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Incidence and determinants of ventilation tubes in early childhood, and the effect of middle ear disease on the neurological development



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English Summary

Conductive hearing loss caused by fluid in the middle ear space called middle ear effusion (MEE) is an important concern about otitis media (OM). Prolonged hearing disability in early childhood can affect especially the linguistic and cognitive development.

The most common treatment for recurrent OM and persistent MEE in numerous countries is the insertion of ventilation tubes (VT) in the tympanic membrane. Therefore, we investigated the incidence of VT insertions in Denmark and compared it to other countries. Furthermore, to analyse risk factors of VT in a prospective study cohort, and finally to study the effect of MEE and treatment with VT on the neurological development of the children.

In **study I**, we aimed to investigate the incidence of VT in Denmark using registry data. We compared this to findings in the COPSAC₂₀₁₀ cohort and analyzed determinants of VT insertions using the cohort data. This is a solid outcome compared to former studies using a more unclear and varied definition of OM.

We found an incidence of 35/1000 for all children 0-15 years of age in Denmark. This is to our knowledge the highest in the world. The prevalence of VT was 24% for children 0-3 years of age, which is comparable to the COPSAC₂₀₁₀ cohort where we found a prevalence of 29% before the age of 3. Determinants of VT were older siblings, children also suffering from persistent wheeze and family history of middle ear disease.

In **Study II**, we investigated the relation between maternal antibiotic intake during pregnancy and the children's risk of acute otitis media (AOM) or VT in early childhood. The consumption of antibiotics is increasing worldwide. A concern is that this may have possible adverse longterm consequences. Antibiotic treatment during pregnancy can alter the maternal bacterial colonization, which have raised the hypothesis that an inappropriate exposure in pregnancy can initiate a propensity for disease in the child. In the COPSAC₂₀₁₀ cohort, 37% of the mothers were treated with antibiotics during pregnancy.

We found that maternal antibiotic intake during pregnancy was significantly associated with increased risk of AOM and VT before the age of 3. Especially treatment late in pregnancy

increased the risk of VT; we also observed a dose-response relationship with an increased risk of AOM with increasing number of treatments. The effect late in pregnancy points towards a potential mechanism of altered microbiome.

In **Study III**, we explored the possible association between MEE and the neurological development of children in the COPSAC₂₀₁₀ cohort. We used several developmental endpoints: age at achievement of gross motor milestones, language development at 1 and 2 years of age, cognitive score at 2.5 years and a general development score based on a questionnaire regarding: fine motor, gross motor, problem solving, communication and personal-social skills at 3 years of age.

We found slightly lower language scores for children with MEE in the 1-year language test. Regarding the cognitive scores, we found that children who had had VT inserted scored lower than children with MEE or children without middle ear disease. In conclusion, children with MEE at 1 year had slightly lower language scores at 1-year, but they were not delayed in other ways when examining several neurological endpoints up to the age of 3. Furthermore, our data do not support an effect of treatment with VT on the neurological development.

In conclusion, Denmark has a very high incidence of VT insertions and to our knowledge the highest in the world. From our prospective birth cohort study, we found that older siblings, history of persistent wheeze and family history of otitis media were determinants of VT insertions. We also found that maternal antibiotics during pregnancy increased the risk of OM. Furthermore, children with MEE are delayed in their early language development, but without a long-lasting effect.

Danish Summary (Dansk resumé)

Konduktivt høretab forårsaget af væske i mellemøret er en vigtig bekymring i forhold til otitis media. Langvarig hørenedsættelse i den tidlige barndom tænkes at kunne påvirke især den sproglige og kognitive udvikling. Gentagne mellemørebetændelser og vedvarende væskeansamling i mellemøret behandles i mange lande med dræn i trommehinden.

Derfor undersøgte vi incidensen af drænanlæggelser i Danmark og sammenlignede med andre lande. Derudover analyserede vi risikofaktorer for drænanlæggelse med udgangspunkt i en prospektiv fødselskohorte, og til sidst undersøgte vi betydningen af væske i mellemøret samt behandling med dræn på børnenes neurologiske udvikling.

I **studie I** undersøgte vi incidensen af drænanlæggelser i Danmark ved brug af registerdata. Vi sammenlignede dette med resultater fra COPSAC₂₀₁₀ kohorten og ved brug af data fra kohorten analyserede vi determinanter for drænanlæggelse. Dette er et solidt endepunkt sammenlignet med tidligere studier, som har brugt mere uklare og varierede definitioner af mellemørebetændelse.

Vi fandt en incidens på 35/1000 for alle 0-15 årige børn i Danmark. Dette er så vidt vi ved ud fra tidligere publicerede studier om dette, den højeste incidens i verden. Prævalensen af dræn var 24% for alle 0-3 årige børn, og dette kan sammenlignes med COPSAC₂₀₁₀ kohorten, hvor vi fandt en prævalens på 29% før 3 års alderen. Determinanter for drænanlæggelse var ældre søskende, børn med vedvarende episoder med hvæsen, og en familiehistorie med mellemøre sygdom.

I studie II undersøgte vi forholdet mellem moderens antibiotikaindtag i graviditeten og børnenes risiko for mellemørebetændelse og drænanlæggelse i den tidlige barndom. Antibiotika forbruget i verden er stigende og en bekymring er om dette kan have uheldige langsigtede konsekvenser. Antibiotikabehandling i graviditeten kan ændre moderens bakteriekolonisering og hypotesen er at dette muligvis kan medføre en øget sygdomstilbøjelighed hos barnet.

I COPSAC₂₀₁₀ kohorten blev 37% af mødrene behandlet med antibiotika i graviditeten. Vi fandt, at moderens antibiotikaindtag i graviditeten var signifikant associeret med øget risiko

for mellemørebetændelse og dræn inden 3 års alderen. Specielt behandling sent i graviditeten øgede risikoen for dræn. Vi observerede også et dosis-respons forhold med en øget risiko for mellemørebetændelse med stigende antal behandlinger. Den sene effekt i graviditeten tyder på en potentiel mekanisme med et ændret mikrobiom.

I **Studie III** undersøgte vi en mulig sammenhæng mellem væske i mellemøret og den neurologiske udvikling af børnene i COPSAC₂₀₁₀ kohorten. Vi brugte flere udviklings mål: alder for opnåelse af de store motoriske milepæle, sprog udvikling i 1 og 2 års alderen, kognitiv score i 2.5 års alderen og en generel udviklingsscore baseret på et spørgeskema omkring fin motorik, grov motorik, problem løsning, kommunikation og personlig-sociale evner i 3 års alderen.

Vi fandt en lidt lavere sprogscore for børnene med væske i mellemøret i 1 års alderen. I forhold til de kognitive scorer fandt vi at børnene som havde fået dræn scorede lavere end dem med væske i mellemøret eller uden mellemøre sygdom. Konklusionen er, at børn med væske i mellemøret i 1 års alderen scorede lidt lavere i sprogtesten i 1 års alderen, men de var ikke forsinket på andre områder, når vi undersøgte forskellige neurologiske udviklings mål op til 3 års alderen. Derudover understøtter vores data ingen effekt af behandling med dræn på den neurologiske udvikling.

Konklusion: Danmark har en meget høj incidens af drænanlæggelser og ifølge vores fund er det den højeste i verden. Fra vores prospektive kohorte studie fandt vi, at ældre søskende, vedvarende episoder med hvæsen og en familiehistorie med mellemøresygdom var determinanter for drænanlæggelse. Vi fandt også at moderens antibiotika indtag i graviditeten øger risikoen for mellemøresygdom. Derudover har vi vist at børn med væske i mellemøret er forsinket i deres tidlige sproglige udvikling, men der ses i studiet ingen langtidseffekt.

Abbreviations

OM: Otitis Media
AOM: Acute otitis media
MEE: Middle ear effusion
VT: Ventilation tubes
COPSAC – COpenhagen Prospective Studies on Asthma in Childhood
COPSAC ₂₀₁₀ – the COPSAC 2010 birth cohort (700 unselected children)
ICD10: International Classification of Diseases Version 10
ENT: Ear-Nose-Throat
RTI: Respiratory Tract Infection
UTI: Urinary Tract Infection
PCA: Principal Component Analysis
PPCA: Proportional Principal Component Analysis
ASQ-3: Ages-and-Stages-Questionnaire
HR: Hazard Ratio
IQR: Inter Quartile Range
SAS: Statistical Analysis System
SD: Standard deviation
CI: Confidence Interval
RCT: Randomized Controlled Trial

1. Introduction

1.1. Otitis Media - Prevalence and Treatment

Otitis media (OM) is an inflammatory process within the middle ear space, presenting as either an acute otitis media (AOM) episode; which is an acute infection with symptoms e.g. fever and otalgia, or middle ear effusion (MEE); which is an accumulation of fluid without symptoms of acute infection. OM is very common and nearly all children experience MEE in early childhood (1,2). Recurrent AOM is defined as 3 or more episodes within 6 months or more than 4 episodes in a year (3).

MEE may occur as an inflammatory response following AOM, spontaneously because of poor Eustachian tube function or during an upper respiratory infection (1). MEE most often resolve spontaneously; 75% resolving within 3 months without treatment (4). MEE decreases the mobility of the tympanic membrane, which can result in a mild to moderate conductive hearing loss of on average 20dB (5). There is a general concern that this level of hearing impairment in early childhood can result in delayed language and cognitive development (6– 9).

OM causes a significant burden of disease and is a common reason for contact with the health care system. It is important to find the most effective treatment of OM. Antibiotics to treat AOM are most used in children under two years of age with bilateral AOM, or with both AOM and otorrhoea (10). Treatment of MEE remains controversial. Treatment of MEE with both oral and nasal steroids have been evaluated. A Cochrane review found a short-term effect of steroid treatment on MEE resolution, but no effect on symptoms and no studies with long-term evaluation (11). There may be a short-term benefit of topical intranasal steroids in children with adenoidal hypertrophy or for children with allergic rhinitis and MEE, although the magnitude of the effect is small (12,13). Treatment with antibiotics for MEE have been evaluated in a Cochrane study (14). A small benefit for resolution of the effusion was found, but adverse effects using long-term treatment with antibiotics such as bacterial resistance do not way up the small benefit; with a conclusion not to recommend antibiotics. Other treatments such as antihistamines, decongestants and montelukast have been evaluated but

without finding significant effect on effusion resolution (15,16). Another treatment option is surgical treatment with ventilation tubes (VT).

The recommendations for treatment with VT in Denmark was announced in 2015 from The Danish Health Authority in a national guideline regarding this topic (17,18). VT are recommended when:

1) Persistent MEE with hearing impairment of at least 25dB with or without delayed language development. If MEE persist and the child has no hearing impairment, but other symptoms than delayed language development probably due to MEE, then VT insertion may be considered anyway.

2) VT are recommended for recurrent AOM with MEE between the episodes. If there are many AOM episodes but no MEE between them; then VT can be considered anyhow.

In **Paper I**, we address the subject of VT by calculating the incidence of VT insertions in Denmark and compare it to other westernized countries.

1.2. Risk Factors of Otitis Media

As OM is very common, it is important to investigate reasons for acquiring the disease, either to be able to prevent or to reduce the risk of the OM. Traditionally, risk factors of OM have been explained by environmental exposures combined with a heritability factor, see Figure 1 Causality Diagram.

A meta-analysis of 24 studies investigating risk factors of chronic and recurrent OM was published in 2014 (19). Confidence in the results is hampered by lack of reproducibility between the studies, only few of the studies have prospective data collection and the definition of OM varies between the studies. The following factors were found to be significant risk factors of chronic and recurrent OM:

Allergy/atopy, upper respiratory tract infection, snoring, low social status, acute otitis media episode, passive smoking. Borderline significant increased risk associated with male sex, day-care attendance and siblings in the home/family crowding (19).

In **Paper I**, we analyzed determinants of VT, as it is a treatment for persistent MEE or recurrent AOM. We believe by using VT as outcome that we get a solid marker of severe OM instead of either recurrent OM or MEE and thereby a valid picture of relevant risk factors.

Figure 1. Causality diagram.



1.3. Antibiotics in Pregnancy

The consumption of antibiotics is increasing worldwide, causing concern due to increasing prevalence of antibiotic resistance and potential long-term adverse health effects (20–22). The maternal immune system is suppressed in pregnancy which can make infections more serious both for the mother and the fetus (23,24). These infections are potentially preventable with antibiotic treatment (25). Antibiotics may change the maternal bacterial colonization (26,27), and result in an unfavorable bacterial ecology which can affect the earliest colonization of the child by vertical transmission (28,29). This unfavorable microbiome may trigger lasting disease processes in susceptible offspring.

Prescribing drugs during pregnancy generally presents a challenge to the physician; diseases need to be treated, whilst protecting the fetus against possible harm.

In the Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC₂₀₁₀) birth cohort, we chose to investigate the exposure of antibiotic treatments during pregnancy. The antibiotics might affect the developing fetus while still in the uterus or it can alter the composition of the vaginal ecosystem which can be transferred to child at birth (26). It is important to study if such alterations can affect the risk of disease in the offspring. In a former study from COPSAC, it was reported that more than one-third of the women were prescribed oral antibiotics during pregnancy (30). This finding is consistent with what they have found in other studies from westernized societies on drug administration during pregnancy (31–33). Therefore, it is very common exposure in pregnancy and important to examine possible side effects in the long term.

In **Paper II**, we investigated the effect of treatment with antibiotics in pregnancy and the offspring's risk of OM and VT.

1.4. The Impact of Middle Ear Effusion on the Neurological Development

The inflammation in the middle ear space causing MEE has much attention because of a concern that hearing loss can affect the language and cognitive development in early childhood with long-term consequences. Language development accelerates in the first two years of life (34). The conductive hearing loss from OM is thought to complicate the learning of language with a potential reduced speech perception, word- and grammatical comprehension and word production (35). The language development in Denmark has been found slower and with a later acceleration than in comparable countries (36). This is partly due to the structure of the Danish language with many phonetic and prosodic cues but perhaps also because of early daycare attendance. Because of the later acceleration, the period of a conductive hearing loss when having MEE may therefore affect children in Denmark differently than in other countries. Factors affecting early language development are particularly interesting since early-impaired language development may have consequences later in life. There are evidence of correlation between early language development and later literacy (35,36). The question is, if children with MEE, who are delayed in their language development because of hearing impairment, will catch up or if it has long-term consequences.

Another concern about persistent MEE is that hearing impairment may affect the cognitive development. Literacy, language- and cognitive development are related factors, and they are all likely to affect later school performance (37).

Besides the linguistic- and cognitive development children with MEE have been described as being more clumsy (38,39). The accumulated fluid in the middle ear space can affect the vestibular function thus affecting children's balance. This could have impact on the gross

motor development.

In **Paper III**, we wanted to investigate whether MEE and treatment with VT delayed both motor-, language and cognitive development in early childhood in a Danish prospective birth cohort study.

2. Aim and Objectives

The aim of this PhD thesis was to investigate the incidence of VT insertions in Denmark and to study determinants of VT insertions using data from a prospective birth cohort. Furthermore, we aimed to analyze the effect of MEE and treatment with VT on the neurological development of children.

Such insight may increase our knowledge of the extent and the relevance of the use of the VT treatment for OM.

The specific objectives were:

- To study the development of the incidence of VT insertions in Denmark and compare it to other countries
- To study determinants of VT insertions using data from a prospective birth cohort
- To study the association between maternal treatment with antibiotics during pregnancy and the child's risk of both OM and VT
- To study the effect of MEE and treatment with VT on the motor-, language-, cognitive and general development in early childhood

3.1. Design, Setting and Participants

COPSAC2010

All studies in this thesis are based on data from the COPSAC₂₀₁₀, which is an ongoing Danish unselected prospective cohort study. 738 pregnant women were recruited in pregnancy week 24 between 2008 and 2010. Exclusion criteria were gestational age above 26 or any endocrine, heart, kidney or lung diseases other than asthma. The women attended 2 planned visits in pregnancy. At 1 week of age, 700 children were included in a comprehensive program of clinical and objective assessments with prospective data collection at visits to the clinical research unit. The families attended the research unit at 9 planned visits after birth until the age of 3. After the age of 3, the children attend the clinic for an annual visit.

All data were collected according to Good Clinical Practice data management and quality control procedures including external monitoring.

Additional information on the cohort study design, recruitment and baseline characteristics of the participants have previously been described in details (40).

3.2. Ethics Statement

The COPSAC₂₀₁₀ cohort study is conducted in accordance with the Declaration of Helsinki and approved by the Copenhagen Ethics Committee (KF01-289/96) and the Danish Data Protection Agency (2008-41-1754). Oral and written informed consent was obtained from both parents/legal guardians.

The registry-based study was performed on existing data in national registries and was approved by the Danish Data Protection Agency (J.no. 2012-41-0388). Since subjects were not contacted as, part of the study, written informed consent was not required.

3.3. Clinical Outcomes

Ventilation tubes

Information on VT insertions in the first 3 years of life for the children in the COPSAC₂₀₁₀ cohort were extracted from two national registries. The Danish National Patient Registry using the International Classification of Diseases 10th revision [ICD-10] code KDCA20 for all procedures performed at hospitals, and The Danish National Health Service Registry using procedure code 3009 for all procedures performed in community otorhinolaryngology practices (41,42). All information in the Danish national registers are linked with a unique personal identification number (assigned by the Danish Civil Registration System to all people with permanent residency in Denmark), which make it possible to link the procedures to the children in our cohort. The date of the first VT insertion was used in the survival analyses in **Paper II**.

In **Paper I**, we studied VT insertions among all Danish children below 16 years of age between 1st January 1997 and 31st December 2011 in either hospital setting or primary care. To calculate the incidence of VT, we used the number of children in Denmark each year from 1997-2011.

