insufficiency hypothesis. Another biological mechanism underlying the associations between ARM and multiple pregnancy and primiparity may be a disturbed androgen/estrogen balance, as endogenous levels of free oestradiol are increased in first and multiple pregnancies.[39] Free oestradiol levels also increase with increasing body mass index of the mother,[40] and maternal overweight and obesity have consistently been identified as risk factors for ARM.[41]

Although adverse effects of maternal epilepsy and/or antiepileptic drug use on birth defects have already been shown,[42] we found a five times increased risk of ARM in infants/fetuses of mothers with epilepsy during pregnancy. Unfortunately, we were not able to study associations between ARM and the different types and doses of antiepileptic drugs, as no information was available. It is unclear whether congenital anomalies result from teratogenic effects of antiepileptic drugs alone, or are partly the result of the underlying epilepsy itself. Previously, a reduced risk of ARM was shown after periconceptional folic acid supplementation.[19] This may explain the association between ARM and maternal epilepsy during pregnancy as treatment with most antiepileptic drugs was associated with reduced folate serum levels.[43] In agreement with our previous studies on parental risk factors for ARM,[6,15] maternal fever during the first four months of pregnancy was found to be associated with ARM, mainly in infants/fetuses with additional congenital anomalies. A recent meta-analysis reported an increased risk of ARM for maternal pre-existing or gestational diabetes mellitus,[41] but we could not confirm these findings, possibly because of

under-reporting of pre-existing or gestational diabetes among cases and controls and small numbers in the case groups.

Conclusion

This is the first study emphasizing the importance of performing subanalyses on different manifestations of ARM. Our findings suggest the involvement of multiple pregnancy, primiparity, and preeclampsia, factors that are possibly related to placental insufficiency or a disturbed androgen/estrogen balance, as well as fertility treatment, maternal epilepsy, and fever during the first four months of pregnancy in the etiology of ARM. These pregnancy-related disorders mainly seem to play a role in complex phenotypes of ARM in which additional congenital anomalies are present. Only maternal epilepsy and primiparity were found to be involved in the occurrence of isolated ARM. Although these factors increased the risk of ARM 1.5 to 5 times, the absolute risks of having a child with ARM were relatively low, ranging between 0.05% and 0.26% for each potential risk factor. In addition to relative and absolute risks, however, these findings also provide clues about the pathophysiological mechanisms involved in the etiology of ARM, which may in turn guide further research, preventive strategies and health care for ARM patients in the future.

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Chapter 7

Maternal and paternal risk factors for anorectal malformations: a Dutch case-control study

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Abstract

Background Anorectal malformations (ARM) are major congenital malformations that usually require a multitude of surgical procedures at very early age and have a large impact on the lives of patients and their parents. The causes of ARM are still largerly unknown, but it is assumed to have a multifactorial etiology. A few studies focused on environmental risk factors, but evidence is still scarce.

Methods In this Dutch case-control study (1996-2008), we investigated the role of maternal and paternal risk factors in the etiology of ARM. Parents of 85 ARM cases and 650 controls filled in a questionnaire. Controls were children treated with ear ventilation tubes.

Results A higher occurrence of fever during the first trimester of pregnancy was found for case mothers compared to control mothers (odds ratio (OR), 5.1; 95% Confidence Interval (CI), 0.9, 28.1). Maternal occupational exposure to industrial cleaning agents and solvents increased the risk of ARM three times (OR, 2.9; 95% CI, 0.9, 9.3). Overweight (Body Mass Index (BMI) ≥ 25 kg/m^2) before pregnancy also seemed to be associated with ARM (OR, 1.8; 95% CI, 1.1, 2.8), as well as maternal multivitamin use during pregnancy (OR, 1.6; 95% CI, 1.0, 2.7), paternal smoking (OR, 1.8; 95% CI, 1.1, 2.9) and paternal occupational exposure to exhaust fumes (OR, 1.9; 95% CI, 1.0, 3.6). Reported ARM in at least one first or second degree family member greatly increased the risk of having a child with ARM (OR, 40.3; 95% CI, 4.8, 342.8).

Conclusions This study revealed potential risk factors for ARM, including fever during pregnancy, maternal overweight, use of multivitamins, paternal smoking, and occupational exposures, but a familial component seems important as well.

Introduction

Congenital anorectal malformations (ARM) are the most common gastrointestinal anomalies at birth. The worldwide prevalence of ARM is estimated to be 1 in 1500 to 1 in 5000 live births.[1] Anorectal anomalies include a wide range of phenotypes depending on location and size of the defect. They usually require multiple surgical procedures at very early age and have a major impact on the lives of patients and their parents. Additional congenital anomalies are present in approximately 50% of ARM cases, especially anomalies of the heart, kidneys, spine, urogenital tract, and other gastrointestinal atresias, in particular as components of the VACTERL association (Vertebral defects, Anal atresia, Cardiac defects, Tracheo-Esophageal fistula, Renal anomalies, Radial dysplasia, and Limb defects). Not much is known about the etiology of ARM, apart from a few identified genes that have been implicated in syndromes including ARM or were derived from animal studies. Townes-Brocks syndrome, with combinations of anal, renal, limb, and ear anomalies, is caused by mutations in the SALL1 zinc-finger protein.[2] Linkage and mutational analyses in patients with the Currarino syndrome, in which sacral malformations are found along with ARM, identified the homeobox gene HLXB9 as a major locus.[3] Mutant mice with various defects in the signaling pathway of sonic hedgehog exhibit a spectrum of distal hindgut defects mimicking human ARM, while mutant mice lacking Gli2 or Gli3 show imperforate anus with recto-urethral fistula and anal stenosis.[4,5]

Although evidence exists for genetic contribution to ARM based on these studies, nonsyndromic anorectal anomalies rarely aggregate in families.[6] This points towards a substantial role for environmental risk factors. A Japanese study suggested maternal alcohol drinking in early pregnancy as a risk factor for isolated anal atresia,[7] that could not be confirmed by others.[8,9] Cigarette smoking and caffeine intake were found to be associated with anal atresia in the US,[9] whereas in France and Spain, associations with medical drug use during pregnancy were found,[8] in particular use of the benzodiazepine lorazepam.[10] Paternal exposure to occupational hazards, such as organic solvents and metals in vehicle manufacturers seemed to be associated with anal atresia as well.[8,11] Based on a single study in China, it has been suggested that periconceptional folic acid supplementation may reduce the risk of anal atresia.[12] Most of these potential risk factors stem from only one epidemiologic study per risk factor, which is not sufficient for conclusions about causality. Moreover, none of these studies investigated all potential risk factors simultaneously in a structured manner allowing adjustment for confounders. Therefore, the aim of this case-control study was to identify risk factors for ARM among a wide spectrum of familial, pregnancy-related, lifestyle, and occupational factors in both mothers and fathers.

Materials and methods

Study population

Children born between January 1996 and April 2008 who were treated for ARM at the department of Pediatric Surgery of the Radboud University Nijmegen Medical Centre (RUNMC) in the Netherlands were eligible for this study. The medical records were reviewed to retrieve anomaly-specific information to classify ARM according to Levitt and Peña.[13] For boys, the malformations were categorized into perineal fistulas, recto-urethral fistulas (bulbar or prostate), recto-bladderneck fistulas and imperforate anus without fistula and for girls into perineal and vestibular fistulas, and imperforate anus without fistula. Two less common defects, rectal atresia and anal stenosis, were also identified and included. For the diagnosis of the VACTERL association, we used the presence of ARM and at least two additional defects of the VACTERL spectrum as described by Jong et al.[14] and named it ARM-VACTERL in this study. In total, the parents of 132 ARM cases were asked to participate in the study.

The control group was derived from a study on hypospadias[15] and recruited at the Otorhinolaryngology departments of the RUNMC and three general hospitals in the surroundings of Nijmegen, covering the referral area of the ARM cases. All controls were boys with persistent middle ear infection treated with ventilation tubes and born between 1996 and 2004. We randomly selected 1000 controls without major birth defects. This control group was chosen to avoid selective non-response and information bias since they were not informed about their control status. Because middle ear infection is one of the most common diseases in childhood, the control group is likely to be representative of the general population in the area.

Data collection

Between February 2005 and December 2006, the parents of cases and controls known to be alive were sent almost identical questionnaires and similar information letters and leaflets, followed by two reminders after three and seven weeks to increase the response rate. From 2007 onwards, parents of ARM cases were asked to participate in the AGORA project (Aetiologic research into Genetic and Occupational/ Environmental Risk Factors for Anomalies in Children) of the RUNMC at the child's first admission to the hospital. This project aims at building a data- and biobank for questionnaire data and DNA from cases treated for a congenital disorder and their parents.

The questionnaire contained questions about demographic factors, family history of birth defects, and pregnancy history. Questions about health status, prescribed medication, lifestyle, and various exposures at work or during leisure time activities were asked

concerning the three months before conception and during pregnancy for mothers and concerning the three months period before conception for fathers. The specific time windows of exposure during pregnancy were asked for all maternal items. Compared to the questionnaires of controls, those of cases contained a few more items on stress, extreme nausea, caffeine intake, and vaccination during pregnancy, and for some questions, such as dietary intake, different answer categories were used resulting in noncomparability. Therefore, these items were not taken into account in the analyses. Variables included in the analyses were age at childbirth (< or ≥ 35 years), ethnicity, educational level (low: no/primary/lower vocational/intermediate secondary/intermediate vocational education), Body Mass Index (BMI) (< or ≥ 25 kg/m^2), family history of birth defects, defined as two or more 1st or 2nd degree relatives with a birth defect, family history of ARM, defined as one or more 1st or 2nd degree relatives with ARM, time to pregnancy (TTP) (≤ or > 6 months), fertility problems established by a medical doctor, conception with medical treatment, preterm delivery (< 37 weeks), birth weight (≤ or > 2500 grams), parity, chronic diseases, use of folic acid, multivitamins, alcohol (including number of glasses per week), cigarettes (including number per day), and medication (including type of medication) before or during pregnancy, fever (> 38°C) in first trimester of pregnancy, and job exposure during pregnancy to X-rays, cytostatics, anesthetics, industrial cleaning agents and solvents, disinfectants, cosmetics, exhaust fumes, paint/varnish/adhesives/ink/thinner, welding fumes, and pesticides. For certain occupational exposure data, adjustments were made when data indicated that occupational exposure was confused with private use of products, for example pesticides.

The study protocol was approved by the regional Committee on Research Involving Human Subjects and written informed consent was obtained from the parents of all cases and controls.

Statistical analyses

Chi-square tests were used to compare the frequency distributions of background variables among cases and controls. Crude odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for all potential maternal and paternal risk factors using unconditional logistic regression analyses. The variables considered as potential confounders included all determinants that are known or suspected risk factors for ARM from the literature and those covariates that differed between cases and controls in our data. To prevent having too many covariates in the initial multivariable models, we first checked whether a factor changed the risk of ARM by at least 10% in bivariable analyses. In addition, child age at time of filling in the questionnaire was included as well as some background variables. However, those that

shared the causal pathway between the risk factor of interest and the outcome were excluded. All relevant variables were included in multivariable logistic regression models for each potential maternal and paternal risk factor in order to calculate adjusted ORs with 95% CIs. For reasons of precision, covariates were excluded from the multivariable model when exclusion did not change the risk estimate of the potential risk factor by more than 10%. Only risk factors with two or more exposed cases were used in the analyses. Because of a potentially heterogeneous etiology, we performed stratified analyses for ARM cases with and without the VACTERL association, the former being referred to as ARM-VACTERL. Due to small numbers, the results for ARM-VACTERL cases could not be adjusted for confounding.

Results

Ninety-five case parents and 650 control parents returned the questionnaires, resulting in a participation rate of 72% and 65%, respectively. Ten ARM cases were excluded because of chromosomal anomalies (n=1), known syndromes (n=4), cloaca (n=2), and vesicointestinal fissures (n=3). This resulted in 85 ARM cases and 650 controls being included in the study. Most of the ARM cases had a perineal fistula (55%) (Table 1). Almost half of the cases had isolated ARM (total 45%: 40% in boys, 53% in girls) and another 30% (31% in boys, 29% in girls) had only one associated anomaly, which were most often urogenital tract malformations, followed by skeletal malformations. The higher the ARM was located, the more additional malformations were present. Fourteen cases (17%) were diagnosed with the ARM-VACTERL association, which most often included vertebral (79%) and renal anomalies (64%), followed by cardiovascular defects (43%) and esophageal atresia/tracheo-esophageal fistula (38%). Only one case showed an upper limb anomaly (7%).

All 85 case mothers and 83 case fathers as well as 642 control mothers and 600 control fathers completed the questionnaire. Most case and control parents originated from Europe or The Netherlands (95% and 97%, respectively).

The median age of the children at time of filling in the questionnaire was 5.4 years for cases and 6.5 years for controls (Table 2). Maternal and paternal age at childbirth was not different between the groups, but fathers of an ARM child were lower educated than controls. More ARM children were first borns, were born preterm or had a low birth weight compared to control children. Children with ARM were also more often conceived after a prolonged TTP. More case parents reported a family history of congenital malformations in first or second degree relatives (13.3% vs. 3.5%, respectively), and especially more ARM in at least one family member compared to controls (OR, 40.3; 95% CI, 4.8, 342.8).

Table 1. Classification of Anorectal Malformations and Presence of Other Congenital Malformations.

	Total group (n=85)		Male (n=49)		Female (n=36)	
	No.	**%**	**No.**	**%**	**No.**	**%**
Classification of ARM						
Perineal fistula	47	55.3	24	49.0	23	63.9
Rectourethral fistula: bulbar	4	4.7	4	8.2	-	-
Rectourethral fistula: prostatic	7	8.2	7	14.3	-	-
Vestibular fistula	12	14.1	-	-	12	33.3
Imperforate anus without fistula	7	8.2	6	12.2	1	2.8
Rectal atresia	3	3.5	3	6.1	-	-
Anal stenosis	5	5.9	5	10.2	-	-
Other congenital malformations						
Urogenital tract malformations	34	40.0	21	42.9	13	36.1
Skeletal malformations	23	27.1	16	32.7	7	19.4
Gastro-intestinal tract malformations	11	12.9	8	16.3	3	8.3
Circulatory tract malformations	12	14.1	7	14.3	5	13.9
Limb malformations	5	5.9	4	8.2	1	2.8
Central nervous system malformations[a]	3	3.6	3	6.3	0	0
ARM-VACTERL	14	16.5	11	22.4	3	8.3

ARM, anorectal malformations; VACTERL, vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula and/or esophageal atresia, renal anomalies, radial dysplasia, and limb defects.

[a]Data on central nervous system malformations was missing in 1 boy.

Table 2. Maternal, paternal and child characteristics of the case and control study population.

	Cases (n=85)[a]	Controls (n=650)[a]	*P*[b]
Age child at time study, median (range)	5.4 (0.0-15.1)	6.5 (1.6-9.3)	0.067[c]
Age at childbirth ≥ 35 years			
Mother, no. (%)	12 (14.1)	102 (16.0)	0.653
Father, no. (%)	30 (36.1)	219 (36.7)	0.924
Educational level, low			
Mother, no. (%)	52 (61.9)	397 (62.0)	0.982
Father, no. (%)	60 (73.2)	362 (60.6)	0.028
Parity: first live born, no. (%)	44 (52.4)	263 (40.5)	0.037
Preterm delivery (< 37 weeks), no. (%)	12 (14.5)	59 (9.5)	0.156
Birth weight ≤ 2500 grams, no. (%)	11 (13.8)	50 (8.1)	0.090
TTP > 6 months, no. (%)	26 (32.9)	135 (21.4)	0.022
Family history of birth defects, no. (%)	11 (13.3)	21 (3.5)	0.001
Family history of ARM, no. (%)	6 (7.2)	1 (0.2)	< 0.001

ARM, anorectal malformations; *P*, p-value; TTP, time to pregnancy.

[a]The maximum number of missing values was 6 in cases and 30 in controls, except for family history of birth defects and ARM with 53 missings in controls.

[b]Chi-square test.

[c]Mann-Whitney U test.

Maternal risk factors for ARM are presented in Table 3. More case mothers were overweight before pregnancy (BMI ≥ 25kg/m^2) (OR, 1.8; 95% CI, 1.1, 2.8). Three mothers of ARM cases reported fever in the first trimester of pregnancy (3.7%) versus only four of the 624 control mothers (0.6%), resulting in a five times increased risk of ARM (OR, 5.1; 95% CI, 0.9, 28.1). Excluding the six cases and one control that had a family history of ARM did not change these results, whereas excluding ARM-VACTERL cases strengthened the association with fever (OR, 6.3; 95% CI, 1.1, 35.1). Maternal chronic diseases were only weakly associated with ARM.

The intake of folic acid supplements before and during pregnancy was 72% in case and control mothers, not resulting in an increased risk of ARM. The same was true for folic acid use just before pregnancy up until the first trimester (58% in both groups). In the ARM-VACTERL group (n=14), the percentage of folic acid use in the total period was only 57%, resulting in a crude OR of 0.5 (95% CI, 0.2, 1.5). Multivitamins, however, were taken 9% more often by case mothers compared to controls, resulting in an increased risk of 1.6 (95% CI, 1.0, 2.7), which was slightly weakened by excluding the ARM-VACTERL cases (OR, 1.4; 95% CI, 0.8, 2.4), in which the risk was highest (OR, 3.4; 95% CI, 1.1, 10.2). These results remained the same when only the intake before conception and in the first trimester of

pregnancy was taken into account. Alcohol intake, smoking of cigarettes, and the use of medication before or during pregnancy were not associated with ARM, or with ARM-VACTERL, irrespective of the number of glasses of alcohol, number of cigarettes, the type of medication, and the period of use before and during pregnancy.

Most job exposures in mothers, including exposure to X-rays, cytostatics, paints, disinfectants, cosmetics, and exhaust fumes did not reveal an increased risk of ARM. Exposure to industrial cleaning agents and solvents used in a variety of jobs ranging from professional cleaners to nurses, however, was more prevalent among case mothers, resulting in an increased risk of ARM (OR, 2.9; 95% CI, 0.9, 9.3). Moreover, two mothers in the ARM-VACTERL group (14.3%) were exposed to cytostatics compared to 10 (1.6%) controls (OR, 10.2; 95% CI, 2.0, 51.8).

Table 3. Associations between maternal factors and anorectal malformation.

	Cases (n=85)[a] No. (%)	**Controls (n=642)[a] No. (%)**	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**
BMI before pregnancy ≥ 25kg/m^2	32 (38.1)	160 (26.0)	1.8 (1.1-2.8)	-
Fever in 1st trimester of pregnancy	3 (3.7)	4 (0.6)	6.0 (1.3-27.5)	5.1 (0.9-28.1)[b]
Chronic disease	12 (14.1)	59 (9.2)	1.6 (0.8-3.2)	-
Fertility problems mother	6 (7.1)	63 (9.8)	0.7 (0.3-1.7)	-
Conception with medical treatment	4 (4.8)	43 (6.8)	0.7 (0.2-2.0)	-
Before or during pregnancy use of:				
Folic acid tablets	61 (71.8)	456 (71.6)	1.0 (0.6-1.7)	-
Multivitamins	24 (28.9)	129 (20.3)	1.6 (1.0-2.7)	-
Alcohol	34 (40.0)	260 (40.9)	1.0 (0.6-1.5)	-
Cigarettes	19 (22.4)	171 (26.8)	0.8 (0.5-1.3)	-
Medication	46 (54.1)	365 (57.6)	0.9 (0.6-1.4)	-
Job exposure during pregnancy:				
X-rays	2 (2.4)	20 (3.2)	0.7 (0.2-3.2)	0.6 (0.1-2.6)[c]
Cytostatics	2 (2.4)	10 (1.6)	1.5 (0.3-6.9)	-
Industrial cleaning agents and solvents	5 (6.0)	12 (1.9)	3.2 (1.1-9.4)	2.9 (0.9-9.3)[b]

BMI, body mass index; CI, confidence interval; OR, odds ratio.

'-' means that the association was not confounded by any covariate.

[a]The maximum number of missing values was 4 in cases and 27 in controls.

[b]Adjusted for family history of ARM and paternal smoking.

[c]Adjusted for maternal multivitamin use.

Exposure to occupational hazards was more frequently seen among fathers than in mothers and a few of these paternal exposures showed an association with ARM (Table 4). In the univariable analyses paints/varnish/adhesives/ink/thinners, welding fumes and exhaust fumes seemed to increase the risk of ARM in offspring. After controlling for confounders, however, only exhaust fumes remained associated with ARM (OR, 1.9; 95% CI, 1.0, 3.6). In separate analyses, excluding the six cases and one control that had a family history of ARM, the results for maternal and paternal occupational exposures were the same.

Fathers smoking cigarettes in the three months before conception also seemed to be at risk of having a child with ARM (OR, 1.8; 95% CI, 1.1, 2.9). Numbers of cigarettes smoked was similar between the smoking case and control fathers. Paternal alcohol use did not influence the risk of ARM, and neither did the existence of a chronic disease, like asthma or rheumatic diseases. In contrast to mothers, fertility problems among fathers occurred more frequently in the case group (6.0%) compared to controls (2.8%) (OR, 2.2; 95% CI, 0.8, 6.1). These problems especially seemed to increase the risk of having a child with the ARM-VACTERL association (crude OR, 5.7; 95% CI, 1.2, 27.5).

Table 4. Associations between paternal factors and anorectal malformation.

	Cases (n=83)[a] No. (%)	**Controls (n=600)[a] No. (%)**	**Crude OR (95% CI)**	**Adjusted OR (95% CI)**
BMI before pregnancy ≥ 25kg/m^2	40 (50.0)	313 (54.4)	0.8 (0.5-1.3)	-
Chronic disease	7 (8.5)	41 (7.0)	1.2 (0.5-2.9)	-
Fertility problems father	5 (6.0)	17 (2.8)	2.2 (0.8-6.1)	-
Smoking 3 months before conception	41 (50.0)	210 (35.7)	1.8 (1.1-2.9)	-
Alcohol use 3 months before conception	70 (85.4)	479 (81.7)	1.3 (0.7-2.5)	-
Job exposure 3 months before conception:				
Exhaust fumes	14 (17.3)	59 (10.0)	1.9 (1.0-3.6)	-
Industrial cleaning agents and solvents	5 (6.8)	37 (6.4)	1.1 (0.4-2.8)	0.6 (0.2-1.7)[b]
Paint/ varnish/ adhesives/ ink/ thinner	7 (9.0)	31 (5.4)	1.7 (0.7-4.1)	1.4 (0.6-3.7)[c]
Welding fumes	7 (8.8)	30 (5.1)	1.8 (0.8-4.2)	1.3 (0.5-3.3)[d]

BMI, body mass index; CI, confidence interval; OR, odds ratio.

'-' means that the association was not confounded by any covariate.

[a]The maximum number of missing values was 10 in cases and 26 in controls.

[b]Adjusted for family history of ARM, maternal BMI before pregnancy, paternal smoking and paternal job exposure to exhaust fumes.

[c]Adjusted for family history of ARM.

[d]Adjusted for family history of ARM and paternal job exposure to exhaust fumes.

Discussion

This study investigating a wide spectrum of potential parental risk factors for ARM in children using structured questionnaires, revealed risk factors such as maternal fever, overweight, multivitamin use, paternal smoking, and occupational exposures, as well as a familial component.

To interpret these results properly, the strengths and weaknesses of our study have to be discussed. We studied a representative group of ARM cases for this referral hospital as we compared them to all 264 ARM cases born in or referred to the same hospital between 1974 and 1995.[16] Both groups present similar gender and ARM type distributions with higher percentages of boys, presumably because of a more complex embryologic development in boys and more missed or delayed diagnoses in girls. The only difference between our study and the older group of cases is the number of isolated cases, 45% versus 33%, respectively, which can be explained by our exclusion of cloacas, known syndromes, and deceased cases. The latter may have resulted in survival bias, assuming different risk factors in ARM cases with multiple congenital anomalies. A group of 174 ARM cases in France, however, consisted of 50% isolated cases, with urogenital and skeletal malformations being the most prevalent additional anomalies, similar to our study.[17] The control parents appear to be representative for the general population as well. The percentages of, for example, smoking, alcohol consumption, and folic acid supplementation are similar to those found in the general population and in studies investigating pregnant women in the Netherlands.[18-20] Another strong point of our study is the wide range of risk factors that was investigated in both mother and father allowing thorough adjustment for confounders, which is unique in studying ARM.

All risk factors studied were self-reported, so random misclassification of exposure and recall bias cannot be excluded, especially since parents were asked to recall events that had occurred some time ago. Because the control parents were not aware of their control status, however, severe recall bias is not very likely, except for family history of birth defects and ARM. This may have caused overestimation of the ORs for these factors, whereas random misclassification could have resulted in underestimation of other risks. Differential misclassification due to recall times is not probable as small differences in recall time between cases and controls did not seem to be relevant in the multivariable models. The relatively small sample size of cases made it difficult to provide reliable findings for less frequently occurring risk factors and for the ARM-VACTERL group. Although the gender distribution in our study differed between cases (58% boys) and controls (100% boys), we do not expect this to have biased our results, because no indications exist that the chances of being exposed to the investigated risk factors of ARM during pregnancy differ by gender of

the expected child. Even the results for low birth weight did not change substantially in a subanalysis of only boys (data not shown).

This is the first time that maternal fever is indicated as a potential risk factor for ARM. Another epidemiologic study investigating maternal influenza and congenital abnormalities in Hungary did not reveal an increased risk of ARM.[21] Antifebrile treatment could have masked an existing association, however, because they only found associations in mothers who had not received antifebrile treatment. We cannot assess whether the increased risk observed in our study is indicative of a causal relation or was due to reporting bias. However, we expected a greater percentage of women with fever in the control group than in the general population, because infections, often associated with fever, tend to run in families of children with persistent ear infections.[22] The few women who reported fever in the first trimester of pregnancy might have been those with serious fever or fever for a long episode, that could really have an effect on the development of the anorectal area. In animals, hyperthermia has been shown to cause cell death, membrane and vascular disruption, and placental infarction.[23] Moreover, a relation has been observed between fever and neural tube defects and congenital renal anomalies in humans.[24-26]

In contrast to a Chinese study,[12] use of folic acid supplements was not associated with ARM. Surprisingly, multivitamin intake before and during the sensitive period of pregnancy appeared to increase the risk of having a child with ARM, especially for children with the ARM-VACTERL association. Eight out of the 24 case mothers reported to have taken other than specific pregnancy multivitamins, which contain higher amounts of vitamin A than advised during pregnancy. As information on dose and frequency of use is lacking, one could only speculate that the high vitamin A intake could have had detrimental effects on embryogenesis, such as shown in the study by Rothman and co-workers.[27] An overdose of etretinate, a long acting vitamin A analogue, induced ARM in murine embryos by disrupting the retinoic-acid signaling pathway.[28]

Mothers of children with ARM were more likely to be overweight (BMI $\geq$ 25 kg/m^2) than control mothers. This result is supported by a study in the US that showed an association between anorectal atresia and maternal obesity (BMI $\geq$ 30 kg/m^2).[29] Paternal smoking in the three months prior to conception seemed to give a relatively small increased risk of having a child with ARM. So far, paternal smoking has only been found as a risk factor for other congenital malformations, such as cleft lip and/or cleft palate, hypospadias, and ventricular septal defect.[19,30,31] The proposed effects of maternal smoking[9] and drinking[7] on ARM could not be confirmed in our study.