To compare the incidence of VT to other developed countries we searched PubMed using the search words: "ventilation tubes and incidence", "grommets and incidence" "tympanostomy tubes and incidence". We also used the search word "prevalence" in the same combinations to be sure we captured all studies with a reported incidence also in studies focusing on the prevalence. The incidence of VT on Iceland was calculated from two registries where data are available online: <u>www.sjukra.is</u> and <u>www.hagstofa.is</u> with data from 2011 (43,44).

Otitis media

A structured daily symptom diary was filled out by the parents from birth to 3 years of age, and validated by the research pediatricians during clinical visits. It included clinical symptoms and medical treatments in three categories: lung symptoms, infections and eczema. To be included in the analyses, we required diaries that were completed at least 90% of the time from birth to 3 years of age. All OM episodes were captured in the infection category of the diary. An episode was defined as one or more concurrent days of parental registration of OM

and the first day registered in the child's first episode was used in the survival analyses. OM diagnoses were verified by interviews at clinic visits. Most often children had been diagnosed at their private physician or by an ear-nose-throat (ENT) specialist and received antibiotics to treat the OM episode. Otherwise, episodes registered by parents that were not clearly OM were deleted at visits at the research clinic from the diary. The OM diagnosis therefore corresponds to doctor diagnosed acute episodes of middle ear infection.

Middle ear effusion

To diagnose MEE we used tympanometry (MT10, Interacoustics, Denmark). Children were examined by the doctors in the COPSAC clinic at scheduled visits at one, two and three years of age with tympanometry measurement. A flat curve was interpreted as MEE and the diagnosis was verified with otoscopy. If the child had MEE, at least in one of the ears they were classified as having MEE in the analyses.

3.4. Other Clinical Variables

Antibiotics in pregnancy

Detailed information on maternal antibiotic use during pregnancy and the first year after birth was obtained from The Danish National Prescription Registry, which included records on all drugs filled at the pharmacy and this information are linked with a unique person identification number. The information was validated during interviews with the mothers at the COPSAC research clinic. At 1-week postpartum prescriptions from pregnancy was validated and at the 1-year visit in the clinic information regarding the prescriptions from birth until 1-year post-partum was validated. This double check procedure minimized both recall bias and excluded antibiotics collected at the pharmacy, but not taken by the participants. The prescriptions from birth until the children were 1 year of age were used to analyze the effect of treatment after pregnancy. This to investigate if the mechanism was associated to pregnancy.

The administration of antibiotics were divided into usage in each of the three pregnancy trimesters: first trimester (\leq 14 weeks of gestation), second trimester (>14 – \leq 26 weeks of gestation), and third trimester (>26 weeks of gestation). Furthermore, the antibiotics were categorized by the most likely treatment indication based on ATC (Anatomical Therapeutic

Chemical) code: Respiratory Tract Infection (RTI) antibiotics (J01CAxx excl. J01CA08, J01CExx, J01FAxx, J01CRxx) and Urinary Tract Infection (UTI) antibiotics (J01CA08, J01EBxx, J01XExx). Other antibiotics, which could not be categorized into one of these groups, were excluded due to low numbers.

Covariates

Information regarding maternal age at birth, gestational age, maternal asthma, maternal smoking during pregnancy, delivery method, birth weight, sex and older children in the household were obtained by personal interview at the 1-week visit in the COPSAC clinic. Older siblings include both biological siblings and half siblings, with primary address in the home of the child. Days solely breastfed and age at beginning of daycare were obtained longitudinally from clinical interviews during the first year of life.

For the study of determinants of VT (**Paper I**), from the lung symptoms category we captured children with recurrent wheeze episodes, who have been treated with inhaled corticosteroids according to a predefined algorithm (45). These children were diagnosed with persistent wheeze. Furthermore, for the study of determinants of VT, information about household income was obtained at the 2-year visit in the clinic so the data represented the circumstances between the age of 1 and 2 to avoid the year the mother had been on maternity leave resulting in a lower income than normally. Household income was categorized into low; below 50.000 Euro, medium; 50.000 – 110.000 Euro and high; above 110.000 Euro.

In **Paper II** to reflect the situation in pregnancy, information regarding the household income were obtained at the 1-week visit. Maternal educational level were likewise obtained from interviews at the 1-week visit and were categorized as: low; elementary school or college graduate, medium; tradesman or medium length or high; university.

In **Paper III** the information regarding maternal age, maternal education level and household income were obtained from interviews when the children were 2 years of age and combined as a composite measure of the child's social circumstances defined as the z-scores first component of a Principal Component Analysis (PCA) (explaining 55% of the variance). Information regarding the family members' history of middle ear disease was obtained from interviews at 3 years of age. Information obtained after the 1-week visit was not acquired for all 700 children but the numbers included in the analyses are shown in the tables.

3.5. The Neurological Developmental Assessments in the COPSAC₂₀₁₀ cohort

Gross motor milestones

At the child's first clinical visit (1 week of age) the parents were given a registration form, with thorough instructions, based on World Health Organization milestones registration (46) and the Denver Development Index (47). Dates of achievement of 13 predefined milestones were registered prospectively by the parents. Implementation of the milestone registration started after the first 500 children were born, and some of the milestones of these children were therefore registered retrospectively. For the analyses in this study, only the gross motor milestones: sit alone, stand help, crawl, stand-alone, walk with help and walk alone were used to analyze the association with MEE and VT; because the achievement of these milestones could be delayed by poor balance.

Language development

Language development was assessed with the Danish version of The MacArthur Bates Communicative Developmental Inventory (48). The assessment was performed by a webbased questionnaire filled out by parents around the child's 1-year (Words and gesticulation) and 2-year birthday (Words and sentences). The 1-year questionnaire evaluates language comprehension, early word production and gestural communication; we used the scores regarding word production and word comprehension in the analyses. The 2-year questionnaire assesses vocabulary, grammatical skills, syntax and morphology and we used the word production score for the analyses.

Cognitive development

Cognitive development was assessed at 2.5 years of age, using the cognitive part of the third edition of the Bayley Scales of Infant and Toddler Development (49). During the examinations, the examiner presented a series of test materials to the child and observed the child's responses and behavior. Based on its performance, the child was given a composite score, which was standardized by use of a normalization material of age corrected means of 100 and a standard deviation of 15 (range 50-150). 10 examiners performed examinations and inter-examiner consistency in performance was validated by inspection of video recordings.

Ages and Stages Questionnaire - ASQ-3

The parent-completed Ages & Stages Questionnaires®, Third Edition (ASQ-3) consists of 5 categories; fine motor, gross motor, personal-social, communication and problem solving skills. Parents completed the questionnaire prior to the 3 years visit. At the 3-year visit in the clinic, the responses were subsequently verified. We combined the scores of the 5 categories in a statistical model and used this combined score as a measure of the general development at the age of 3.

3.6. Statistical Analyses

All variables were tested for normal distribution and models were verified by visual inspection of residual plots. Associations between categorical variables and VT insertions in the first study, maternal antibiotics in the second study and children with or without middle ear disease in the third study were analyzed by chi-squared tests or Fisher's exact test where expected counts in any cells were less than 5. Household income was analyzed with a Cochran-Armitage Trend Test. Normal distributed continuous variables were tested with t-test. Variables not normally distributed were analyzed using Wilcoxon rank-sum test. A significance level of 5% was used in all analyses. All estimates were reported with 95% confidence intervals (CI). Missing data were treated as missing observations. PCA and PPCA analyses were performed using the statistical software package R (50) version 3.2.1 with the package pcaMethods (51) and visualized using the package ggplot2 (52). R version 3.2.3(50) was used for calculating genetic risk scores. All other analyses were analyzed using SAS version 9.4 for Windows (SAS Institute Inc., Cary, NC, US).

Paper I - Genetic risk score

The heritability was tested using a genetic risk score. The score were generated from 21 polymorphic genetic variants which was found to be associated with recurrent OM in a large questionnaire based genome-wide study (n=121,810) (53). The genetic variants were weighted according to their odds ratios. The risk alleles were summed and z-score transformed given the risk score.

To generate the risk score for the children in the cohort, DNA was purified from blood cells and multiple single-nucleotide polymorphisms were genotyped genome-wide using the high

throughput Illumina HumanOmniExpressExome bead chip platform (Illumina, Inc., San Diego, CA, USA). Genotyping was performed at AROS Applied Biotechnology AS, Aarhus, Denmark. Genotyping quality control was made by removal of sex mismatches, duplicates, ethnic outliers, and Hardy-Weinberg equilibrium (p>10-6) outliers. A genotyping call rate of at least 95% was required. Correct familial relations were verified by Mendel error rates and identityby-descent analyses. Quality control was performed using PLINK software (54,55). The HumanOmniExpressExome chip was imputed to the 1000 genomes phase 3 imputation panels (CEU individuals) using Mach 1.0, Markov Chain Haplotyping, and IMPUTE2.

Paper I and II - Cox proportional hazard regression

The effect of maternal antibiotic use on the age of the first AOM episode or VT insertion was quantified in terms of hazard ratios (HR) by Cox proportional hazards regression. The children were retained in the analysis from birth until age of event or age 3 years, whichever came first. Possible confounders were chosen from results based on factors influencing maternal intake of antibiotics during pregnancy. Additionally, we also adjusted for presence of older siblings and family history of middle ear disease a priori, since these factors are known to affect the risk of AOM and VTs (19). The stratified analyses were also performed using Cox proportional hazard regression. No interaction was calculated.

Cox proportional hazard regression was also used in the multivariate analyses in Paper I.

Paper III - Principal component analysis

For the analyses in **Paper III**, we divided the children into three groups: children who had received VT, children with untreated MEE and children with no middle ear disease. We excluded children with a neurological diagnosis, as well as those born <37 weeks of gestation or with birth weights <2500 g in all analyses. The language data for the 1-year test was log-transformed after adding a pseudo count of 1 for the purpose of statistical analysis. Results are presented as medians with interquartile range (IQR) on the original scale. No transformation of the data were needed for the 2-year language data or the cognitive test. General linear models were used to analyze the association between untreated MEE, treatment with VT compared to children with no disease and their language- and cognitive development. Associations between the clinical predictors (MEE and VT) and neurological

endpoints were adjusted for sex; boys have more middle ear disease than girls and the neurological development are different for the two sexes (19,56). Age of gross motor milestone achievement were analyzed using a probabilistic PCA (PPCA) model. This model included the full dataset with missing values, assuming that the missing values were missing at random. Missing data were otherwise treated as missing observations. Children received a score for each of the 5 areas of the ASQ-3 and we combined these statistically in a PCA model and used the combined score as a general development score at the age of 3. All the study outcomes were combined in a PCA model and analyzed for association to MEE and VT with the age of 1, so the exposure was before all of the neurological endpoints.

4. Results

4.1. Paper I - Incidence and Determinants of Ventilation Tubes in Denmark

Registry data

In this paper, we studied the incidence of VT in Denmark for children 0-15 years old between 1997 and 2011. The total number of person years for children <16 was 15.593.538. The average number of children living in Denmark was 1,039,569 and 288,224 received VT in the study period. Several of the children received VT more than once, which made the total number 514,575 VT insertions. The average number of VT insertions was 36,196 annually. For all Danish children under 16 years the incidence was 35/1,000. During the observation period, there has been an overall increase in VT insertions: Figure 1. Among children having VT insertions 53% received tubes one time, 24% two times, 11% three times and 12% of the children have VT more than 3 times.

Most children have VT insertions in early childhood: Figure 2. The national prevalence of VT in the first 3 years of life was 24% in children born in 2009. In the children aged 0-3 years the number of VT insertions increased from 62/1,000 to 108/1,000, 43% increase, corresponding to an increased by 2.94 per year, SE 0.27, p<0.0001. A sex difference was found in the numbers of VT. Boys more often received VT compared to girls, respectively 57% and 43%. The hospitals account for 4% of the procedures and the private ENT-clinics for 96%: Figure 3. We compared the incidence of 35/1000 in Denmark for children below 16 years of age with incidences from other developed countries reported in former studies: Figure 4.



Figure 1. Incidence of ventilation tubes in Denmark.

<u>Figure legend</u>: The Incidence (ventilation tubes/1000 children 0-3 years of age) from 1997-2011 in Denmark.

Figure 2. Age distribution of children 0-15 years who received VT between 1997-2011 in Denmark.





Figure 3. Number of VT placed in Private ENT-clinics compared to Hospitals.



Figure 4. The incidence of ventilation tubes in Denmark compared to other countries.

<u>Figure legend</u>: The overall incidence of ventilation tubes in Denmark of 35/1000 for children 0-15 years of age compared to incidences in other developed countries published in the literature (43,44,57–62).

COPSAC2010 cohort

We further wanted to analyze determinants of treatment with VT and used COPSAC₂₀₁₀ longitudinal data. First, we describe the prevalence of middle ear disease in the cohort. The mean age of the first VT insertion was 16 months (SD 4.90). The youngest child was 5.8 months old. The accumulated prevalence of VT was 5% (n=37) after one year, 22% (n=151) after 2 years and 29% (n=205) after 3 years: Figure 5.

OM episodes in the first 3 years of life among children in the cohort were captured in the daily disease diary. 74% (N=515) of the cohort had information of OM episodes for >90% of the first 3 years of life. Of these 67% (n=346) had at least one OM episode from age 0-3 years: Figure 5. MEE was found in 52% (292/563) at the 1 year clinic visit; 37% (221/595) at the age of 2 years and 27% (n=163/454) at the 3 years visit. In total 66% (n=454/614) of the children had MEE at least once before the age of 3: Figure 5.

Figure 5. Venn diagram of the otitis media disease continuum and prevalence in COPSAC₂₀₁₀



Determinants of ventilation tube insertion

We found that a main determinant for VT insertion was family history of middle ear disease. Maternal history persisted in the multivariate analysis; HR 2.07, [1.45-2.96] and for siblings; HR 3.02, [2.11-4.32], Table 1. To analyze the heritability factor further we used a genetic risk score, but we did not observe a significant association between this risk score and time to receiving VT before the age of 3; HR 0.97, 95% CI [0.84-1.12], p=0.68. Older children in the home were also associated with a higher risk of VT; OR 1.58, 95% CI, [1.13-2.21], p=0.0072, but this association was not found in the multivariate analysis. Children with persistent wheeze also demonstrated a significant increased risk of VT insertions, OR 1.62, 95% CI, [1.08-2.43], p-value of 0.0191, but the association did not persist in the multivariate analysis. On average the children who received VT before the age of 3 had started 14 days earlier in daycare compared to children without VT, p=0.0577. There was no association between sex and VT in the COPSAC cohort and no associations were found between VT and maternal age, maternal smoking during pregnancy, household income, maternal asthma, delivery by cesarean section, length of exclusive breast feeding, gestational age, birth weight or season of birth.

	Participants	Tubes	No Tubes	P value	Adjusted HR (95% CI)
All	700	205	495		
Maternal-related characteristics					
Maternal age at birth, mean years (range)	698	32.3 (21.5- 48.3)	32.2 (19.1- 44.0)	0.9261	-
Maternal smoking during pregnancy, % (n)	696	8% (16)	8% (38)	0.9539	-
Maternal asthma, % (n)	697	27% (55)	26% (129)	0.7897	-
Household income *	677			0.7333	-
- Low % (n)	56	10% (19)	8% (37)		-
- Medium % (n)	366	54% (108)	54% (258)		-
- High % (n)	255	37% (73)	38% (182)		-
Child-related characteristics					
Cesarean section, 151 children,% (n)	700	22% (46)	21% (105)	0.7195	-
Mean number of days exclusively breastfed, mean (range)	692	103.4 (0- 255)	103.0 (0-266)	0.8660	-
Mean GA weeks mean (range)	700	39.7 (33.0- 42.3)	39.9 (29.4- 42.3)	0.1429	-
Mean birth weight kg, mean (range)	700	3.5 (1.9- 5.2)	3.5 (1.3-5.0)	0.9797	-
Sex, boys, % (n)	700	56% (111)	49%(249)	0.1117	-
Mean days at beginning of daycare (range)	688	320 (156- 946)	334 (180- 1154)	0.0577	-
Older siblings present, %(n)	700	64%(132)	53% (264)	0.0072	1.34 (0.86; 2.05)
Season of birth, % (n)	700			0.2768	-
Winter	215	26% (55)	32% (160)		-
Spring	186	26% (52)	27% (134)		-
Summer	149	22% (46)	21% (103)		-
Fall	150	25% (52)	20% (98)		-
Child with persistent wheeze any time before the age of 3 years	662	25% (48)	17% (79)	0.0191	1.18 (0.79; 1.76)
Family history of middle ear disease:	T	T	T		1
- Paternal % (n)	615	22% (38)	11% (48)	0.0002	1.48 (0.97; 2.27)
- Maternal % (n)	643	34% (62)	17% (79)	<0.0001	2.07 (1.45; 2.96)
- Siblings % (n)	518	59% (90)	25% (92)	<0.0001	3.02 (2.11; 4.32)

Table 1. Determinants of ventilation tubes.

* Household income at age 2: Low (Below 50.000 Euro), Medium (50.000-110.000), High (Above 110.000)

4.2. Paper II - Antibiotics in Pregnancy Increase the Children's Risk of Otitis Media and Ventilation Tubes

Baseline characteristics

Information on VT insertions was available for all 700 children in the cohort since it was obtained from the Danish registers. The incidence of VT insertions before 3 years of age was 29% (n=205). Information on maternal antibiotic intake during pregnancy was available for 699 children. The prevalence of any maternal antibiotic use during pregnancy was 37% (n=256). In the first year after birth 41% (n=283) of the mothers were treated with antibiotics. For the analyses of OM we only used data from children with full follow-up and >90% valid diary information in the first three years of life. 73% (n=514) of the children were included in the analyses of OM. 67% (n=346) of the children had at least one OM episode in this period.

We compared the children with adequate diary information to those that did not participate in the OM analyses and found that only maternal smoking during pregnancy was significantly different between these two groups. There were fewer non-Caucasians, low educated mothers and families with lower income in the group of children with adequate diary information; but the differences were not significant: Table 1. We found no difference in the prevalence of antibiotics administration between mothers of children with full diary information and incomplete diary information regarding maternal antibiotic intake (p = 0.83). The percentage of mothers treated with antibiotics was 37% in both the OM analyses and the VT analyses even though the numbers of participants in the OM analyses were lower. Potential predictors for antibiotic use in pregnancy are described in Table 2. Maternal educational level demonstrated a trend of association with antibiotic use (p=0.08); therefore, all analyses were adjusted for this as well as older siblings and parental history of middle ear disease as these factors are known to increase the risk of OM (19).

Table 1. Cohort baseline

	Full COPSAC ₂₀₁₀	Dropout	OM Study cohort	P Value
Ν	700	186	514	
Maternal age at birth, mean (SD)	32.3 (4.3)	32.0 (4.9)	32.3 (4.1)	0.399
Maternal asthma, % (N) ^a	26% (184)	30% (56)	25% (128)	0.163
Maternal smoking in pregnancy, % (N)	8% (54)	12% (22)	6% (32)	0.014
Caesarean section, % (N)	22% (151)	24% (44)	21% (107)	0.420
Maternal education level, % (N) ^b	-	-	-	0.375
Low	26% (184)	30% (56)	25% (128)	
Medium	46% (322)	43% (80)	47% (242)	
High	28% (194)	27% (50)	28% (144)	
Household income, % (N) ^c	-	-	-	0.310
Low	10% (67)	12% (23)	9% (44)	
Medium	53% (370)	52% (97)	53% (273)	
High	37% (262)	35% (66)	38% (196)	
Older siblings, % (N)	57% (396)	56% (104)	57% (292)	0.833
Gestational age in weeks, mean (SD)	40 (1.7)	40 (1.5)	40 (1.7)	0.473
Birth weight, mean (SD)	3.5 (0.5)	3.5 (0.6)	3.5 (0.5)	0.750
Male sex, % (N)	51% (360)	55% (102)	50% (258)	0.278
Race, Caucasian, % (N)	96% (670)	94% (174)	97% (496)	0.089
Days exclusively breastfed, mean (SD)	103 (60)	95 (62)	106 (59)	0.082
Age at daycare start in months, mean (SD)	10.9 (3.1)	10.9 (3.4)	10.8 (2.9)	0.882

^a History of doctor diagnosed asthma
^b Low (elementary school or college graduate), Medium (tradesman or medium length), High (university)
^c Low (Below 50.000 Euro), Medium (50.000-110.000), High (Above 110.000)

	All	Antibiotics		P Value	
		Yes	No		
All % (N)	100% (699)	37% (256)	63% (443)	-	
Maternal age at birth, mean (SD)	32.3 (4.3)	32.2 (4.7)	32.3 (4.2)	0.67	
Asthma history % (N) *	26% (184)	30% (76)	24% (108)	0.13	
Smoking in pregnancy % (N)	8% (54)	10% (25)	7% (29)	0.12	
Older children % (N)	57% (395)	61% (155)	54% (240)	0.10	
Maternal educational level, % (N) **					
Low	26% (184)	29% (74)	25% (110)		
Medium	46% (322)	48% (124)	45% (198)		
High	28% (193)	23% (58)	30% (135)		
Household annual income, % (N) ***	*			0.14	
Low	10% (67)	12% (31)	8% (36)		
Medium	53% (370)	54% (138)	52% (232)		
High	37% (261)	34% (87)	39% (174)		

Table 2. Predictors of maternal antibiotic intake during pregnancy.

* History of maternal doctor diagnosed asthma

** Low (elementary school or college graduate), Medium (tradesman or medium length), High (university)

*** Low (Below 50.000 Euro), Medium (50.000-110.000), High (Above 110.000)

Maternal antibiotic use and risk of otitis media

Maternal use of antibiotics during pregnancy was associated with an increased risk of OM in the child during the first 3 years of life; adjusted hazard ratio (aHR) 1.30, 95% CI, [1.04-1.63], p=0.02. Overall 74% (n=138) had OM before the age of three if the mother had received antibiotics in pregnancy compared to 64% (n=208) if the mother had not been treated. In children born to mothers treated in the third pregnancy trimester 77% (n=56) had OM, compared to 66% (n=290) if the mother had not been treated; though non-significant after adjustment; aHR 1.34 [0.98-1.83], p=0.06. In children born to mothers treated in the second pregnancy trimester 78% (n=67) had OM, compared to 65% (n=279) if the mother had not been treated; aHR 1.40, 95% CI, [1.06-1.85], p = 0.02. We found no association between treatment in first trimester and risk of OM. In children born to mothers treated with RTI antibiotics in pregnancy 81% (n=83) had OM, compared to 64% (N=263) if the mother had not been treated; aHR 1.45, 95% CI, [1.11-1.89], p = 0.006. There was no effect of UTI antibiotics on the risk of OM. We observed no effect of antibiotic treatment in the year after pregnancy, nor when restricting the analysis to those mothers, who did not receive any antibiotics in pregnancy: Table 3.

	ОМ	Unadjusted		Adjusted		VT	Unadjusted		Adjusted	
		HR	Р	HR	Р		HR	Р	HR	Р
	N=514	[CI] ^d	Value	[CI]	Value	N=699	[CI]	Value	[CI]	Value
Maternal AB										
in	187	1.36		1.30		256	1.32		1.14	
Pregnancy	(37%)	[1.10-1.69]	0.005	[1.04-1.63]	0.02	(37%)	[1.00-1.74]	0.05	[0.83-1.56]	0.42
RTI ^e	103	1.56		1.45		142	1.33		1.09	
antibiotics	(20%)	[1.22-1.99]	<0.001	[1.11-1.89]	0.006	(20%)	[0.97-1.83]	0.08	[0.76-1.58]	0.64
UTI ^f	112	1.22		1.26		148	1.55		1.56	
antibiotics	(22%)	[0.95-1.56]	0.12	[0.97-1.63]	0.09	(21%)	[1.14-2.10]	0.005	[1.10-2.19]	0.01
1. trimester	88	1.19		1.16		124	1.17		1.14	
AB	(17%)	[0.90-1.55]	0.22	[0.87-1.54]	0.30	(18%)	[0.83-1.65]	0.36	[0.78-1.68]	0.50
2. trimester	86	1.43		1.40		114	1.55		1.38	
AB	(17%)	[1.10-1.87]	0.01	[1.06-1.85]	0.02	(16%)	[1.12-2.15]	0.009	[0.94-2.02]	0.10
3. trimester	73	1.43		1.34		92	1.96		1.60	
AB	(14%)	[1.08-1.91]	0.01	[0.98-1.83]	0.06	(13%)	[1.40-2.75]	<0.001	[1.08-2.36]	0.02
3.trimester	37	1.93		1.84		49	2.32		1.56	
RTI	(7%)	[1.34-2.76]	<0.001	[1.24-2.74]	0.003	(7%)	[1.54-3.50]	<0.001	[0.95-2.56]	0.08
3.trimester	38	1.05		1.06		46	1.61		1.61	
UTI	(7%)	[0.70-1.58]	0.81	[0.70-1.62]	0.77	(7%)	[1.01-2.59]	0.05	[0.96-2.70]	0.07
Maternal AB										
post	203	1.20		1.15		283	1.25		1.28	
pregnancy	(40%)	[0.97-1.48]	0.10	[0.92-1.44]	0.23	(41%)	[0.95-1.64]	0.11	[0.94-1.75]	0.12
Maternal AB										
post										
pregnancy										
but not in	115	0.94	0.55	0.91	0.40	158	0.96		1.00	0.00
pregnancy	(22%)	[0.73-1.22]	0.66	[0.69-1.19]	0.49	(23%)	[0.67-1.33]	0.78	[0.69-1.46]	0.99
Dose-		1.24		1.20			1.26		1.15	
response		[1.08-1.43]	0.003	[1.04-1.40]	0.02		[1.06-1.51]	0.01	[0.94-1.41]	0.17

Table 3. Associations between maternal antibiotics (AB)^a and risk of otitis media (OM)^b and ventilation tubes (VT)^c in the children during the first 3 years of life.

^{*a*} AB = Antibiotics

^b OM = Acute otitis media

^c VT = Ventilation Tubes

^d HR = Hazard Ratio, CI = Confidence Interval

eRTI = Respiratory Tract Infection

fUTI = Urinary Tract Infection
Maternal antibiotic use and risk of ventilation tubes

Maternal antibiotic use in pregnancy was associated with an increased risk of VT in the children during the first 3 years of life, though not significant in the overall analysis; aHR 1.14, 95% CI, [0.83-1.56], p = 0.42. This corresponds to 34% (n=87) of children had VT if mothers had been treated with antibiotics and 27% (N=118) had VT if the mother had not been treated. In children born to mothers treated in the third pregnancy trimester 46% (n=42) had VT, compared to 27% (n=163) if the mother had not been treated; aHR 1.60, 95% CI, [1.08-2.36], p=0.02: Figure 1. We found no associations between treatment in first or second trimester and risk of VT. In children born to mothers treated with UTI antibiotics in pregnancy 39% (n=57) had VT, compared to 27% (n=148) if the mother had not been treated; aHR 1.56, 95% CI, [1.10-2.19], p = 0.01. No significant association between VT and maternal treatment with RTI antibiotics. We found no effects of post pregnancy treatment in the mother on the risk of VT in the children: Table 3.

Figure 1. The risk of ventilation tubes in the child if the mother received antibiotics (AB) in third trimester.



Dose-response relationship

We observed a dose-response relationship between the number of antibiotic treatments during pregnancy and the risk of OM; per-level aHR 1.20, 95% CI, [1.04-1.40], p=0.02. The risk of VT was also increased before adjustment: Figure 2, but the effect was not significant after adjustment; per-level aHR 1.15, 95% CI, [0.94-1.41], p=0.17.

Figure 2. Dose-response relationship between the number of maternal antibiotic treatments in pregnancy and the risk of ventilation tubes in childhood.



Stratification for mode of delivery

The stratification resulted in higher risk of both OM and VT after treatment with antibiotics during pregnancy in children with vaginal delivery, though not significant. The risk of OM was significantly increased if the mother had received antibiotics and gave birth by vaginal delivery; aHR 1.70, 95% CI, [1.01-2.89], p=0.05. The risk of OM was not significantly increased had the child been born by caesarean section; aHR 1.23, 95% CI, [0.95-1.59], p=0.11. The risk of VT was nearly significant and with a doubled risk if the mother had received VT and given birth by vaginal delivery, aHR 1.97, 95% CI, [0.97-3.98], p=0.06. There was no association

between mothers treated with antibiotics and undergoing caesarean section and the children's risk of VT; aHR 0.96, 95% CI, [0.67-1.38], p=0.81.

4.3. Paper III - Middle Ear Effusion and Ventilation Tubes and the Effect on the Neurological Development in Early Childhood

Baseline characteristics

Seven hundred children were included in the COPSAC₂₀₁₀ cohort at birth and information on VT insertions was available for all 700 children. We excluded 37 children from the study because of either a neurological diagnosis, gestational age <37 weeks or birth weight <2500 grams: Figure 1. Of the 537 children with a tympanometry measurement at 1 year of age 51% (276) had MEE, at 2 years of age 37% (208) of the 563 children with measurements had MEE and at 3 years of age 26% (153) of the 578 children with measurements had MEE: Figure 2 and 3. Before 1 year of age 5% (35) of the children had VT inserted. Before 2 years of age 26% (173) of the children had VT inserted and before 3 years of age 29% (192) of the children in this study population of the cohort had had VT inserted. Figure 1 illustrates how many children participated in the analysis of each neurological endpoint. Table 1 shows the baseline characteristics for children, who had either MEE or were treated with VT compared to children with no middle ear disease before the age of 3. We found more boys with both MEE and VT compared to girls and as sex is a known determinant of the neurodevelopment of children (56,63,64), all further analyses were adjusted for sex. Season of birth was associated with risk of MEE, but season of birth was not associated with VT insertion. We found that older siblings increased the risk of treatment with VT, but did not associate with MEE.

Figure 1. Flowchart.



Figure 2. Prevalence of middle ear effusion measured by tympanometry at 1, 2 and 3 years of age in the COPSAC₂₀₁₀ cohort.



Figure 3. Kaplan-Meier curve illustrating time to first ventilation tube insertion in the COPSAC₂₀₁₀ cohort.



Table 1. Baseline characteristics of the COPSAC₂₀₁₀ cohort: the children with middle ear effusion (MEE), ventilation tubes (VT) or children without middle ear disease before the age of 3.

	No middle ear disease ≤ 3 y N=146	Middle ear effusion ≤ 3 y n=312	P value	Ventilation tubes ≤ 3 y n=192	P value		
Pregnancy and birth							
Maternal age at birth, mean (SD)	31.7 (4.2)	32.5 (4.3)	0.073	32.3 (4.5)	0.207		
Delivery, vaginal, % (n)	81.5 (119)	80.5 (251)	0.141	79.7 (153)	0.141		
Length in cm at birth, mean (SD)	52.2 (2.1)	52.1 (2.2)	0.430	52.2 (2.0)	0.895		
Birth weight in kg, mean (SD)	3.6 (0.5)	3.6 (0.5)	0.442	3.6 (0.5)	0.684		
Head circumference in cm at birth, mean (SD)	35.2 (1.3)	35.0 (1.7)	0.310	35.2 (1.6)	0.857		
Sex, boys, % (n)	41.8 (61)	52.6 (164)	0.032	55.7 (107)	0.011		
Race, Caucasians, % (n)	94.5 (138)	96.2 (300)	0.425	97.4 (187)	0.173		
Season of birth, % (n) Winter Spring Summer fall	23.3 (34) 21.2 (31) 34.9 (51) 20.6 (30)	36.5 (114) 29.2 (91) 14.4 (45) 19.9 (62)	<0.0001	26.0 (50) 26.6 (51) 22.9 (44) 24.5 (47)	0.109		
Exposures							
Smoking during pregnancy, % (n)	6.9 (10)	6.7 (21)	0.963	6.8 (13)	0.977		
Older siblings, % (n)	50.0 (73)	57.1 (178)	0.158	67.2 (129)	0.001		
Duration of solely breastfeeding in days, mean (SD)	104.3 (60.8)	103.4 (57.1)	0.834	104.6 (60.6)	0.903		
Age at start in daycare in months, mean (SD)	11.3 (3.5)	10.9 (3.0)	0.219	10.6 (2.9)	0.047		
Social circumstances PCA score *	- 0.05 (1.0)	0.08 (1.0)	0.194	- 0.09 (1.0)	0.710		

Gross motor milestones were not associated with MEE or VT

499 children (71%) had information on at least one gross motor milestone and information regarding MEE and VT before the age of 1 year: Figure 1. We used the PC1 score, which explained 64.9% of the variance in the original variables, representing the overall gross motor

development, to analyze the associations with otitis media: Figure 4. There were no significant differences in the age of milestone achievement between children with either MEE, VT or children without middle ear disease: Table 2 and Figure 4.

Figure 4. PPCA biplot of the gross motor milestone scores of each child and loadings of the neurological variables. Ellipses illustrate the scores (95% CI) of children with middle ear effusion, treatment with ventilation tubes and no disease.



<u>Figure legend</u>: The PC1 explains 64.9%. The figure shows that there is no difference between children with middle ear effusion, ventilation tubes and no disease in the age of achieving the gross motor milestones.

Table 2. Associations between middle ear effusions (MEE) or treatment with ventilation tubes (VT) and neurological development.

Gross Motor Milestones PPCA									
PC1	N=499	Estimate	95% CI	P value					
$VT \le 1$ year	34	0.31	[(-0.38)-1.00]	0.373					
MEE at 1 year	253	(-0.04)	[(-0.39)-0.31]	0.816					
No VT, no MEE ≤ 1 year	212	reference	-	-					
Word production at 1 year									
	N=307	Median	IQR	P value					
$VT \le 1$ year	21	6	[2-9]	0.734					
MEE at 1 year	161	3	[1-7]	0.015					
No VT, no MEE ≤ 1 year	125	5	[2-8]	reference					
Word comprehension at 1 year									
	N=307	Median	IQR	P value					
$VT \le 1$ year	21	45	[21-77]	0.998					
MEE at 1 year	161	37	[22-65]	0.032					
No VT, no MEE ≤ 1 year	125	48	[28-85]	reference					
Word production at 2 years									
	N=496	Median	IQR	P value					
$VT \le 2$ years	130	226	[86-362]	0.159					
MEE at 1 or 2 years	173	238	[114-345]	0.249					
No VT, no MEE ≤ 2 years	193	269	[153-365]	reference					
Cognitive test at 2.5 years									
	N=584	Estimate	95% CI	P value					
$VT \le 2.5$ years	169	(-2.04)	[(-4.09)-0.00]	0.050					
MEE at 1 or 2 years	258	(-0.10)	[(-1.97)-1.76]	0.914					
No VT, no MEE \leq 2.5 years	157	reference	-	-					
ASQ-3 PCA at 3 years									
PC1	N=437	Estimate	95% CI	P value					
$VT \le 3$ years	124	(-0.96)	[(-3.89)-1.97]	0.520					
MEE ever (at 1, 2 or 3 years)	219	(-1.49)	[(-4.14)-1.16]	0.270					
No VT, No MEE \leq 3 years	94	reference		-					
Combined PCA of all neurological assessments									
PC1	N=530	Estimate	95% CI	P value					
$VT \le 1$ year	35	0.41	[(-0.29)-1.10]	0.249					
MEE at 1 year	270	(-0.16)	[(-0.50)-0.18]	0.361					
No VT, No MEE ≤ 1 year	225	reference	-	-					
PC2	N=530	Estimate	95% CI	P value					
$VT \le 1$ year	35	(-0.06)	[(-0.52)-(-0.40)]	0.801					
MEE at 1 year	270	(-0.25)	[(-0.48)-(-0.02)]	0.037					
No VT, No MEE ≤ 1 year	225	reference	-	-					

Decreased language development at 1-year was associated with MEE but with no longterm effect.