Exposure to a few occupational hazards revealed an increased risk of ARM, such as maternal exposure to industrial cleaning agents and solvents and paternal exposure to

exhaust fumes. Some associations between paternal exposure to occupational hazards and ARM have been found earlier, such as for organic solvents and metals in vehicle manufacturers.[8,11] Organic solvents have also been associated with birth defects in general.[32] The question remains, however, whether the rather inaccurate assessment of exposure to occupational agents of the parents reflects the real exposure.

Our study indicates an association of ARM with prolonged TTP and probably with established paternal fertility problems, supporting the existing literature on the association between subfertility and ARM. Some studies demonstrated higher rates of ARM children born after assisted reproductive techniques[33-35] and others showed a prolonged TTP in couples that had a child with the VACTERL association.[36] We could not replicate these results, but we did find an association between ARM-VACTERL and established paternal fertility problems. Adding up all of the evidence, it is still not clear whether the fertility problem itself or the related fertility treatment plays a role in the etiology of ARM.

In addition to potential environmental risk factors for ARM, we found an indirect indication for a genetic role in the embryologic development of the anorectal area. A reported history of ARM in first or second degree relatives was strongly associated with ARM in offspring. This association may be overrated because of selective inquiry about ARM in case families, but our results are supported by the study of Falcone Jr and coworkers,[37] which showed an increased heritability of certain types of ARM.

Conclusion

The results from our study suggest a role in the etiology of ARM for maternal fever in the first trimester of pregnancy, maternal overweight, use of multivitamins, paternal smoking, and job exposures to industrial cleaning agents in women and exhaust fumes in men. In addition, primiparity, fertility problems, and a family history of congenital malformations or ARM seem to increase the risk of ARM even more. However, these results need to be confirmed in other studies before firm conclusions can be drawn.

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Chapter 8

Parental subfertility, fertility treatment and the risk of congenital anorectal malformations

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Abstract

Background Fertility treatment seems to play a role in the etiology of congenital anorectal malformations, but it is unclear whether the underlying parental subfertility, ovulation induction, or the treatment itself is involved. Therefore, we investigated the odds of anorectal malformations among children of subfertile parents who conceived with or without treatment compared with fertile parents.

Methods We performed a case-control study among 380 cases with anorectal malformations treated at three departments of Pediatric Surgery in The Netherlands and 1,973 population-based controls, born between August 1988 and August 2012. Parental questionnaires were used to obtain information on fertility-related issues and potential confounders.

Results In singletons, increased risks of anorectal malformations were observed for parents who underwent intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF) treatment compared with fertile parents (odds ratio = 2.4 [95% confidence interval = 1.0-5.9] and odds ratio = 4.2 [1.9-8.9], respectively). For subfertile parents who conceived after IVF treatment, an elevated risk was also found when they were compared with subfertile parents who conceived without treatment (3.2 [1.4-7.2]). Among children of the latter category of parents, only the risk of anorectal malformations with other major congenital malformations was increased compared with fertile parents (2.0 [1.3-3.3]). No associations were found with intrauterine insemination or use of hormones for ovulation induction.

Conclusions We found evidence of a role of ICSI and IVF treatments in the etiology of anorectal malformations. However, subfertility without treatment increased only the risk of anorectal malformations with additional congenital malformations.

Introduction

Congenital anorectal malformations are major anomalies of the gastrointestinal system, with a prevalence of approximately 1 in 3000 births worldwide.[1] Anorectal malformations are characterized by disrupted embryonic development of the anus and rectum in the 4th to 8th week after conception, often also involving other organs, such as the bladder or vagina. Although the physical and psychosocial impact is large for patients with anorectal malformations and their parents,[2] knowledge about the potential risk factors is still limited.

In the past decade, the risks of congenital malformations following assisted reproductive techniques (ART), including in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), received large amounts of scientific attention. Most studies showed an increased prevalence of congenital malformations after ART, but they investigated only heterogeneous patient groups consisting of broad categories of congenital malformations, such as all gastrointestinal malformations.[3,4] Four studies, specifically focused on anorectal malformations, found ART to increase the risk of anorectal malformations more than 3 times compared with spontaneous conceptions,[5-8] whereas two studies did not.[9,10] Clearly increased risks of anorectal malformations were not found when all fertility treatments, also including intrauterine insemination (IUI) and ovulation induction, were analyzed together.[10,11] However, two studies showed an association between anorectal malformations and ovulation induction without ICSI or IVF,[10,12] whereas one study did not.[13] The role of parental subfertility was investigated in only one study, which suggested an association between anorectal malformations and paternal but not maternal subfertility.[11]

Limitations of the previous studies, however, were low case numbers, moderately to poorly characterized cases, nonspecific or pooled exposure groups, lack of information on potential confounders, and/or different methods of data collection among cases and controls. Furthermore, none of these studies combined the effects of parental subfertility, prolonged time to pregnancy, and types of fertility treatment within one study. Therefore, we aimed to elucidate whether the excess risk of anorectal malformations is attributable to the underlying parental subfertility, the hormones used for ovulation induction, or the ART procedure itself. We performed a case-control study to investigate the role of parental subfertility and prolonged time to pregnancy without fertility treatment or with a range of fertility treatments in the etiology of nonsyndromic anorectal malformations. We also assessed these effects in subgroups of isolated anorectal malformations and anorectal malformations with one or more other major congenital malformations as etiologic heterogeneity was suggested previously.[10,14]

Methods

Study Population

AGORA (Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children) is a large data- and biobank with clinical and questionnaire data and DNA samples from children with congenital malformations or childhood cancer, population-based control children, and their parents (www.AGORAproject.nl). In total, 448 cases with anorectal malformations were derived from the AGORA databank. These cases were diagnosed shortly after birth and treated at the departments of Pediatric Surgery of the Radboud university medical center in Nijmegen, the Sophia Children's Hospital – Erasmus Medical Centre in Rotterdam, or the University Medical Center Groningen in the Netherlands. They were born between August 1988 and August 2012 and recruited from 2007 onward. Pediatric surgeons, clinical geneticists and researchers reviewed the medical records to obtain information on anorectal malformation phenotypes and additional congenital malformations.

Phenotypes of anorectal malformations were classified according to the international Krickenbeck criteria[15] and additional congenital malformations were divided into major and minor malformations according to the EUROCAT classification.[16] Cases with syndromic forms of anorectal malformations, chromosomal abnormalities, or cloacal exstrophy were excluded from the study (n=41, 9%). The remaining cases were divided in isolated anorectal malformations with or without minor congenital malformations and anorectal malformations with one or more additional major congenital malformations. The latter subgroup also included cases with the VACTERL association (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal, and Limb defects), defined as cases with anorectal malformations and the presence of at least two other features of this association.[17] Eighty of the cases with anorectal malformations in this study (21%) were also included in our previous study, in which a wide range of potential risk factors for anorectal malformations were evaluated, such as parental lifestyle factors, occupational exposures and pooled exposure groups of fertility-related issues.[11]

Control children were also derived from the AGORA databank (n=2,196). They were born between January 1990 and March 2011 and randomly sampled and recruited in 2010-2011 via 39 municipalities in geographical areas that covered the referral areas of the three hospitals where the cases were treated. Control children with major congenital malformations, chromosomal abnormalities or syndromes were excluded based on the questionnaire data (n=56, 3%). In addition, 7 cases and 2 controls were excluded because a sibling was already, leaving 400 cases and 2,138 controls.

Ethical approval for the study protocol of the AGORA data- and biobank was granted by the regional Committee on Research involving Human Subjects. All parents of cases and controls gave informed consent for the study.

Data collection and definitions

Cases and controls were all between 0 and 22 years of age. Their parents were invited to participate in AGORA. Both parents were asked to fill in a questionnaire with questions about demographic factors, family history of congenital malformations, and health and lifestyle before and during pregnancy. We used these questionnaires to obtain information about fertility issues and characteristics of cases and controls. Mothers were asked whether their pregnancies were planned, whether they or their partners had received any type of fertility treatment, and about the number of months between the start of trying to conceive and conception. Both mothers and fathers were asked whether they were ever diagnosed with subfertility by a physician. For this study, subfertile parents were defined as parents that had a time to pregnancy of more than 12 months, were diagnosed with either maternal or paternal subfertility, or both. We assumed that parents with an unplanned pregnancy were fertile. Parents were excluded from the analyses when information on time to pregnancy or parental subfertility was unknown (20 cases [5%] and 165 controls [8%]), leaving 380 cases and 1,973 controls for the final analyses. In addition, the following types of fertility treatment were evaluated: ICSI, IVF, intrauterine insemination (IUI; with or without donor), ovulation induction only, and surgical (e.g. removal of adhesions in fallopian tube) or other treatment. Parents were classified according to the above priority sequence if more than one treatment was reported. In the Netherlands, treatment for subfertility typically starts after 12 months of trying to conceive.

Infant and parental characteristics included sex and year of birth of the child, maternal and paternal ethnicity (Dutch vs. other), maternal and paternal age at delivery, maternal and paternal education, anorectal malformations in first degree relatives of the child, multiple pregnancy (vs. singleton pregnancy), parity (nulliparity vs. multiparity), periconceptional folic acid use (any use of folic acid supplements or multivitamins containing folic acid in 4 weeks before through 10 weeks after conception), maternal pre-existing and gestational diabetes, and maternal prepregnancy body mass index (BMI). The latter was calculated using reported weight (in kg) and height (in m) before conception and categorized in 4 groups (<18.5, 18.5-24.9, 25.0-29.9, and ≥30.0 kg/m^2).

Statistical analyses

Statistical analyses were performed using SPSS 20.0 for Windows (IBM SPSS, Chicago, IL, USA). Logistic regression analyses were used to calculate crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for associations between anorectal malformations and parental fertility-related issues. The parents were divided into three groups: fertile parents who conceived spontaneously (reference), subfertile parents who conceived without treatment, and subfertile parents who conceived after any type of fertility treatment. We also distinguished among the types of fertility treatment and performed subgroup analyses that included singletons and nulliparous women only. The latter was done because multiple pregnancy seemed to be associated with both anorectal malformations and fertility treatment and because a previous study showed divergent results for the association between anorectal malformations and fertility treatment in singleton compared with multiple pregnancies.[7] In addition, associations between anorectal malformations and classes of time to pregnancy (1-3, 4-6, 7-12, >12 months) were assessed in singletons conceived spontaneously as a measure of fecundity, to study whether increased risks of anorectal malformations occurred with increasing time to pregnancy. Potential confounders included year of birth of the child (continuous), maternal age at delivery, maternal education, pre-existing diabetes mellitus, and prepregnancy BMI, because these factors were previously identified in the literature as risk factors or found to be associated with anorectal malformations in this study. Potential confounding factors that changed the ORs in bivariable analyses were included in the multivariable models, from which they were excluded when the OR did not change more than 10% upon removal. Only maternal age at delivery and BMI, in two analyses, were found to be true confounders. We repeated the analyses for isolated anorectal malformations and anorectal malformations with one or more other major congenital malformations. Finally, we performed sensitivity analyses by including and excluding unplanned pregnancies and infants with affected first degree relatives.

Results

Response rates were 57% among cases and 34% among controls. After applying the exclusion criteria, maternal questionnaire data were available for 380 cases and 1,973 controls and paternal questionnaire data for 350 cases and 1,762 controls. In total, 230 cases had isolated anorectal malformations (61%) and 150 cases had anorectal malformations with one or more additional major congenital malformations (39%). Among the latter, 40 cases fulfilled the criteria for VACTERL. Vertebral (14%), cardiac (11%), and renal malformations (10%) were the most commonly associated major congenital malformations. The majority of

cases were diagnosed with perineal fistulas (56%), followed by rectourethral fistulas (14%) in boys and vestibular fistulas (13%) in girls. We did not observe substantial differences between cases and controls in sex of the child, maternal ethnicity, multiple pregnancy, and nulliparity (Table 1). However, cases as well as their mothers were slightly younger and more often had a first degree relative with anorectal malformations compared with controls. In addition, low education, periconceptional folic acid use, pre-existing or gestational diabetes, and pre-pregnancy overweight or obesity were more common among case mothers than among control mothers. The distributions of paternal characteristics, including age at delivery, ethnicity, and education, were similar to those of the maternal characteristics.

Table 1. Characteristics of cases with anorectal malformations and controls, The Netherlands, 1988-2012.

	Cases (n=380) No. (%)[a]	**Controls (n=1,973) No. (%)[a]**
Sex of child		
Boy	185 (48.7)	975 (49.4)
Girl	195 (51.3)	998 (50.6)
Year of birth of child		
1988-1990	10 (2.6)	58 (2.9)
1991-1995	77 (20.3)	413 (20.9)
1996-2000	91 (23.9)	494 (25.0)
2001-2005	84 (22.1)	464 (23.5)
2006-2010	103 (27.1)	540 (27.4)
2011-2012	15 (3.9)	4 (0.2)
Maternal age at delivery (years)		
<25	25 (6.6)	119 (6.1)
25-29	133 (35.0)	613 (31.2)
30-34	161 (42.4)	867 (44.2)
≥35	61 (16.1)	364 (18.5)
Maternal ethnicity		
Dutch	336 (90.1)	1,812 (91.8)
Other	37 (9.9)	161 (8.2)
Maternal education[b]		
Low	97 (25.5)	335 (17.0)
Middle	164 (43.2)	908 (46.2)
High	119 (31.3)	724 (36.8)
Anorectal malformations in 1st degree relatives	5 (1.4)	0 (0.0)
Multiple pregnancy	18 (4.8)	78 (4.0)

	Cases (n=380) No. (%)[a]	Controls (n=1,973) No. (%)[a]
Nulliparity	147 (38.9)	771 (39.2)
Periconceptional folic acid use	233 (67.9)	1,126 (65.6)
Diabetes mellitus		
Pre-existing	5 (1.4)	10 (0.5)
Gestational	12 (3.3)	38 (2.0)
Pre-pregnancy body mass index[c]		
Underweight (<18.5)	19 (5.2)	65 (3.5)
Normal (18.5-24.9)	232 (63.9)	1,319 (71.2)
Overweight (25.0-29.9)	74 (20.4)	351 (18.9)
Obese (≥30.0)	38 (10.5)	118 (6.4)

[a]Numbers do not add up to total numbers due to missing values (<1% in all variables, except for ARM in 1st degree relatives [11%, mainly due to missing paternal questionnaires], folic acid use [13%, mainly due to exclusion of mothers who used supplements outside the advised period], pre-existing diabetes mellitus [3%], gestational diabetes [2%] and body mass index [6%].

[b]Low: no, primary, lower vocational, or intermediate secondary education; middle: intermediate vocational or higher secondary education; high: higher vocational or academic education.

[c]Body mass index in weight (kg)/height $(m)^2$.

Approximately 20% of all parents reported parental subfertility with or without fertility treatment, ranging from 18% in control parents to 21% in cases parents. The adjusted odds ratios for anorectal malformations among children of subfertile parents with and without fertility treatment for all births and for singleton births only are shown in Table 2. The risk of having a child with anorectal malformations seemed to be slightly increased for subfertile parents who conceived without treatment (OR = 1.3 [95% CI = 0.9–1.8]) compared with fertile parents. This increased risk was apparent in nulliparous women only (OR = 1.6 [0.9-2.7]). Subfertile parents included parents with a TTP >12 months and parents who ever had a diagnosis of parental subfertility with a normal TTP (≤ 12 months), unplanned pregnancy, or unknown TTP. No association with anorectal malformations was observed among the latter group of parents (OR = 1.2 [0.7-2.1]). One-third of these parents reported that the reason for subfertility was unknown (33%), while the reported reasons included polycystic ovary syndrome (11%) and irregular menstrual cycles (9%) as the main groups. In subanalyses stratifying on mutually exclusive groups of maternal (13 cases and 60 controls), paternal (9 cases and 27 controls) and couple subfertility without fertility treatment (1 case and 5 controls), only the OR for subfertility of the father without treatment deviated from unity (OR = 1.8 [0.8-3.9]).

Approximately 8% of all infants were born after any type of fertility treatment. These births seemed to be associated with a slightly increased risk of anorectal malformations, as compared with births among fertile parents (OR = 1.3 [0.9–1.8]). Ovulation induction was the most common treatment among controls, whereas parents of cases most often underwent IVF treatment. Surgical or other fertility treatments were not reported by case parents. When we restricted the analyses to ICSI, IVF, IUI, and ovulation induction, a slightly higher OR was found for any fertility treatment (OR = 1.4 [1.0-2.1]). This increased risk was due only to the relatively strong associations between anorectal malformations and ICSI or IVF treatments (OR = 2.0 [0.9-4.5] and OR = 2.7 [1.4-5.4], respectively), however, since no associations were observed between anorectal malformations and treatments that did not involve gamete manipulation, including IUI and ovulation induction. Most risk estimates were stronger for singleton births only, especially the estimates for ICSI and IVF treatment compared with fertile parents (OR = 2.4 [1.0-5.9] and OR = 4.2 [1.9-8.9], respectively).

For methodological reasons explained above, the further analyses were performed among singletons only. The adjusted odds ratios for isolated anorectal malformations and anorectal malformations with one or more additional major congenital malformations among children of subfertile parents with and without fertility treatment are presented in Table 3. No associations were observed between isolated anorectal malformations and parental subfertility without treatment or with any type of fertility treatment in singletons, except for IVF treatment compared with fertile parents (OR = 2.6 [1.0-7.3]). In contrast to isolated anorectal malformations, the risk of anorectal malformations with one or more additional malformations was increased for parental subfertility without fertility treatment (OR = 2.0 [1.3-3.3]), especially for subfertile parents with a TTP longer than 12 months (OR = 2.3 [1.3-3.9]). For ICSI or IVF treatments, the risks of anorectal malformations with other congenital malformations were increased five- to eight-fold. The risk estimates for anorectal malformations with additional major congenital malformations were similar or slightly stronger when cases with the VACTERL association were excluded. Sensitivity analyses performed by restricting the above-mentioned analyses to planned pregnancies or to infants without first degree relatives with anorectal malformations only, did not yield different results.

The risks of anorectal malformations and subgroups of anorectal malformations among children of subfertile parents who conceived with fertility treatment were also compared with subfertile parents who conceived without treatment to estimate the risk of treatment beyond the underlying subfertility. Overall, smaller risk estimates were observed when we used subfertile parents without treatment as the reference group (Table 4) compared with using fertile parents as reference group (Table 3). Interestingly, however, the

risk of anorectal malformations was still increased for subfertile parents who underwent IVF when they were compared with subfertile parents who conceived without treatment (OR = 3.2 [1.4-7.2]), whereas a lower OR with a broader confidence interval was found after ICSI treatment (OR 1.8 [0.7-4.8]). Comparable risk estimates were observed for subgroups of anorectal malformations, including isolated anorectal malformations and anorectal malformations with other congenital malformations.

To investigate whether fecundity plays a role in the occurrence of anorectal malformations, we assessed whether increased risks of anorectal malformations with increasing classes of time to pregnancy could be observed in singletons conceived spontaneously excluding unplanned pregnancies and pregnancies with unknown time to pregnancy (Table 5). For anorectal malformations as a group, a pattern of increasing risks of anorectal malformations with increasing time to pregnancy seemed to be present, but this may completely be due to the increased risk of anorectal malformations with additional congenital malformations after a time to pregnancy longer than 12 months, indicating parental subfertility. No pattern of increasing risks of isolated anorectal malformations with increasing time to pregnancy was observed.

Table 2. Adjusted odds ratios for anorectal malformations among children of subfertile parents with and without fertility treatment compared to fertile parents for all births and for singletons births only.

	All births			Singleton births		
	Cases (n=380) No. (%)	Controls (n=1,973) No. (%)	Adjusted OR (95% CI)[a]	Cases (n=359) No. (%)	Controls (n=1,894) No. (%)	Adjusted OR (95% CI)[a]
Fertile parents[b]	299 (78.7)	1,626 (82.4)	1.0	286 (79.7)	1,585 (83.7)	1.0
Subfertile parents without fertility treatment	46 (12.1)	195 (9.9)	1.3 (0.9-1.8)	45 (12.5)	190 (10.0)	1.3 (0.9-1.9)
Time to pregnancy > 12 months	30 (7.9)	124 (6.3)	1.3 (0.9-2.0)	30 (8.4)	121 (6.4)	1.4 (0.9-2.1)
Diagnosis of parental subfertility only[c]	16 (4.2)	71 (3.6)	1.2 (0.7-2.1)	15 (4.2)	69 (3.6)	1.2 (0.7-2.1)
Subfertile parents with fertility treatment[d]	35 (9.2)	152 (7.7)	1.3 (0.9-1.8)	28 (7.8)	119 (6.3)	1.3 (0.8-2.0)
Intracytoplasmic sperm injection	8 (2.1)	22 (1.1)	2.0 (0.9-4.5)	7 (1.9)	16 (0.8)	2.4 (1.0-5.9)
In vitro fertilisation	13 (3.4)	26 (1.3)	2.7 (1.4-5.4)	12 (3.3)	16 (0.8)	4.2 (1.9-8.9)
Intrauterine insemination[e]	6 (1.6)	31 (1.6)	1.1 (0.4-2.4)	5 (1.4)	25 (1.3)	1.1 (0.4-2.9)
Ovulation induction only	8 (2.1)	56 (2.8)	0.8 (0.4-1.6)	4 (1.1)	46 (2.4)	0.5 (0.2-1.3)
Surgical or other treatment	0 (0.0)	15 (0.8)	-	0 (0.0)	15 (0.8)	-

[a]Adjusted for year of birth, maternal age at delivery, maternal education, pre-existing diabetes mellitus, and pre-pregnancy body mass index if >10% change in OR upon removal. We did not calculate ORs when < 3 cases or controls were exposed.

[b]Reference category.

[c]These parents were diagnosed with subfertility by a physician. The pregnancy was unplanned or the time to pregnancy was ≤ 12 months or unknown.

[d]Numbers of types of treatment do not add up to total number due to missings in two controls.

[e]Intrauterine insemination with or without donor sperm.

Table 3. Adjusted odds ratios for subgroups of anorectal malformations among children of subfertile parents with and without fertility treatment compared to fertile parents for singleton births only.

	Controls (n=1,894) No. (%)	Isolated ARM (n=222) No. (%)	Adjusted OR (95% CI)[a]	ARM with other anomalies[b] (n=137) No. (%)	Adjusted OR (95% CI)[a]
Fertile parents[c]	1,585 (83.7)	188 (84.7)	1.0	98 (71.5)	1.0
Subfertile parents without fertility treatment	190 (10.0)	21 (9.5)	0.9 (0.6-1.5)	24 (17.5)	2.0 (1.3-3.3)
Time to pregnancy > 12 months	121 (6.4)	13 (5.9)	0.9 (0.5-1.6)	17 (12.4)	2.3 (1.3-3.9)
Diagnosis of parental subfertility only[d]	69 (3.6)	8 (3.6)	1.0 (0.5-2.1)	7 (5.1)	1.6 (0.7-3.7)
Subfertile parents with fertility treatment[e]	103 (5.4)	13 (5.9)	1.1 (0.6-1.9)	15 (10.9)	2.4 (1.3-4.2)
Intracytoplasmic sperm injection	16 (0.8)	2 (0.9)	-	5 (3.6)	4.6 (1.6-13.1)
In vitro fertilisation	16 (0.8)	5 (2.3)	2.6 (1.0-7.3)	7 (5.1)	8.3 (3.3-21.2)
Intrauterine insemination[f]	25 (1.3)	3 (1.4)	1.0 (0.3-3.4)	2 (1.5)	-
Ovulation induction only	46 (2.4)	3 (1.4)	0.6 (0.2-1.8)	1 (0.7)	-

[a]Adjusted for year of birth, maternal age at delivery, maternal education, pre-existing diabetes mellitus, and pre-pregnancy body mass index if >10% change in OR upon removal. We did not calculate ORs if < 3 cases or controls were exposed.

[b]Also including ARM cases with the VACTERL association.

[c]Reference category.

[d]These parents were diagnosed with subfertility by a physician. The pregnancy was unplanned or the time to pregnancy was ≤ 12 months or unknown.

[e]Numbers of types of treatment do not add up to total number due to missings in two controls. Total numbers do not include surgery and other treatments.

[f]Intrauterine insemination with or without donor sperm.

Table 4. Adjusted odds ratios for anorectal malformations among children of subfertile parents with fertility treatment compared to subfertile parents without treatment for singleton births only.

	Controls (n=293) No. (%)	Total ARM cases (n=73) No. (%)	Adjusted OR (95% CI)[a]	Isolated ARM (n=34) No. (%)	Adjusted OR (95% CI)[a]	ARM with other anomalies[b] (n=39) No. (%)	Adjusted OR (95% CI)[a]
Subfertile parents without fertility treatment[c]	190 (64.8)	45 (61.6)	1.0	21 (61.8)	1.0	24 (61.5)	1.0
Subfertile parents with fertility treatment[d]	103 (35.2)	28 (38.4)	1.1 (0.7-1.9)	13 (38.2)	1.1 (0.5-2.4)	15 (38.5)	1.2 (0.6-2.3)
Intracytoplasmic sperm injection	16 (5.5)	7 (9.6)	1.8 (0.7-4.8)	2 (5.9)	-	5 (12.8)	2.4 (0.8-7.2)
In vitro fertilisation	16 (5.5)	12 (16.4)	3.2 (1.4-7.2)	5 (14.7)	2.8 (0.9-8.5)	7 (17.9)	3.5 (1.3-9.3)

[a]Adjusted for year of birth, maternal age at delivery, maternal education, pre-existing diabetes mellitus, and pre-pregnancy body mass index if >10% change in OR upon removal. We did not calculate ORs if < 3 cases or controls were exposed.

[b]Also including ARM cases with the VACTERL association.

[c]Parents who had a time to pregnancy > 12 months and/or were diagnosed with subfertility by a physician; reference category.

[d]Fertility treatment include intracytoplasmic sperm injection, *in vitro* fertilisation, intrauterine insemination, and ovulation induction.

Table 5. Adjusted odds ratios for associations between anorectal malformations and time to pregnancy for singletons conceived without fertility treatment.

Time to pregnancy (months)	Controls (n=1,582)[a] No. (%)	All ARM cases (n=297)[a] No. (%)	Adjusted OR (95% CI)[b]	Isolated ARM (n=194) No. (%)	Adjusted OR (95% CI)[b]	ARM with other anomalies (n=103) No. (%)	Adjusted OR (95% CI)[b]
1-3[c]	1,043 (65.9)	176 (59.3)	1.0	120 (61.9)	1.0	56 (54.4)	1.0
4-6	247 (15.6)	51 (17.2)	1.2 (0.9-1.7)	33 (17.0)	1.2 (0.8-1.7)	18 (17.5)	1.4 (0.8-2.4)
7-12	171 (10.8)	40 (13.5)	1.4 (0.9-2.0)	28 (14.4)	1.4 (0.9-2.2)	12 (11.7)	1.3 (0.7-2.5)
>12	121 (7.6)	30 (10.1)	1.5 (1.0-2.3)	13 (6.7)	0.9 (0.5-1.7)	17 (16.5)	2.6 (1.5-4.6)

[a]Total numbers were not the same as in Tables 2 and 3 as unplanned pregnancies and pregnancies after fertility treatment or with unknown time to pregnancy were excluded.

[b]Adjusted for year of birth, maternal age at delivery, maternal education, pre-existing diabetes mellitus, and pre-pregnancy body mass index if >10% change in OR upon removal. We did not calculate ORs if < 3 cases or controls were exposed.

[c]Reference category.

Discussion

Previous studies found indications for a role of parental fertility-related issues in the etiology of anorectal malformations.[5-8,10-12] This large case-control study provided additional evidence that these factors are associated with an increased risk of anorectal malformations in offspring, including both isolated anorectal malformations and anorectal malformations with one or more additional congenital malformations. ICSI and IVF treatments, in particular, seemed to play a role in the etiology of anorectal malformations, compared both to fertile parents and to subfertile parents who conceived without treatment. Although much smaller than the impact of ICSI and IVF treatment, subfertility itself seems to have an effect on the occurrence of anorectal malformations, but only for anorectal malformations with additional congenital malformations.

Our study population consisted of large numbers of well-characterized cases and population-based controls. The prevalence of associated congenital malformations in 39% of the cases in our study was slightly lower than in previous studies, but these included syndromic and chromosomal forms of anorectal malformations and/or terminations of pregnancies as well.[18-20] The relatively large number of cases enabled subanalyses on isolated anorectal malformations and anorectal malformations with additional congenital malformations separately. Unfortunately, we could not perform separate analyses on more specific phenotypes of anorectal malformations, VACTERL, multiple births or first pregnancies only, as the numbers of case parents who underwent fertility treatment in these subgroups were too small for reliable analyses. Population-based controls were a representative group, as they were recruited via municipalities in comparable geographical areas as the cases and were of similar age.

Another strength of our study was the combination of information on several fertility-related issues, including the diagnosis of parental subfertility, time to pregnancy, and type of fertility treatment, within one study. Furthermore, information on several potential maternal and paternal confounders was used in our analyses. These data were more comprehensive than in previous studies on anorectal malformations and assisted conception.[6-8] Additional analyses on the underlying reasons for parental subfertility were not feasible, as this information was often unknown.

Despite the relatively low response rate, we do not think that selection bias was a major issue, because the parents were probably unaware of the potential association between fertility issues and anorectal malformations because the questionnaires covered many pregnancy-related topics, such as pregnancy complications and lifestyle during pregnancy. Moreover, the rates of fertility treatment in the control group were representative for the Dutch general population,[21] which probably excludes selection due to a higher or

lower participation rate among control parents who underwent fertility treatment. A limitation of this study was the possibility of misclassification due to the self-reported nature of the data with the concomitant potential for recall problems, especially since the range of the time interval between childbirth and filling out the questionnaires was 0 to 22 years. However, this range was similar for cases and controls, and we do not expect parental subfertility and fertility treatment to be highly susceptible to recall errors, as these are major life events. In addition, the assumption that parents with an unplanned pregnancy were fertile may have resulted in non-differential misclassification, but this seemed negligible as sensitivity analyses showed that the results were similar when unplanned pregnancies were excluded. In many studies on congenital malformations, the effects of fertility treatment may be underestimated when only live-born cases and controls are included in the study, as subfertile parents who receive fertility treatment may be more likely to be offered prenatal screening, possibly resulting in a termination of pregnancy. However, anorectal malformations can hardly be diagnosed prenatally.[22] Therefore, the potential for underestimation due to inclusion of live-borns only is limited in the analyses on isolated anorectal malformations. However, the results on anorectal malformations with other congenital malformations may be underestimated if pregnancies were terminated because of severe associated malformations.

We found increased risks of anorectal malformations for subfertile parents who received ICSI or IVF treatment compared with fertile parents who conceived spontaneously, with even larger effects in singletons only, which is consistent with previous studies.[7,8] Interestingly, ICSI and IVF treatments were still associated with increased occurrences of anorectal malformations when the analyses were restricted to subfertile parents with or without treatment, which has never been done before. This may point to an added effect of gamete manipulation, including culture medium and timing of fertilization, and/or of the hormones involved in ovulation induction as part of ICSI and IVF treatment, over and above possible adverse effects of the underlying parental subfertility. However, no association was found between anorectal malformations and the use of ovulation induction hormones without ICSI or IVF. This is in line with a large population-based study,[13] but not with two register-based studies.[10,12] Furthermore, the associations may still be confounded by the indication for ICSI or IVF treatment, as some of the subfertile parents who conceived after ICSI or IVF may be more severely subfertile than those who conceived without treatment. This indicates that, although subfertile parents who conceived without treatment were used as the reference group, the underlying parental subfertility and/or its causal factors could still play a role. This was confirmed by our finding that subfertility without treatment led to an increased risk of anorectal malformations with additional major congenital malforma-

tions. Disturbed epigenetic processes as a consequence of ICSI or IVF and/or male subfertility may also be explanations for their involvement in the etiology of anorectal malformations,[23,24] as was shown for the Beckwith-Wiedemann syndrome.[25]

Previously, increased risks of anorectal malformations after ICSI or IVF were found in singletons, whereas no associations were found in multiple births.[7] Due to small numbers, we could not calculate risk estimates for multiple births, but we did find higher odds ratios in singleton births only than in singleton and multiple births combined. We did not find indications for changes over time in the risk of anorectal malformations after fertility treatment as the risk estimates for IVF treatment were similar when the analyses were stratified into before and after 2000. We were unable to stratify the analyses for more time windows or for ICSI treatment due to small numbers. The association between anorectal malformations and IVF was observed among isolated anorectal malformations, but was much more pronounced for anorectal malformations with one or more additional congenital malformations, which was also shown by Zwink *et al*.[8] Furthermore, subfertile parents who conceived without treatment were found to have an increased risk of anorectal malformations with additional major congenital malformations, but not isolated anorectal malformations. Cases with additional congenital malformations more often have severe forms of anorectal malformations, such as rectourethral fistulas or cloacas, which may imply stronger associations of parental subfertility and in vitro fertilization with severe forms of anorectal malformations.

Parental subfertility without treatment doubled the risk of anorectal malformations with additional congenital malformations. Subfertile parents were defined as those who had a TTP > 12 months and/or were ever diagnosed with maternal and/or paternal subfertility. In some parents in the latter group, however, fertility might have been restored prior to this pregnancy, which could have led to underestimation of the effects due to non-differential misclassification. Therefore, we performed separate analyses for these two groups and observed stronger associations between anorectal malformations and a TTP > 12 months, which we considered most reliable. In addition, we found only an association between anorectal malformations and parental subfertility without treatment in nulliparous women, which may point to a difference in the severity of parental subfertility between nulliparous and multiparous women and their partners in this study. This was not in line with a previous study on heterogeneous patient groups with congenital malformations that found similar results in nulliparous women as in the total group of women, including multiparous women as well.[3] In our previous small study, we evaluated the role of parental subfertility in the etiology of anorectal malformations; we found paternal subfertility to potentially increase the risk of anorectal malformations, but not maternal subfertility.[11] This is in line with the finding for subfertility of the father in the present study, in which only 21% of the cases were

derived from the previous study. In the latter, however, the subfertile groups included parents with and without fertility treatment and no analyses on subgroups of anorectal malformations were performed. In our time to pregnancy analyses, we found weak indications that fertile parents who conceive within 3 months have the smallest risk of having a child with anorectal malformations, since the odds ratios for almost all other classes of time to pregnancy were above unity.

In conclusion, we found evidence for a role of ICSI and IVF treatment in the etiology of anorectal malformations, even compared with subfertile parents who conceived without treatment. We also found indications for an independent role of subfertility in the etiology of anorectal malformations with other congenital malformations. Future studies are needed to unravel the underlying biological mechanisms through which the development of the anorectal channel may be disrupted by these factors. The findings of this study on anorectal malformations, in combination with the existing literature on ART and other congenital malformations, may also provide guidance to counseling of subfertile couples.

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Part 3

Genetic factors and gene-environment interaction

Chapter 9

No major role for periconceptional folic acid use and its interaction with the *MTHFR C677T* polymorphism in the etiology of congenital anorectal malformations

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Abstract

Background Both genetic and nongenetic factors are suggested to be involved in the etiology of congenital anorectal malformations (ARM). Maternal periconceptional use of folic acid supplements were inconsistently suggested to play a role in the prevention of ARM. Therefore, we investigated independent associations and interactions of maternal periconceptional folic acid supplement use and the infant and maternal *MTHFR* (methylenetetrahydrofolate reductase) *C677T* polymorphisms with the risk of ARM and subgroups of ARM.

Methods A case-control study was conducted among 371 nonsyndromic ARM cases and 714 population-based controls born between 1990 and 2012 using maternal questionnaires and DNA samples from mother and child. Cases were treated for ARM at departments of Pediatric Surgery of the Radboud university medical center, Sophia Children's Hospital-Erasmus Medical Centre Rotterdam, and the University Medical Center Groningen in The Netherlands and hospitals throughout Germany.

Results No association with folic acid use was present (odds ratio = 1.1; 95% confidence interval: 0.8–1.4) for ARM as a group. Infant and maternal *MTHFR* C*677T* polymorphisms were weakly associated with isolated ARM in particular. Lack of folic acid supplement use in combination with infants or mothers carrying the *MTHFR* C*677T* polymorphism did not seem to increase the risk of ARM or subgroups of ARM. The relative excess risks due to interaction did not clearly indicate interaction on an additive scale either.

Conclusion This first study investigating interactions between periconceptional folic acid supplement use and infant and maternal *MTHFR C677T* polymorphisms in the etiology of ARM did not provide evidence for a role of this gene-environment interaction.

Introduction

Anorectal malformations (ARM) are common congenital malformations of the digestive system, but are still rare with a prevalence of 2 to 6 cases per 10,000 births worldwide.[1] ARM are characterized by disturbed embryonic development of the hindgut in weeks 4 to 8 after conception and include a wide range of malformations of the rectum and anus, often also involving the urogenital tract. Despite improved surgical treatment, ARM patients encounter lifelong functional and psychosocial problems.[2,3] The etiology for most forms of ARM remains to be elucidated, but is assumed to be an interplay between genetic and nongenetic factors. No major gene is identified yet, while only a few nongenetic risk factors have consistently been found to increase the risk of ARM, including assisted reproductive techniques,[4-8] multiple pregnancy,[7-11] maternal prepregnancy overweight or obesity,[12-15] and pre-existing diabetes mellitus.[15-20]

Maternal periconceptional use of folic acid supplements plays an important role in the prevention of neural tube defects.[21] Other studies also showed decreased risks of some other congenital malformations, such as congenital heart defects and orofacial clefts, after maternal folic acid supplement use.[22,23] Inconsistent results were, however, observed for ARM.[14,20,24-26] A few studies did not find an association between ARM and folic acid use,[14,26] whereas others found slightly reduced risks.[20,24,25]

The B-vitamin folate is a one-carbon donor and as such essential for biosynthesis of nucleotides and the remethylation of homocysteine to methionine, an important step that donates methylgroups to DNA, RNA, proteins, and lipids. Therefore, folate is important for rapid cell division and gene expression, essential for cellular growth and differentiation during embryogenesis. Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme in the folate metabolism and catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (active form of folate), which leads to the remethylation of homocysteine. The *MTHFR C677T* polymorphism reduces MTHFR enzyme activity, leading to decreased plasma levels of active folate and increased levels of homocysteine.[27] Use of folic acid supplements during embryonic development may compensate for reduced folate levels due to genetic polymorphisms in the *MTHFR* gene.

Associations between ARM and the *MTHFR C677T* polymorphism have never been studied, but may exist as they do for other congenital malformations.[28,29] Previous studies on orofacial clefts and congenital heart malformations showed synergistic associations of the *MTHFR C677T* polymorphism and a lack of folic acid use,[23,30] but gene-environment interactions have never been evaluated for ARM either. However, the inconsistent findings regarding an association between ARM and folic acid use may be explained by differences in genetic background. Therefore, we aimed to investigate independent associations and

interactions of the *MTHFR C677T* polymorphism and folic acid supplementation with the risk of ARM. As previous studies showed indications for etiologic heterogeneity among different subgroups of ARM,[8,31] we also studied these associations and interactions in subgroups of ARM.

Materials and methods

Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children (AGORA) is a large data- and biobank with clinical and questionnaire data and DNA samples from children with congenital malformations or childhood cancer, control children, and their parents coordinated by the Radboud university medical Center (Radboudumc) (www.AGORAproject.nl). In the current case-control study, cases were live born children treated for ARM at the Departments of Pediatric Surgery of the Radboudumc, Sophia Children's Hospital-Erasmus MC Rotterdam (EMC), or the University Medical Center Groningen (UMCG) in The Netherlands. From 2007 onwards, the parents of ARM cases were asked to participate in AGORA at the child's first admittance to the Radboudumc. The parents of older cases treated at the Radboudumc and cases treated at the EMC or UMCG were invited to participate by mail. We collected blood or saliva samples from cases born between January 1990 and April 2012, and from their parents, as well as parental questionnaire information concerning demographics, family history, and health and lifestyle before and during pregnancy. From cases born before 1990, blood or saliva samples were collected, but parental questionnaires were not. CURE-Net, a German Network for Congenital Uro-REctal malformations, provided DNA samples with year of birth and ethnicity of 150 additional German ARM cases and their mothers. They were recruited through the German self-help organization for ARM patients (SoMA e.V.) and pediatric surgical departments throughout Germany. These cases were included in the genetic analyses to increase power, but were excluded from the folic acid and interaction analyses. ARM cases with syndromic forms of ARM, chromosomal abnormalities, or cloacal exstrophy were excluded, but other major congenital malformations may be present among the cases. Our population-based controls were children born between January 1990 and December 2010, randomly sampled from 39 municipalities in the same geographical areas as the cases. Control children with major congenital malformations were excluded from the analyses. To ensure independent analyses, only one case or control per family was included. The parents of control children filled in the same questionnaires as the case parents and provided saliva samples from both mother and child. The regional Committee on Research involving Human Subjects approved the AGORA protocol and the Ethics Committee of the University of Bonn

and the University of Heidelberg approved the CURE-Net protocol. Written informed consent was obtained from all children and/or parents.

Pediatric surgeons, clinical geneticists, and researchers reviewed the medical records of the ARM patients to obtain clinical information on ARM phenotype and additional congenital malformations. ARM phenotypes were classified according to the Krickenbeck criteria[32] and other minor and major congenital malformations according to the EUROCAT classification.[33] For the main analyses, we created two subgroups of ARM: isolated ARM cases (with or without minor other congenital malformations) and ARM cases with one or more additional major congenital malformations. The latter group also included ARM cases with the VACTERL association (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal, and Limb defects). ARM-VACTERL cases were defined as ARM cases with at least two other congenital malformations included in the VACTERL association.[34]

Blood was collected in EDTA containing tubes and saliva in Oragene containers (DNA Genotek Inc., Ottawa, Canada). DNA was extracted from blood or saliva using standard methods. Samples were genotyped for the *MTHFR C677T* polymorphism (rs1801133) using the 5'-nuclease Taqman single nucleotide polymorphism genotyping assay (C_1202883_20, Applied Biosystems, Foster City, CA). Polymerase chain reactions (PCRs) were carried out in 96 or 384 wells plates in a 5 µl reaction volume containing 10 ng genomic DNA, 2 µl Taqman universal PCR master mix, 0.05 µl assay mix, and 1.95 µl milli-Q. The PCR consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 denaturation cycles at 92˚C for 15 seconds and annealing and extension at 60°C for 60 seconds. After PCR, allele-specific fluorescence was measured on ABI 7500 FAST and 7900 HT instruments (Applied Biosystems) for 96 or 384 wells plates, respectively. In each plate, four blanks were added and five wells were loaded with internal controls for quality control purposes. Genotyping was performed in a laboratory recognized and granted accreditation for quality control by the coordinating committee for improvement of quality control of laboratory research in health care.

In the maternal questionnaires, mothers were asked about their use of folic acid or multivitamin supplements in the 3 months before conception and during pregnancy, including information about exact weeks before and/or during pregnancy and brand name of the multivitamin. Users were defined as mothers reporting use of folic acid supplements or multivitamins containing folic acid in the 4 weeks before conception and/or the first 10 weeks after conception. Nonusers were mothers who reported no use during the entire period of 3 months before conception until the end of pregnancy. Mothers who did not know the period of use or began using more than 10 weeks after conception, were excluded from the analyses. Infant and maternal characteristics extracted from the questionnaires

included gender, year of birth, ethnicity of the child (European vs. non European), maternal age at delivery, maternal education (low: no/primary/lower vocational/intermediate secondary education; middle: intermediate vocational/higher secondary education; high: higher vocational/academic education), ARM in first degree relatives of the child, fertility treatment (also including ovulation induction), multiple pregnancy (singleton vs. multiple), parity (nulliparity vs. multiparity), pre-existing diabetes, and maternal prepregnancy body mass index. The latter was calculated using reported weight (kg) and height (m) before conception and categorized in 4 groups (<18.5, 18.5-24.9, 25.0-29.9, and ≥30.0 kg/m^2).

Statistical analyses were performed using SPSS 20.0 for Windows (IBM SPSS, Chicago, IL, USA). The *MTHFR C677T* genotype frequencies in controls were tested for deviation from Hardy Weinberg equilibrium (HWE). Univariable and multivariable logistic regression analyses were used to calculate crude and adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for independent associations between ARM and periconceptional folic acid use and infant and maternal *MTHFR C677T* genotypes. The homozygous wild type (*CC*) was used as reference category. As MTHFR enzyme activity is already reduced by 35% in the heterozygous genotype (*CT*),[27] the analyses were repeated by assuming a dominant effect (*CT/TT* vs. *CC*). In addition, the role of interactions between periconceptional folic acid use and *MTHFR C677T* genotypes in the etiology of ARM was examined. We calculated adjusted ORs for ARM among the following groups for both infant and maternal genotypes and different models of inheritance: *MTHFR 677CC* with periconceptional folic acid use (reference), *MTHFR 677CC* without periconceptional folic acid use, *MTHFR C677T* polymorphism (*CT*, *TT* or *CT/TT*) with periconceptional folic acid use, *MTHFR C677T* polymorphism without periconceptional folic acid use (interaction). We also calculated relative risks due to interaction (RERIs) to assess interaction on an additive scale using the method and Excel spreadsheet (www.epinet.se) developed by Andersson et al.[35] We considered ethnicity of the child, maternal age at delivery, maternal education, multiple pregnancy, fertility treatment, pre-existing diabetes, and prepregnancy body mass index as potential confounders in the analyses that included folic acid use, because these factors were previously reported as risk factors or found to be associated with ARM in this study. Only potential confounding factors that changed the OR of the determinant of interest in bivariable analyses were included in the full multivariable logistic regression models to prevent instable models due to too many covariates. Because risk estimates for infant *MTHFR C677T* genotypes may also be mediated by maternal genetic effects as these may partially determine uterine environment,[36-38] we included infant and maternal *MTHFR C677T* genotypes in multivariable analyses of the *MTHFR C677T* polymorphism only and in the interaction analyses. For reasons of precision, we excluded factors from the full models

when the OR did not change more than 10% upon removal. The above-mentioned analyses were repeated for subgroups of ARM.

Results

Response rates were 57% among cases and 34% among controls. After applying the exclusion criteria and exclusion of one control-mother pair because of Mendelian errors, 371 cases and 714 controls were eligible for the entire case-control study. The genetic analyses were performed using DNA samples of 643 cases and 714 controls, including German cases and Dutch cases without questionnaire information. Genotyping was completed successfully in more than 97% of the infant and maternal DNA samples. Of the total 643 cases, 373 had isolated ARM (58%) and 265 were ARM cases with other major congenital malformations (42%), including 83 ARM-VACTERL cases (Table 1). Perineal fistulas were the most common ARM phenotype, with the highest percentage in isolated ARM cases (58%). Vertebral (14%), cardiac (11%), and/or renal malformations (14%) were most commonly associated with ARM. No substantial differences were observed between cases and controls regarding gender, year of birth, nulliparity, and pre-existing diabetes (Table 2). Non-European descent, young maternal age, low maternal education, ARM in first degree relatives, fertility treatment, multiple pregnancy, and maternal obesity were more common among cases than controls.

In total, 69% of all mothers reported use of folic acid or multivitamin supplements in the period of 4 weeks before through 10 weeks after conception. Of these mothers, 69% took supplements containing folic acid only, 9% took multivitamins, and 22% took both folic acid supplements and multivitamins. Use of folic acid was similar in cases and controls, resulting in an OR of 1.1 (95% CI: 0.8–1.4) after adjustment for maternal education. We did not find associations between folic acid use and the subgroups of isolated ARM and ARM with other major congenital malformations either.

Table 3 shows the independent associations between ARM and subgroups of ARM and infant and maternal *MTHFR C677T* genotypes. Genotype frequencies of *MTHFR C677T* were in HWE among control mothers ($P > 0.05$), but not among control children ($P = 0.03$). The homozygous *TT* variant was present in approximately 10 to 12% of children and mothers, but this variant did not increase the risk of ARM (OR = 0.9; 95% CI: 0.6–1.3 and OR = 1.1; 95% CI: 0.7–1.5, respectively). However, both infant and maternal heterozygosity (*CT*) slightly increased the risk of ARM compared to the wild type (OR = 1.3; 95% CI: 1.0–1.7 and OR = 1.2; 95% CI: 0.9–1.5, respectively). Assuming dominant effects, the combined infant or maternal *CT* and *TT* genotypes were slightly more common among cases than among controls as well. These weak associations were observed for isolated ARM, but not for ARM

with other major congenital malformations. No differences in risk estimates were observed after mutual adjustment for infant and maternal *MTHFR C667T* genotypes. When the analyses were restricted to Dutch cases and controls only, the risk estimates were slightly lower.

Table 1. Different phenotypes of anorectal malformations, The Netherlands and Germany, 1953-2012.

	Total ARM cases (n=643)[a,b]	Isolated ARM cases[c] (n=373)	ARM cases with other major defects (n=265)
	No. (%)	No. (%)	No. (%)
Phenotypes of ARM			
Perineal fistula	293 (45.6)	215 (57.6)	78 (29.4)
Vestibular fistula	103 (16.0)	53 (14.2)	50 (18.9)
ARM without fistula	32 (5.0)	16 (4.3)	16 (6.0)
Rectourethral fistula			
Bulbar	50 (7.8)	24 (6.4)	26 (9.8)
Prostate	46 (7.2)	17 (4.6)	29 (10.9)
Unspecified	30 (4.7)	13 (3.5)	16 (6.0)
Rectovesical fistula	16 (2.5)	5 (1.3)	11 (4.2)
Cloaca	30 (4.7)	7 (1.9)	23 (8.7)
Anal stenosis	14 (2.2)	11 (2.9)	3 (1.1)
Rare types[d]	16 (2.5)	8 (2.1)	8 (3.0)
Type unknown	13 (2.0)	4 (1.1)	5 (1.9)
Additional major congenital malformations			
ARM-VACTERL	83 (12.9)	-	83 (31.3)
Vertebral malformations	87 (13.6)	-	87 (32.8)
Cardiac defects	73 (11.4)	-	73 (27.5)
Tracheo-Esophageal atresia	51 (8.0)	-	51 (19.2)
Renal malformations	88 (13.8)	-	88 (33.2)
Radial limb defects	35 (5.5)	-	35 (13.2)

[a]Numbers of isolated ARM cases and ARM cases with other major defects do not add up to total number of ARM cases due to missing information about additional major congenital malformations in 5 ARM cases.

[b]ARM cases include 493 cases from The Netherlands and 150 cases from Germany.

[c]Isolated ARM cases may have additional minor congenital malformations.

[d]Including rectovaginal fistula, rectal atresia, rectal stenosis, postanal sinus, dorsal cloaca-like defect with complex H-fistula.

Table 2. Characteristics of cases with anorectal malformations and controls, The Netherlands, 1990-2012.

	Cases (n=371) No. (%)[a]	Controls (n=714)[b] No. (%)[a]
Gender of child		
Male	180 (48.5)	331 (46.4)
Female	191 (51.5)	383 (53.6)
Year of birth of child		
1990-1995	81 (21.8)	158 (22.1)
1996-2000	96 (25.9)	197 (27.6)
2001-2005	84 (22.6)	168 (23.5)
2006-2010	100 (27.0)	191 (26.8)
2010-2012	10 (2.7)	0 (0.0)
Ethnicity of child		
European	337 (91.3)	676 (94.7)
Non European	32 (8.7)	37 (5.2)
Maternal age at delivery		
<25 years	27 (7.3)	28 (3.9)
25-29 years	123 (33.2)	217 (30.4)
30-34 years	156 (42.0)	346 (48.5)
≥35 years	65 (17.5)	123 (17.2)
Maternal education		
Low	98 (26.4)	112 (15.8)
Middle	156 (42.0)	339 (47.7)
High	117 (31.5)	260 (36.6)
ARM in 1st degree relatives	5 (1.5)	0 (0.0)
Fertility treatment	33 (9.0)	21 (3.0)
Multiple pregnancy	18 (4.9)	21 (2.9)
Nulliparity	144 (39.0)	282 (39.7)
Pre-existing diabetes mellitus	3 (0.9)	2 (0.3)
Pre-pregnancy body mass index		
Underweight (<18.5 kg/m^2)	18 (5.1)	23 (3.4)
Normal (18.5-24.9 kg/m^2)	228 (64.8)	487 (71.7)
Overweight (25.0-29.9 kg/m^2)	72 (20.5)	127 (18.7)
Obese (≥30.0 kg/m^2)	34 (9.7)	42 (6.2)

[a]Numbers do not add up to total numbers due to missing values (<1% missings in all variables, except for ARM in 1st degree relatives with approximately 10% missings [mainly due to missing paternal questionnaires] and pre-pregnancy diabetes mellitus and body mass index with approximately 5% missings).