Completed language assessment at 1-year and information regarding MEE and VT before the age of 1 was obtained for 44% (305) of the children: Figure 1. MEE at the age of 1 was associated with a lower word production at the concurrent language assessment (median 3, IQR [1-7]), compared with no middle ear disease (median 5, IQR [2-8]), corresponding to a reduction of 24%, 95% CI [5%–39%], p=0.015. MEE at the age of 1 was also associated with a lower word comprehension (median 37; IQR [22-65]), compared with no middle ear disease (median 48; IQR [28-85]), corresponding to a reduction of 21%, 95% CI [2%-37%], p=0.032. No associations were found between children who had received VT and the 1-year language scores (Table 2).

Completed language assessment at 2 years and information on MEE at 1 or 2 years or VT insertions before the age of 2 years was obtained for 71% (496) of the children: Figure 1. We found no associations between MEE or VT and the 2-year language scores: Table 2.

The cognitive score was lower for children treated with VT

Completed cognitive test at the age of 2.5-years and information regarding MEE at the age of 1 or 2 years and VT before the age of 2.5 years was obtained for 83% (584) of the children: Figure 1. VT insertion was associated with a lower composite score; adjusted β -coefficient - 2.04; 95% CI [-4.09-0.00]; 0.050, Table 2. However, there were no differences between children with MEE at either 1 or 2 years and children without disease concerning the cognitive scores at 2.5 years.

The general development assessment (ASQ-3) was not affected by MEE or treatment with VT

Completed ASQ-3 assessment at the age of 3 years and information regarding MEE at age 1, 2 or 3 years or VT before 3 years of age was obtained for 62% (437) of the children: Figure 1. The five categories in the ASQ-3 questionnaire were analyzed together as one measure of the child's overall development at 3 years using a PCA. The PC1 score explained 48.1% of the variation in the data (Figure 5), therefore we used this PC for the analysis. There were no differences between children with MEE at either 1, 2 or 3 years of age, VT before the age of 3

and children without disease concerning the general development of the child at age 3 years: Table 2.

Figure 5. PCA biplot of the ASQ-3 scores of each child and loadings of the neurological variables. Ellipses illustrate the scores (95% CI) of children with middle ear effusion, treatment with ventilation tubes and children with no disease.



<u>Figure legend:</u> The ASQ-3 consists of 5 categories; fine motor development, gross motor development, personal-social skills, communication and problem solving. When the scores are analyzed together as one measure of the child's development it results in a PC1 score, which explains 48.1% of the variation.

Combined neurological PCA showed decreased score for children with MEE

To analyze effects of middle ear disease and all later neurological endpoints, we examined MEE at age 1 year and VT before 1 year in a combined PCA model of all neurological measures. The PC1 explained 36.9% of the variation in the neurological data and the PC2 explained 18.0% of the variation (Figure 6). MEE at 1 year was significantly associated with a lower PC2-value; adjusted estimate -0.25; 95% CI [(-0.48)-(-0.02)]; p=0.037; but was not associated with PC1 and no significant associations were found for VT before 1 year of age.

Figure 6. PCA biplot of the neurological development scores of each child and loadings of the neurological variables. Ellipses illustrate the scores (95% CI) of children with middle ear effusion, treatment with ventilation tubes and no disease.



<u>Figure legend:</u> Gross motor milestones (sit alone, stand with help, stand alone, walk with help, walk alone), language development at 1 and 2 years, cognitive score at 2.5 years of age and the ASQ-3 scores (problem solving, communication, personal-social, fine motor, gross motor) at 3 years comparing children with middle ear effusion, ventilation tubes and children without middle ear disease.

5. Discussion

5.1. Principal Findings

The incidence of VT in Denmark is very high. Compared with incidences reported from other westernized countries it is to our knowledge the highest in the world. From registry data, we found that 24% of children received VT before the age of 3. In the COPSAC₂₀₁₀ birth cohort we investigated determinants of VT insertions and found older siblings, a history of persistent wheeze and family history of otitis media increased the risk of receiving VT. After adjusting for co-significant factors, only maternal and sibling history of middle ear disease increased the risk significantly.

We analyzed the association between maternal antibiotic intake in pregnancy and the offspring's risk of both OM and VT. We found an overall increased risk and a dose-response relationship regarding OM. We found an association between treatment with antibiotics late in pregnancy and increased risk of VT for the offspring.

Finally, we investigated the impact of MEE and treatment with VT on the neurological development in early childhood. We found that children with MEE had slightly lower language scores at 1-year of age, but they were not delayed in any other ways when examining several neurological endpoints up to the age of 3. In addition, our data do not support an effect of treatment with VT on the neurological development.

5.2. Treatment of Otitis Media with Ventilation tubes

We found that 24% of Danish children had been treated with VT before the age of 3. We found an even higher rate in our cohort with 29% of the children. A possible explanation is that we followed the children very closely and if we found MEE, the parents were recommended to contact an ENT-specialist.

In the United States the American Academy of Otolaryngology – Head and Neck Surgery Foundation have made a clinical practice guideline (1,65). It resembles the Danish guideline. VT are recommended: if MEE are bilateral and persistent for ≥3 months and the child has either documented hearing difficulties or other symptoms that are likely attributable to MEE, e.g. vestibular problems, poor school performance, behavioral problems, ear discomfort, or reduced quality of life. If children have recurrent AOM, both in the US and in Denmark VT are only recommended if the child also have periods with MEE between the AOM episodes. The effect of treatment with VT for recurrent AOM is based on sparse evidence. There are few studies that have found only a small reduction in AOM episodes with VT treatment (66,67). The benefit of treatment with VT for recurrent AOM could be to reduce systemic antibiotic therapy. If a child have an AOM episode and have VT, they can be treated with local treatment in the form of antibiotic eardrops.

Usually MEE resolves spontaneously. The percentage of children treated in Denmark are comparable to the natural course of the disease; as at least 25% of MEE episodes persist for 3 months. This could indicate that too many children are treated. 5-10% of children not treated will have persistent MEE for a year (1), which could be the group of children to aim to find and treat. With the natural course of MEE in mind, it seems reasonable with a longer period of watchful waiting than 3 months and treatment with VT reserved for persistent MEE, which does not resolve after perhaps 6-9 month or for children with severe symptoms.

As the guidelines for treatment with VT are similar, it is interesting to discuss what can explain the differences in the incidence between the westernized countries. Treatment is free in Denmark like in many of the other countries. The prevalence of MEE is likely very equal between Denmark and especially the other Scandinavian countries. One difference between Denmark, Sweden and Norway is that the children attend daycare institutions a little later in the two other countries because of longer maternity/paternity leave (68). Early childhood is a critical time for middle ear disease with a high prevalence of MEE around the age of 1 year and VT insertions that peak at the age of 1.5 years. A later start in daycare institutions in the other countries could have great impact (please refer to Figure 2 in the result section of Paper III showing the prevalence of MEE in COPSAC cohort at age 1, 2 and 3, and Figure 3 showing the age of first VT insertion). Another difference is that The Health Care Systems are organized differently. In Denmark, you do not need referral to have an appointment with an ENT-specialist. For most other examinations and treatments with specialists in Denmark, the general practitioner function as a gatekeeper. There might be a financial incentive to treat for the private ENT-specialist. At the hospital the doctors receive a fixed salary and are not paid per procedure. In the US, doctors are paid for their treatments as well, but if the parents do

not have health insurance with full coverage, they might be hesitant with the procedure to avoid the costs.

5.3. Risk Factors of Otitis Media

In former studies when investigating risk factors of OM, a very varied diagnosis have been used e.g. recurrent ear infections (69) and with diagnostic variations from >3 (70) or >5 episodes (71), continuous otorrhea for both >2 months (72) and >3 months (73), VT insertions (74–76) and several other definitions (63). The studies evaluate different environmental exposures such as daycare attendance, breastfeeding, crowding, tobacco exposure, sex, gestational age, socioeconomic status among other factors but there is lack of reproducibility between the studies. This can be because of the variation in the diagnosis of OM or because of variations in definition of the exposures. It might not be possible to compare e.g. low socio-economic class in Denmark and USA as the societal benefits and costs of living despite the same monthly payment probably cannot be compared.

Lack of reproducibility can also be due to difference in study design; the majority of studies on OM are either retrospective, cross-sectional or case-control. We would expect them to be less accurate without the close follow-up that the clinical cohort study provides.

The following factors have been found to be significant risk factors of OM in a meta-analysis: Allergy/atopy, upper respiratory tract infection, snoring, low social status, acute otitis media episode, passive smoking. Borderline significant increased risk associated with male sex, daycare attendance and siblings in the home/family crowding (63).

In **Paper I** using VT as the best marker of severe OM, we found that older siblings in the household increased the risk of VT insertion. A history of persistent wheeze also increased the risk of VT. This is probably like the upper respiratory tract infections found in other studies. A family history of middle ear disease was the most dominant factor predicting VT insertion and the factor that remained significant after adjusting for the co-significant factors. This result is in agreement with findings in former studies as it has been shown that OM is 40-70% heritable and recently genome-wide association studies have been published on genetic variants influencing the proneness of OM (77,78).

We calculated a genetic risk score for the children in the COPSAC₂₀₁₀ cohort for receiving VT before the age of 3, but we did not find any association. This could be explained by a

phenotypic difference. The genetic risk score is calculated from results from a questionnairebased-study where the phenotype was those with recurrent AOM. This is different from the children in our cohort (as well as in Denmark) who primarily receive VT for persistent MEE. Intriguingly, this may also be indicative of many Danish children might receive VT actually to treat other symptoms found in combination with MEE; e.g. decreased sleep quality, delayed speech development or decreased quality of life. We use VT as a solid marker of OM and it is probably the best marker of middle ear disease, but maybe not all severe disease; it might also reflect other problems. Finally, it could be because of lack of power that we did not find an association.

The causality diagram we have presented shows that the hypothesis is; that OM probably occur as an interaction between several candidate genes modulated by different environmental factors. Heritability is an important factor and not really possible to change (77,78). Therefore, former studies have searched for risk factors either to modify or to find atrisk-children whom could be followed and assed more closely. In our study, we found that the family history of middle ear disease was the only factor that remained significant in the multivariate analysis. It could be discussed, if the causality diagram should be extended by another factor, which describes a doctor seeking / family behavior. If older siblings or the mother have had VT the risk is increased. This might not only be because of genetics, but could also be because of increased stress for the family with more children in the home. The quality of life can be even more reduced with less amount of sleep and more days with sick children to make the family even more desperate for a solution. This could cause a doctor-seeking-behavior which is important for the clinician to be aware of as this is not an indication for the procedure.

Potential side effects of the treatment with VT

The most common sequela of VT insertion is otorrhea, which 16% of children have within the first 4 weeks following the procedure and 26% at any time while the VT remains in place (65). There are other potential side effects of the procedure: risk of undergoing anesthesia, tympanosclerosis and permanent perforation of the tympanum. Up to 2% suffer from permanent perforation and most undergo new surgery to repair the defect (myringoplasty) (79,80). In a Swedish study, they compared hearing ability from patients formerly treated with VT because of MEE with patients who had had episodes of middle ear disease but had not

been treated with VT and a control group of healthy individuals with no documented middle ear disease. They found that those who had been treated with VT had a significantly higher prevalence of elevated thresholds for solitary frequencies (81).

5.4. Antibiotic Intake During Pregnancy and the Risk of Otitis Media and Ventilation Tubes for the Offspring

In **Paper II**, we found increased risk of OM in children born to mothers who had been exposed to antibiotics in pregnancy. The effect of pregnancy antibiotics on the risk of VT was especially driven by treatment in third trimester, without significant effects of treatment in other pregnancy trimesters. We found no association between maternal antibiotic intake the year after pregnancy and the child's risk of neither OM nor VT. Furthermore, there was no association between treatments the year after and not having received any antibiotics during pregnancy. This suggests that the associations we found are not an expression of maternal disease susceptibility or a doctor-seeking behavior. Therefore, we suggest a possible mechanism must be sought in pregnancy.

Antibiotic treatments are indicators of maternal infections and the triggered immune response and following inflammation during pregnancy may affect the fetus (82). We found the strongest effect of antibiotic treatment on the offspring's risk of disease with treatments late in pregnancy. This could be explained by the fetus' own immune system especially develops in the later part of pregnancy (83). The pregnant woman's immune system is suppressed during pregnancy to prevent rejection or mounting an inappropriate immune response against the child (84). Some infections are particularly dangerous during pregnancy because they can harm the fetus (23).

The effect of treatment late in pregnancy also suggests a possible mechanism mediated by vertical transmission of unfavorable microbiota between mother and child. The dose-response relationship as well as the equal effects of RTI and UTI antibiotics point toward a microbial mechanism rather than an inherited propensity for infections, which could have been an alternate explanation for the observed associations. We found that the risk of OM was associated primarily with the use of RTI antibiotics, but not significantly with UTI antibiotics, although the estimate were increased. The risk of VT was associated with UTI antibiotics and

not significantly with RTI antibiotics. This divergence could indicate differences in the immune phenotypes associated with these two conditions: children with acute OM episodes and children with MEE, which are those that primarily are treated with VT. An explanation could be that different types of antibiotics favor different microbial profiles that may each modulate the immune system in different ways towards susceptibility to either acute or chronic middle ear disease.

We stratified the analyses for mode of delivery and found that children with vaginal delivery were affected more by the maternal antibiotic intake compared with children born by caesarean section. This could indicate that microbial alterations caused by the antibiotics lead to the increased disease susceptibility by vertical transmission of an unfavorable microbiome during birth.

The study is observational, and we cannot claim causality of the antibiotics. However, the stratification in pregnancy trimesters and in the dose-response relation from antibiotics allows us to speculate in possible mechanisms.

Prescribing antibiotics during pregnancy presents a challenge to the physician because some infections indeed warrant treatment, while protecting the fetus against possible side effects remains a high priority. Potential long-term health consequences for the child may warrant caution, when prescribing antibiotics to the pregnant woman.

5.5. Middle Ear Effusion, Treatment with Ventilation Tubes and the Neurological Development of the Children

A concern of persistent MEE is that the hearing ability can be reduced in early childhood. If the child has hearing loss, it can delay speech and language development and early language development is related to later academic achievements (37), which means it can have longterm consequences.

The Danish language development is later than for instance children learning to speak English as the Danish language is more difficult to pronounce (36). This could lead to different consequences for children with OM in Denmark regarding their language development compared to other countries. In our study, we found that the presence of MEE at one year of age resulted in lower language scores both regarding word comprehension and word production. Children who had been treated with VT were not delayed compared to children without middle ear disease. This could indicate an effect of VT. However, we found no difference in the 2-year language scores between children with MEE, VT or no middle ear disease. The early delay does not seem to have long-term consequences. This finding is in line with results described in other studies, investigating the association between middle ear disease and literacy. MEE can cause a slight delay in early language development probably because of the hearing impairment, but the children catch up (7,8,85). The relation between MEE and hearing loss during early childhood and later academic skills have been investigated and no association found (8,34,37,85,86). A randomized controlled trial comparing prompt insertion of VT (3 months with effusion) and delayed (up to 9 months delayed) found no difference between the groups at annually evaluations up to the age of 9-11 years (86–89). They recommended a more conservative approach for otherwise healthy children, with watchful waiting regarding VT insertions. In conclusion, our findings is supported by previous studies investigating the effect of MEE on language development and have shown no long-term effects on language development (86–90).

We found a 2 point lower cognitive score for children with VT before the age of 2.5 compared to children without middle ear disease. We found no delay for children with untreated MEE. Children treated with VT scored lower, which could indicate that VT did not have beneficial effect on the cognitive development. It could also be due to confounding by indication; the children treated with VT were those who had the most symptoms of delayed development. In the ALSPAC prospective cohort study from UK they analyzed the effect of persistent MEE with hearing loss on the IQ scores of children and found lower scores at the age of 4 (91). The effect did not have long-term consequences, as they did not find significant associations at 8 years of age. This finding is consistent with other studies (91,92). Therefore, it seems very likely that the children delayed in the cognitive test in our cohort because of middle ear disease will catch up, like they have seen in these other studies.

Another concern about MEE is that it can affect the balance of the children and delay the motor development (38,39). We found no difference regarding the age of achievement of the gross motor milestones when comparing children with MEE to children treated with VT or children without middle ear disease before the age of 1. Children with MEE have been

described as being clumsier than children without MEE (38), but it does not seem to have consequences for their early motor development as their milestones were not delayed compared to healthy children. Nor did we find association between middle ear disease and motor development evaluated as part of the ASQ-3 at the age of 3. Other small studies have evaluated the effect of MEE on the balance of the children but evaluating methods, study design and results vary (93,94), making the conclusion unclear. We do not find evidence for the claim that MEE affect children's motor development comparing children with treated and untreated MEE to healthy controls.

The combined PCA analysis of all neurological endpoints showed that children with MEE at 1 year had a decreased language score and cognitive score, independent of motor development, represented in PC2 (please refer to the figure in the result section of **Paper III**). No association was found for VT, which could be because of few children treated with VT before 1 year of age. The result of this combined neurological PCA supports the findings of the univariate analyses.