[b]No questionnaire data were available for one control child.

Table 3. Associations between anorectal malformations and the MTHFR 677C>T polymorphism, The Netherlands and Germany, 1953-2012.

	Controls (n=714) No. (%)	Cases[a] (n=643) No. (%)	OR (95% CI)[b,c]	Isolated ARM[d] (n=373) No. (%)	OR (95% CI)[b,c]	ARM with other major defects (n=265) No. (%)	OR (95% CI)[b,c]
Infant *MTHFR 677C>T*[e]							
CC	326 (47.5)	260 (42.7)	1.0 (ref.)	146 (41.4)	1.0 (ref.)	112 (44.6)	1.0 (ref.)
CT	276 (40.2)	291 (47.8)	1.3 (1.0-1.7)	170 (48.2)	1.4 (1.0-1.8)	118 (47.0)	1.2 (0.9-1.7)
TT	84 (12.2)	58 (9.5)	0.9 (0.6-1.3)	37 (10.5)	1.0 (0.6-1.5)	21 (8.4)	0.7 (0.4-1.2)
CT/ TT	360 (52.5)	349 (57.3)	1.2 (1.0-1.5)	207 (58.7)	1.3 (1.0-1.7)	139 (55.4)	1.1 (0.8-1.5)
Maternal *MTHFR 677C>T*[f]							
CC	335 (48.1)	227 (44.2)	1.0 (ref.)	122 (40.8)	1.0 (ref.)	105 (49.5)	1.0 (ref.)
CT	285 (40.9)	232 (45.1)	1.2 (0.9-1.5)	141 (47.2)	1.4 (1.0-1.8)	89 (42.0)	1.0 (0.7-1.4)
TT	77 (11.0)	55 (10.7)	1.1 (0.7-1.5)	36 (12.0)	1.3 (0.8-2.0)	18 (8.5)	0.7 (0.4-1.3)
CT/ TT	362 (51.9)	287 (55.8)	1.2 (0.9-1.5)	177 (59.2)	1.3 (1.0-1.8)	107 (50.5)	0.9 (0.7-1.3)

[a]Numbers of isolated ARM cases and ARM cases with other major defects do not add up to total number of ARM cases due to missing information about additional major congenital malformations in 5 ARM cases.

[b]Similar results were found when cases and controls born before 1990 were excluded or when non-Europeans were excluded.

[c]Similar results were observed when the odds ratios of infant *MTHFR 677C>T* genotypes were adjusted for maternal genotypes and maternal *MTHFR 677C>T* genotypes were adjusted for infant genotypes.

[d]Isolated ARM cases may have additional minor congenital malformations.

[e]*MTHFR C677T* genotype data were available for a total of 686 controls and 609 cases, including 353 isolated ARM cases and 251 ARM cases with other major defects.

[f]*MTHFR C677T* genotype data were available for a total of 697 control mothers and 514 case mothers, including 299 mothers of isolated ARM cases and 212 mothers of ARM cases with other major defects.

The effects of interactions of periconceptional folic acid supplement use and *MTHFR C677T* genotypes on the risk of ARM and subgroups of ARM are shown in Tables 4 and 5, respectively. Lack of periconceptional folic acid use in combination with an infant carrying the *CT* or *TT* genotype did not seem to increase the risk of ARM (OR = 1.3; 95% CI: 0.8–2.0 and OR = 0.9; 95% CI: 0.4–2.1, respectively) compared to folic acid supplement users with infants carrying the wild type. Nevertheless, the RERIs of 0.5–0.6 provided weak indications for additivity in the joint associations, although statistical significance was not reached. Neither additivity nor increased risks of ARM were suggested when mothers did not use folic acid supplements and carried the *CT* or *TT* genotype (OR 1.0; 95% CI: 0.6–1.6, RERI 0.2; 95% CI: -0.4–0.7 and OR = 1.3; 95% CI: 0.5–2.9, RERI 0.6, 95% CI -0.6–1.7, respectively). Similar results were observed for isolated ARM, although with slightly stronger risk estimates and maternal RERIs. For ARM with other major congenital malformations, none of the risk estimates or RERIs were suggestive for a role of interaction between lack of folic acid supplement use and infants or mothers carrying the *CT* or *TT* genotype (ORs and RERIs ranging from 0.6 to 1.1 and -0.1 to 0.7, respectively). The results for all interaction analyses were similar after adjusted for infant and maternal *MTHFR C677T* genotypes.

None of the risk estimates for isolated ARM differed when only isolated ARM with perineal fistulas were studied. No substantial differences were observed either when ARM-VACTERL cases were excluded from the analyses on ARM with other congenital malformations. In addition, restriction of the analyses to children without first-degree relatives with ARM or to children from European descent only did not yield different results.

Table 4. Interactions of the MTHFR 677C>T polymorphism and maternal periconceptional folic acid supplement use with the risk of anorectal malformations, The Netherlands, 1990-2012.

	Periconceptional folic acid use		No periconceptional folic acid use		
	No. cases / controls[a]	**Adjusted OR (95% CI)[b,c]**	**No. cases / controls[a]**	**Adjusted OR (95% CI)[b,c]**	**RERI (95% CI)**
Infant *MTHFR 677C>T*					
CC	100/190	1.0 (ref.)	40/101	0.7 (0.4-1.1)	
CT	93/161	1.1 (0.8-1.6)	50/69	1.3 (0.8-2.0)	0.5 (-0.1-1.1)
TT	19/58	0.7 (0.4-1.2)	9/16	0.9 (0.4-2.1)	0.6 (-0.2-1.5)
CT/ TT	112/219	1.0 (0.7-1.4)	59/85	1.2 (0.8-1.8)	0.6 (0.0-1.1)
Maternal *MTHFR 677C>T*					
CC	99/195	1.0 (ref.)	47/97	0.8 (0.5-1.3)	
CT	89/174	1.0 (0.7-1.5)	44/74	1.0 (0.6-1.6)	0.2 (-0.4-0.7)
TT	23/53	0.9 (0.5-1.5)	10/15	1.3 (0.5-2.9)	0.6 (-0.6-1.7)
CT/ TT	112/227	1.0 (0.7-1.4)	54/89	1.0 (0.7-1.6)	0.2 (-0.3-0.8)

[a]Questionnaire data in combination with genotype data were available for 344 cases, 346 case mothers, 685 controls, and 696 control mothers. Numbers in the columns do not add up to these total numbers, mainly due to exclusion of mothers who used folic acid supplements outside the advised period.

[b]Adjusted for maternal education. Other potential confounders did not change the OR >10% upon removal.

[c]Similar results were observed when the odds ratios of infant *MTHFR 677C>T* genotypes combined with folic acid supplement use were adjusted for maternal genotypes and the odds ratios of maternal *MTHFR 677C>T* genotypes combined with folic acid supplement use were adjusted for infant genotypes.

Table 5. Interactions of the MTHFR 677C>T polymorphism and maternal periconceptional folic acid supplement use with the risk of anorectal malformations in phenotypic subgroups, The Netherlands, 1990-2012.

	Periconceptional folic acid use		No periconceptional folic acid use		
	No. cases / controls[a]	Adjusted OR (95% CI)[b,c]	No. cases / controls[a]	Adjusted OR (95% CI)[b,c]	RERI (95% CI)
Isolated ARM					
Infant *MTHFR 677C>T*					
CC	57/190	1.0 (ref.)	23/101	0.7 (0.4-1.1)	
CT	59/161	1.2 (0.8-1.9)	33/69	1.5 (0.9-2.6)	0.6 (-0.2-1.4)
TT	14/58	0.9 (0.5-1.7)	6/16	1.1 (0.4-2.9)	0.6 (-0.6-1.8)
CT/ TT	73/219	1.1 (0.8-1.7)	39/85	1.4 (0.9-2.3)	0.6 (-0.1-1.3)
Maternal *MTHFR 677C>T*					
CC	56/195	1.0 (ref.)	25/97	0.8 (0.5-1.3)	
CT	57/174	1.2 (0.8-1.8)	32/74	1.3 (0.8-2.2)	0.4 (-0.4-1.1)
TT	16/53	1.1 (0.6-2.1)	8/15	1.8 (0.7-4.6)	1.0 (-0.7-2.8)
CT/ TT	73/227	1.2 (0.8-1.7)	40/89	1.4 (0.9-2.3)	0.5 (-0.2-1.2)
ARM with other major congenital malformations					
Infant *MTHFR 677C>T*					
CC	43/190	1.0 (ref.)	17/101	0.7 (0.4-1.4)	
CT	34/161	0.9 (0.6-1.5)	17/69	1.1 (0.6-2.0)	0.4 (-0.4-1.2)
TT	5/58	0.4 (0.1-1.0)	3/16	0.8 (0.2-3.0)	0.7 (-0.4-1.9)
CT/ TT	39/219	0.8 (0.5-1.3)	20/85	1.0 (0.6-1.9)	0.5 (-0.2-1.2)
Maternal *MTHFR 677C>T*					
CC	43/195	1.0 (ref.)	22/97	0.9 (0.5-1.6)	
CT	32/174	0.8 (0.5-1.4)	12/74	0.6 (0.3-1.3)	-0.1 (-0.8-0.6)

	Periconceptional folic acid use		No periconceptional folic acid use		
	No. cases / controls[a]	**Adjusted OR (95% CI)[b,c]**	**No. cases / controls[a]**	**Adjusted OR (95% CI)[b,c]**	**RERI (95% CI)**
TT	7/53	0.6 (0.3-1.4)	2/15	0.6 (0.1-2.7)	0.0 (-1.1-1.2)
CT/ TT	39/227	0.8 (0.5-1.2)	14/89	0.6 (0.3-1.2)	-0.1 (-0.8-0.6)

[a]Questionnaire data in combination with genotype data were available for 214 isolated ARM cases, 218 mothers of isolated ARM cases, 130 ARM cases with other major congenital malformations, 128 mothers of ARM cases with other major congenital malformations, 685 controls, and 696 control mothers. Numbers in the columns do not add up to these total numbers, mainly due to exclusion of mothers who used folic acid outside the advised period.

[b]Adjusted for maternal education, except for associations between ARM with other major congenital malformations and infant *MTHFR 677C>T*, in which maternal education was not identified as a confounder. Other potential confounders did not change the OR >10% upon removal.

[c]Similar results were observed when the odds ratios of infant *MTHFR 677C>T* genotypes combined with folic acid supplement use were adjusted for maternal genotypes and the odds ratios of maternal *MTHFR 677C>T* genotypes combined with folic acid supplement use were adjusted for infant genotypes.

Discussion

This is the first study investigating the role of gene-environment interactions between maternal periconceptional folic acid supplement use and infant and maternal *MTHFR 677CT/TT* genotypes in the etiology of ARM. No major role was observed for this gene-environment interaction. We did not find evidence for an independent association between ARM and periconceptional folic acid supplement use either and only found weak associations with the infant and maternal *MTHFR C677T* polymorphisms, for isolated ARM in particular.

Major strengths of this study are the relatively large numbers of well-characterized ARM cases and population-based controls. The large case number enabled us to study subgroups of ARM separately, including isolated ARM, isolated ARM with perineal fistulas only, and ARM with one or more other major congenital malformations with and without ARM-VACTERL. However, the numbers were too small to study more detailed phenotypic subgroups of ARM. Approximately 42% of the cases had associated congenital malformations, which is in line with previous studies that reported rates of 40-70%, usually including syndromic or chromosomal forms of ARM as well.[8,39] Our population-based controls were randomly sampled via municipalities in the same geographical areas as the cases and were of similar age. Although the response rate was relatively low, the control group seems to be representative as most maternal characteristics, such as multiple pregnancy and fertility treatment, were similar to the general Dutch pregnant population,[40] except for maternal education. Control mothers were slightly higher educated than case mothers, which may be due to a lower participation rate among low educated control mothers. Therefore, we adjusted the analyses that included folic acid use for maternal education. This may, however, have led to overadjustment due to a strong correlation between folic acid supplement use and maternal education.

In this study, 69% of all mothers used folic acid supplements 4 weeks before conception and/or in the first 10 weeks after conception, which was similar to the findings in another recent Dutch study.[41] We used a more inclusive definition of folic acid supplement use than consistent use only to include larger numbers of cases and controls while accepting some degree of random misclassification. As the majority of women in The Netherlands are aware of the correct period of folic acid supplement use, however, we expected the resulting bias towards the null to be small. This was confirmed in a subanalyses restricted to women who definitely used folic acid supplements consistently during the advised period, in which an OR of 1.2 (0.8-1.8) was found after adjustment for maternal education.

An additional strength of this study was the possibility to investigate combinations of detailed questionnaire data on folic acid use with infant and maternal *MTHFR* genotype

data. This is unique in etiologic research on ARM, as to our knowledge we were the first to study this gene-environment interaction. Detailed information was also collected on potential confounders that were used in the multivariable analyses. Except for maternal education, none of these proved to be true confounders in any of the associations and neither did infant or maternal *MTHFR C667T* genotypes. Selection bias due to the relatively low response rates does not seem to be an issue in the genetic analyses either as the distribution of *MTHFR* genotypes is probably unrelated to response.

A limitation of this study was the potential for misclassification as information on folic acid supplement use was collected retrospectively using self-reported data. As pregnancy is a major life event and a great deal of attention is given to periconceptional folic acid use, however, we expect women to be able to adequately recall this information. In addition, the time interval between childbirth and filling out the questionnaires did not differ substantially between cases and controls. Deviation from HWE was not expected beforehand among the population-based control children. This phenomenon may rather be explained by genetic selection than by genotyping problems, as several researchers found selection in favor of the *T* allele in the last decade. They hypothesized that periconceptional folic acid use might have influenced genetic selection in favor of the *MTHFR C677T* polymorphism.[42-44] Our finding of deviation from HWE among control children but not among control mothers strengthens this hypothesis as folic acid supplements were not used in the period that the control mothers were born. Genetic confounding can arise in case-control designs due to population stratification, but this is minimal in ethnically homogeneous groups, such as the Dutch and German populations.[45]

We did not find an association between ARM and folic acid supplement use, as in other European studies.[14,26] In contrast, two non-European studies found slightly decreased risks.[20,25] One of these latter studies was from China, where the prevalence of folate deficiency is high compared to other populations,[46] which may explain the different results. We were the first to observe weak associations between ARM and infant and maternal *MTHFR C677T* polymorphisms and to investigate their interaction with periconceptional folic acid supplement use. The latter did not provide evidence that the *MTHFR C677T* polymorphism is involved in the etiology of ARM especially when no folic acid supplements are used in the critical time period of embryonic development of the anorectal channel. This result deviates from the findings in studies on other congenital malformations, such as neural tube defects, cleft lip and palate and congenital heart defects.[23,30,47] Even if future studies with larger ARM patient series would show similar risk estimates with smaller confidence intervals, the role of this gene-environment interaction seems to be marginal. Folate acts in many biochemical processes, including DNA synthesis and methylation, but

this study suggests that it is not substantially involved in the development of the anorectal channel. The precise pathogenic mechanisms by which a lack of folic acid and reduced MTHFR enzyme activity act during embryogenesis are not fully understood. Several researchers hypothesized that a disturbed folate and/or homocysteine metabolism affects neural crest cell formation, leading to the above-mentioned neural-crest cell associated malformations.[30,48,49] Both *in vivo* and *in vitro* experiments also suggested that altered levels of folate and/or homocysteine lead to abnormalities in neural crest cell outgrowth, differentiation and migration.[48,50] This may explain why periconceptional folic acid use and the *MTHFR C677T* polymorphism do not play a major role in the etiology of ARM, which does not depend on neural crest cell formation.

In conclusion, while the ultimate goal is to provide women who try to conceive with individualized lifestyle recommendations, we did not find evidence that periconceptional use of folic acid supplements reduces the risk of having a child with ARM, not even for women who carry the *MTHFR C677T* polymorphism.

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Chapter 10

Rare coding variants and the risk of congenital anorectal malformations: an exome chip association study

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Abstract

Anorectal malformations (ARM) are rare congenital malformations, resulting from disturbed development of the hindgut. Evidence regarding the genetic etiology of ARM is still scarce. Therefore, we performed genotyping of 598 Caucasian ARM patients and 1,931 Caucasian population-based controls using the Illumina Human Exome BeadChip, which contains >240K rare coding variants. Single variant and gene-based analyses were performed. Statistically significant single variant results after Bonferroni correction ($p < 1.13*10^{-6}$) were validated in patients using targeted resequencing with the molecular inversion probe (MIP) technique. Sanger sequencing was used to verify the results with a second validation method. An additional 175 Caucasian ARM patients and 3,201 controls as well as 307 Han Chinese patients and 466 controls were used to replicate the single variant results that were confirmed in the validation study. In total, 55 single variants reached statistical significance in the discovery study. Only three of these, residing in the *CLCN1*, *LRBA*, and *ZNF423* genes, were validated, but the replication analyses in Caucasian and Han Chinese patients and controls did not confirm the results for these variants. In conclusion, we did not find evidence for associations between ARM and rare coding variants with large effect sizes captured by the exome chip. Future studies will need large sample sizes to identify common and rare variants with small to moderate effects, while stratifying on phenotypically homogeneous groups of ARM patients.

Introduction

Congenital anorectal malformations (ARM) are major congenital malformations, resulting from disturbed development of the rectum and anus. Although ARM are one of the most frequently occurring malformations of the gastrointestinal tract, the prevalence of 2 to 6 in 10,000 live births is relatively low (see URLs). ARM encompass a broad range of different phenotypes in both males and females, which are mostly classified according to the type of fistula to neighboring organs. In approximately 50% of the patients, ARM present with additional congenital malformations, such as vertebral, cardiac and/or renal malformations.[1,2] Multiple surgical corrections are required during the first years of a patient's life. Despite major improvements in the care and treatment of ARM patients in the past decade, a substantial number of patients face lifelong physical and psychosocial problems.[3]

Our current understanding of the embryology and etiology of ARM is limited. A syndrome caused by a fully penetrant mutation in a single gene, such as Currarino or Townes-Brocks syndrome, is identified in approximately 10% of the patients.[1] In the remaining patients, the involvement of both genetic and non-genetic factors in the occurrence of ARM seems likely. Previous studies consistently found non-genetic risk factors, such as fertility treatment,[4-7] maternal overweight or obesity,[8-10] and pre-existing diabetes,[11-13] to be associated with ARM. Familial aggregation of ARM was shown in some studies,[10,14] which may point to genetic factors. So far, genetic research into ARM has mainly focused on candidate genes that are involved in embryonic signaling pathways, such as sonic hedgehog (SHH), wingless-type integration site (WNT), and fibroblast growth factor (FGF) signaling.[15] However, human ARM studies did not provide substantial evidence to support a contribution of these genes.[16-21] Hypothesis-generating approaches through genome-wide studies seem valuable to acquire new knowledge and hypotheses, as these have been successful in the identification of genetic variants for many multifactorial disorders, such as Hirschsprung's disease.[22]

Wong *et al.* performed the only genome-wide association study for ARM in a small sample of 175 patients. This study did not yield any associated common single nucleotide variants, nor did it suggest a role for common copy number variants (CNV) in the etiology of ARM.[23] However, the authors did identify an excess of rare CNVs, observing many different rare CNVs in individual isolated ARM patients, but not in controls nor in healthy individuals from the Database of Genomic Variants.[23] This suggests the importance of rare variation in the etiology of ARM. In the current study, we aimed to identify rare genetic variants for ARM by exome-wide genotyping of numerous known rare coding variants in the largest sample of well-characterized ARM patients in genetic ARM research so far.

Materials and Methods

Discovery exome chip association study

Study population

AGORA (Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children) is a large data- and biobank with DNA samples and clinical and questionnaire data from children with congenital malformations or childhood cancer, control children, and their parents. AGORA is a multicentre effort coordinated by the Radboud university medical center (Radboudumc) in Nijmegen, The Netherlands. For the current study, AGORA provided 429 blood or saliva samples from live born Caucasian patients who were treated for ARM at the departments of Surgery-Pediatric Surgery of the Amalia Children's Hospital-Radboudumc, the Sophia Children's Hospital-Erasmus MC Rotterdam (EMC), and the University Medical Center Groningen (UMCG) in The Netherlands. The German Network for Congenital Uro-REctal malformations (CURE-Net) provided 169 additional DNA samples from ARM patients of Caucasian ancestry. These patients were recruited through the German self-help organization for ARM patients (SoMA e.V.) and pediatric surgical departments throughout Germany. DNA samples from ARM patients with chromosomal abnormalities or syndromes with a known genetic cause were not included in the study population.

Pediatric surgeons, clinical geneticists, and researchers reviewed the medical records of the ARM patients extensively to obtain clinical information on ARM phenotypes and associated congenital malformations. We classified ARM phenotypes according to the Krickenbeck criteria[24] and divided the congenital malformations in major and minor malformations according to the EUROCAT classification.[25] The phenotypic characteristics of the ARM patients are shown in Supplemental Table 1.

Controls were derived from the Nijmegen Biomedical Study (NBS), a population-based survey conducted by the Department for Health Evidence and the Department of Laboratory Medicine of the Radboudumc (see URLs). In total, 22,451 age and sex stratified randomly selected adult inhabitants of the municipality of Nijmegen received an invitation to fill out a postal questionnaire on items such as lifestyle and medical history, and to donate two blood samples. The response to the questionnaire was 43% (n=9,350), and 69% (n=6,468) of the responders donated blood samples. For the current study, DNA samples of 1,931 Caucasian controls were used. The Arnhem-Nijmegen Regional Committee on Research Involving Human Subjects approved the AGORA and NBS study protocols and the Ethics Committees of the University of Bonn and the University of Heidelberg approved the CURE-Net study protocol. Written informed consent was obtained from all participants and parental consent for children under 18 years of age.

Genotyping and quality control

Using standard methods, DNA was extracted from blood collected in EDTA-containing tubes or saliva specimens collected in Oragene containers (DNA Genotek Inc., Ottawa, Canada). The DNA samples were genotyped using the Illumina Human Exome BeadChip (v1.1), which contains 242,901 markers throughout the exome. The majority of these markers (~220,000) are rare coding variants (nonsynonymous, splice site, stop gain, and stop loss variants) and a small proportion are non-coding variants. These markers were selected based on their occurrence in approximately 12,000 sequenced genomes and exomes from multiple populations of primarily European ancestry (see URLs). Approximately 20% of the markers have a minor allele frequency (MAF) >1% and 80% of the markers a MAF <1%. Genotyping and calling was performed at the Erasmus MC in Rotterdam, The Netherlands, as part of the ExomeChip Rainbow Project (RP10) of Biobanking and Biomolecular Research Infrastructure Netherlands (BBMRI-NL) (see URLs). Regular GenomeStudio (GS) clustering and calling was followed by re-calling of 'no-calls' only using zCall,[26] for patient and control samples separately. zCall was especially designed as a tool for calling rare variants from array data.

We applied quality control procedures to GS called data using PLINK v1.07 (see URLs). Samples and markers that did not meet quality control criteria were removed from the zCall genotype data, which were used for the analyses. In total, 2,467 of the 2,529 patient and control samples (97.5%) were successfully genotyped with call rates > 99% and could be included in the analyses. We excluded 11 samples (3 patients and 8 controls) based on sex discordance between genotype data and clinical information, 3 samples (1 patient and 2 controls) with aberrant heterozygosity rates (> ±4 SD from the mean), 4 patients based on relatedness, and 2 patient duplicates. To identify population outliers, multidimensional scaling (MDS) analysis was carried out by calculating the pairwise identity-by-state from LD pruned markers, leading to 3 population outliers (3 patients) being excluded from further analyses. Regarding the markers, 241,385 of the 242,901 (99.4%) were successfully genotyped with a call rate > 95%. Markers with Hardy-Weinberg Equilibrium *p*-values < 0.0001 in controls were excluded (n=208). As a result, 241,177 genotyped markers in 558 patients and 1,886 controls remained for further analyses.

Statistical analyses

Statistical analyses were performed using PLINK v1.07, R statistical software v3.0.3, and SPSS v20.0. We used support files obtained from the Illumina website and the University of Michigan, as well as the UCSC genome browser (see URLs) and Alamut software version 2.3 to assess variant types, amino acid changes, and genes.

Single variant analyses

To reduce potential false-positives and have sufficient power to replicate a statistically significant variant in smaller replication samples, we restricted the single variant analyses to variants with a minor allele frequency (MAF) >0.4%, which corresponds to at least 20 minor alleles in the combined patient and control sample. Based on this criterion, 178,417 variants, including 134,298 monomorphic variants, were excluded, leaving 44,119 variants for the statistical analyses. We tested the associations between ARM and each variant using Fisher's exact tests in both an allelic and additive genetic model, assuming Hardy Weinberg equilibrium. We used an exact method as the asymptotic score test is known to be too liberal and does not provide robust results when variant allele counts are low. As genotypic effects were not evaluated for Y chromosome and mitochondrial variants, 44,022 variants remained for these analyses. Potential of bias due to population stratification was assessed using the quantile-quantile plot (QQ plot) and by calculating the genomic control inflation factor, which was defined as the regression coefficient of the observed to expected –log *p* values from the Fisher's exact test. Test statistics were adjusted for the genomic control inflation factor. We performed subgroup analyses on isolated ARM and ARM with additional congenital malformations separately for apparently statistically significant findings (55 variants) in the total ARM group. A *p*-value below $1.13*10^{-6}$ was considered statistically significant in the single variant analyses, which corresponds to a Bonferroni correction for 44,119 tests (number of variants tested).