We evaluated the impact of MEE on the neurological development of Danish children regarding language-, cognitive- and motor development. We found a slight delay on the early language scores of MEE but no long-lasting effect; which is in line with results from former studies (8,85,89). We found a lower cognitive score for children treated with VT. We cannot conclude if treatment with VT had any beneficial effects, but we demonstrate no long-term positive differences between children treated with VT and untreated children. In the US guideline they concluded that no RCTs have found significant impact of VT on speech-, language- or cognitive outcomes in line with findings in our study (65). A quote from the **Cochrane** study; **"GROMMETS (VENTILATION TUBES) FOR HEARING LOSS ASSOCIATED WITH OTITIS MEDIA WITH EFFUSION IN CHILDREN"** (95) is here presented to substantiate this conclusion:

"The hearing-deprived period is rapidly compensated for by the flexibility of development in children. No study that randomised children to grommets versus 'watchful waiting/active monitoring' demonstrated a significant effect on any developmental outcome in either group compared with 'normal' non-otitis media with effusion controls".

5.6. Interpretation of the Study

This study addresses the impact of OM in early childhood. Most children will have episodes of middle ear disease in early childhood – either acute and/or persistent MEE, and in Denmark, at least 24% of the children will have VT insertion before the age of 3.

We have discussed risk factors of OM to find factors that make some children at-risk of middle ear disease and to treatment with VT insertion. This is a relevant topic not only to the families of children with recurrent OM or persistent MEE; but also to many clinicians: general practitioners, ENT-surgeons and pediatricians.

The first clinical guidelines from Danish Health Authorities about VT treatment for OM was recently published. The evidence this guideline is built upon is weak.

As the treatment is so popular and used in Denmark although the evidence of the effect of the procedure on the neurological development is vague, we wonder if there is either over-use of the procedure or other effects of the treatment. OM has a possible effect on not only the child but the entire family e.g. sleep disruption and parents that become stressed because they are struggling to balance demands of their work and many days at home with a sick child. This can affect the quality of life for the child and the family. It might help children sleep better or be less irritable which would be small change with great importance to a stressed family. These quality of life parameters are difficult to evaluate. Frequent infections and sleep problems because of otitis media may result in reduced quality of life for the child and the effect of VT on the quality of life for the child and family, the amount and quality of sleep as well as days home from daycare before and after treatment. However, these are not the indications for treatment of OM with VT, and therefore should not be the reason for Denmark having such a high incidence. The effect of treatment with VT on the quality of life should be investigated further, as this might be an explanation for the popularity of the procedure.

Altogether, this seems to indicate a more conservative approach and watchful waiting before treating MEE with VT. The new Danish guidelines are not very clear and this makes it interpretable. It could be a future solution to make the guidelines less flexible and have recommendations for VT based on strong evidence. It might also be an idea with a gatekeeper

function, which means referral from the general practitioner after a period of watchful waiting before contact with the ENT-specialist.

5.7. Strengths and Limitations

The strength of our data relates to the close longitudinal surveillance at the COPSAC clinical research unit. We have frequent clinical assessments by experienced study-doctors, which assures consistency in definitions of conditions, diagnoses, and data capture methods and reduce risk of misclassification. Furthermore, the Danish registries strengthen the study. They contain information regarding all VT procedures and contacts with the health care system linked to a personal identification number. Therefore, we have information regarding VT procedures on all 700 children in the cohort. The registries do not contain information on the VT indication (middle ear effusion, recurrent acute otitis media or other reason), or for how long the VT was in place.

Acute OM episodes were captured in the prospective diaries filled out by the parents during the first years of life. The longitudinal assessments from birth assure robust clinical endpoints and improved statistical power from the time of first OM. It is a unique dataset since it is difficult to get accurate information regarding small children's disease type and frequency. Some parents did not manage to fill out the diary every day for 3 years, which caused 186 children to be excluded from the OM analyses. It could weaken our OM endpoint that not all children had full follow up. The children were not seen in the clinic with symptoms of acute middle ear disease, which means that the diagnosis could be less accurate. Most of the episodes had been diagnosed by the private physician of the children or an ENT-specialist. The diagnosis captured in the disease diary was validated by the study pediatricians by interviews at clinic visits. Our finding of a prevalence of 67% with OM before the age of 3 is comparable to the finding in another Danish study which found a slightly smaller incidence probably due to the retrospective interview based design of their study (97).

MEE was diagnosed by the tympanometry measurement, which is an objective, wellrecognized test and easy to perform. A limitation is the nature of an annual spot measurement in the COPSAC clinic. Children may have had periods of MEE between the examinations, which we would not have captured and therefore regarded them as having no middle ear disease.

However, most children have MEE over longer periods of time (1), making the annual measurements an acceptable measure of MEE burden. We captured middle ear effusion among a high percentage of the children comparable to findings in other studies (1), and it would be unrealistic to have closer follow up on such a large population. We classified the children with at least one ear affected with MEE as having MEE to have more statistical power. We assume that most of the children we found with unilateral MEE probably had bilateral MEE in the beginning. The effect and symptoms of unilateral MEE are milder compared to bilateral effusion. This could have affected the analyses of the association to the neurological measurements. As our results are in line with those from former studies, we do not believe this have affected our results particularly.

Season of birth was associated with MEE but not with VT. This could be due to the study design with annual measurements, since children were assessed in the same season every year, and children assessed during the winter are probably more likely to suffer from ongoing MEE (Refer to Table 1 in the 3. study, describing the baseline characteristics).

VT is placed because of either recurrent OM or persistent MEE. In **Paper I**, we used VT as outcome for OM to analyze risk factors, as we assumed that children receiving VT are those with most severe OM. We could have used the OM diagnosis from the disease diaries as we did for the analyses in **Paper II**, but as discussed above, the OM diagnosis could be a more varied disease phenotype, because the children were not seen in our research clinic with these symptoms. Like former studies with varied definitions of OM, we found that OM and MEE are not as solid markers of severe middle ear disease as VT insertions, especially in Denmark and in our cohort as we have this very high prevalence of the procedure, why we chose to use VT in all the studies as outcome.

We have very accurate information regarding several possible confounders because of the close follow up with interviews with the families of their social status and home environment at each clinical visit. We had very accurate information about maternal intake of antibiotics during pregnancy for the second study. High intake of antibiotics could be caused by a disease propensity in the mother, which the child would likely inherit. It could otherwise be due to maternal doctor-seeking behavior, which would also be conferred to the child. Therefore, we performed the analyses of maternal intake of antibiotics the year after pregnancy in relation

to both OM and VT. Especially the analysis of mothers receiving antibiotics the year after pregnancy but not in pregnancy with no associations to the clinical outcomes supports the pregnancy effect of antibiotics and that these factors are not confounding the association between mothers' antibiotic use in pregnancy and OM or VT in the young child. We also stratified into pregnancy trimesters and analyzed the dose-response relation from antibiotics as well as mode of delivery, which allows us to speculate in possible mechanisms. The study is observational, and we cannot claim causality of the antibiotics.

We did a comprehensive neurological data collection with both registration of age at achievement of milestones, language tests at 1 and 2 years of age, an objective cognitive test at the age of 2.5 and a general development assessment at 3 years of age. The tests we chose to evaluate the children in the cohort are standardized and validated, which makes our results both reliable and comparable to other studies. It is a unique collection of standardized neurological developmental tests performed prospectively in a large cohort of children. Parents filled out the milestones registration, the language assessments and the ASQ-3 questionnaire. Studies have shown excellent correlation between parents and professionals evaluations of the skills (98,99).

Not all children completed all tests, which could limit the statistical power. Missing information on specific milestones was handled by the PPCA model. A single missing milestone would otherwise have excluded the child in a traditional PCA model.

A limitation is the study's observational design. Only a randomized trial on either VT insertion or a conservative approach with watchful waiting can truly evaluate the efficacy of the VT insertion with respect to later developmental endpoints.

6. Conclusions and Perspectives

Conclusion

Denmark have to our knowledge the world's highest incidence of VT insertions. We found that older siblings in the household, children with persistent wheeze or with a family history of middle ear disease has the highest risk of treatment with VT. In addition, we found an association between antibiotic intake in pregnancy and an increased risk of both OM and VT in the offspring. Prescribing antibiotics during pregnancy must be under careful consideration to avoid unnecessary treatments and possible long-term consequences for the child. We found no long-term consequences of MEE on the neurological development in early childhood. Altogether, a more conservative approach with watchful waiting for children with MEE before VT insertions seems recommendable.

Future research

As Denmark hold the world record in treatment with VT, it would be reasonable to set up a RCT for children before VT insertions. It would be interesting to investigate the effect on less examined factors as sleep quality and duration, quality of life for the family and days home with sick children because of OM before and after treatment with VT. A RCT could compare treatment after 3 months of MEE with 6-9 month of watchful waiting before insertion of VT. Recommendations from the guidelines with vague evidence must be evaluated in a Danish setting in future research projects for instance treatment with VT for recurrent acute OM episodes with and without MEE as the quality of the evidence is low and sparse.

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Paper I

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Incidence and Determinants of Ventilation Tubes in Denmark

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Abstract

Background and objectives

Many children are treated for recurrent acute otitis media and middle ear effusion with ventilation tubes (VT). The objectives are to describe the incidence of VT in Denmark during 1997–2011 from national register data, furthermore, to analyze the determinants for VT in the Copenhagen Prospective Studies on Asthma in Childhood₂₀₁₀ (COPSAC₂₀₁₀) birth cohort.

Methods

The incidence of VT in all children under 16 years from 1997–2011 were calculated in the Danish national registries. Determinants of VT were studied in the $COPSAC_{2010}$ birth cohort of 700 children.

Results

Nationwide the prevalence of VT was 24% in children aged 0 to 3 three years, with a significant increase over the study period. For all children 0–15 years, the incidence of VT was 35/ 1,000. In the VT population, 57% was male and 43% females. In the COPSAC₂₀₁₀ birth cohort, the prevalence of VT during the first 3 years of life was 29%. Determinants of VT were: maternal history of middle ear disease; aHR 2.07, 95% CI [1.45–2.96] and siblings history of middle ear disease; aHR 3.02, [2.11–4.32]. Paternal history of middle ear disease, presence of older siblings in the home and diagnosis of persistent wheeze were significant in the univariate analysis but the association did not persist after adjustment.

Conclusion

The incidence of VT is still increasing in the youngest age group in Denmark, demonstrating the highest incidence recorded in the world. Family history of middle ear disease and older siblings are the main determinants for VT.



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Abbreviations: VT, Ventilation Tubes; AOM, Acute Otitis Media; COPSAC₂₀₁₀, COpenhagen Prospective Study on Asthma in Childhood₂₀₁₀; ENT, Ear-Nose-Throa; HR, Hazard Rati; Cl, Confidence interval; SE, Standard Erro; PCV, Pneumococcal Conjugated Vaccine.

Introduction

Otitis media is a common infection in early childhood and a common cause of children's health care utilization, partly from ventilation tube (VT) insertion [1]. Denmark did not have an official guideline for ventilation tube insertions until 2015. Now the indications are similar to the US guideline that recommend VT when middle ear effusion is persistent for over 3 months and accompanied by either documented hearing loss (>20 dB) or speech, language, learning, balance problems, ear discomfort, or reduced quality of life among others [2,3]. Recurrent acute otitis media is defined as either a minimum of three episodes of acute otitis media (AOM) in a six months period or more than three episodes in a year [4,5]. VT insertion is not recommended for children with recurrent AOM without middle ear effusion [2]. Benefits of VT must be balanced against the associated risk of complications including the general anesthesia [6–8].

A meta-analysis on risk factors for otitis media has shown that upper airway tract infections, low social status and attending daycare increases the risk of otitis media, and also a borderline significant association between increased otitis media and both male sex and living with siblings [9]. To our knowledge no former analysis of determinants of VT insertions have been performed. Previous studies have focused on risk factors for otitis media.

The rate of VT insertions varies greatly across developed countries, and does not directly reflect socioeconomic affluence [10-16]. The incidence is under 10/1000 in United Kingdom, Canada and United States but over 30/1000 in Iceland and Denmark, S1 Fig.

The aim of this study is to analyze the rate of VT insertions in Denmark over time and compare with other countries. Furthermore, we will utilize data from the Copenhagen Prospective Studies on Asthma in Childhood₂₀₁₀ (COPSAC₂₀₁₀) birth cohort [17] to analyze determinants for VT in Danish children.

Methods

This is a 2-step study design where we use Danish registry data to calculate and describe the development of the incidence of ventilation tube insertions. Furthermore, we use data from the COPSAC₂₀₁₀ cohort study to analyze determinants of ventilation tubes.

Ethics

The registry-based study was performed on existing data in national registries and was approved by the Danish Data Protection Agency (J.no. 2012-41-0388). Since subjects were not contacted as part of the study, written informed consent was not required. The COPSAC₂₀₁₀ cohort study was performed according to the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (2008-41-2599), and written informed consent was obtained from all families.

Study Population

Registry data. The study includes the total number of Danish children who had VT between 1st January 1997 and 31st December 2011 in either hospital setting or primary care. The National Patient Registry contains data on all hospital contacts linked with a unique personal identification number (assigned by the Danish Civil Registration System to all people with permanent residency in Denmark), dates of admission and discharge and diagnoses at discharge classified according to the international classification of diseases (ICD-10) [18,19]. Cases of VT insertions were identified as children below 16 years with the ICD-10 diagnosis: KDCA20. Information regarding procedures performed in the private Ear-Nose-Throat
(ENT) surgeon clinics was obtained from The National Health Insurance Service Registry [20]. The VT procedure codes extracted were 3009 and 3109, but to calculate the number of children having VT only the code for the first ear was used (3009).

To calculate the incidence of ventilation tubes, we used the number of children in Denmark each year from 1997–2011.

COPSAC₂₀₁₀ **cohort.** COPSAC₂₀₁₀ is a birth cohort of 700 children [17]. Mothers were recruited during pregnancy and children were included at one week of age and followed prospectively in our research unit for thorough clinical phenotyping. The children have currently been followed by the study pediatricians with scheduled visits at 1 week, 1, 3, 6, 12, 18, 24, 30, 36, 48, 60 months.

Determinants of ventilation tube insertion. Information regarding maternal age at birth, maternal smoking during pregnancy, maternal asthma, delivery method, gestational age, birth weight, sex and older children in the household was obtained by personal interview at the 1 week visit in the COPSAC clinic. Older siblings include biological siblings and half siblings, with primary address in the home of the child. Days solely breastfed and age at beginning of daycare were obtained longitudinally from clinical interviews during the first year of life. Information regarding the household income was obtained at the 2-year visit in the clinic and data from the child's 1-year birthday until 2 years was used to avoid the year the mother had been on maternity leave resulting in a lower income than normally. Information regarding the family members' history of middle ear disease was obtained from interviews at age 3 years. Information obtained after the 1-week visit was not acquired for all 700 children. The numbers included in the analysis are shown in Table 1.

Genetic risk score. The otitis media gene risk score was based on 21 polymorphic genetic variants found to be associated with recurrent otitis media in childhood in a large question-naire based genome-wide association study (n = 121,810) [21]. The variants were weighted according to their odds ratios, and risk alleles were summed and z-score transformed.

Genotyping of the COPSAC₂₀₁₀ **cohort.** DNA was purified from blood cells from the children and multiple single-nucleotide polymorphisms were genotyped genome-wide using the high throughput Illumina HumanOmniExpressExome bead chip platform (Illumina, Inc., San Diego, CA, USA). Genotyping was performed at AROS Applied Biotechnology AS, Aarhus, Denmark. Genotyping quality control included removal of gender mismatches, duplicates, ethnic outliers, and Hardy-Weinberg equilibrium ($p>10^{-6}$) outliers. A genotyping call rate of at least 95% was required. Correct familial relations were verified by Mendel error rates and identity-by-descent analyses. All quality control was performed using PLINK software [22,23]. The HumanOmniExpressExome chip was imputed to the 1000 genomes phase 3 imputation panels (CEU individuals) using Mach 1.0, Markov Chain Haplotyping, and IMPUTE2.

Clinical predictors. A disease diary was filled out daily by the parents from birth to 3 years of age, and validated by the research pediatricians during clinical visits, including clinical symptoms or medical treatments in three categories: lung symptoms, infections and eczema. We required completed diaries during >90% of the period to be included in the analysis. The lung symptom category have been used to capture children with recurrent wheeze episodes and who have been treated with inhaled corticosteroids according to a predefined algorithm [24]. These children were diagnosed with persistent wheeze. AOM episodes and likewise persistent middle ear effusion are indications for VT, see causality diagram S2 Fig. These factors were not used in the analysis regarding determinants of VT but to describe disease prevalence in the cohort. All AOM episodes from birth to 3 years of age were captured in the infection category of the diary.

Table 1. Determinants of ventilation tubes.

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	Participants Tubes		No Tubes	P value	Adjusted HR (95% CI)	
All	700	205	495			
Maternal-related characteristics						
Maternal age at birth, mean years (range)	698	32.3 (21.5–48.3)	32.2 (19.1–44.0)	0.9261	-	
Maternal smoking during pregnancy, % (n)	696	8% (16)	8% (38)	0.9539	-	
Maternal asthma, % (n)	697	27% (55)	26% (129)	0.7897	-	
Household income *	677			0.7333	-	
- Low % (n)	56	10% (19)	8% (37)		-	
- Medium % (n)	366	54% (108)	54% (108) 54% (258)		-	
- High % (n)	255	37% (73)	38% (182)		-	
Child-related characteristics						
Cesarean section, 151 children,% (n)	700	22% (46)	(46) 21% (105)		-	
Mean number of days exclusively breastfed, mean (range)	692	103.4 (0–255)	103.0 (0–266)	0.8660	-	
Mean GA weeks mean (range)	700	39.7 (33.0–42.3)	39.9 (29.4–42.3)	0.1429	-	
Mean birth weight kg, mean (range)	700	3.5 (1.9–5.2)	3.5 (1.3–5.0)	0.9797	-	
Sex, boys, % (n)	700	56% (111)	49%(249)	0.1117	-	
Mean days at beginning of daycare (range)	688	320 (156–946)	334 (180–1154)	0.0577	-	
Older siblings present, %(n)	700	64%(132)	53% (264)	0.0072	1.34 (0.86; 2.05)	
Season of birth, % (n)	700			0.2768	-	
Winter	215	26% (55)	32% (160)		-	
Spring	186	26% (52)	27% (134)		-	
Summer	149	22% (46)	21% (103)		-	
Fall	150	25% (52)	20% (98)		-	
Child with persistent wheeze anytime before the age of 3 years	662	25% (48)	17% (79)	0.0191	1.18 (0.79; 1.76)	
Family history of middle ear disease:						
- Paternal % (n)	615	22% (38)	11% (48)	0.0002	1.48 (0.97; 2.27)	
- Maternal % (n)	643	34% (62)	17% (79)	<0.0001	2.07 (1.45; 2.96)	
- Siblings % (n)	518	59% (90)	25% (92)	<0.0001	3.02 (2.11; 4.32)	

* Household income at age 2: Low (Below 50.000 Euro), Medium (50.000–110.000), High (Above 110.000)

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To diagnose middle ear effusion we used a flat curve at tympanometry measurement. Children were examined at age one, two and three years with a tympanometry (MT10, Interacoustics, Denmark).