Gene-based analyses

We performed gene-based analyses using the sequence kernel association (SKAT) test in R and the gene annotation file from the Illumina website (see URLs).[27] SKAT has been shown to be a powerful method when both harmful and protective variants with different magnitudes of effect occur in one gene. We tested 13,864 genes with at least two variants that passed quality control and were polymorphic in either patients or controls. The analyses were performed using default settings.[27] A *p*-value below $3.61*10^{-6}$ was declared statistically significant in the gene-based analyses, corresponding to a Bonferroni correction for 13,864 tests (number of genes tested). The Globaltest R package method, which was developed by one of the co-authors (JG) and is nearly identical to the SKAT method,[28] was used to visualize the individual contribution of each variant to the *p*-value of the identified genes. We also performed conditional analyses using SKAT to consider whether the gene-based test results were driven by the most statistically significant variant in a gene as determined in the single variant analyses by adjustment for the allele count of this variant.

Validation and replication studies

Study populations

To validate the results of the 55 variants that were found to be statistically significantly associated with ARM in the single variant analyses, the DNA samples from all patients that passed quality control in the discovery study were re-genotyped by targeted resequencing using the molecular inversion probe (MIP) technique.[29,30] For 7 of the 558 samples, the amount of DNA was insufficient or the quality was too low for further genotyping, leaving 551 patient DNA samples for validation. We validated the 55 variants in patient samples only, as most of these variants were rare or absent in controls. In a second validation step, 10 of these variants were validated again using Sanger sequencing to verify the results of the targeted resequencing technique. For each variant, 5 patients were re-genotyped.

A combined set of 175 additional Caucasian ARM patients from AGORA and CURE-Net and 3 unrelated Caucasian population-based control series served as replication sample. The control series included participants of the Genome of The Netherlands Consortium (GoNL study) (n=498) (see URLs), the Dutch-Belgian Randomized Lung Cancer Screening Trial (NELSON study) (n=1,061),[31] and the Utrecht Health Project (n=2,908).[32] A second replication sample was derived from the University of Hong Kong, China, and consisted of DNA samples from 307 Han Chinese ARM patients and 466 Han Chinese healthy controls. The above-mentioned patient classifications in the discovery sample were also applied to the Caucasian replication sample (see Supplemental Table 1), but detailed information on type of ARM and associated congenital malformations was not available for the Han Chinese ARM patients. The Ethics Committees in The Netherlands and Germany and at the University of Hong Kong, China, approved all study protocols and written informed consent was obtained from all participants.

Genotyping and quality control

The MIP technique was used to screen the 55 variants that were found in the discovery study in one single reaction by targeted resequencing of 112 bp surrounding the coding variant. MIPs are 70 nucleotide single stranded DNA molecules, including a 30 bp common linker sequence and a locus-specific extension and ligation arm of 40 bp that are complementary to the target DNA. At least two independent MIP probes were designed for each variant to reach good coverage of the genomic region in which the variant occurs. For one variant (exm779484 in *OR1L4*), only one working MIP could be developed. MIP capture and the subsequent polymerase chain reaction (PCR) were performed as described before,[29] with minor modifications. The MIP-captured next generation sequencing (NGS) libraries of 551 ARM patients from the discovery study, 175 additional Caucasian ARM patients, 307 Han

Chinese ARM patients, and 466 Han Chinese controls were sequenced on the Next-Seq500 sequencer (Illumina, San Diego, CA, USA). After sequencing, next generation sequencing (NGS) reads were mapped and MIP arms were trimmed by BWA (version 0.7.7-r441). For all variant positions, the alignment pileups were extracted using a custom made Java program and Samtools (version 0.1.19-44428cd). Positions with less than 10x coverage were marked as missing genotypes due to insufficient coverage. If the reference allele constituted >80% of the base calls for a position, the position was considered to be homozygous reference, whereas the position was considered to be homozygous variant when the variant allele constituted >80% of the base calls. Values in between were considered as heterozygous for the variant allele. After manual checks of the sequences of samples with percentages of total reads in the ranges 10-30% and 70-90%, the scoring of genotypes did not alter. In total, 12 samples (10 validation and 2 replication samples) had missing genotypes for all 55 variants, most likely due to low DNA input. As the MIPs covering the variant exm1131059 in *KIF26A* generated low sequence coverage in almost all samples, we excluded this variant from further analyses. Genotyping was completed successfully in 97.4% of the samples for the remaining 54 variants. Of these, 10 variants (exm1130982 in *KIF26A*, exm1610290 in *CACNA1I*, exm725230 in *JRK*, exm7090 in *MEGF6*, exm949028 in *FUT4*, exm78706 in *COL11A1*, exm1531889 in *FOXS1*, exm2254176 in *SIRPA*, exm1415838 in *PEX11G,* and exm428026 in *LRBA*) were selected for verification by Sanger sequencing with specifically designed primers. PCR was performed in a 12.5 µl reaction volume containing 5 µM of forward and reverse primer, 10 ng DNA and 6.25 µl Readymix (Sigma-Aldrich). The conditions were 95°C (5 min), a 2-step profile including 95°C (30 sec), 60°C (45 sec), and 72°C (40 sec) (two times), and 72°C for 10 min. PCR products were purified using exonuclease treatment. Samples were analyzed on a 3730 (XL) DNA sequencer (Applied Biosystems). The sequences obtained were compared with the reference sequence derived from the UCSC genome browser (Build hg38) using VECTOR NTI software 11.0.

Genotype data based on NGS for the GoNL participants were derived from the online data source (see URLs). The samples from the participants of the NELSON study and the Utrecht Health Project were jointly genotyped in the same BBMRI-NL RP10 project as the patients and controls in our discovery study, using the same Illumina Human Exome BeadChip (v1.1) and GS and zCall for calling. Sample quality control was performed on GenomeStudio calls and low-quality samples were excluded from the zCall data. In total, 106 samples from the NELSON study and 1,160 samples from the Utrecht Health Project were excluded as these had call rates <95% or heterozygosity rates > ±4 SD from the mean, were related to other samples, or were population outliers, leaving 955 samples from the NELSON study and 1,748 samples from the Utrecht Health Project for the analyses. The relatively high

numbers of excluded samples from the Utrecht Health Project was expected as many individuals were related or of non-Caucasian ancestry. The variants that were included in the replication study were successfully genotyped with call rates >99% in both cohorts, except for exm665970 in *CLCN1*. In addition, exm428026 in *LRBA* was not in HWE in either cohort. As the marker quality of these variants was doubtful, we excluded these from the replication analyses in both cohorts.

Statistical analyses

Genotypes and MAFs for the 54 variants in the 541 re-genotyped ARM patients were compared between the discovery study and the validation study. We calculated concordance per variant and for all variants combined. In addition, *p* values for associations between ARM and these 54 variants in the validation study were calculated in an allelic model using Fisher's exact tests and the controls from the discovery study. Fisher's exact *p* values were also calculated for the variants included in the replication study for both the Caucasian and the Han Chinese replication samples. The association analysis results of the validation and replication studies were combined in a meta-analysis using the inverse-variance method and assuming random effect models in Review Manager 5.3.

URLs

International Clearing House for Birth Defects Surveillance and Research. Annual report 2012: with data for 2010, http://www.icbdsr.org/filebank/documents/ar2005/Report2012.pdf; AGORA data- and biobank, www.agoraproject.nl; Exome chip design, http://genome.sph.umich.edu/wiki/Exome_Chip_Design; Nijmegen Biomedical Study, http://www.nijmegenbiomedischestudie.nl/; BBMRI-NL RP10 project, http://www.bbmri.nl/en-gb/activities/rainbow-projects; PLINK v1.07, http://pngu.mgh.harvard.edu/~purcell/plink/; Support and documentation files Human Exome Chip, http://support.illumina.com/array/array_kits/infinium_humanexome_beadchip_kit/downloads.ilmn, ftp://share.sph.umich.edu/HumanExomearray/ProposedContent/coding Content; UCSC genome browser, http://genome.ucsc.edu/; GoNL study, http://www.nlgenome.nl/; NHLBI Exome Sequencing Project; Exome Variant Server, http://evs.gs.washington.edu/EVS/.

Results

Discovery exome chip association study

Single variant analyses

In total, 241,177 markers passed quality control, including 134,298 monomorphic variants (55.7%). A plot of the first two principal components was obtained by multidimensional

scaling analysis for all 558 patients and 1,886 controls (Supplemental Figure 1). No population substructures or differences in clustering between patients and controls or between the two different ARM studies AGORA and CURE-NET were observed. None of the first ten MDS components were associated with patient-control status. However, we found indications for inflation of the observed $-\log_{10}(p\text{-values})$, based on the QQ plot (Supplemental Figure 2) and the genomic control inflation factor of 1.066 after removal of the most statistically significant variants identified (allelic level). Each variant with MAF > 0.4% was tested individually (44,119 variants), and 55 variants were found to be statistically significantly associated with ARM after adjustment for the genomic control inflation factor. The results for these variants are shown in Supplemental Tables 2 (all ARM patients) and 3 (subgroups of ARM patients). The majority of variants identified were rare or even absent in controls. Similar *p* values were observed when an additive genetic model was assumed after adjustment for the corresponding genomic control inflation factor of 1.072 (data not shown).

Gene-based analyses

The gene-based analyses using SKAT yielded 86 statistically significant genes, which are shown in Supplemental Table 4. These results did, however, not lead to new insights as both visualization of the contribution of each variant to the gene-based test signal and conditional analyses on allele count showed that in each gene, one variant contributed to the signal in particular (see Supplemental Table 4). These variants also showed full or borderline statistically significant associations in the single variant analyses. Therefore, we only followed up those variants that were found in the single variant analyses. The figures displaying the contribution of each variant to the gene-based test signal are available on request.

Validation and replication studies

Validation study

Patients with homozygous reference calls in the discovery study using the exome chip were almost always called similarly in the validation study using targeted resequencing with the MIP technique. This resulted in an overall concordance rate of 99.9%. However, patients with heterozygous or homozygous variant calls in the discovery study were mostly called with homozygous reference calls in the validation study. Control samples were not validated, but in light of this information, we excluded 3 variants with higher MAFs among controls than among patients in the discovery study from further analyses (exm230609 in *SPOPL*, exm179650 in *EMILIN1*, and exm779484 in *OR1L4*). For 44 of the remaining 51 variants,

none or only 1 or 2 of the heterozygote or homozygote variant patients in the discovery study had similar genotypes in the validation study, resulting in concordance rates close to 0%. So these 44 variants were not found to be associated with ARM in the first validation study (Supplemental Table 5). To verify the results of the targeted resequencing technique, 9 randomly chosen variants out of these 44 variants were validated again using Sanger sequencing. For each variant, five patients with discordant findings (heterozygous variant call in the discovery study using the exome chip, but homozygous reference call in the validation study using targeted resequencing) were re-genotyped. All calls found in the validation study using targeted resequencing were confirmed, whereas none of the heterozygous variant calls in the discovery study were confirmed using Sanger sequencing as a second validation technique. The remaining 7 variants were included in the replication study and their characteristics are presented in Table 1. Three of these variants (exm665970 in *CLCN1*, exm428026 in *LRBA*, and exm1239020 in *ZNF423*) showed good validation with 100% concordance for heterozygous calls; their association with ARM was confirmed in the validation study (Table 2). One of these variants (exm428026 in *LRBA*) was also included in the second validation step. The results were confirmed in all 5 patients with heterozygous calls submitted to Sanger sequencing. For the other 4 variants (exm2253769 in *NUDT19*, exm1293600 in *MYH13*, exm853474 in *CALHM1*, and exm1017978 in *AVIL*), concordance rates of the heterozygous calls were low (8-30%) and the *p* values in the validation study were high (Table 2). However, we further evaluated all 7 variants in the replication study, assuming that the MAFs in controls might also be lower than found in the discovery study. Subgroup analyses for these 7 variants based on the presence of associated congenital malformations yielded small differences in the results, pointing towards stronger associations with ARM with other birth defects for exm665970 and with isolated ARM for exm428026 and exm1239020 (Table 3).

Replication study

None of the 7 variants included in the replication study was found to be associated with ARM in either of the two replication samples or in the combined analysis (Table 4). Of the three variants that were found to be associated with ARM after validation, exm428026 in *LRBA* showed a much lower MAF in the patients of the Caucasian replication study than in those of the validation study (Tables 2 and 4). More or less similar MAFs in the patients of the validation study and the Caucasian replication study were observed for the variants in *CLCN1* and *ZNF423*. However, the MAFs of these two variants were much lower in the controls of the discovery study than in the Caucasian control populations of the replication study, which were comparable to the NHLBI Exome Sequencing Project with frequency

information of European Americans (MAF is 2.5% for exm665970 in *CLCN1* and 2.1% for exm1239020 in *ZNF423* (see URLs)). The 7 variants included in the replication study were mostly monomorphic in both the Han Chinese patients and controls. Subgroup analyses for isolated ARM and ARM with other congenital malformations were impossible due to small patient numbers in the Caucasian replication sample and lacking detailed phenotypic information for the Han Chinese patients. The meta-analyses showed indications for increased odds ratios (ORs) for several variants, but the wide confidence intervals preclude any conclusions.

Table 1. Characteristics of the single variants derived from the discovery and validation studies that were included in the replication study.

ID	Chr	Position[a]	rs#	Minor / major allele	Variant	Gene
exm665970	7	143017807	rs10282312	G/T	Gly118Trp	*CLCN1*
exm2253769	19	33183265	rs200767135	C/T	Phe133Phe	*NUDT19*
exm1293600	17	10212964	rs35069886	G/C	Asp1614His	*MYH13*
exm853474	10	105218160	rs41317256	T/C	Val117Ile	*CALHM1*
exm428026	4	151242409	rs62346982	G/T	Thr2522Pro, Thr2533Pro	*LRBA*
exm1239020	16	49669726	rs147898137	T/C	Ala1113Thr	*ZNF423*
exm1017978	12	58204606	rs61938187	T/C	Arg184His	*AVIL*

[a]Genome positions are based on human genome build hg19.

Table 2. Associations between single variants and anorectal malformations in the discovery study and the validation study.

ID[a]	Controls (n=1,886) Discovery study[b]		ARM patients (n=558) Discovery study[b]		*P*[c]	ARM patients (n=541)[d] Validation study[e]		*P*[f]
	No. variant Alleles	MAF (%)	No. variant alleles	MAF (%)		No. variant alleles	MAF (%)	
exm665970	2	0.055	30	2.688	3.11×10^{-16}	19[g]	1.943[g]	1.86×10^{-11}
exm2253769	7	0.186	34	3.047	3.81×10^{-15}	4	0.380	0.27
exm1293600	48	1.326	60	5.376	1.83×10^{-12}	19	1.766	0.31
exm853474	24	0.636	38	3.405	3.03×10^{-10}	3	0.282	0.24
exm428026	2	0.055	19	1.706	4.69×10^{-10}	18[g,h]	1.667	2.93×10^{-10}
exm1239020	8	0.212	23	2.065	5.85×10^{-9}	22[g]	2.083	2.41×10^{-9}
exm1017978	10	0.265	22	1.971	1.09×10^{-7}	5	0.473	0.34

ARM: anorectal malformations; MAF: minor allele frequency.

[a]See Table 1 for corresponding chromosome, position, rs-number, minor/major allele, type of variant, and gene.

[b]Discovery study was performed using the Illumina Human Exome BeadChip.

[c]*P* value calculated for an allelic model using Fisher's exact test and adjusted for genomic control.

[d]Seventeen patients were not included in the validation study due to insufficient DNA amount or quality or because the sample dropped out of the MIP validation study.

[e]Validation study was performed using the MIP technique.

[f]*P* value calculated for an allelic model using the controls from the discovery study (2nd and 3rd column of this Table) and Fisher's exact test.

[g]Concordance rates for heterozygous calls were 100%. As 17 patients were not included in the validation study, the number of variant alleles in the validation study is not equal to the number of variant alleles in the discovery study. For exm665970, as a few homozygous variant calls for exm665970 in the discovery study were found to be heterozygous calls in the validation study, the number of variant alleles and the MAF in the validation study was lower.

[h]The heterozygous variant calls of five patients were confirmed using Sanger sequencing.

Table 3. Associations between single variants and ARM subgroups in the validation study.

ID[a]	Controls (n=1,886) Discovery study[b]		Isolated ARM (n=299) Validation study[c]		*P*[d]	ARM with other birth defects n=238) Validation study[c]		*P*[d]
	No. variant alleles	MAF (%)	No. variant alleles	MAF (%)		No. variant alleles	MAF (%)	
exm665970	2	0.055	9	1.679	3.88×10^{-7}	10	2.294	9.75×10^{-9}
exm2253769	7	0.186	4	0.694	0.05	0	0	1
exm1293600	48	1.326	7	1.182	1	11	2.311	0.10
exm853474	24	0.636	3	0.514	1	0	0	0.10
exm428026	2	0.055	13	2.181	5.75×10^{-10}	5	1.050	3.43×10^{-4}
exm1239020	8	0.212	14	2.431	5.19×10^{-8}	8	1.695	1.28×10^{-4}
exm1017978	10	0.265	3	0.517	0.40	2	0.426	0.63

ARM: anorectal malformations; MAF: minor allele frequency.

[a]See Table 1 for corresponding chromosome, position, rs-number, minor/major allele, type of variant, and gene.

[b]Discovery study was performed using the Illumina Human Exome BeadChip.

[c]Validation study was performed using the MIP technique.

[d]*P* value calculated for an allelic model using the controls from the discovery study (2nd and 3rd column of this Table) and Fisher's exact test.

Table 4. Associations between single variants and anorectal malformations in the replication study and the combined analyses.

	Caucasian replication sample						
ID	**MAF (%) ARM patients[a] (n=175)**	**MAF (%) controls GoNL[b] (n=498)**	***P*[c]**	**MAF (%) controls NELSON study[d] (n=955)**	***P*[c]**	**MAF (%) controls Utrecht Health Project[d] (n=1,748)**	***P*[c]**
exm665970[e]	2.047	2.510	0.84	-	-	-	-
exm2253769	0.585	0.389	0.65	0.262	0.29	0.200	0.19
exm1293600	0.857	1.205	0.77	1.361	0.61	1.030	1
exm853474	0.287	0.301	1	0.576	0.71	0.486	1
exm428026[e]	0.571	1.104	0.53	-	-	-	-
exm1239020	1.724	1.670	1	1.259	0.45	1.093	0.29
exm1017978	0.575	0.602	1	0.524	1	0.515	0.70

	Han Chinese replication sample			Meta-analysis[g]	
	MAF (%) ARM patients (n=306)[a,f]	**MAF (%) controls (n=465)[a,f]**	***P*[c]**	***P***	**OR (95% CI)**
exm665970	0.165	0	0.40	0.27	4.96 (0.29-85.83)
exm2253769	0	0	1	0.10	2.19 (0.85-5.65)
exm1293600	0	0	1	0.45	1.21 (0.74-1.96)
exm853474	0.164	0	0.40	0.29	0.59 (0.22-1.57)
exm428026	0	0	1	0.50	4.01 (0.07-222.32)
exm1239020	0	0.108	1	0.26	2.65 (0.48-14.64)
exm1017978	0.658	0.860	0.77	0.63	1.19 (0.59-2.39)

ARM: anorectal malformations; MAF: minor allele frequency.

[a]Analyses performed using MIP technique.

[b]Analyses performed using next-generation sequencing.

[c]*P* value calculated for an allelic model using Fisher's exact test.

[d]Analyses performed using Illumina Human Exome BeadChip.

[e]Variant could not be evaluated in the NELSON study and the Utrecht Health Project due to insufficient marker quality.

[f]One patient sample and one control sample failed the MIP validation study for all variants.

[g]Meta-analysis was performed using the discovery sample after validation, the Caucasian replication sample (with three control cohorts combined) and the Han Chinese replication sample.

Discussion

This first exome chip association study on the role of rare coding variants in the etiology of congenital anorectal malformations did not provide evidence for associations between ARM and rare variants captured by the Illumina Human Exome BeadChip. Three variants were found to be statistically significant in both the discovery study and the validation study, but replication analyses in new series of patients and controls did not confirm these results.

As the prevalence of ARM is low, the inclusion of a relatively large number of 773 Caucasian and 307 Han Chinese ARM patients in this study is unique in the genetic field of ARM. However, the power of this study was insufficient to detect associations with variants with small to moderate effects. We had 80% power to detect single variants with moderate to large effects (ORs of 3.7-8.4 or above) with MAFs of 0.4-2.0% in the discovery sample. The power to replicate variants with these effect sizes in our relatively small Caucasian replication sample was much smaller. Another limitation was the low power for subgroup analyses in the discovery study for specific phenotypic characteristics (e.g. type of fistula) and the inability to perform subgroup analyses in the replication studies based on presence of associated congenital malformations. These analyses seem relevant, as ARM patients comprise a phenotypically heterogeneous group of patients for which heterogeneity in underlying etiologies seems likely.[6] In addition, the scope of this study was limited to the content of the Illumina Human Exome BeadChip, which was based on the coding and splice site variation observed in approximately 12,000 sequenced individuals of mainly European descent. Only ~80% of the initially selected content could be converted into this exome chip. Hence, a large proportion of human exome variation was not covered. Future exome or whole genome sequencing studies are needed to unravel the involvement of exome variation that was not covered by the current chip.

The validation study using two different validation methods targeting the highly associated variants in our discovery study appeared to make a substantial contribution to our investigation. The results of the targeted resequencing technique showed that many heterozygous calls in the exome chip data and their associations with ARM were not confirmed. A second validation step using Sanger sequencing confirmed the results of the targeted resequencing technique and provides evidence for the validity of these results and for the absence of most associations identified in the discovery study. Almost all heterozygous calls detected in ARM patients by targeted resequencing were also found to be heterozygous calls in the zCall exome chip patient data. Goldstein *et al.* introduced the zCall algorithm and showed that application of zCall after a default caller (Illumina's GenCall algorithm and GenomeStudio software) improves rare variant calling (i.e. increases sensitivity), but also increases errors in which homozygous reference variants are called as

heterozygous variants.[26] Hence, our observations in the validation study are in line with the conclusions of Goldstein *et al.* Moreover, patients and controls in our discovery study were genotyped and called separately, which may have resulted in differential misclassification of genotypes and most likely overestimation of positive allele frequency differences between patients and controls. Future case-control studies using exome chip techniques should be aware of this phenomenon, which may be detected by checks for batch effects.[33] Based on the results of Goldstein *et al.*, however, it does not seem likely that we missed potentially strong positive associations with rare variants.

Three variants in the *CLCN1*, *LRBA*, and *ZNF423* genes were confirmed in the validation study. One of these genes, *LRBA*, is located in a region on 4q31.3-q32.3, which was suggested as a susceptibility locus for Hirschsprung's disease using genome-wide linkage analysis in a multigenerational family.[34] Hirschsprung's disease is a congenital malformation of the large intestine, which rarely coexists with ARM. The other two genes have not been associated with ARM in the literature either. None of the three variants was replicated in a small Caucasian patient population, although the MAFs for two of the three variants were comparable to those in the discovery sample. We observed lower MAFs, however, in the Caucasian controls of the discovery study than in controls of the Caucasian replication study and the NHLBI Exome Sequencing Project with frequency information of Caucasians. This may be due to chance or to genotyping errors in controls of the discovery study. In addition, we could not replicate our findings in a Han Chinese replication sample. Caution is needed in the interpretation of these replication results, as the results for one single rare variant in a gene identified in a Caucasian population may not always be generalizable to non-Caucasian populations.

Exome chips have been applied successfully to identify rare variant associations for a number of multifactorial traits, such as blood lipids,[35] insulin secretion,[36] and a rare autoimmune systemic vasculitis (Takayasu arteritis).[37] We failed to identify rare variants for ARM in the current study. A GWAS among a small group of ARM patients did not find common variants to be associated with ARM either.[23] This indicates that major effects of rare or common single nucleotide variants as captured by exome- and genome-wide arrays do not seem to play a role in the development of ARM. ARM appears to comprise a genetically heterogeneous set of malformations, as was also suggested by Wong et al.[38] A potential hypothesis regarding the etiology is that ARM may only be caused by single-gene defects in a, probably small, proportion of patients. Since ARM comprise a wide spectrum of phenotypes, several different monogenic forms of ARM may exist. This hypothesis was also suggested for congenital abnormalities of the kidney and urinary tract (CAKUT).[39] Equally, ARM may be a truly multifactorial disorder with involvement of many genetic as well as non-

genetic risk factors with relatively small effects simultaneously in the majority of patients. Support for this scenario derives from findings of several non-genetic factors being associated with ARM.[15]

In conclusion, the present study among more than 1,000 ARM patients did not yield evidence for associations between ARM and rare coding variants with large effect sizes as captured by an exome chip. Future studies with even larger sample sizes are needed to identify potential common and/or rare genetic variants with small to moderate effects on the occurrence of ARM. For these studies, international collaborations are essential. Genetic studies in phenotypically homogeneous subgroups of ARM patients may further contribute to the elucidation of underlying genetic causes.

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Supplemental Tables

Supplemental Table 1. Phenotypes of anorectal malformation, additional congenital malformations, and gender among patients in the discovery study and the Caucasian replication study.

	ARM patients Discovery study[a] (n=558) No. (%)	ARM patients Caucasian replication study (n=175) No. (%)
Phenotypes of ARM		
Perineal fistula	242 (43.4)	60 (34.3)
Vestibular fistula	89 (15.9)	23 (13.1)
ARM without fistula	29 (5.2)	11 (6.3)
Rectourethral fistula		
Bulbar	43 (7.7)	2 (1.1)
Prostate	35 (6.3)	3 (1.7)
Unspecified	33 (5.9)	28 (16.0)
Rectovesical fistula	17 (3.0)	4 (2.3)
Cloaca	29 (5.2)	5 (2.9)
Anal stenosis	12 (2.2)	2 (1.1)
Rare types[b]	13 (2.3)	12 (6.9)
Type unknown	16 (2.9)	25 (14.3)
Additional major congenital malformations		
Vertebral malformations	86 (15.5)	19 (11.3)
Cardiac malformations	81 (14.6)	27 (16.1)
Tracheo-Esophageal atresia	63 (11.4)	18 (10.7)
Renal malformations	94 (17.0)	31 (18.5)
Radial limb defects	32 (5.8)	1 (5.4)
Subgroups of anorectal malformations		
Isolated ARM	301 (53.9)	93 (53.1)
ARM with other congenital malformations	253 (45.3)	75 (42.9)
Unknown	4 (0.7)	7 (4.0)
Gender		
Male	294 (52.7)	89 (50.9)
Female	264 (47.3)	86 (49.1)

ARM: anorectal malformations.

[a]All patients in the discovery study were Caucasians.

[b]Including rectovaginal fistula, rectal atresia, rectal stenosis, dorsal cloaca-like defect with complex H-fistula.

Supplemental Table 2. All statistically significant associations between anorectal malformations and single variants in the discovery study.

ID	Chr	Position[a]	rs#	Minor / Major allele	Variant	Gene	MAF (%) Controls (n=1,886)	MAF (%) ARM patients (n=558)	*P*[b]
exm681600	8	10470709	rs200642524	A/G	Ser300Leu	*RP1L1*	0	18.550	8.45×10^{-132}
exm1078533	13	108518527	rs201168561	T/C	Gly140Ser	*FAM155A*	0	10.660	2.10×10^{-74}
exm1198894	16	815740	rs139851878	A/G	Arg282Gln	*MSLN*	0.027	11.040	6.13×10^{-74}
exm1531889	20	30433303	rs144561926	T/C	Glu15Lys	*FOXS1*	0	8.513	3.39×10^{-59}
exm1390461	18	60645678	rs199787538	T/C	Arg1390Cys	*PHLPP1*	0	6.989	1.56×10^{-48}
exm7090	1	3428591	rs202242273	T/C	Ala319Thr	*MEGF6*	0.027	6.810	1.30×10^{-45}
exm670492	7	150034066	rs146095396	A/G	Arg39His	*LRRC61*	0	6.093	2.65×10^{-42}
exm1330833	17	43922916	rs144845296	A/G	Arg215Gln	*MAPT-AS1,SPPL2C*	0	5.556	1.40×10^{-38}
exm1130982	14	104640630	rs189768438	T/C	Arg726Cys	*KIF26A*	0.106	6.183	1.18×10^{-37}
exm1335493	17	48267454	rs1800214	C/G	Pro823Ala	*COL1A1*	0.027	4.480	1.16×10^{-29}
exm254596	2	197183374	rs143266673	A/G	Ala747Val	*HECW2*	0	3.943	1.79×10^{-27}
exm1129696	14	103574044	rs200996746	A/G	Arg593Gln	*EXOC3L4*	0	3.763	3.02×10^{-26}
exm1399016	19	1054324	rs114614802	A/G	Arg1237His	*ABCA7*	0	3.680	1.18×10^{-25}
exm688810	8	22476746	rs7845453	A/G	Ala869Thr	*KIAA1967*	0	3.674	1.24×10^{-25}
exm1165387	15	58855862	rs144856280	T/C	Pro443Leu	*LIPC*	0	3.405	8.56×10^{-24}
exm274502	2	232326276	rs61754968	G/C	Glu196Asp	*NCL*	0	3.315	3.51×10^{-23}
exm1415838	19	7553859	rs200516760	T/G	Ser13Stop	*PEX11G*	0	3.226	1.44×10^{-22}
exm441548	5	1077973	rs148501261	T/C	Arg535His	*SLC12A7*	0.027	3.285	2.10×10^{-21}
exm1665335	X	153282018	rs145385375	T/C	Gly369Glu	*IRAK1*	0	4.167	2.11×10^{-21}
exm949028	11	94277959	rs199688248	G/C	Cys220Trp	*FUT4*	0	3.047	2.40×10^{-21}

ID	Chr	Position[a]	rs#	Minor / Major allele	Variant	Gene	MAF (%) Controls (n=1,886)	MAF (%) ARM patients (n=558)	*P*[b]
exm584047	6	145069600	rs144596208	A/G	Val2720Met	*UTRN*	0	2.867	4.01×10^{-20}
exm2254176	20	1918088	rs200783814	A/G	Ala463Ala	*SIRPA*	0.106	3.495	4.99×10^{-20}
exm230609	2	139326516	rs114501427	A/G	Asp349Asn	*SPOPL*	4.454	0	1.02×10^{-18}
exm1131059	14	104642499	rs114955777	T/G	Arg1125Leu	*KIF26A*	0.053	2.867	9.61×10^{-18}
exm42669	1	34330070	rs144223004	T/C	Pro93Leu	*CSMD2, HMGB4*	0	2.419	4.50×10^{-17}
exm78706	1	103470210	rs192313594	T/C	Arg502Gln, Arg579Gln, Arg618Gln, Arg630Gln	*COL11A1*	0	2.419	4.50×10^{-17}
exm913730	11	59382897	rs200837578	T/C	Gly81Ser	*OSBP*	0	2.330	1.83×10^{-16}
exm676304	7	157985057	rs137877535	T/C	Ala154Thr, Ala171Thr	*PTPRN2*	0.080	2.778	2.87×10^{-16}
exm665970	7	143017807	rs10282312	G/T	Gly118Trp	*CLCN1*	0.055	2.688	3.11×10^{-16}
exm658390	7	131241043	rs199547430	A/G	Pro26Ser	*PODXL*	0	2.294	4.82×10^{-16}
exm874064	11	1025358	rs200061527	T/C	Val937Met	*MUC6*	0	2.151	3.02×10^{-15}
exm995133	12	41966841	rs139658899	T/C	Arg496Cys, Arg754Cys	*PDZRN4*	0	2.151	3.02×10^{-15}
exm1368208	17	79892802	rs150914837	A/G	Tyr180Tyr	*PYCR1*	0	2.151	3.02×10^{-15}
exm2253769	19	33183265	rs200767135	C/T	Phe133Phe	*NUDT19*	0.186	3.047	3.81×10^{-15}
exm1580785	21	47566189	rs77740289	T/C	Arg320Gln	*FTCD*	0	2.068	1.15×10^{-14}
exm84805	1	113257761	rs202231915	T/C	Ala55Thr	*PPM1J*	0	2.061	1.22×10^{-14}

ID	Chr	Position[a]	rs#	Minor / Major allele	Variant	Gene	MAF (%) Controls (n=1,886)	MAF (%) ARM patients (n=558)	*P*[b]
exm626311	7	73474781	rs138876104	T/C	Ala537Val, Ala547Val, Ala552Val, Ala566Val, Ala571Val	*ELN*	0	2.061	1.22×10^{-14}
exm658164	7	130417921	rs145868754	A/C	Gly314Cys	*KLF14*	0	2.061	1.22×10^{-14}
exm1103535	14	56137504	rs146550320	G/A	Thr1080Ala, Thr1086Ala, Thr1109Ala	*KTN1*	0.027	2.151	4.92×10^{-14}
exm297681	3	33195264	rs75287757	T/C	Arg287Gln	*SUSD5*	0	1.971	4.96×10^{-14}
exm1577147	21	45855045	rs143528384	T/C	Arg1336Cys	*TRPM2*	0	1.971	4.96×10^{-14}
exm725230	8	143747456	rs74769052	T/C	Gly8Arg	*JRK*	0	1.882	2.01×10^{-13}
exm872472	11	747577	rs138714955	T/C	His32His	*TALDO1*	0	1.882	2.01×10^{-13}
exm870424	11	403150	no rs#	G/A	Ser604Gly	*PKP3*	0	1.808	7.14×10^{-13}
exm1610290	22	40045718	rs59010602	T/G	Ala559Ser, Ala594Ser	*CACNA1I*	0	1.792	8.15×10^{-13}
exm1293600	17	10212964	rs35069886	G/C	Asp1614His	*MYH13*	1.326	5.376	1.83×10^{-12}
exm1510860	19	56487619	rs61734100	G/C	Ile942Met	*NLRP8*	0.106	2.061	4.73×10^{-11}
exm853474	10	105218160	rs41317256	T/C	Val117Ile	*CALHM1*	0.636	3.405	3.03×10^{-10}
exm428026	4	151242409	rs62346982	G/T	Thr2522Pro, Thr2533Pro	*LRBA*	0.055	1.706	4.69×10^{-10}

ID	Chr	Position[a]	rs#	Minor / Major allele	Variant	Gene	MAF (%) Controls (n=1,886)	MAF (%) ARM patients (n=558)	*P*[b]
exm462602	5	76728995	rs145551521	G/A	Leu449Leu	*WDR41*	0.159	2.061	6.62×10^{-10}
exm1239020	16	49669726	rs147898137	T/C	Ala1113Thr	*ZNF423*	0.212	2.065	5.85×10^{-9}
exm1560265	20	62324609	rs139221232	T/C	Arg1013Trp, Arg989Trp	*RTEL1,RTEL1-TNFRSF6B*	0.186	1.902	1.77×10^{-8}
exm179650	2	27305610	rs188761759	T/G	Ala391Ser	*EMILIN1*	1.935	0	2.45×10^{-8}
exm1017978	12	58204606	rs61938187	T/C	Arg184His	*AVIL*	0.265	1.971	1.09×10^{-7}
exm779484	9	125486542	rs143746640	G/A	Ile92Val	*OR1L4*	1.988	0.090	2.77×10^{-7}

ARM: anorectal malformations; MAF: minor allele frequency.

[a]Genome positions are based on human genome build hg19.

[b]*P* value calculated for an allelic model using Fisher's exact test and adjusted for genomic control.

Supplemental Table 3. Associations between subgroups of anorectal malformations and single variants in the discovery study.

ID[a]	MAF (%) Controls (n=1,886)	MAF (%) Isolated ARM (n=301)	*P* [b] Isolated ARM	MAF (%) ARM with other birth defects (n=253)	*P*[b] ARM with other birth defects
exm681600	0	21.760	5.34×10^{-112}	14.820	4.86×10^{-68}
exm1078533	0	12.290	3.61×10^{-62}	8.893	1.37×10^{-40}
exm1198894	0.027	13.100	3.10×10^{-63}	8.367	5.30×10^{-36}
exm1531889	0	10.300	5.73×10^{-52}	6.522	8.24×10^{-30}
exm1390461	0	7.807	2.38×10^{-39}	6.126	5.03×10^{-28}
exm7090	0.027	8.140	1.74×10^{-39}	5.336	3.76×10^{-23}
exm670492	0	7.807	2.38×10^{-39}	4.150	3.85×10^{-19}
exm1330833	0	6.478	1.11×10^{-32}	4.545	6.52×10^{-21}
exm1130982	0.106	6.977	1.66×10^{-30}	5.336	1.98×10^{-20}
exm1335493	0.027	4.817	4.55×10^{-23}	4.150	6.27×10^{-18}
exm254596	0	4.983	3.18×10^{-25}	2.767	5.74×10^{-13}
exm1129696	0	5.316	7.08×10^{-27}	1.976	1.87×10^{-9}
exm1399016	0	4.485	9.48×10^{-23}	2.778	5.48×10^{-13}
exm688810	0	4.817	2.13×10^{-24}	2.372	3.28×10^{-11}
exm1165387	0	4.485	9.48×10^{-23}	2.174	2.48×10^{-10}
exm274502	0	3.488	8.10×10^{-18}	3.162	9.97×10^{-15}
exm1415838	0	3.821	1.85×10^{-19}	2.569	4.35×10^{-12}
exm441548	0.027	3.523	1.09×10^{-16}	3.036	6.79×10^{-13}
exm1665335	0	5.374	5.62×10^{-20}	2.786	1.12×10^{-9}
exm949028	0	3.654	1.23×10^{-18}	2.372	3.28×10^{-11}
exm584047	0	3.156	3.53×10^{-16}	2.569	4.35×10^{-12}

ID[a]	MAF (%) Controls (n=1,886)	MAF (%) Isolated ARM (n=301)	*P* [b] Isolated ARM	MAF (%) ARM with other birth defects (n=253)	*P*[b] ARM with other birth defects
exm2254176	0.106	3.322	1.92×10^{-13}	3.755	7.39×10^{-14}
exm230609	4.454	0	8.62×10^{-11}	0	3.22×10^{-9}
exm1131059	0.053	2.492	5.13×10^{-11}	3.360	1.31×10^{-13}
exm42669	0	3.322	5.35×10^{-17}	1.383	7.89×10^{-7}
exm78706	0	2.824	1.53×10^{-14}	1.976	1.87×10^{-9}
exm913730	0	2.824	1.53×10^{-14}	1.779	1.40×10^{-8}
exm676304	0.080	3.821	2.01×10^{-16}	1.581	9.31×10^{-6}
exm665970	0.055	2.159	2.52×10^{-9}	3.360	2.19×10^{-13}
exm658390	0	2.211	2.20×10^{-11}	2.429	2.58×10^{-11}
exm874064	0	2.658	1.00×10^{-13}	1.581	1.05×10^{-7}
exm995133	0	2.658	1.00×10^{-13}	1.581	1.05×10^{-7}
exm1368208	0	3.156	3.53×10^{-16}	0.988	4.41×10^{-5}
exm2253769	0.186	3.821	6.26×10^{-14}	2.174	1.99×10^{-6}
exm1580785	0	2.326	4.32×10^{-12}	1.594	9.99×10^{-8}
exm84805	0	2.824	1.53×10^{-14}	1.186	5.90×10^{-6}
exm626311	0	2.824	1.53×10^{-14}	1.186	5.90×10^{-6}
exm658164	0	2.492	6.59×10^{-13}	1.581	1.05×10^{-7}
exm1103535	0.027	2.824	2.03×10^{-13}	1.383	5.02×10^{-6}
exm297681	0	2.658	1.00×10^{-13}	1.186	5.90×10^{-6}
exm1577147	0	2.824	1.53×10^{-14}	0.988	4.41×10^{-5}
exm725230	0	2.326	4.32×10^{-12}	1.383	7.89×10^{-7}
exm872472	0	1.827	1.20×10^{-9}	1.976	1.87×10^{-9}

ID[a]	MAF (%) Controls (n=1,886)	MAF (%) Isolated ARM (n=301)	*P* [b] Isolated ARM	MAF (%) ARM with other birth defects (n=253)	*P*[b] ARM with other birth defects
exm870424	0	1.839	1.13×10^{-9}	1.600	9.73×10^{-8}
exm1610290	0	2.159	2.82×10^{-11}	1.383	7.89×10^{-7}
exm1293600	1.326	5.980	3.88×10^{-10}	4.545	1.16×10^{-5}
exm1510860	0.106	2.492	9.23×10^{-10}	1.581	2.36×10^{-5}
exm853474	0.636	4.319	4.77×10^{-10}	2.372	8.98×10^{-4}
exm428026	0.055	2.159	2.38×10^{-9}	1.190	1.26×10^{-4}
exm462602	0.159	1.993	8.80×10^{-7}	2.174	9.10×10^{-7}
exm1239020	0.212	2.326	2.34×10^{-7}	1.786	7.85×10^{-5}
exm1560265	0.186	2.013	1.81×10^{-6}	1.600	1.79×10^{-4}
exm179650	1.935	0	7.43×10^{-5}	0	2.73×10^{-4}
exm1017978	0.265	2.658	5.76×10^{-8}	1.186	1.02×10^{-2}
exm779484	1.988	0.166	4.82×10^{-4}	0	2.95×10^{-4}

ARM: anorectal malformations; MAF: minor allele frequency.

[a]See Supplemental Table 2 for corresponding chromosome, position, rs-number, minor/major allele, type of variant, and gene.

[b]*P* value calculated for an allelic model using Fisher's exact test and adjusted for genomic control in entire sample.

Supplemental Table 4. All statistically significant associations between anorectal malformations and genes in the discovery study.

Gene	Number of variants on exome chip	*P*[a]	Adjusted *P*[b]
RP1L1	53	1.82×10^{-160}	0.338
FAM155A	6	5.32×10^{-102}	0.343
MSLN	21	5.05×10^{-95}	0.466
FOXS1	4	2.48×10^{-78}	0.473
PHLPP1	8	2.83×10^{-74}	0.083
KIF26A	21	3.05×10^{-65}	0.014[c]
LRRC61	3	7.94×10^{-54}	0.679
MAPT-AS1, SPPL2C	22	2.68×10^{-50}	0.982
EXOC3L4	13	2.89×10^{-38}	0.056
COL1A1	9	1.41×10^{-36}	0.731
HECW2	5	7.29×10^{-35}	0.607
NCL	4	7.66×10^{-30}	0.166
IRAK1	4	2.88×10^{-29}	1.000
FUT4	3	4.94×10^{-28}	0.231
PEX11G	6	6.30×10^{-28}	0.519
SIRPA	3	2.03×10^{-27}	0.444
MEGF6	31	1.10×10^{-26}	0.368
KIAA1967	9	4.06×10^{-26}	0.337
ELN	13	9.82×10^{-24}	4.04×10^{-5}
SLC12A7	16	1.41×10^{-23}	0.588
LIPC	9	3.13×10^{-23}	0.723
OSBP	2	4.29×10^{-21}	0.593
PYCR1	4	7.33×10^{-21}	0.266
NUDT19	7	1.07×10^{-20}	0.310
TRPM2	30	6.76×10^{-20}	0.039
UTRN	37	4.81×10^{-19}	0.206
TALDO1	5	3.99×10^{-18}	0.310
PODXL	11	8.68×10^{-18}	0.044
FTCD	12	1.04×10^{-17}	0.389
COL11A1	21	4.35×10^{-17}	0.477
CENPC1	5	2.18×10^{-16}	0.570
KLF14	3	1.25×10^{-15}	0.377
KTN1	7	1.90×10^{-15}	0.858
CSMD2, HMGB4	4	2.03×10^{-15}	0.295
SUSD5	13	2.25×10^{-15}	0.445

Gene	Number of variants on exome chip	P^a	Adjusted P^b
TAF4	3	2.25×10^{-14}	0.637
GLP2R	7	3.76×10^{-14}	0.158
PITX2	2	6.54×10^{-14}	0.592
PTPRN2	19	6.58×10^{-14}	0.684
PPM1J	9	1.50×10^{-13}	0.119
MYH13	13	3.87×10^{-13}	0.686
MED16	13	4.85×10^{-13}	0.696
HDAC5	7	8.09×10^{-13}	0.003
CLCN1	11	9.07×10^{-13}	0.778
PDZRN4	16	1.47×10^{-12}	0.150
QRFP	5	3.61×10^{-12}	0.157
C6orf120	2	4.43×10^{-12}	0.347
NLRP8	24	5.37×10^{-12}	0.425
PKP3	13	5.94×10^{-12}	0.949
FGFR4	8	6.51×10^{-12}	0.231
CALHM1	6	7.17×10^{-12}	0.235
PAX7	3	9.68×10^{-11}	0.724
C5orf25	3	1.46×10^{-10}	0.590
LEFTY2	2	1.70×10^{-10}	0.590
JRK	12	3.50×10^{-10}	0.458
ABHD10	5	3.86×10^{-10}	0.770
ABCA7	32	6.36×10^{-10}	0.024
RTEL1, RTEL1-TNFRSF6B	24	1.64×10^{-9}	0.213
MUC6	28	2.04×10^{-9}	0.916
CACNA1I	9	4.67×10^{-9}	0.133
WAC	5	2.66×10^{-8}	0.884
PTRF	6	3.67×10^{-8}	0.309
MRPL44	3	4.46×10^{-8}	0.360
WDR41	9	5.13×10^{-8}	0.686
PRSS57	5	6.33×10^{-8}	0.488
WDR74	3	7.99×10^{-8}	0.588
ZNF423	13	8.22×10^{-8}	0.739
AVIL	11	8.65×10^{-8}	0.430
CAND2	27	1.32×10^{-7}	0.005
NPTX1	2	1.62×10^{-7}	0.107
VWDE	34	1.81×10^{-7}	0.190

Gene	Number of variants on exome chip	*P*[a]	Adjusted *P*[b]
PVR	10	2.01×10^{-7}	0.011
BMP6	5	2.21×10^{-7}	0.293
VPS4A	3	2.77×10^{-7}	0.695
TRMT1	5	3.23×10^{-7}	0.492
FASN	27	3.25×10^{-7}	0.017
TBCC	8	3.67×10^{-7}	0.241
TMEM201	6	4.01×10^{-7}	0.021
MGAT3	5	4.14×10^{-7}	0.409
MSI2	4	4.40×10^{-7}	0.351
CRHR2	7	6.67×10^{-7}	0.381
OSBPL6	6	1.02×10^{-6}	0.117
MAN1C1	5	1.70×10^{-6}	0.482
ZNF507	5	2.28×10^{-6}	0.644
GAS6	12	3.20×10^{-6}	0.206
PAX1	4	3.24×10^{-6}	0.689

[a] *P* value based on SKAT analyses.

[b] *P* value after adjustment for the allele count of the most statistically significant variant in the single variant analyses.

[c] *P* value adjusted for the two variants in the *KIF26A* gene (exm1130982 and exm1131059) that were found to be statistically significant in the single variant analyses.

Supplemental Table 5. Associations between anorectal malformations and single variants in the discovery study and the validation study.

ID[a]	MAF (%) controls (n=1,886) Discovery study[b]	MAF (%) ARM patients (n=558) Discovery study[b]	*P*[c]	MAF (%) ARM patients (n=541)[d] Validation study[ef]	*P*[g]
exm681600	0	18.550	8.45×10^{-132}	0	1
exm1078533	0	10.660	2.10×10^{-74}	0	1
exm1198894	0.027	11.040	6.13×10^{-74}	0	1
exm1531889	0	8.513	3.39×10^{-59}	0.101	0.21
exm1390461	0	6.989	1.56×10^{-48}	0	1
exm7090	0.027	6.810	1.30×10^{-45}	0	1
exm670492	0	6.093	2.65×10^{-42}	0	1
exm1330833	0	5.556	1.40×10^{-38}	0	1
exm1130982	0.106	6.183	1.18×10^{-37}	0.095	1
exm1335493	0.027	4.480	1.16×10^{-29}	0	1
exm254596	0	3.943	1.79×10^{-27}	0	1
exm1129696	0	3.763	3.02×10^{-26}	0	1
exm1399016	0	3.680	1.18×10^{-25}	0	1
exm688810	0	3.674	1.24×10^{-25}	0	1
exm1165387	0	3.405	8.56×10^{-24}	0	1
exm274502	0	3.315	3.51×10^{-23}	0	1
exm1415838	0	3.226	1.44×10^{-22}	0	1
exm441548	0.027	3.285	2.10×10^{-21}	0	1
exm1665335	0	4.167	2.11×10^{-21}	0	1
exm949028	0	3.047	2.40×10^{-21}	0	1
exm584047	0	2.867	4.01×10^{-20}	0	1

ID[a]	MAF (%) controls (n=1,886) Discovery study[b]	MAF (%) ARM patients (n=558) Discovery study[b]	*P*[c]	MAF (%) ARM patients (n=541)[d] Validation study[ef]	*P*[g]
exm2254176	0.106	3.495	4.99×10^{-20}	0	0.58
exm42669	0	2.419	4.50×10^{-17}	0	1
exm78706	0	2.419	4.50×10^{-17}	0	1
exm913730	0	2.330	1.83×10^{-16}	0	1
exm676304	0.080	2.778	2.87×10^{-16}	0	1
exm665970	0.055	2.688	3.11×10^{-16}	1.943	1.86×10^{-11}
exm658390	0	2.294	4.82×10^{-16}	0.095	0.22
exm874064	0	2.151	3.02×10^{-15}	0	1
exm995133	0	2.151	3.02×10^{-15}	0	1
exm1368208	0	2.151	3.02×10^{-15}	0	1
exm2253769	0.186	3.047	3.81×10^{-15}	0.380	0.27
exm1580785	0	2.068	1.15×10^{-14}	0.196	0.05
exm84805	0	2.061	1.22×10^{-14}	0	1
exm626311	0	2.061	1.22×10^{-14}	0	1
exm658164	0	2.061	1.22×10^{-14}	0	1
exm1103535	0.027	2.151	4.92×10^{-14}	0	1
exm297681	0	1.971	4.96×10^{-14}	0	1
exm1577147	0	1.971	4.96×10^{-14}	0	1
exm725230	0	1.882	2.01×10^{-13}	0	1
exm872472	0	1.882	2.01×10^{-13}	0	1
exm870424	0	1.808	7.14×10^{-13}	0	1
exm1610290	0	1.792	8.15×10^{-13}	0	1

ID[a]	MAF (%) controls (n=1,886) Discovery study[b]	MAF (%) ARM patients (n=558) Discovery study[b]	*P*[c]	MAF (%) ARM patients (n=541)[d] Validation study[ef]	*P*[g]
exm1293600	1.326	5.376	1.83×10^{-12}	1.766	0.31
exm1510860	0.106	2.061	4.73×10^{-11}	0.093	1
exm853474	0.636	3.405	3.03×10^{-10}	0.282	0.24
exm428026	0.055	1.706	4.69×10^{-10}	1.667	2.93×10^{-10}
exm462602	0.159	2.061	6.62×10^{-10}	0	0.35
exm1239020	0.212	2.065	5.85×10^{-9}	2.083	2.41×10^{-9}
exm1560265	0.186	1.902	1.77×10^{-8}	0.192	1
exm1017978	0.265	1.971	1.09×10^{-7}	0.473	0.34

ARM: anorectal malformations; MAF: minor allele frequency.

[a]See Supplemental Table 2 for corresponding chromosome, position, rs-number, minor/major allele, type of variant, and gene.

[b]Discovery study was performed using the Illumina Human Exome BeadChip.

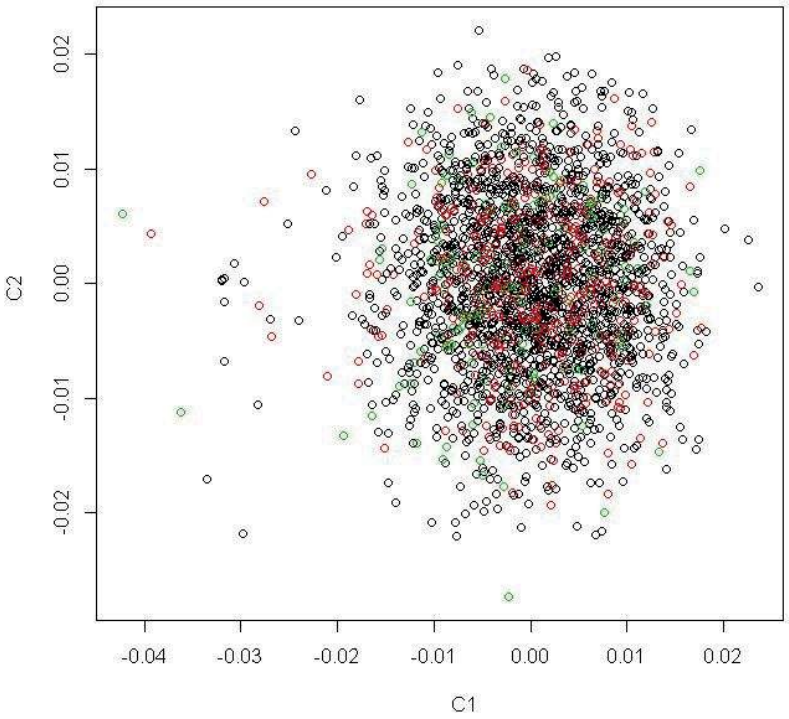
[c]*P* value calculated for an allelic model using Fisher's exact test and adjusted for genomic control.