Statistics

Registry data. Data from The National Patient Registry and The National Health Insurance Service Registry was combined to evaluate the total number of VT insertions in Denmark from 1997–2011. We described the development over time in the number of VT procedures and calculated the difference between the numbers of procedures in hospitals compared to private ENT-clinics. Changes over time were analyzed by linear regression analysis.

COPSAC₂₀₁₀ **data**. Univariate associations between predictors and ventilation tubes ever (0-3 years) were examined. Associations between categorical variables and VT insertions were analyzed by chi-squared tests or Fisher's exact test where expected counts in any cells were less than 5. Household income was analyzed with a Cochran-Armitage trend test. Normal distributed continuous variables were tested with t-test. Variables not normally distributed were analyzed using Wilcoxon rank-sum tests. Univariate significant determinants of VT insertions

were thereafter analyzed in Cox proportional hazards regression models to adjust for all other significant determinants. All adjusted results were expressed as hazard ratios (HR) with 95% confidence intervals (CI). A significance level of 5% was used in all analysis. Missing data were not imputed. R version 3.2.3 [25] was used for calculating genetic risk scores. SAS version 9.4 for Windows (SAS Institute Inc., Cary, NC, US) was used for all other analyses.

Results

Registry data

The total number of person years for children 0–15 years of age from 1997–2011 was 15.593.538. The average number of children living in Denmark was 1,039,569 in the study period. The number of children <16 years having VT from 1997–2011 was 288,224 and several of the children received VT more than once, which made the total number 514,575 of VT insertions. The average number of VT insertions was 36,196 annually.

Most children have VT insertions in early childhood (Fig 1). The national prevalence of VT in the first 3 years of life was 24% in children born in 2009. For all Danish children under 16 years, the incidence was 35/1,000. During the observation period, there has been an overall increase in VT insertions (Fig 2). Especially in the children aged 0–3 years the number of VT





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Incidence of ventilation tubes in Denmark



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insertions increased from 62/1,000 to 108/1,000, 43% increase, corresponding to an increased by 2.94 per year, SE 0.27, p<0.0001. There was a sex difference. Boys more often received VT compared to girls, respectively 57% and 43%. Among children having VT insertions, 53% received tubes one time, 24% two times, 11% three times and 12% of the children had VT more than 3 times. The hospitals account for 4% of the procedures and the private ENT-clinics for 96% (Fig 3).

COPSAC₂₀₁₀ cohort

The mean age of the first VT insertion was 16 months (SD 4.9). The youngest child was 5.8 months old. The accumulated prevalence of VT was 5% (n = 37) after one year, 22% (n = 151) after 2 years, and 29% (n = 205) after 3 years, very similar to the national prevalence of VT.

AOM episodes in the first 3 years of life among children in the cohort were captured in the daily disease diary; 74% (N = 515) of the cohort had information of AOM episodes for >90% of the first 3 years of life. Of these, 67% (n = 346) had at least one AOM episode from age 0–3 years.

Flat tympanometry curves was found in 52% (292/563) at the 1 year clinic visit, 37% (221/595) at the age of 2 years, and 27% (n = 163/454) at the 3 years visit. In total, 66% (n = 454/614) of the children had middle ear effusion at least once before the age of 3.



Fig 3. Number of ventilation tubes placed in private ENT-clinics compared to hospitals.

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Determinants of ventilation tube insertion

A main determinant for VT insertion was family history of middle ear disease. Maternal history persisted in the adjusted analysis; HR 2.07, [1.45–2.96] and likewise history of middle ear disease among siblings; HR 3.02, [2.11–4.32] (Table 1). Older children in the home were also associated with a higher risk of VT; OR 1.58, 95% CI, [1.13–2.21], p = 0.0072, but not in the adjusted analysis. A diagnosis of persistent wheeze was also associated with a significant higher risk of VT insertions, OR 1.62, 95% CI, [1.08–2.43], p = 0.0191, but the association did not persist in the adjusted analysis. On average the children who received VT before the age of 3 had started 14 days earlier in daycare compared to children without VT, p = 0.0577. There was no association between sex and VT in the COPSAC cohort and no associations were found between VT and maternal age, maternal smoking during pregnancy, household income, maternal asthma, delivery by cesarean section, length of exclusive breast feeding, gestational age, birth weight, season of birth.

Using a genetic risk score, we did not observe a significant association between this risk score and time to receiving VT before the age of 3; HR 0.97, 95% CI [0.84–1.12], p = 0.68.

Discussion

Main findings

The annual incidence in children during the observation period 1997–2011 is to our knowledge the highest incidence reported from any country and without obvious reasons. Children in the COPSAC₂₀₁₀ birth cohort had a period prevalence of VT of 29% before the age of 3, comparable to the national prevalence of 24% in the same period. Determinants for VT insertion were AOM, family history of middle ear disease and older children in the home.

Strengths and limitations

The Danish registries are unique because they contain information regarding all procedures and contacts with the health care system linked to personal identification numbers for the complete population. Thereby all VT insertions in Denmark, both procedures performed in the hospital setting and in ENT-clinics could be included in the analysis. The registries do not contain any information of the VT indication, so we were not able to evaluate whether all children fulfilled the criteria for VT insertion.

The COPSAC₂₀₁₀ birth cohort has been followed prospectively from birth and the collected data on the 700 children has been linked to the registry data for information on all VT procedures. This is a robust outcome compared to former studies that have used either recurrent AOM or middle ear effusion with more varied and unclear definitions.

The COPSAC₂₀₁₀ data contains information of several exposures in early life and prospective disease diaries which provide information on all AOM episodes during the first 3 years of life. It is a unique dataset because of the difficulty in getting accurate information regarding small children's diseases. In addition, we obtained annual tympanometry measurement, though this is limited by the nature of a spot measurement, and children may have experienced middle ear effusion between the examinations. However; we captured middle ear effusion among a high percentage of the children. As the majority of children experience middle ear effusion during their first three years of life, this could almost be considered a natural phenomenon. We observed a difference between sex regarding VT and probably non significant because of lack of power. This is a limitation, but the sex difference for otitis media has been described in other studies [9].

Interpretation

The incidence of VT in Denmark is the highest compared to available data from other developed countries [10–15,26] (S1 Fig) and with a continuing increase over the study period (Fig 2).

In the United States 6.8% of children have had VT before the age of 3 associated with their insurance status and access to treatment [2]. In the United Kingdom otitis media prevalence decreased after the introduction of the Pneumococcal Conjugated Vaccine (PCV) vaccine in the national vaccine program [27]. PCV vaccine was introduced in 2007 in Denmark yet the VT rate has only been increasing since (Fig 2).

We would expect the Danish population to be comparable to especially the other Nordic countries with similar disease prevalence. Still, very different incidences of VT insertions are observed (S1 Fig). The Danish population has free access to ENT specialists like in Island and they have a similar incidence of VT. [28,29]. In Sweden, the general practitioner is gate keeper with referral being needed to be examined by an ENT surgeon and have a 3–4 fold lower incidence of VT [30].

Children receive VT for either middle ear effusion or recurrent AOM. 67% of all the COP-SAC children had had at least one AOM episode before the age of three and AOM was associated with a significantly increased risk of VT. We found that middle ear effusion was present at one, two and three years of age among 51%, 37% and 27% respectively. We found that 66% of the children had middle ear effusion at minimum one of the annual measurements, but this was not found significantly associated with VT. Middle ear effusion could be considered a natural phenomenon [31] with a high prevalence in early childhood and is not alone an indication for VT insertion. Middle ear effusion can cause impaired hearing and ventilation tubes leads to a small hearing improvement [32], however, the overall beneficial effects of VT are debated. A randomized controlled trial concluded no long term beneficial effects of ventilation tubes on language, speech or cognitive development [33–35], though children with middle ear effusion and a delayed speech development might benefit from VT insertion [32,36–38]. Frequent infections and sleep problems because of otitis media may result in reduced quality of life for the child and the whole family, but the effect of VT on this is ambiguous [39,40].

Family history of middle ear disease was among the most dominant factors predicting VT insertion. It has been shown that otitis media is 40–70% heritable and recently genome-wide association study studies have been published on genetic variants influencing the proneness of otitis media [41,42]. We did not find any association between the genetic risk score and receiving VT in the COPSAC₂₀₁₀ cohort before the age of 3. This can be explained by phenotypic differences (the genetic risk score is done from a questionnaire based study, both an inherent strength and weakness) or lack of power. The genetic risk score phenotype is based on those suffering from recurrent AOM may therefore represent differences compared to risk of VT as children often receive VT for persistent middle ear effusion.

Intriguingly, this may also be indicative of the fact that many children in the COPSAC₂₀₁₀ cohort (as well as in Denmark) receive VT for other reasons than otitis media; e.g. decreased sleep quality, delayed speech development or decreased quality of life.

Other determinants of VT included older children in the home and early start in daycare. These factors are both accompanied by increased exposure to pathogens and infections early in life. In Denmark, children are typically attending daycare centers within the first year of life. Our study is consistent with findings in previous literature. A study from the United States also found that daycare attendance was a risk factor of VT [43], and a recent meta-analysis analyzing risk factors of otitis media found association to both siblings and attending daycare [9].

Children diagnosed with persistent wheeze had a higher rate of VT insertions. The association between recurrent upper airway infections and middle ear effusion have been shown in other studies [9,44], and it is probably due to a higher propensity for pathogen colonization in the airways in general in these children [45].

It has previously been shown that males suffer from more AOM than girls [46]. This was confirmed in the data from the national registries with a ration of approx. 3:2.

Children in the COPSAC₂₀₁₀ cohort had a slightly higher prevalence of VT insertion, 29% compared to 24% in the national registers among children born in the same year as the COP-SAC₂₀₁₀ cohort. This higher prevalence could be explained by the close follow-up of the COP-SAC₂₀₁₀, where children with middle ear effusion at the yearly tympanometry measurements were recommended to get an appointment at an ENT-clinic.

Conclusion

In Denmark, we have the highest incidence of VT insertions reported worldwide with a continuing increase. This suggests that health-care practices rather than evidence-based guidelines determine the use of VT. Heritability, children with persistent wheeze and older siblings in the home significantly influence the risk of VT. The determinants of VT are comparable to previous studies on either AOM or middle ear effusion, but this is the first study using VT as outcome.

Supporting Information

S1 Fig. The incidence of ventilation tubes in Denmark compared to other countries. The overall incidence of ventilation tubes in Denmark of 35/1000 for children 0–15 years of age compared to incidences in other developed countries published in the literature [10–15,28,29]. (PDF)

S2 Fig. Causality diagram. (PDF)

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Author Contributions

Conceptualization: TMP JS ARCMJ HB.

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Paper II

Antibiotics in Pregnancy Increase the Children's Risk of Otitis Media and Ventilation Tubes

Antibiotics in Pregnancy Increase the Children's Risk of Otitis Media and Ventilation Tubes

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ABSTRACT

Objectives

To study the association between antibiotic intake in pregnancy and the development of otitis media and ventilation tubes in the offspring under the hypothesis that antibiotics in pregnancy may alter the offspring's propensity for disease.

Methods

Data from the 700 children in the Copenhagen Prospective Studies on Asthma in Childhood₂₀₁₀ unselected birth cohort study were used. Information on maternal antibiotic use and other exposures during pregnancy was collected prospectively from interviews and validated in national registries. otitis media episodes were registered in a prospective diary for 3 years. Information regarding children's ventilation tubes was obtained from national registries.

Results

514 children had diary information and were included in the analysis regarding otitis media episodes. For ventilation tubes analysis 699 children were included. 37% of the mothers received antibiotics during pregnancy, and this was associated with increased risk of otitis media; aHR 1.30, 95% CI, [1.04-1.63], p=0.02. The risk of receiving ventilation tubes was especially associated with third trimester antibiotics; aHR 1.60, 95% CI [1.08-2.36], p=0.02. The risk of otitis media increased with increasing number of treatments; per level aHR 1.20 [1.04-1.40], p=0.02 but for ventilation tubes this association was not significant after adjustment.

Conclusion

Maternal antibiotics during pregnancy associates with increased risk of otitis media and ventilation tube insertions in the offspring. Antibiotics late in pregnancy mainly

contributed to these effects pointing towards potential transmission of an unfavorable microbiome from mother to child.

Abbreviations:

 $COPSAC_{2010} = COpenhagen$ Prospective Studies on Asthma in Childhood₂₀₁₀

- aHR = Adjusted hazard ratio
- CI = Confidence interval
- OM = Otitis media
- VT = Ventilation tubes
- RTI = Respiratory tract infection
- UTI = Urinary tract infection
- AB = Antibiotics

INTRODUCTION

The consumption of antibiotics is increasing worldwide, causing concern in the medical community due to increasing prevalence of bacterial antibiotic resistance and numerous potential long-term adverse health effects (1–3). Often infections are more serious if contracted in pregnancy, both for the mother and the fetus, in part due to a suppressed maternal immune response (4,5). These infections and related complications are potentially preventable with antibiotic treatment (6), leaving the prescription of antibiotics during pregnancy a complex clinical decision. Antibiotics may perturb the maternal bacterial colonization (7,8), and a subsequent unfavorable maternal bacterial ecology may trigger lasting disease processes in perinatal life in susceptible offspring or affect the earliest colonization of the child by vertical transmission (9,10).

Otitis media (OM) is one of the most common infections in early childhood (11). Middle ear effusion and recurrent acute otitis media are often treated with ventilation tubes (VT). Some aspects of the pathogenesis of OM are well described, including the anatomic, genetic and infectious microbiological components (12–17), while others remain obscure, including environmental triggers of disease susceptibility. The objective of this study was to analyze the effect of maternal antibiotic intake during pregnancy on the risk of OM and VTs in early childhood. We hypothesized that antibiotic consumption in pregnancy can increase the child's risk of OM, and examined possible mechanisms by analyzing effects of treatment in different pregnancy trimesters, number of treatments and furthermore treatment after birth.

METHODS

Study population

The Copenhagen Prospective Studies on Asthma in Childhood 2010 cohort (COPSAC₂₀₁₀) (18) is an ongoing unselected birth cohort where mothers were recruited at pregnancy week 24 in 2008-2010. It is a single-center study. Exclusion criteria were maternal chronic cardiac, endocrine, renal or pulmonary disease other than asthma. Seven hundred children were included at one week of age and were followed prospectively by study pediatricians with scheduled visits at 1 week, 1, 3, 6, 12, 18, 24, 30 and 36 months.

Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and was approved by the Local Ethics Committee (H-B-2008-093), and the Danish Data Protection Agency (2015-41-3696). Both parents gave written informed consent before enrolment.

Primary Exposure: Maternal Antibiotics during Pregnancy

Detailed information on maternal antibiotic use during pregnancy and one year after birth was collected prospectively from The Danish National Prescription Registry (19), which records all prescription drugs purchased at Danish pharmacies, and linked to the recipient's unique personal identification number assigned by the Danish Civil Registration System (20). Intake of collected medication was validated by interviews with participants. Treatments administered in hospitals or abroad were obtained through the interviews. Oral antibiotic use during pregnancy and in the first year after birth was analyzed as a dichotomized variable (yes/no), and for the pregnancy exposure also as categorized by the most likely treatment indication based on ATC (Anatomical Therapeutic Chemical) code; Respiratory Tract Infection (RTI) antibiotics (J01CAxx excl. J01CA08, J01CExx, J01FAxx, J01CRxx) and Urinary Tract Infection (UTI) antibiotics (J01CA08, J01EBxx, J01XExx). Other antibiotics, which could not be categorized into one of these groups, were excluded due to low numbers. Analyses were further stratified by treatment trimester; first trimester was defined as the first 14 weeks of gestation, second trimester as 15th-26th weeks of gestation, third trimester as 27th week of gestation until birth. Numbers of treatments were analyzed as 0, 1, or 2 or more treatments to investigate possible dose-response effects.

Otitis Media

All episodes of OM were registered in a structured daily symptom diary from birth to 3 years of age. Children with >90% valid diary information during the first three years of life were included in the analyses. An episode was defined as one or more concurrent days of parental registration of OM and the first day registered in the child's first episode was used in the survival analysis. OM diagnoses were verified by interviews at clinic visits and held up against antibiotic treatments with information from registers. The children were not seen in our research clinic with acute ear infections, but diagnosed by either an ear-nose-throat specialist or their general practitioner. The OM diagnosis therefore corresponds to doctor diagnosed acute episodes of middle ear infection.