[d]Seventeen patients were not included in the validation study due to insufficient DNA amount or quality or because the sample dropped out of the MIP validation study.

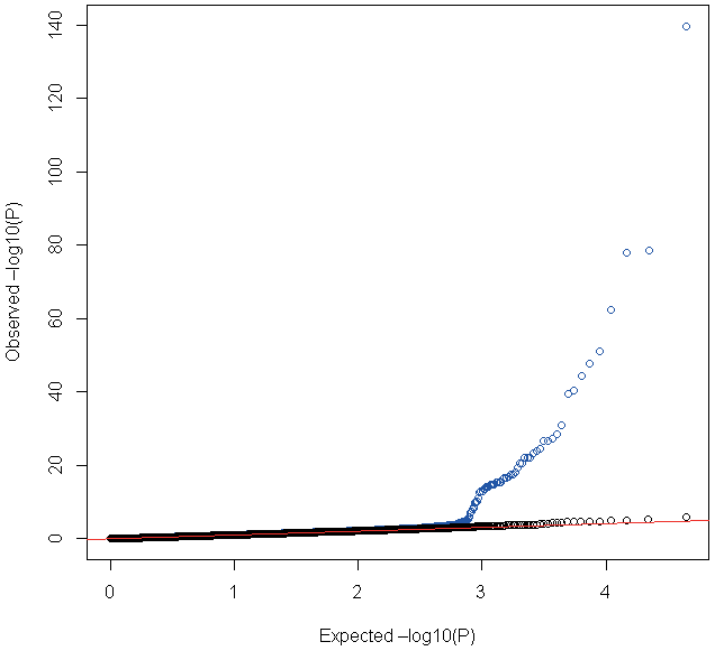
[e]Validation study was performed using the MIP technique.

[f]Concordance rates of heterozygous calls were close to 0% for the majority of the variants, except for exm2253769, exm1293600, exm853474, and exm1017978 with concordance rates of 8-30% and exm665970, exm428026, and exm1239020 with concordance rates of 100%.

[g]*P* value calculated using the controls from the discovery study (2nd column of this Table).



Supplemental Figure 1. The first two principal components obtained by multidimensional scaling analysis for controls (black), anorectal malformation patients AGORA (red), anorectal malformation patients CURE-Net (green).



Supplemental Figure 2. Quantile-quantile (QQ) plot for the unadjusted single variant analyses on anorectal malformations in the discovery study (allelic effects) before (blue) and after (black) removing identified variants. The red line represents the null hypothesis of no association. Genomic control inflation factor was: λ = 1.066.

Part 4

General discussion and summaries

Chapter 11

General discussion

This thesis presents the results of our research project with the main aim to obtain more knowledge on the involvement of genetic and non-genetic risk factors in the etiology of congenital anorectal malformations (ARM). Etiologic research into ARM received relatively little scientific attention in the past decades, particularly genetic research on ARM. We performed several case-control studies on potential non-genetic risk factors using parental questionnaire and registry-based data. The main findings from these studies are summarized in Table 1. In addition, we were the first to study the assumed role of a gene-environment interaction in the development of ARM, and we performed an exome chip association study on rare variants in the by far largest sample of ARM cases described in genetic studies on this congenital malformation so far. However, no evidence was found for associations between ARM and rare genetic variants with large effect sizes captured by the Illumina Human Exome BeadChip in this study. Before we will further discuss our findings in light of the objectives and the existing literature, some methodological issues will be addressed. Thereafter, we will provide directions for future research and consider the clinical implications.

Table 1. Main findings from studies on non-genetic risk factors for anorectal malformations.

Potential risk factor	**Association with anorectal malformations?**		
	Our studies	**Other studies**	**Global conclusion**
Fertility-related factors			
Parental subfertility without treatment	+	NI	(+)
ICSI or IVF treatment	+	+	+
Hormonal treatment	+/-	+/-	+/-
Other reproductive treatments	(-)	NI	(-)
Pregnancy-related factors			
Nulliparity	+/-	+/-	+/-
Multiple pregnancy	+	+	+
Maternal overweight or obesity	+	+	+
Maternal epilepsy	+	NI	(+)
Fever during first trimester	+	NI	(+)
Preeclampsia	+	(+)	(+)
Lifestyle factors			
Maternal folic acid supplement use	-	+/-	(-)
Paternal smoking	+/-	(-)	+/-
Parental job exposures	+	(+)	(+)

+ positive association; (+) possible association; - no association; (-) probably no association; +/- inconsistent findings; NI not investigated

Methodological issues

Study design

All studies on potential risk factors described in this thesis were based on case-control designs with retrospectively collected data. Compared to randomized controlled trials, the paradigm of epidemiologic study designs, this observational design provides lesser amounts of evidence for causal inferences based on the study results. However, it is a very commonly used design that has proven to be efficient to assess associations between exposures and outcome, particularly when the outcome under study is rare, as is the case for ARM. Several potential biases may be associated with observational studies, mainly related to the recruitment of the study participants and the methods of data collection. These issues are addressed below.

Study populations

The recruitment of cases and/or controls is an important step both in studying prevalence rates and in designing case-control studies. The ARM cases studied within the realm of this thesis were mainly derived from AGORA and/or CURE-Net in The Netherlands and Germany, respectively. A major strength of these data sources was that cases were well-characterized as they were reviewed by experienced clinical geneticists, pediatric surgeons, and ARM researchers. The detailed case characterization enabled us to calculate prevalence rates of different ARM phenotypes and associated congenital malformations. These prevalence rates partly depend on the inclusion and exclusion criteria for the cases, as was reflected in the studies described in this thesis. We included live born cases without chromosomal abnormalities or known genetic syndromes in most studies (Chapters 3, 7, 8, 9 and 10), whereas live born and stillborn cases and induced abortions without chromosomal abnormalities or known genetic syndromes were included in the European Surveillance of Congenital Anomalies (EUROCAT) study (Chapter 6), and live born cases including those with chromosomal abnormalities or known genetic syndromes in the ARM-NET study (Chapter 4). Consequently, the prevalence of associated congenital malformations ranged between 39% and 55% in the first-mentioned studies, whereas it was 64% in the EUROCAT study and 72% in the ARM-NET study. Therefore, we need to be aware of potential differences in prevalence rates, due to variation in the inclusion and exclusion criteria for the cases. The consequences of the inclusion of live born cases only need to be considered in studies on potential risk factors for ARM as well. Isolated ARM is seldom fatal as more than 99% of the isolated ARM cases were found to be live born.[1] In addition, terminations of pregnancy among fetuses with isolated ARM are scarce as ARM can hardly be diagnosed prenatally.[2] Therefore, the potential for selection due to the inclusion of live born participants only is

limited in analyses on isolated ARM. In contrast, stillbirth and induced abortion rates are higher for ARM with multiple congenital malformations, because of severe associated birth defects. Therefore, the results for ARM with other defects may be underestimated when stillbirth and/or induced abortion rates are higher among cases exposed to the determinant under study compared to non-exposed cases. In our studies, this may be the case for couples who underwent ICSI or IVF treatment or for mothers with pre-existing diabetes mellitus as they may have been offered prenatal screening more easily than other couples.

Three types of controls were used in our studies on non-genetic risk factors, including malformed controls with chromosomal or known genetic syndromes ('genetic controls') (Chapter 6), hospital-based controls with otitis media with effusion (Chapters 3 and 7) and population-based controls (Chapters 8, 9 and 10). 'Genetic' or hospital-based controls are often used to reduce selective non-response and the potential for recall bias in studies on non-genetic risk factors. Recall bias occurs when the parents of cases and controls remember or report certain exposures differently, which may lead to differential misclassification in population-based studies. On the other hand, selection could be introduced in hospital-based studies when certain exposures are positively or negatively associated with the disorder of the controls, leading to underestimation or overestimation of the risk estimates, respectively. This problem was avoided in our studies using the population-based control group established especially for these ARM studies and for future studies within the AGORA data- and biobank. A limitation of this control group is, however, that the parents on average seem to be somewhat higher educated than the general Dutch population, probably due to lower response rates among low-educated parents. Therefore, maternal education was included as potential confounder in the multivariable regression analyses. The potential for recall bias cannot fully be excluded, although many potential risk factors studied do not seem to be prone to recall bias (e.g. fertility treatment and chronic diseases) and recall bias appears to be overrated in this area of research.[3] So next to practical reasons, the choice for the most suitable control group in non-genetic studies may depend on the potential risk factor under study. We consider malformed or hospital-based controls appropriate only if the risk factor is prone to recall bias and is not associated with the control disorder, whereas population-based controls are preferred in all other situations, if chosen accurately. Ideally, the control population represents the geographical area and age distribution of the source population of the cases to minimize bias due to geographical or generational effects. We consider this to be achieved for all three control populations used in our studies.

The selection of cases and controls in similar geographical areas is also essential to limit bias due to population stratification in genetic association studies. This form of

selection bias arises when differences in the allele frequencies of the genetic variants between cases and controls are due to systematic ancestry differences rather than real associations between the genetic variants and the disorder under study. We attempted to reduce this kind of bias by selecting or performing subanalyses on cases and controls of European ancestry only in our genetic associations studies (Chapters 9 and 10). In addition, we performed multidimensional scaling analyses in order to exclude population outliers in the exome chip association study (Chapter 10). Therefore, this kind of bias seems to be limited in our studies.

Data collection

Information on potential non-genetic risk factors for ARM was obtained using self-administered parental questionnaires (Chapters 3, 7, 8 and 9) and register-based data (Chapter 6). Of these two methods, we consider retrospective data collection by means of parental questionnaires the superior approach because of the efficient way of collecting a large amount of data. More importantly, we were able to collect detailed information on important issues and information on timing of exposure to a wide range of potential risk factors, whereas only a limited amount of relevant information is usually available in register-based studies,[4] such as EUROCAT. A disadvantage of questionnaire data may be the potential for differential misclassification due to recall bias, as discussed in the previous paragraph. We expect non-differential misclassification due to recall problems in parents of both cases and controls, however, to be more likely and to have led to underestimation of the results. Recall errors seem to be less of a problem in our study using register-based data, because information on exposure was mainly recorded from already existing hospital sources. Although more timely and detailed data can be collected and recall problems are negligible in prospective cohort studies, this design is not suited for studies on ARM as it would be far too expensive and time-consuming to collect data for a sufficient number of cases.

Data on genetic variants were obtained through genotyping of case and control DNA samples (Chapters 9 and 10). Genotyping with the Illumina Human Exome BeadChip and calling (using zCall software) of the rare variants in the discovery phase of our exome chip association study were challenging procedures because of the innovative nature of this chip. Several rare variants were found to be associated with ARM. The case samples were re-genotyped for the variants identified in a validation study using targeted resequencing with the molecular inversion probe (MIP) technique. This validation study appeared to be an important step in our study, as many heterozygous calls for the associated variants in the exome chip data were not confirmed in the validation study, and neither were the

associations between ARM and these variants. The separate genotyping and calling of case and control samples in the discovery study possibly caused differential misclassification of genotypes. This may have resulted in overestimation of positive allele frequency differences between cases and controls. We elaborated further on this issue in Chapter 10.

Our findings in light of the objectives and the existing literature

1. To increase knowledge on definitions and prevalence of ARM phenotypes and associated congenital malformations

In the past decades, different international classification systems for ARM were proposed to define ARM phenotypes. We consider the comprehensive Krickenbeck classification to be the most useful system, just like the majority of pediatric surgeons and other medical professionals (Chapter 4).[5] The WHO International Classification of Diseases (ICD) and the Human Phenotype Ontology (HPO), however, do not follow the Krickenbeck criteria and their classifications for ARM are far from comprehensive. As several studies, such as the EUROCAT study (Chapter 6), are based on ICD codes, comparisons between studies concerning ARM phenotypes are difficult or even impossible. In addition, a harmonized diagnostic classification system for the VACTERL (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal and Limb defects) association is still lacking but warranted, as we stressed in Chapter 5. Genetic and/or epidemiological evidence for certain risk factors in specific VACTERL phenotypes will probably help to define inclusion criteria for clinical features involved in VACTERL. Recent studies found indications for genetic aberrations in VACTERL cases,[6-9] but a major contributing factor is not identified yet.

The prevalences of ARM phenotypes and associated congenital malformations were assessed in several studies in this thesis, which add information to the existing literature.[10-12] Perineal fistula is the most commonly occurring ARM phenotype. Overall, the percentage of perineal fistula is higher in isolated ARM than in ARM with other congenital malformations. On the other hand, the more complex phenotypes, such as rectourethral fistula or cloaca, are more common among ARM with other defects compared to isolated ARM. The same pattern was observed among ARM cases treated in the Radboudumc between 1974 and 1995, although these cases were not completely classified according to the Krickenbeck criteria.[10] Comparisons with some other studies are impossible as less detailed classification systems were used.[11-13] In addition, the occurrence of associated congenital malformations in ARM cases also differed substantially among our studies, mainly due to differences in the inclusion and exclusion of cases as described above. Associated malformations were mostly

congenital malformations that are described in the VACTERL association. The ARM-NET consortium represents a valuable resource of ARM cases as multiple centers in several European countries are involved (Chapter 4). However, 72% of the cases within the ARM-NET register having other congenital malformations seems relatively high. A possible explanation would be that cases with only minor additional malformations were also classified as ARM with other congenital malformations, whereas these cases were classified as isolated ARM in most other studies. On the other hand, this relatively high proportion of ARM cases with other congenital malformations may be a consequence of referral of more complex cases to some of the participating centers within ARM-NET.

2. To study a wide range of potential non-genetic risk factors for ARM

The role of several potential non-genetic risk factors in the etiology of ARM was summarized in our systematic review (Chapter 2) and investigated in a number of studies described in this thesis (see Table 1). Strong indications for fertility-related factors increasing the risk of ARM were found in our studies (Chapters 6, 7 and 8) and in the existing literature.[14-17] Although ICSI/IVF treatment in particular was found to be associated with ARM, this was not the case in the EUROCAT study (Chapter 6). However, we consider the evidence for a positive association between ICSI/IVF treatment and ARM to be stronger than the evidence for a lack of association, because four out of five other studies on ARM identified relatively strong associations with ICSI/IVF treatment as well.[14-18] The reasons for the negative finding in the EUROCAT study may be: 1) the use of 'genetic controls', which may have resulted in underestimated risk estimates, as associations have previously been found between ICSI/IVF treatment and chromosomal abnormalities,[19] and 2) the low power to identify this underestimated risk as the numbers of case and control parents who underwent ICSI or IVF treatment were low in the EUROCAT database. Interestingly, we observed that the association between ARM and ICSI or IVF treatment remained when subfertile parents who conceived without treatment were used as the reference group (Chapter 8). This suggests that ICSI or IVF treatment plays a role above the possible effect of the underlying parental subfertility. However, confounding by indication cannot be excluded as subfertile parents who conceived after ICSI or IVF may be more severely subfertile than those who conceived without treatment. Therefore, we expect both parental subfertility and ICSI or IVF treatment to be involved in the etiology of ARM. This was confirmed by the finding that parental subfertility without treatment was associated with ARM with additional congenital malformations. Underlying disturbed epigenetic mechanisms may explain the involvement of parental subfertility, mainly male subfertility, and ICSI/IVF treatment in the etiology of

ARM. Epigenetic modifications refer to changes in DNA that occur through other modifications than variation in the DNA sequence. Children conceived through ICSI/IVF treatment were found to have altered DNA methylation levels in imprinted genes and an increased risk of imprinting disorders compared to children conceived spontaneously.[20]

Novel findings were the relatively strong associations between ARM and maternal fever during the first trimester of pregnancy (Chapters 3, 6, and 7) and maternal epilepsy during pregnancy (Chapter 6), which need further clarification. Preeclampsia was found to be associated with ARM with other congenital malformations in particular. Although preeclampsia arises after the relevant time window for hindgut development, it may be a consequence of dysfunction of the placenta in early pregnancy, a condition in which the nutrient and hormone supply to the embryo may be insufficient. The associations with low birth weight, preterm delivery, and multiple pregnancy, as well as a possible association with nulliparity, also suggest a potential role for placental dysfunction in early pregnancy. We found the association with multiple pregnancy for ARM with other congenital malformations, but not for isolated ARM (Chapter 6). Furthermore, mean weight of the placenta was found to be significantly lower for ARM with other congenital malformations compared to controls, but not for isolated ARM compared to controls.[12] These studies support the hypothesis that placental dysfunction is involved in the etiology of ARM with other congenital malformations.

Several studies, including ours, showed increased risks of having a child with ARM for mothers with overweight or obesity and for mothers with pre-existing diabetes mellitus.[21-26] We studied pre-existing diabetes mellitus in the EUROCAT study only (Chapter 6), and did not identify an association in relatively small numbers of case mothers with pre-existing diabetes. However, the characteristics of the study population in Chapters 8 en 9 showed higher rates of pre-existing diabetes among case mothers than among control mothers. Hyperglycemia or poor glycemic control in diabetic mothers as well as in overweight or obese mothers may explain the increased risks of ARM.

This thesis provided less evidence for a role of parental lifestyle factors in the etiology of ARM. Although maternal folic acid supplement use turned out to be effective in the prevention of neural tube defects, a major role in the prevention of ARM can be excluded based on the results in Chapter 9 and other studies.[24,27-29] These studies did not observe associations with folic acid supplement use either, or found marginal effects only. Furthermore, inconsistent results or non-specific findings were observed for ARM and paternal smoking or various parental occupational exposures.

Overall, the effects of non-genetic risk factors were more pronounced in ARM cases with other major congenital malformations than in isolated ARM cases. Possibly, these risk

factors are not unique for ARM, but result in other congenital malformations as well. In addition, ARM cases with other congenital malformations frequently suffer from more complex ARM phenotypes, such as rectourethral fistulas or persistent cloaca, as discussed above. Therefore, another explanation for associations being observed in ARM cases with other malformations in particular may be that non-genetic factors have stronger effects in more complex forms of ARM than in mild forms of ARM.

3. To identify a potential gene-environment interaction and rare genetic variants that contribute to the development of ARM

We were the first to study the role of a single nucleotide polymorphism (SNP) in the *MTHFR* gene (*MTHFR C677T*) and its interaction with maternal folic acid supplement use in the etiology of ARM. The *MTHFR C677T* polymorphism leads to decreased enzyme activity and consequently to reduced folate levels, while folate is essential for cell division and gene expression during embryogenesis. Elevated risks of neural tube defects, orofacial clefts, and congenital heart defects among mothers with the polymorphism and a lack of folic acid supplement use were reported before,[30-32] but an important role in the etiology of ARM does not exist for this polymorphism and its gene-environment interaction with folic acid supplements. We elaborated on potential reasons for this finding in Chapter 9.

Our systematic review (Chapter 2) showed that knowledge about the involvement of genetic factors in the etiology of ARM is scarce. Previous genetic studies on ARM were mainly focused on candidate genes involved in embryonic signaling pathways, but did not yield any substantial evidence. No common genetic variants were identified in the first and only small genome-wide association study,[33] and neither were common copy number variants (CNVs). The study did, however, identify a role for many different rare CNVs in individual isolated ARM cases, which were not found in controls. To identify rare genetic variants, we used a hypothesis-free approach studying 242,901 exome-wide rare variants with the Illumina Human Exome BeadChip. Fifty-five rare coding variants were found to be associated with ARM in the discovery study. Three of these variants were validated in the same ARM cases using the MIP validation study. However, replication analyses in new Caucasian and Han Chinese replication samples of cases and controls did not confirm these variants as being associated with ARM. Therefore, major single rare or common variants captured by genome- and exome-wide arrays do not seem to play a role in the development of ARM on the population level. However, genetic variants occurring in small numbers of cases and variants with moderate or small effects could still play a role, as our power was probably insufficient to identify these. The genetic etiology of ARM seems to be

heterogeneous, as was also suggested previously.[33] In Chapter 10, we considered the contribution of both monogenic and multifactorial forms of ARM. As ARM comprise a wide spectrum of phenotypes, several etiologic mechanisms and pathways may be involved.

Finally, both our genetic and non-genetic studies showed the importance of international collaborations to increase the power to detect potential contributors to the etiology of ARM, to be able to differentiate between ARM phenotypes in the analyses, and to replicate results in other patient cohorts. This was also emphasized in Chapter 3.

Conclusions

In conclusion, this thesis shows strong indications for associations between ARM and non-genetic factors, such as ICSI or IVF treatment, maternal fever during the first trimester of pregnancy, and maternal overweight or obesity. The underlying parental subfertility or maternal infection, however, could also play a role. In contrast, maternal folic acid supplement use or its interaction with the *MTHFR C677T* polymorphism does not have a major role in the etiology of ARM, while the potential role of placental insufficiency needs further elucidation. Rare variants with large effect sizes captured by the Illumina Human Exome BeadChip do not seem to be involved in the occurrence of ARM. As ARM are most likely a genetically heterogeneous set of malformations, monogenic as well as multifactorial forms need to be investigated in future studies.

Opportunities for future research

The findings in this thesis provide several directions for future research to increase our knowledge on the underlying biological mechanisms in the development of ARM. Major efforts have been done to collect questionnaire and clinical data and DNA samples of large numbers of ARM cases and population-based controls and their parents within AGORA and ARM-NET. This represents a valuable resource for new research opportunities for ARM.

Non-genetic studies

To investigate our hypothesis that disturbed epigenetic processes may be an explanation for the associations of ARM with parental subfertility and ICSI or IVF treatment, studies on DNA methylation patterns may be valuable. We suggest to assess whether DNA methylation levels are disturbed (hyper- versus hypomethylation) in some genes using a candidate-gene approach or a genome-wide methylation assay on DNA samples of cases and controls who were conceived either with or without ICSI/IVF treatment, also taking into account the fertility status of the parents.

The effects of maternal fever and maternal epilepsy during the first part of pregnancy need to be disentangled from potential effects of maternal infection and/or drug use (e.g. antibiotic, anti-inflammatory or antiepileptic drugs) on the development of ARM. Clarification may come from future studies that, for example, differentiate the risks for mothers with fever or epilepsy between those who did and did not use medication. As the prevalences of these exposures are relatively low, large sample sizes are essential in case-control studies with collection of detailed information on these risk factors and other potential teratogenic drugs, for which knowledge on their role in the etiology of ARM is limited. Information on medical drug use during pregnancy was collected using the maternal AGORA questionnaire, but a previous study showed that, depending on the type of medical drug, maternal recall of prescriptive drug use was moderate to poor using this retrospective questionnaire.[34] Probably, more valid results can be obtained by linking AGORA data to Dutch pharmacy registers, a study that will be undertaken in the future.

The association between ARM and preeclampsia also needs confirmation in future studies, which should also include chronic hypertension, as Bánhidy *et al.*[35] found an association between ARM and preeclampsia superimposed on chronic hypertension in a relatively small study sample. Future studies may also clarify the possible role for placental dysfunction in early pregnancy in the etiology of ARM with other congenital malformations, as they have for hypospadias.[36,37] As only indirect indications exist so far, an initial step may be to evaluate potential placental pathologies, such as abnormal size, weight or thickness of the placenta, infarction, and/or calcifications,[38] in a group of placentas of ARM cases with other congenital malformations and healthy controls. In addition, low levels of Human Chorionic Gonadotropin (HCG) measured in the first 9-10 weeks of pregnancy may emphasize the role of placental dysfunction early in pregnancy.[39-41]

Furthermore, the role of parental life style and occupational factors could be evaluated in more detail. The distribution of Food Frequency Questionnaires (FFQs) among mothers of cases and controls is planned for the near future, to evaluate possible associations between ARM and maternal intake of nutrients and vitamins during the first part of pregnancy. Two previous studies showed the value of etiologic research using FFQs. In these studies, a higher dietary glycemic index was found among ARM case mothers compared to control mothers without diabetes mellitus, which supports the role of maternal hyperglycemia in the etiology of ARM.[42,43] As basic information on the occupation of both parents has already been collected in AGORA, one or more Job Exposure Matrices (JEMs) could be applied to study the role of parental occupational exposure in the etiology of ARM. For example, the JEM that was developed by our research group some years ago[44] may be

used to assess the effects of occupational exposures to endocrine disruptors on the development of ARM.

The majority of the studies mentioned above may be performed using the already collected data on ARM cases and population-based controls within AGORA, which will be extended with additional data from Dutch pharmacy registers and FFQs. In addition, new ARM cases will be recruited through continuous data collection within AGORA and ARM-NET and from other university medical centers in The Netherlands to increase the power of these future studies.

Genetic studies

Both monogenic and multifactorial forms of ARM need to be considered in future genetic studies. Exome and whole genome sequencing seem promising techniques to study small homogeneous groups of cases to identify genetic variants that may cause ARM in some of these patients. These groups should comprise selected cases, such as familial ARM cases, males with perineal fistula behind a bucket-handle, or females with a cloaca or an isolated vestibular fistula. To evaluate whether *de novo* mutations are involved in the occurrence of ARM, these techniques may be applied to case-parent triads. In addition, targeted resequencing using the MIP technique that we used in the study described in Chapter 10 for validation and replication of single variants seems very promising for sequencing of multiple candidate genes. This is currently being done for both ARM and VACTERL in one experiment within the Radboud university medical center. This experiment may yield causal variants in candidate genes that may explain the occurrence of ARM in a small part of the patients.

For ARM cases in whom no single gene seems to play a causal role, many common and rare variants with moderate or small effects in combination with non-genetic factors may contribute to the occurrence of ARM. Additional genome-wide screening procedures will be needed to investigate the contribution of these potential genetic risk factors. The only genome-wide association study (GWAS) on ARM did not yield any common SNPs that were associated with ARM.[33] As this study was performed among 175 cases only and used a relatively low-density array containing approximately 500,000 SNPs, it seems promising to perform another GWAS in our larger patient series using a newer array containing at least 1,000,000 SNPs. Furthermore, the genotyping data of the Illumina Human Exome BeadChip (Chapter 10) may also be used to perform future analyses, such as pathway association analyses and repeated analyses using larger sample sizes with additional data of new ARM cases. Before that, however, it is essential to repeat the calling procedure for all cases and controls combined to reduce the potential for batch effects.[45] New technologies, such as exome or whole genome sequencing, will create even more data, but these methods are not

yet feasible in large cohorts of cases and controls due to the high costs and the need for large processing capacity.

Expression studies and functional studies will be needed to further evaluate genetic variants that may be discovered in the studies suggested above. Additional evidence for the involvement of these genes in the development of ARM may be derived from expression studies in human anorectal tissue or hindgut tissue of animal embryos with ARM. Functional studies in animal models may also add knowledge about the function of specific genes and about the involvement of potential pathways. A few animal models for ARM have been reported, such as retinoic acid induced ARM in mice,[46] ethylenethiourea induced ARM in rats,[47] and Danforth's short tail mice.[48]

Clinical implications

The most commonly asked questions of parents with a child with ARM are: 'What is the diagnosis?', 'What is the best treatment and clinical care for my child?', 'What is the recurrence risk in future pregnancies?', 'What is the cause?' and 'What can I or anybody do to prevent having a child with ARM?'. Therefore, the ultimate objectives of research on ARM are to improve diagnostics, clinical care, outcome, and quality of life, to better guide parents of a child with ARM or couples with a child wish regarding their future pregnancies, and to develop preventive measures for ARM.

In the coming years, registration of ARM patients in the ARM-NET registry will continue and several new centers within Europe will join ARM-NET or already joined recently. One of the major aims of the ARM-NET consortium is to provide better clinical care and outcome assessment, as several aspects of treatment and management of ARM patients are still debated among pediatric surgeons and health care professionals. This ARM-NET registry is a valuable basis for discussions on harmonized diagnostic classifications and the best treatment options for specific phenotypes, which may lead to improvement of the management of patients with ARM.

To improve guidance of parents who have a child with ARM regarding future pregnancies, it is essential to provide knowledge about the recurrence risk of ARM. For parents who have a child with a syndromic form of ARM, such as the autosomal dominant Townes-Brocks syndrome, the recurrence risk can be up to 50%. For parents who have a child with ARM without a chromosomal abnormality or known genetic syndrome, the recurrence risk is much lower and estimated to be 1-2%. Results from future genetic studies may provide more precise estimations for individual patients and their parents.

Couples who conceived after ICSI/IVF treatment, diabetic mothers, and mothers with overweight or obesity were consistently found to have an increased risk of having a child

with ARM. These 'higher risk groups' should be guided and informed about the potential risks before conception, although absolute risks are low and future studies are still needed to unravel the underlying mechanisms. The risk estimates that were found in this thesis were presented as odds ratios, which can be interpreted as relative risks since ARM is a rare anomaly. Assuming an odds ratio of 2.5 for the above-mentioned risk factors, the absolute risk of having a child with ARM for these couples changes from 0.03% (1 in 3000) to 0.08% (1 in 1200). Previous studies, however, showed that the above-mentioned risk factors are not unique for ARM, but increase the risk of other congenital malformations as well.[15,22,23,49] This also needs to be considered when guiding couples regarding future pregnancies.

The implication of these risk factors at the population level depends on the prevalence of a given risk factor. The population attributable fraction (PAF) presents the proportion of cases in a population that are affected due to the risk factor under study and can be calculated as follows: PAF = p*(OR − 1) / (p*(OR–1) +1). Assuming ORs of 2.5 and a prevalence of 2% for ICSI or IVF treatment versus 6% for maternal obesity, the PAFs are 3% and 8%, respectively. The latter suggests that 8% of the ARM cases could be prevented if maternal obesity would be ruled out completely.

Effective preventive measures, such as folic acid supplementation to prevent neural tube defects, do not seem to be available for the prevention of ARM yet, but the increased knowledge about the etiology of ARM provided within this thesis may be a first step to better guide couples with a child wish and the parents of patients with ARM.

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Chapter 12

Summary

Samenvatting

About the author

PhD portfolio

List of publications

Dankwoord

Summary

Congenital anorectal malformations (ARM) are defects in the development of the anus and the rectum, which occur in approximately 1 per 3,000 live births worldwide. One or more additional major congenital malformations are present in 40 to 70% of the ARM patients, mostly malformations of the VACTERL (Vertebral, Anal, Cardiac, Tracheo-Esophageal, Renal, and Limb defects) association. Multiple surgical procedures are usually required in the first years of a patient's life to restore bowel function. Nevertheless, this disorder often accounts for substantial lifelong psychosocial and physical problems, such as constipation, fecal soiling, and sexual problems. Because of these problems, primary prevention of ARM is needed, but this requires extensive knowledge about the etiology of ARM. Therefore, the main objective of this thesis is to obtain more insight into the contribution of genetic and non-genetic risk factors to the occurrence of ARM.

Part 1: Introduction, prevalence, and definitions

After a brief general introduction in Chapter 1, we present an extensive systematic review of the existing literature about the genetic and non-genetic etiology of ARM in Chapter 2. Assisted reproductive techniques, multiple pregnancy, preterm delivery, low birth weight, maternal overweight or obesity, and pre-existing diabetes were consistently found to be associated with ARM. Furthermore, we showed that human research into the genetic etiology of ARM is remarkably scarce and that most studies were based on a candidate-gene approach. Although animal studies demonstrated the contribution of genes or targets in embryonic signaling pathways, such as the sonic hedgehog, wingless-type integration site, fibroblast growth factor, and bone morphogenetic protein signaling pathways, human studies did not provide substantial evidence for the involvement of genes in these pathways in the etiology of ARM. The first small genome-wide association study (GWAS) on ARM did not yield any single nucleotide polymorphisms (SNPs) or common copy number variants (CNVs), but did identify many different rare CNVs in individual isolated ARM cases, which were not found in controls. We concluded that large cohorts of ARM patients from international collaborations are needed to conduct future genetic studies with hypothesis-generating approaches, such as GWAS to elucidate the role of common variants and exome or whole genome sequencing to study rare variants.

To this end, the ARM-NET consortium described in Chapter 3 was established, with involvement of clinical geneticists, epidemiologists, pediatric surgeons, and members of patient organizations within Europe. Chapter 4 presents the first results on the prevalence of ARM phenotypes, additional congenital malformations, and surgical procedures within the

ARM-NET consortium among 203 ARM patients. Perineal fistula was the most prevalent ARM phenotype and 72% of the ARM cases had at least one additional major congenital malformation. In Chapter 5, we stressed the importance of a harmonized diagnostic classification system for the VACTERL association, which often includes ARM, to improve treatment and follow-up of VACTERL patients.

Part 2: Non-genetic factors

Chapter 6 describes the results of a register-based case-control study on pregnancy-related risk factors of ARM using data from 17 registries of the European Surveillance of Congenital Anomalies (EUROCAT). In this relatively large study, maternal epilepsy, fertility treatment, multiple pregnancy, primiparity, preeclampsia, and maternal fever during pregnancy were identified as potential risk factors for ARM, primarily for ARM with additional major congenital malformations. It remained unclear, however, whether epilepsy, fertility treatment, and fever themselves play a role in the etiology of ARM or the pharmacological treatment or underlying disorders, such as parental subfertility and maternal infection. The associations between ARM and multiple pregnancy, primiparity, and preeclampsia may reflect situations of placental dysfunction in early pregnancy, which may result in insufficient supply of nutrients and hormones to the fetus.

In Chapter 7, a wide range of potential maternal and paternal risk factors for ARM was evaluated using case-control data from the AGORA (Aetiologic research into Genetic and Occupational/environmental Risk factors for Anomalies in children) data- and biobank. This small study revealed indications that the risk of ARM was increased with a family history of ARM, maternal fever during the first trimester of pregnancy, maternal overweight, maternal multivitamin use, paternal smoking, and parental occupational exposures. No associations were observed between ARM and most lifestyle factors, such as folic acid supplement use, maternal smoking, and maternal and paternal alcohol use.

The role of parental subfertility and a range of fertility treatments in the etiology of ARM was studied in more detail in a larger AGORA study population in Chapter 8. We did not find associations between ARM and hormonal treatment for ovulation induction or intrauterine insemination. However, for parents who conceived after intracytoplasmic sperm injection (ICSI) or *in vitro* fertilization (IVF) treatment, an increased risk of having a child with ARM was identified compared to fertile parents. The risk of ARM was also elevated when parents who underwent ICSI or IVF were compared to subfertile parents who conceived without treatment. Parental subfertility without treatment was only associated with ARM with additional congenital malformations. As these associations may be confounded by

indication, we concluded that both ICSI or IVF treatment and the underlying parental subfertility play a role in the etiology of ARM.

Part 3: Genetic factors and gene-environment interaction

Independent associations and interactions of maternal periconceptional folic acid supplement use and infant and maternal *MTHFR* (methylenetetrahydrofolate reductase) *C677T* polymorphisms with the risk of ARM were investigated in Chapter 9. Infant and maternal *MTHFR* C*677T* polymorphisms were only weakly associated with ARM, while no association was observed between ARM and maternal folic acid supplement use. No evidence was provided for a role of the gene-environment interaction in the etiology of ARM either.

Chapter 10 describes our exome chip association study on rare coding variants using the Illumina Human Exome BeadChip in the largest sample of ARM cases studied so far, with the aim to identify new rare genetic variants that contribute to the occurrence of ARM. In total, 55 variants were statistically significant in the discovery study. Only three of these variants in the *CLCN1*, *LRBA*, and *ZNF423* genes were validated using targeted resequencing with the molecular inversion probe (MIP) technique. However, replication analyses of these variants in Caucasian and Han Chinese replication samples did not confirm these results. Therefore, we did not find evidence for strong associations between ARM and rare genetic variants captured by this exome chip.

Part 4: General discussion

In the general discussion in Chapter 11, we considered several methodological issues that were associated with the case-control studies described in this thesis. These issues were mainly related to the study design, the recruitment and inclusion of the study participants, and the methods of data collection. We discussed, for example, the advantages and disadvantages of the three types of controls that were used in the different studies and the potential impact of recall problems when using parental questionnaire data in a case-control design. The findings from our studies were also summarized in light of the main objectives of the thesis and the existing literature. Furthermore, directions for future research were provided, such as studies on DNA methylation patterns in cases and controls who were born as a result of ICSI or IVF treatment, studies on potential teratogenic medical drugs, and studies on nutrition and parental occupational exposures. In addition, exome or whole genome sequencing and genome-wide screening procedures, such as a large GWAS, are needed to identify the contribution of as yet unknown genes or pathways. The ultimate goal of etiologic research on ARM is primary prevention and improved genetic counseling, but

effective preventive measures do not seem to be available for the prevention of ARM yet. However, the increased knowledge on the etiology of ARM provided in this thesis represents a first step to better guide couples with a child wish and the parents of patients with ARM.

Samenvatting

Aangeboren anorectale malformaties (ARM) zijn defecten in de ontwikkeling van de anus en de endeldarm, die wereldwijd in ongeveer 1 per 3.000 levendgeborenen voorkomen. Naast een anorectale malformatie heeft 40 tot 70% van de patiënten ook één of meerdere andere aangeboren aandoeningen, vaak behorend tot de VACTERL (Vertebrale, Anale, Cardiale, Trachea-oEsofageale, Renale en Ledemaat aandoeningen) associatie. Meestal zijn meerdere chirurgische ingrepen in de eerste levensjaren van de patiënt noodzakelijk om de darmfunctie te herstellen. Desondanks leidt deze aandoening vaak tot blijvende psycho-sociale en fysieke problemen, zoals obstipatie, fecale incontinentie en seksuele problemen. Vanwege deze problemen is primaire preventie van ARM nodig, maar dit vereist uitgebreide kennis over de etiologie van ARM. Daarom is het belangrijkste doel van dit proefschrift om meer inzicht te krijgen in de rol van genetische en niet-genetische risicofactoren voor het ontstaan van ARM.

Deel 1: Inleiding, prevalentie en definities

Na een korte algemene inleiding in hoofdstuk 1, beschrijven we in hoofdstuk 2 een uitgebreide systematische review van de bestaande literatuur over de genetische en niet-genetische etiologie van ARM. Daarin werden consistent associaties gevonden tussen ARM en vruchtbaarheidsbehandelingen, meerlingzwangerschappen, vroeggeboorte, laag geboortegewicht, overgewicht of obesitas van de moeder en reeds vóór de zwangerschap bestaande diabetes. Bovendien bleek dat humaan onderzoek naar de genetische etiologie van ARM opmerkelijk schaars is en dat de meeste genetische studies gebaseerd waren op een kandidaatgen benadering. Hoewel dierstudies aantoonden dat genen en eiwitten in embryonale signaleringsroutes, zoals de Sonic Hedgehog (SHH), wingless-type integration (WNT), fibroblast growth factor (FGF) en bone morphogenetic protein (BMP) signaleringsroutes, een rol spelen in de etiologie van ARM, leverden humane studies geen substantieel bewijs voor de betrokkenheid van genen in deze signaleringsroutes bij het ontstaan van ARM. In de eerste kleine genoomwijde associatie studie (GWAS) naar ARM werden geen polymorfismen of veelvoorkomende zogenaamde kopie nummer variaties (CNVs) gevonden. Wel werden veel verschillende zeldzame CNVs geïdentificeerd in individuele geïsoleerde ARM-patiënten, die niet werden gevonden in controles. We concludeerden dat grote cohorten met ARM-patiënten nodig zijn om toekomstige genetische studies met een hypothese-genererende aanpak uit te voeren, zoals een GWAS om de rol van polymorfismen op te helderen en exoom- of genoom-sequencing om zeldzame varianten te bestuderen. Hiervoor zijn internationale samenwerkingsverbanden essentieel.

In dat kader werd het ARM-NET consortium opgericht, dat beschreven is in hoofdstuk 3. In dit consortium zijn klinisch genetici, epidemiologen, kinderchirurgen en leden van patiënten-organisaties uit Europa betrokken. Hoofdstuk 4 laat de eerste resultaten zien met betrekking tot de prevalentie van ARM fenotypes, additionele aangeboren aandoeningen en chirurgische ingrepen in 203 ARM-patiënten binnen het ARM-NET consortium. Een perineale fistel was het meest voorkomende ARM fenotype en 72% van de patiënten had naast ARM één of meerdere andere aangeboren aandoeningen. In hoofdstuk 5, hebben we het belang benadrukt van een geharmoniseerd diagnostisch classificatiesysteem voor de VACTERL associatie om de behandeling en de follow-up van VACTERL-patiënten te kunnen verbeteren.

Deel 2: Niet-genetische factoren

Hoofdstuk 6 beschrijft de resultaten van een patiënt-controle onderzoek naar zwangerschap-gerelateerde risicofactoren voor ARM. In deze studie hebben we gebruik gemaakt van gegevens uit 17 Europese registraties van EUROCAT (een Europees samenwerkingsverband van registraties van aangeboren aandoeningen). In deze relatief grote studie werden epilepsie bij de moeder, vruchtbaarheidsbehandelingen, meerling-zwangerschappen, de eerste zwangerschap, pre-eclampsie en koorts tijdens de zwangerschap geïdentificeerd als potentiële risicofactoren voor ARM. Deze werden voornamelijk gevonden voor ARM-patiënten met andere aangeboren aandoeningen. Het blijft echter onduidelijk of de epilepsie, vruchtbaarheidsbehandeling of koorts zelf een rol speelt in de etiologie van ARM of de farmacologische behandeling of onderliggende oorzaken, zoals verminderde vruchtbaarheid van de ouders en een infectie bij de moeder. De associaties tussen ARM en meerlingzwangerschappen, de eerste zwangerschap en pre-eclampsie duiden mogelijk op placenta-disfunctie in een vroege fase van de zwangerschap. Placenta-disfunctie kan leiden tot onvoldoende toevoer van voedingsstoffen en hormonen naar de foetus.

In hoofdstuk 7 evalueerden we een breed scala aan potentiële maternale en paternale risicofactoren voor ARM met behulp van patiënt-controle gegevens uit de AGORA (Aetiologisch onderzoek naar Genetische en Omgevings Risicofactoren voor Aandoeningen bij kinderen) data- en biobank. In deze kleine studie werden aanwijzingen gevonden voor een verhoogd risico op ARM bij het voorkomen van ARM in de familie, koorts tijdens het eerste trimester van de zwangerschap, maternaal overgewicht, gebruik van multivitaminen door de moeder, roken door de vader en beroepsmatige blootstellingen bij de moeder en/of vader. Er werden geen associaties gezien tussen ARM en een aantal leefstijlfactoren tijdens de zwangerschap, zoals het gebruik van foliumzuur, roken door de moeder en alcoholgebruik door moeder en/of vader.

De rol van verminderde vruchtbaarheid van de ouders en verschillende vruchtbaarheidsbehandelingen in het ontstaan van ARM werd in een grotere AGORA studiepopulatie en in meer detail bestudeerd in hoofdstuk 8. Hormonale behandeling voor ovulatie-inductie en intra-uteriene inseminatie waren niet geassocieerd met ARM. Het risico op een kind met ARM was echter wel verhoogd voor ouders die na een intracytoplasmatische sperma-injectie (ICSI) of *in vitro* fertilisatie (IVF) behandeling zwanger werden, vergeleken met vruchtbare ouders. Dit risico was nog steeds verhoogd wanneer de ouders die ICSI of IVF ondergingen, werden vergeleken met verminderd vruchtbare ouders die zonder behandeling zwanger waren geworden. Verminderde vruchtbaarheid zonder behandeling was alleen geassocieerd met ARM als ook andere aangeboren aandoeningen bij het kind aanwezig waren. Omdat confounding door medische indicatie een rol kan spelen in deze associaties, hebben we geconcludeerd dat zowel ICSI- of IVF-behandeling als de onderliggende verminderde vruchtbaarheid een rol spelen bij het ontstaan van ARM.

Deel 3: Genetische factoren en gen-omgeving interactie

Onafhankelijke associaties en interacties tussen periconceptioneel gebruik van foliumzuursupplementen en *MTHFR* (methyleentetrahydrofolaat reductase) *C677T* polymorfismen bij moeder en kind werden onderzocht in hoofdstuk 9. *MTHFR C677T* polymorfismen bij moeder en kind waren slechts zwak geassocieerd met ARM, terwijl er geen verband werd gezien tussen ARM en het gebruik van foliumzuursupplementen door de moeder. Ook werd er geen bewijs gevonden voor een rol van deze gen-omgeving interactie in de etiologie van ARM.

Hoofdstuk 10 beschrijft onze exome chip associatiestudie naar zeldzame, coderende genetische varianten met de Illumina Human Exome BeadChip in de grootste ARM-populatie die tot nu toe bestudeerd is. Het doel was om nieuwe, zeldzame genetische varianten te identiferen die bijdragen aan het ontstaan van ARM. In totaal waren er 55 varianten statistisch significant geassocieerd met ARM in de initiële studie. Slechts drie van deze varianten, in de *CLCN1*, *LRBA* en *ZNF423* genen, konden we valideren met behulp van de moleculaire inversie probe (MIP) techniek. Deze resultaten werden echter niet gerepliceerd in nieuwe DNA samples van Kaukasische en Han-Chinese ARM-patiënten. Dus we hebben geen bewijs gevonden voor sterke associaties tussen ARM en zeldzame, genetische varianten die we met deze exome chip konden bestuderen.

Deel 4: Algemene discussie

In de algemene discussie in hoofdstuk 11 gaan we in op een aantal methodologische aandachtspunten gerelateerd aan de patiënt-controle onderzoeken beschreven in dit

proefschrift. Deze aandachtspunten hebben betrekking op het onderzoeksontwerp, de onderzoekspopulaties en de methoden van dataverzameling. We bespreken bijvoorbeeld de voor- en nadelen van de drie soorten controlepopulaties in de verschillende studies en de mogelijke impact van herinneringsproblemen bij het gebruik van gegevens uit vragenlijsten in het patiënt-controle onderzoek. Tevens worden de bevindingen van onze studies samengevat in het licht van de belangrijkste doelstellingen van dit proefschrift en de bestaande literatuur. Ook worden suggesties gedaan voor richtingen van toekomstig onderzoek, zoals studies naar mogelijke verschillen in DNA-methylatie tussen patiënten en controles die zijn geboren na een ICSI- of IVF-behandeling, onderzoek naar mogelijke teratogene medicijnen en studies naar voeding en beroepsmatige blootstelling van de ouders. Bovendien zijn exoom- of genoom-sequencing en genoomwijde studies, zoals een grote GWAS, nodig om de bijdrage van nog onbekende genen te identificeren. Het uiteindelijke doel van etiologische onderzoek naar ARM is primaire preventie en verbeterde genetische counseling. Tot op heden lijken effectieve, preventieve maatregelen nog niet beschikbaar. De toegenomen kennis over de etiologie van ARM, beschreven in dit proefschrift, is echter een eerste stap naar een betere begeleiding van stellen met een kinderwens en van ouders van patiënten met ARM.

About the author

Lotte Wijers was born on January 27th, 1985 and grew up in Afferden (Limburg), The Netherlands. In 2003, she obtained her secondary school (VWO) diploma at the Elzendaalcollege in Boxmeer. From 2003 through 2009, she studied Biomedical Sciences, with a major in Epidemiology and minors in Reproduction and Pathobiology, at the Radboud University in Nijmegen. She conducted her Bachelor internship at the Department of Hematology, Radboud university medical center (Radboudumc) and her Master internship at the Department for Health Evidence, Radboudumc. In addition, she performed a minor internship in Reproduction at the Department for Health Evidence and the Department of Obstetrics and Gynaecology, Radboudumc and another minor internship at the Department of Psychiatry of the University Hospital Gasthuisberg in Leuven, Belgium. During her last year of study, she obtained a PhD grant for talented Biomedical Sciences students from the Radboudumc. After graduation in 2009, she started her PhD project on genetic and non-genetic risk factors for congenital anorectal malformations at the Department for Health Evidence of the Radboudumc, of which the results are described in this thesis. During this period, she was also appointed as a teacher of Biomedical Sciences and Medicine and she was a member of the PhD Council of the Radboud Institute for Health Sciences for two years. In November 2014, Lotte took up her current position as an epidemiologist at the Department of Drug Monitoring of the Trimbos-institute in Utrecht.

PhD Portfolio

Name PhD student: C.H.W. Wijers	PhD period: October 2009 – August 2014
Department: Department for Health Evidence	Promotor: Prof.dr. H.G. Brunner
Graduate School: Radboud Institute for Health Sciences	Co-promotors: Dr.ir N. Roeleveld, dr. I.A.L.M. van Rooij, dr. I. de Blaauw

	Year(s)	ECTS
TRAINING ACTIVITIES		
a) Courses & Workshops		
- Certificate Advanced English, Radboud University	2009-2010	3.6
- Workshop Anorectal Malformations, Radboudumc	2010	0.6
- Principles of epidemiologic data-analyses, NIHES, Rotterdam	2010	0.7
- Basiscursus Regelgeving en Organisatie voor Klinisch Onderzoekers (BROK), Radboudumc	2010	1.4
- Genome-wide association analysis, NIHES, Rotterdam	2010	1.4
- From DNA variation to phenotype, Molecular Medicine, Rotterdam	2011	1.4
- SNPs and Human Diseases, Molecular Medicine, Rotterdam	2011	2.0
- Management for PhD students, Radboud University	2011	3.0
- Cursus begeleiden werkgroepen, Radboudumc	2011-2012	0.6
- Introductiecursus voor docenten, Radboudumc	2012	0.1
- Career management for PhD students, Radboud University	2013	0.6
- ARM-Net meeting and workshops, Radboudumc	2013	0.6
b) Seminars & lectures		
- Seminars and colloquia at the Department for Health Evidence, Radboudumc	2009-2014	NA
c) Symposia & congresses		
- Werkgroep Epidemiologisch Onderzoek Nederland (WEON), Amsterdam (oral presentation)	2009	0.6
- WEON, Nijmegen (oral presentation)	2010	0.6
- International Paediatric Colorectal Club, Padua, Italy (oral presentation)	2010	0.6
- WEON, IJmuiden (poster presentation)	2011	0.6
- European Society of Human Genetics (ESHG), Amsterdam	2011	1.2
- EUROCAT symposium on congenital malformations, Antwerpen, Belgium (oral presentation)	2011	0.3
- Symposium Vereniging Anusatresie, Arnhem (oral presentation)	2011	0.3
- RIHS PhD Retreat, Wageningen (poster presentation)	2011	0.6
- CURE-Net Conference, Heidelberg, Germany (oral presentation)	2011	0.6
- RIHS Symposium, Nijmegen	2011, 2012	0.6
- WEON, Rotterdam	2012	0.6

- RIHS PhD Retreat, Wageningen (organisation)	2012, 2013	1.2
- WEON, Utrecht (poster presentation)	2013	0.6
- EUROCAT symposium, Groningen (oral presentation)	2013	0.3
- Voorjaarsoverleg Nederlandse Vereniging voor Toxicologie – Sectie Teratologie en Reproductie Toxicologie, Den Bosch (oral presentation)	2013	0.3
- RIHS Symposium, Nijmegen (laptop presentation)	2013	0.3
- European Epidemiology Congress, Aarhus, Denmark (poster presentation)	2013	0.9
- WEON, Leiden (oral presentation)	2014	0.6
d) Other		
- Weekly journal club Epidemiology at the Department for Health Evidence, Radboudumc	2009-2014	4.5
TEACHING ACTIVITIES		
e) Lecturing		
- Teacher in BSc course Inleiding Hoofdvakken 1 (5INH1), Biomedical Sciences, Radboud University	2009-2010	NA
- Teacher in BSc course Research Methodology 1 (5OMB1), Biomedical Sciences, Radboud University	2011	NA
- Teacher in BSc course Introduction Biomedical Sciences (5IBMW), Biomedical Sciences, Radboud University	2013	NA
- Teacher in MSc course Risk Assessment (5MG01), Biomedical Sciences, Radboud University	2010-2012	NA
f) Supervision of internships / other		
- Supervisor of a group of BSc students writing a research proposal (5KMP5), Medicine, Radboud University	2010-2012	NA
- Organizer orientation days for secondary schools, Radboud University	2010-2013	NA
- Co-supervisor of two Msc internships, Biomedical Sciences, Radboud University	2012, 2014	NA
TOTAL		31.3

List of publications

Wijers CHW, van Rooij IALM, Rassouli R, Wijnen MH, Broens PM, Sloots CEJ, Brunner HG, de Blaauw I, Roeleveld N. Parental subfertility, fertility treatment, and the risk of congenital anorectal malformations. *Epidemiology* 2015;26:169-176.

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