Ventilation Tubes

Information on all ventilation tube insertions in the first 3 years of life were extracted from two national registries; The Danish National Patient Registry using the

International Classification of Diseases 10th revision [ICD-10] code KDCA20 for all procedures performed at hospitals, and The Danish National Health Service Registry using procedure code 3009 for all procedures performed in community otorhinolaryngology practices (21,22). The date of the first ventilation tube insertion was used in the survival analysis.

Covariates

Information regarding maternal asthma (doctor diagnosed), any maternal smoking during pregnancy (yes/no), older siblings in the household (defined as both biological and half siblings, with primary address in the home of the child), maternal age, maternal educational level (low (elementary school or college graduate), medium (tradesman or medium length), high (university)) and household income (low (below 50.000 euro), medium (50.000-110.000), high (above 110.000)) was obtained by personal interview at the 1-week clinical visit in the COPSAC research unit. Information regarding maternal and paternal history of middle ear disease was obtained from interviews at age 3 years.

Statistics

Baseline characteristics describing women with antibiotic use in pregnancy were analyzed by chi-squared test for dichotomized variables. Normally distributed continuous variables were tested using Student's t-test. Variables not normally distributed were analyzed using Wilcoxon rank-sum test. The effect of maternal antibiotic use on age at first OM episode or ventilation tube insertion was quantified in terms of hazard ratios by Cox proportional hazards regression. The children were retained in the analysis from birth until age of event or age 3 years, whichever came

first. Possible confounders were chosen from results based on factors influencing maternal intake of antibiotics during pregnancy, Table 1. Additionally, we also adjusted for older siblings and family history of middle ear disease a priori, since these factors are known to affect the risk of OM and VTs (13,23,24). The stratified analyses were also performed using Cox proportional hazard regression. No interaction was calculated. A significance level of 5% was used in all analyses. SAS version 9.4 for Windows (SAS Institute Inc., Cary, NC, US) was used for all data analysis.

RESULTS

Information on VT insertions was obtained from registries and available for all 700 children in the cohort, and information on maternal antibiotic intake during pregnancy was available for 699 children. The prevalence of any maternal antibiotic use during pregnancy was 37% (n=256). In the first year after birth 41% (n=283) of the mothers were treated with antibiotics. The incidence of VT insertions before 3 years of age was 29% (n=205).

A total of 73% (n=514) of the children had full follow-up and >90% valid diary information in the first three years of life and was included in the analyses of OM, 67% (n=346) had at least one OM episode in this period. We compared the children with adequate diary information to those that did not participate in the OM analysis and found that only maternal smoking during pregnancy was significantly different between these groups. There were fewer non-Caucasians, low educated mothers and families with lower income in the group of children with adequate diary information; but the differences were not significant (Table 1, online). The percentages of mothers treated with antibiotics did not change much in the OM analysis even though the number was lower than in the VT analysis. We found no difference in the prevalence of antibiotics administration between mothers of children with full diary information and incomplete diary information regarding maternal antibiotic intake (p = 0.83). Potential predictors for antibiotic use in pregnancy are described in Table 2. Maternal educational level demonstrated a trend of association with antibiotic use (p=0.08), therefore all analyses were adjusted for this as well as older siblings and parental history of middle ear disease. We also analyzed the effect of being born pre-term and of breastfeeding

duration, but we found no association between these factors and the risk of OM or VT in our cohort.

Maternal Antibiotic Use and Risk of Acute OM

Maternal antibiotics during pregnancy was associated with an increased risk of OM in the child during the first 3 years of life; adjusted hazard ratio (aHR) 1.30, 95% CI, [1.04-1.63], p=0.02. Overall 74% (n=138) had OM before the age of three if the mother had received antibiotics in pregnancy compared to 64% (n=208) if the mother had not been treated. In children born to mothers treated in the third pregnancy trimester 77% (n=56) had OM, compared to 66% (n=290) if the mother had not been treated; though non-significant after adjustment; aHR 1.34 [0.98-1.83], p=0.06. In children born to mothers treated in the second pregnancy trimester 78% (n=67) had OM, compared to 65% (n=279) if the mother had not been treated; aHR 1.40, 95% CI, [1.06-1.85], p = 0.02. We found no association between treatment in first trimester and risk of OM. In children born to mothers treated with RTI antibiotics in pregnancy 81% (n=83) had OM, compared to 64% (N=263) if the mother had not been treated; aHR 1.45, 95% CI, [1.11-1.89], p = 0.006. There was no effect of UTI antibiotics on the risk of OM. We observed no effect of antibiotic treatment in the year after pregnancy, nor when restricting the analysis to those mothers, who did not receive any antibiotics in pregnancy (Table 3).

Maternal Antibiotic Use and Risk of VTs

Maternal antibiotic use in pregnancy was likewise associated with an increased risk of VT in the children during the first 3 years of life, though not significant in the overall analysis; aHR 1.14, 95% CI, [0.83-1.56], p = 0.42. This corresponds to 34% (n=87) of

children had VT if mothers had been treated with antibiotics and 27% (N=118) had VT if the mother had not been treated. In children born to mothers treated in the third pregnancy trimester 46% (n=42) had VT, compared to 27% (n=163) if the mother had not been treated; aHR 1.60, 95% CI, [1.08-2.36], p=0.02 (Figure 1). We found no associations between treatment in first or second trimester and risk of VT. In children born to mothers treated with UTI antibiotics in pregnancy 39% (n=57) had VT, compared to 27% (n=148) if the mother had not been treated; aHR 1.56, 95% CI, [1.10-2.19], p = 0.01. No significant association found to RTI antibiotics. Again, we found no effects of post pregnancy treatment in the mother on the risk of VT in the children (Table 3).

Dose-Response Relationship

We observed a dose-response relationship between the number of antibiotic treatments during pregnancy and the risk of OM; per-level aHR 1.20, 95% CI, [1.04-1.40], p=0.02. The risk of VT was also increased before adjustment (Figure 2) but the effect was not significant after adjustment; per-level aHR 1.15, 95% CI, [0.94-1.41], p=0.17.

Stratification

Stratification for mode of delivery resulted in higher risk of both OM and VT after pregnancy antibiotics in children with vaginal delivery, though not significant. OM: caesarean section; aHR 1.23, 95% CI, [0.95-1.59], p=0.11, vaginal delivery; aHR 1.70, 95% CI, [1.01-2.89], p=0.05. VT: caesarean section; aHR 0.96, 95% CI, [0.67-1.38], p=0.81, vaginal delivery, aHR 1.97, 95% CI, [0.97-3.98], p=0.06.

DISCUSSION

Primary Findings

Maternal antibiotic intake during pregnancy was associated with an increased risk of OM and VT in early childhood, especially after treatment in the third trimester. We furthermore observed a dose-response relationship with an increased risk of OM with increasing number of treatments.

Strengths and Limitations

The COPSAC₂₀₁₀ birth cohort has been followed prospectively from birth and the collected data on the 700 children has been linked to the registry data for information on all maternal antibiotic prescriptions and the children's ventilation tube procedures. The Danish registries are unique because they contain information regarding all procedures, contacts and medical prescriptions within the health care system linked by a personal identification number. Maternal antibiotic intake during pregnancy was obtained from registries, but validated and extended at interviews with the mother to minimize recall bias and to avoid including antibiotics collected at the pharmacy but not administered to the mother. In the COPSAC₂₀₁₀ cohort 29% received VT before the age of 3. This is a high prevalence; but it is a known phenomenon in Denmark (11).

The COPSAC₂₀₁₀ data contains information of several exposures in early life and the prospective diaries provide information on all OM episodes during the first years of life. The longitudinal assessments from birth assure robust clinical endpoints and improved statistical power from the time of first OM and VT onset clearly distinguishing the populations. It is a unique dataset since it is difficult to get accurate information regarding small children's disease type and frequency. Some parents did not manage to

fill out the diary everyday for 3 years which caused 186 children to be excluded from the OM analysis. The differences between the children in the analysis and in the dropout group is compared in Table 1, online. It could weaken our OM endpoint that not all children had full follow up; however, we had full data on VT.

High intake of antibiotics could be caused by a disease propensity in the mother, which the child would likely inherit, or it could be due to maternal doctor-seeking behavior, which would also be conferred to the child. To account for this, we performed the analyses of maternal intake of antibiotics the year after pregnancy in relation to the clinical outcomes, both with and without the mothers who also received antibiotics in pregnancy. Especially the analysis of mothers receiving antibiotics the year after pregnancy but not in pregnancy with no such associations suggest a pregnancy effect of antibiotics and that these factors are not confounding the association between mothers antibiotic use in pregnancy and OM or VT in the young child.

The study is observational, and we cannot claim causality of the antibiotics, however the stratification in pregnancy trimesters and in the dose-response relation from antibiotics as well as mode of delivery allows us to speculate in possible mechanisms.

Interpretation

The risk of OM was increased in children born to mothers who had been exposed to antibiotics in pregnancy; however, this effect was not present after treatment in the first trimester and with no effects of treatment after birth. The effect of pregnancy antibiotics on the risk of VT was especially driven by treatment in third trimester, without significant effects of treatment in other pregnancy trimesters or after birth. Therefore, we suggest a possible mechanism must be sought in pregnancy. We previously reported associations between maternal antibiotic intake during pregnancy and risk of childhood

asthma (25). However, in a follow-up study in national registry data it appeared that both antibiotics before, during and after pregnancy was equally associated with the child's asthma risk, and therefore maternal antibiotic use was interpreted as a marker of disease propensity, which was inherited by the child rather than a causal agent for asthma itself (26). In the present study, we found no association between maternal antibiotic intake the year after pregnancy and the child's risk of neither OM nor VT especially when looking at the association between treatment the year after and not having received any antibiotics during pregnancy. This suggests that the associations are not just an expression of maternal disease susceptibility or a doctor-seeking behavior transferred to the child.

Overall, the effect was isolated to treatment during pregnancy, especially in the third trimester of the pregnancy period. Antibiotics can alter the composition of the microbiome and a disturbance in the maternal bacterial ecology would presumably have the greatest impact on vertical transmission if occurring shortly before birth (7). Thus, after antibiotic treatment the newborn child may receive a different and perhaps non-favorable composition of microbiota during birth, which could enact a wide range undesirable of metabolic and immunologic effects, ultimately leading to disease (27,28). We stratified the analyses for mode of delivery, as the vertical bacterial transmission can be interrupted, when giving birth by caesarean section. We found that children with vaginal delivery were affected more by the maternal antibiotic intake compared with children born by caesarean section. This could indicate that microbial alterations caused by the antibiotics in the mother lead to the increased disease susceptibility by vertical transmission of an unfavorable composition during birth. The dose-response relationship as well as the equal effects of RTI and UTI antibiotics point toward a

microbial mechanism rather than an inherited propensity for airway infections, which could have been an alternate explanation for the observed associations. We found that the risk of OM was associated primarily with the use of RTI antibiotics, but not significantly with UTI antibiotics, although the estimate was increased, and that the risk of VT was associated with UTI antibiotics and not significantly with RTI antibiotics. This divergence could indicate differences in the immune phenotypes associated with these two conditions. An explanation could be that different types of antibiotics favor different microbial profiles that may each modulate the immune system in different ways towards susceptibility to either acute or chronic middle ear disease. Antibiotic treatments are an indicator of maternal infection and the triggered immune response and following inflammation during pregnancy may affect the fetus (29). This could also explain the stronger effects of antibiotic treatment in the end of pregnancy, as the fetus' own immune system especially develops in the later part of pregnancy (30). The pregnant woman's immune system is suppressed during pregnancy to prevent rejection or mounting an inappropriate immune response against the child (31). Some infections are particularly dangerous during pregnancy because they can harm the fetus (4).

Prescribing antibiotics during pregnancy presents a challenge to the physician because some infections indeed warrant treatment, while protecting the fetus against possible side effects still remains a high priority (32). Long-term health effects in the child may further warrant caution, when prescribing antibiotics to the pregnant woman.

Conclusion

Maternal antibiotic intake during pregnancy was associated with an increased risk of OM and VTs in early childhood. The timing of the intake suggests a mechanism mediated by a disturbed vertical transmission of microbiota between mother and child. Care must be taken to avoid unnecessary or inappropriate treatments due to potential long-term consequences for the child.

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	All	Antibiotics	P Value			
		Yes	No			
All % (N)	100% (699)	37% (256)	63% (443)	-		
Maternal age at birth, mean (SD)	32.3 (4.3)	32.2 (4.7)	32.3 (4.2)	0.67		
Asthma history % (N) ^a	26% (184)	30% (76)	24% (108)	0.13		
Smoking in pregnancy % (N)	8% (54)	10% (25)	7% (29)	0.12		
Older children % (N)	57% (395)	61% (155)	54% (240)	0.10		
Maternal educational level, % (N) ^b						
Low	26% (184)	29% (74)	25% (110)			
Medium	46% (322)	48% (124)	45% (198)			
High	28% (193)	23% (58)	30% (135)			
Household annual income, % (N) ^c						
Low	10% (67)	12% (31)	8% (36)			
Medium	53% (370)	54% (138)	52% (232)			
High	37% (261)	34% (87)	39% (174)			

Table 2. Predictors of maternal antibiotic intake during pregnancy

^a History of maternal doctor diagnosed asthma

^b Low (elementary school or college graduate), Medium (tradesman or medium length), High (university) ^c Low (Below 50.000 Euro), Medium (50.000-110.000), High (Above 110.000)

	ОМ	Unadiusted		Adiusted		VT	Unadiusted		Adiusted	
		HR	Р	HR	Р	. –	HR	Р	HR	Р
	N=514	[CI] ^d	Value	[CI]	Value	N=699	[CI]	Value	[CI]	Value
Maternal AB										
in	187	1.36		1.30		256	1.32		1.14	
Pregnancy	(37%)	[1.10-1.69]	0.005	[1.04-1.63]	0.02	(37%)	[1.00-1.74]	0.05	[0.83-1.56]	0.42
RTI ^e	103	1.56		1.45		142	1.33		1.09	
antibiotics	(20%)	[1.22-1.99]	<0.001	[1.11-1.89]	0.006	(20%)	[0.97-1.83]	0.08	[0.76-1.58]	0.64
UTI ^f	112	1.22		1.26		148	1.55		1.56	
antibiotics	(22%)	[0.95-1.56]	0.12	[0.97-1.63]	0.09	(21%)	[1.14-2.10]	0.005	[1.10-2.19]	0.01
1. trimester	88	1.19		1.16		124	1.17		1.14	
AB	(17%)	[0.90-1.55]	0.22	[0.87-1.54]	0.30	(18%)	[0.83-1.65]	0.36	[0.78-1.68]	0.50
2. trimester	86	1.43		1.40		114	1.55		1.38	
AB	(17%)	[1.10-1.87]	0.01	[1.06-1.85]	0.02	(16%)	[1.12-2.15]	0.009	[0.94-2.02]	0.10
3. trimester	73	1.43		1.34		92	1.96		1.60	
AB	(14%)	[1.08-1.91]	0.01	[0.98-1.83]	0.06	(13%)	[1.40-2.75]	<0.001	[1.08-2.36]	0.02
3.trimester	37	1.93		1.84		49	2.32		1.56	
RTI	(7%)	[1.34-2.76]	<0.001	[1.24-2.74]	0.003	(7%)	[1.54-3.50]	<0.001	[0.95-2.56]	0.08
3.trimester	38	1.05		1.06		46	1.61		1.61	
UTI	(7%)	[0.70-1.58]	0.81	[0.70-1.62]	0.77	(7%)	[1.01-2.59]	0.05	[0.96-2.70]	0.07
Maternal AB										
post	203	1.20		1.15		283	1.25		1.28	
pregnancy	(40%)	[0.97-1.48]	0.10	[0.92-1.44]	0.23	(41%)	[0.95-1.64]	0.11	[0.94-1.75]	0.12
Maternal AB										
post										
pregnancy										
but not in	115	0.94		0.91		158	0.96		1.00	
pregnancy	(22%)	[0.73-1.22]	0.66	[0.69-1.19]	0.49	(23%)	[0.67-1.33]	0.78	[0.69-1.46]	0.99
Dose-		1.24		1.20			1.26		1.15	
response		[1.08-1.43]	0.003	[1.04-1.40]	0.02		[1.06-1.51]	0.01	[0.94-1.41]	0.17

Table 3. Associations between maternal antibiotics $(AB)^a$ and risk of otitis media $(OM)^b$ and ventilation tubes $(VT)^c$ in the children during the first 3 years of life.

^{*a*} AB = Antibiotics

^b OM = Otitis media

 $^{c}VT = Ventilation Tubes$

^d HR = Hazard Ratio, CI = Confidence Interval

^eRTI = Respiratory Tract Infection

^fUTI = Urinary Tract Infection


Figure 1. The risk of ventilation tubes in the child if the mother received antibiotics (AB) in third trimester.



Figure 2. Dose-response relationship between the number of maternal antibiotic treatments in pregnancy and the risk of ventilation tubes in childhood.

Supplementary material Paper II

Antibiotics in Pregnancy Increase the Children's Risk of Otitis Media and Ventilation Tubes

TABLE 1, online. Cohort baseline

	Full COPSAC ₂₀₁₀	Dropout	OM Study cohort	P Value
Ν	700	186	514	
Maternal age at birth, mean (SD)	32.3 (4.3)	32.0 (4.9)	32.3 (4.1)	0.399
Maternal asthma, % (N) ^a	26% (184)	30% (56)	25% (128)	0.163
Maternal smoking in pregnancy, % (N)	8% (54)	12% (22)	6% (32)	0.014
Caesarean section, % (N)	22% (151)	24% (44)	21% (107)	0.420
Maternal education level, % (N) ^b	-	-	-	0.375
Low	26% (184)	30% (56)	25% (128)	
Medium	46% (322)	43% (80)	47% (242)	
High	28% (194)	27% (50)	28% (144)	
Household income, % (N) ^c	-	-	-	0.310
Low	10% (67)	12% (23)	9% (44)	
Medium	53% (370)	52% (97)	53% (273)	
High	37% (262)	35% (66)	38% (196)	
Older siblings, % (N)	57% (396)	56% (104)	57% (292)	0.833
Gestational age in weeks, mean (SD)	40 (1.7)	40 (1.5)	40 (1.7)	0.473
Birth weight, mean (SD)	3.5 (0.5)	3.5 (0.6)	3.5 (0.5)	0.750
Male sex, % (N)	51% (360)	55% (102)	50% (258)	0.278
Race, caucasian, % (N)	96% (670)	94% (174)	97% (496)	0.089
Days solely breastfed, mean (SD)	103 (60)	95 (62)	106 (59)	0.082
Age at daycare start in months, mean (SD)	10.9 (3.1)	10.9 (3.4)	10.8 (2.9)	0.882

^a History of doctor diagnosed asthma

^b Low (elementary school or college graduate), Medium (tradesman or medium length), High (university)

^c Low (Below 50.000 Euro), Medium (50.000-110.000), High (Above 110.000)

Paper III

Middle Ear Effusion and Ventilation Tubes and the effect on the Neurological development in early childhood Middle ear effusion and ventilation tubes and the effect on the neurological development in early childhood

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Abbreviations:

- MEE = Middle Ear Effusion
- VT = Ventilation Tubes
- $COPSAC_{2010} = COpenhagen$ Prospective Studies on Asthma in Childhood₂₀₁₀
- ASQ-3 = Ages & Stages Questionnaires®, Third Edition
- PPCA = Proportional Principal Component Analysis
- PC = Principal Component
- PCA = Principal Component Analysis
- IQR = Interquartile range
- CI = Confidence Interval
- SD = Standard deviation

Key Points

Question: How does middle ear effusion and treatment with ventilation tubes affect children's neurological development?

Findings: In this cohort study of 700 children we found slightly lower language scores at 1-year of age in children with concurrent middle ear effusion but no long-term effects. We also found a 2-point lower cognitive score in children with ventilation tubes at the age of 2 compared to children without middle ear disease.

Meaning: Middle ear disease was not associated with any long-term effects on developmental outcomes.

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ABSTRACT

Importance: Children with middle ear effusion are often treated with ventilation tubes to alleviate potential adverse effects on their motor, language and cognitive development.

Objectives: To investigate the effect of middle ear effusion and treatment with ventilation tubes on childhood neurological development.

Design: Children participated in the Copenhagen Prospective Studies on Asthma in Childhood unselected birth cohort study, recruited in 2008-2011.

Setting: Children was followed by study pediatricians with regular visits to the research unit from pregnancy until 3 years of age.

Participants: The cohort consists of 700 children. Children with both information regarding middle ear disease and neurological endpoints were included in the analyses.

Main outcome and measure: Middle ear effusion was diagnosed using tympanometry at age 1, 2 and 3 years. Information regarding ventilation tubes from age 0-3 years was obtained from national registries. We assessed age at achievement of gross motor milestones, language scores at 1 and 2 years, cognitive score at 2.5 years and general development score at the age of 3 years using standardized quantitative tests.

Results

Middle ear effusion was associated with lower word production at 1 year: median 3, IQR [1-7] compared to healthy: median 5, IQR [2-8]), and lower word comprehension at 1 year in children with middle ear effusion: median 37; IQR [22-65]; compared to healthy: median 48, IQR [28-85]). Ventilation tubes was not associated with language development at 1 year. Children with ventilation tubes had a lower cognitive score at 2.5 years than healthy children; β -coefficient -2.04; CI [(-4.09)-0.00]; p=0.050, whereas there was no difference between children with middle ear effusion and healthy children. No differences were found between children with middle ear disease and healthy

children regarding the gross motor milestones, word production at 2 years or the general developmental score at 3 years.

Conclusion and relevance

Children with middle ear effusion were delayed in the 1-year language scores but not affected in any other neurological endpoints up to the age of 3. Our data do not support a beneficial developmental effect of ventilation tubes.

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INTRODUCTION

Neurological development including motor and language skills, and cognitive function can be affected by both genetic makeup and environmental factors^{1,2}. Early life influences play a significant role in shaping neurological development³ and may be important determinants of a child's subsequent academic achievements^{4–7}.

Otitis media is an inflammatory process within the middle ear space, presenting as either an acute infection or as middle ear effusion (MEE); a long-lasting condition with accumulation of fluid without symptoms of acute infection⁸. MEE is believed to impair children's balance and it has been speculated that prolonged MEE in early childhood can affect motor development^{9,10}. Furthermore, MEE decreases the mobility of the tympanic membrane, which can result in a mild to moderate conductive hearing loss¹¹ and potentially delay language development^{12–14}.

Prolonged MEE is often treated with ventilation tubes (VT). Treatment with VT has been shown to increase the short-term hearing ability, but with no apparent long-term effects¹⁴. Recent studies investigating the effect of MEE on language and cognitive development have shown conflicting results^{11,15}.

The aim of this study was to investigate the associations between untreated MEE and children treated with VT compared to children with no middle ear disease. We used the neurological assessments: age at achievement of gross motor milestones, scores in language- and cognitive tests as well as general development of the children in the Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC₂₀₁₀) birth cohort¹⁶.

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METHODS

Study Population

COPSAC₂₀₁₀ is an ongoing unselected prospective birth cohort study of 738 pregnant women and their children recruited in pregnancy week 24 from 2008-2010¹⁶. The key exclusion criteria were chronic disease other than asthma or lack of fluency in Danish. Seven-hundred children were enrolled in the study at one week of age, excluding children with severe congenital abnormalities. For the present study, we excluded children with: a neurological diagnosis, as well as those born <37 weeks of gestation or with birth weights <2500 g in all analysis. The children were followed in the COPSAC research clinics with 9 planned visits during the first three year of life.

Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and was approved by the Local Ethics Committee (H-B-2008-093), and the Danish Data Protection Agency (2015-41-3696). Both parents gave written informed consent before enrolment.

Middle ear effusion and ventilation tubes

MEE was assessed using tympanometry performed by the research doctors in the COPSAC unit at scheduled visits at age one, two and three years. A flat curve was interpreted as MEE. Information regarding VT insertions was obtained from two Danish registries, The Danish National Hospital Register¹⁷, which contains all hospital records on VT insertions (ICD-10 diagnosis: KDCA20), and The Danish National Health Service Register (procedure code: 3009)¹⁸, which contains VT procedures performed in private Ear-Nose-Throat surgeon clinics. All procedures are linked with a personal identification number of the child, leaving no missing observations¹⁹.

Neurological developmental assessment

Milestones

The parents received a registration form at the 1-week clinical visit, with thorough instructions, based on The Denver Development Index²⁰ and World Health Organization milestones registration²¹. Dates of achievement of 13 predefined milestones were registered by the parents. Only the gross motor milestones: sit alone, stand with help, crawl, stand alone, walk with help and walk alone were used to analyze the association with MEE and VT because the achievement of these could be delayed by poor balance^{10,22}.

Language development

Language development was assessed with the Danish version of The MacArthur Bates Communicative Developmental Inventory²³. The assessment was performed by a web-based questionnaire filled out by parents around the child's 1-year (Words and gesticulation) and 2-year birthday (Words and sentences). From the 1-year questionnaire, we used the scores regarding word production and word comprehension. From the 2-year questionnaire, we used the word production score for the analyses.

Cognitive development

Cognitive development was assessed at 2.5 years of age, using the cognitive part of the third edition of the Bayley Scales of Infant and Toddler Development²⁴. During the examinations, the examiner presented a series of test materials to the child and observed the child's responses and behavior. Based on its performance, the child was given a composite score, which was standardized by use of a normalization material of age corrected means of 100 and a standard deviation of 15 (range 50-

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150). Examinations were performed by 10 trained examiners and inter-examiner consistency in performance was validated by inspection of video recordings.

General neurological development

The parent-completed Ages & Stages Questionnaires®, Third Edition (ASQ-3) consists of 5 categories; fine motor, gross motor, personal-social, communication and problem solving skills. Parents completed the questionnaire prior to the 3 years visit, where the responses were subsequently verified. Children received a score for each category of the ASQ-3 and we combined the 5 areas statistically and used the combined score as a general development score at the age of 3.

Covariates

Information regarding maternal age at birth, smoking during pregnancy, maternal asthma, delivery method, gestational age, birth weight, sex and older siblings in the household was obtained by personal interview at the 1 week visit in the COPSAC clinic. Duration of exclusive breastfeeding and age at beginning of daycare were obtained longitudinally from clinical interviews during the first year of life. Household income, maternal age and level of education were obtained from interviews when the children were 2 years of age and combined as a composite measure of the child's social circumstances defined as the z-scored first component of a PCA (explaining 55% of the variance).

Statistics

Differences in the baseline characteristics between children with MEE, VT and with no middle ear disease were determined by Chi-square test, t-test, or Wilcoxon rank-sum test. All variables were tested for normal distribution and models were verified by visual inspection of residual plots. For the analysis, we split the children into three groups: children who had received VT, children with

untreated MEE and children with no middle ear disease. The language data for the 1-year test was log-transformed after adding a pseudocount of 1 for the purpose of statistical analysis. Results were presented as medians with interquartile range (IQR) on the original scale. No transformation of the data was needed for the 2-year language data or the cognitive test. General linear models were used to analyze the association between untreated MEE, treatment with VT compared to children with no disease and their language- and cognitive development. As sex is a known determinant of neurodevelopment^{25–27}, all associations were adjusted for sex.

Age of milestone achievement was analyzed using a probabilistic principal component analysis (PPCA) (eFigur 1). This model included the full dataset with missing values, assuming that the missing values were missing at random. Missing data were otherwise treated as missing observations. The 5 areas of the ASQ-3 were combined in a PCA model, which was used to extract underlying principal components (PCs) that described the systematic part of the variation across the ASQ-3 score variables in fewer uncorrelated variables (eFigur 2). All the study outcomes were combined in a PCA model and analyzed for association with MEE and VT at age 1 year. A significance level of 0.05 was used in all analyses. All estimates were reported with 95% confidence intervals (CI). The data processing was conducted using SAS version 9.4 for Windows (SAS Institute Inc., Cary, NC, USA) and PCA and PPCA analyses were performed using the statistical software package R²⁸ version 3.2.1 with the package pcaMethods²⁹ and visualized using the package ggplot2³⁰.

RESULTS

Baseline Characteristics

A total of 700 children were included in the $COPSAC_{2010}$ cohort at birth and information on VT insertions was available for all 700 children. We excluded 37 children from the study because of

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neurological diagnosis, low gestational age or birth weight (Figure 1). Of the 537 children with a tympanometry measurement at 1 year 276 (51%) had MEE, at 2 years 208 of 563 children (37%) had MEE and at 3 years 153 of 578 children (26%) had MEE (eFigure 3 and 4). Before 1 year of age 35 children (5%) had VT inserted, before 2 years of age 173 children (26%) had VT inserted and before 3 years of age 192 children (29%) had VT inserted. Figure 1 illustrates how many children participated in the analysis of each neurological endpoint. Table 1 shows the baseline characteristics for children, who had either MEE or were treated with VT compared to children with no middle ear disease before the age of 3. We found more boys with both MEE and VT compared to girls. Season of birth was associated with the risk of MEE, but not VT insertion. We found that older siblings were associated with treatment with VT, but not with MEE.

Gross motor milestones

499 children (71%) had information on at least one gross motor milestone and information regarding MEE and VT before the age of 1 year (Figure 1). We used the PC1 score, which explained 64.9% of the variance in the original variables, representing the overall gross motor development, to analyze the associations (eFigure 1). There were no significant differences in the age of milestone achievement between children with either MEE, VT or children without middle ear disease (Table 2 and eFigure 1).

Language development at 1 and 2 years

Completed language assessment at 1 year and information regarding MEE and VT before the age of 1 year was obtained for 305 children (44%) (Figure 1). MEE at age 1 year was associated with a lower word production at the concurrent language assessment (median 3, IQR [1-7]), compared with no middle ear disease (median 5, IQR [2-8]), corresponding to a reduction of 24%, 95% CI [5%–39%], p=0.02. MEE at age 1 year was also associated with a lower word comprehension

(median 37; IQR [22-65]) compared with no middle ear disease (median 48; IQR [28-85]), corresponding to a reduction of 21%, 95% CI [2%-37%], p=0.03. No associations were found between children who had received VT and the 1-year language scores (Table 2). Completed language assessment at 2 years and information on MEE at 1 or 2 years or VT insertions before the age of 2 years was obtained for 496 children (71%) (Figure 1). We found no associations between MEE or VT and the 2-year language scores (Table 2).

Cognitive score

Completed cognitive test at 2.5 years and information regarding MEE at age 1 or 2 years and VT before 2.5 years was obtained for 584 children (83%) (Figure 1). VT insertion was associated with a lower composite score; adjusted β -coefficient -2.04; 95% CI [-4.09-0.00]; 0.05, (Table 2). However; there were no differences between children with MEE at either 1 or 2 years and children without disease with regards to the cognitive scores at 2.5 years.

General development assessment (ASQ-3)

Completed ASQ-3 assessment at 3 years and information regarding MEE at age 1, 2 or 3 years or VT before 3 years of age was obtained for 437 children (62%) (Figure 1). The ASQ-3 PCA resulted in a PC1 score, which explained 48.1% of the variation in the data (eFigure 2). There were no differences between children with MEE at either 1, 2 or 3 years of age, VT before the age of 3 and children without disease with regards to the general development of the child at age 3 years (Table 2).

Combined PCA of neurological endpoints

To analyze effects of middle ear disease and all later neurological endpoints, we examined MEE at age 1 year and VT before 1 year in a combined PCA model of all neurological measures. PC1

explained 36.9% of the variation in the neurological data and PC2 explained 18.0% of the variation (Figure 2). MEE at 1 year was significantly associated with a lower PC2-value; adjusted estimate - 0.25; 95% CI [(-0.48)-(-0.02)]; p=0.04; but was not associated with PC1 and no significant associations were found for VT before 1 year of age.

DISCUSSION

Primary Findings

We found slightly lower language scores at age 1-year among children with concurrent MEE compared to children treated with VT and without disease, but no differences in the 2-year language scores. A reduced cognitive score at 2.5 years was found in children, who had received VT but not in children who had had MEE. No differences were found between children with MEE or VT and those without disease for gross motor milestone achievement or for the general development ASQ-3 score at 3 years of age.

Strengths and Limitations

The children in the COPSAC₂₀₁₀ cohort have been followed prospectively since birth with several neurological assessments to evaluate the development of the children. We have both early and late measures of the motor development. We have evaluated the language development both at age 1 and 2 years and as part of the ASQ-3 at 3 years. Furthermore, we evaluated the cognitive development of the children by the objective cognitive test in the research clinic. This provides a unique collection of standardized neurological developmental tests performed prospectively in a large cohort of children.

Not all children completed all tests (Figure 1), which could limit the statistical power. Missing information on specific milestones was handled by the PPCA model. A single missing milestone would otherwise have excluded the child in a traditional PCA model.

MEE was diagnosed by the tympanometry measurement, which is an objective, well recognized test and easy to perform. The limitation is the nature of an annual spot measurement in the COPSAC clinic, as children may have had periods of MEE between the examinations. However, most children have MEE over longer periods of time, making the yearly measurements an acceptable

measure of MEE burden. Season of birth was associated with MEE but not with VT. This could be due to the study design with annual measurements, since children were assessed in the same season every year (Table 1).

The Danish registries strengthen the study. They contain information regarding all VT procedures and contacts with the health care system linked to a personal identification number. Therefore, we have information regarding VT procedures on all 700 children in the cohort. The registries do not contain information on the VT indication (middle ear effusion, recurrent acute otitis media or other reason), or for how long the VT was in place, which may limit our interpretation. Another limitation is the study's observational design. Only a randomized trial can truly evaluate the efficacy of the VT insertion with respect to later developmental endpoints.

Interpretation

At 1 year of age, presence of MEE resulted in a lower word production and a lower word comprehension, whereas children, who had been treated with VT was equal to children without middle ear disease. This could indicate that the VT insertion is beneficial for the short-term hearing ability; however, at 2 years of age we found no difference between the children with MEE or VT and children without disease regarding their language development. This observation is supported by previous literature describing MEE to cause a mild hearing impairment, which often dissolves spontaneously¹⁴ and MEE should probably be regarded as a natural phenomenon in most cases. We found a 2 point lower score in the cognitive test at the age of 2.5 years for children treated with VT compared to healthy children, but no association with untreated MEE. This could indicate that treatment with VT did not have beneficial effects on the cognitive development of the children. Alternatively, it could indicate confounding by indication; the children treated with VT were those, who had the most symptoms of delayed development.

We observed no associations between early middle ear disease and neither the gross motor milestones nor the general development measured by ASQ-3³¹. The combined PCA analysis of all recorded neurological measures showed that children with MEE at 1 year both had lower language and cognitive scores represented in PC2, independent of gross motor development (PC1) (Figure 2). No association was found for VT in the combined PCA analysis, which could be because of few children treated with VT before 1 year of age. The result of this combined neurological PCA supports the individual endpoint analyses.

The literature shows conflicting results regarding the beneficial effects of treatment with VT on neurodevelopmental outcomes^{11,15,32–34}. In the American study by Paradise et al., children with MEE were randomized to treatment either promptly with VT or after 9 months if MEE still persisted, and they were followed until the age of 9-11 years with developmental evaluations. They found no difference between children in the two groups regarding cognitive, language, speech, or psychosocial development^{35,36}. Developmental outcomes may vary between countries. It is known that Danish children generally score lower in early language tests and it has been hypothesized that the nature of the Danish sound structure is more difficult to learn than other languages³⁷. Other factors potentially accounting for discrepancies between studies could be cultural differences such as early daycare attendance³⁷. We furthermore compared children treated with VT to both children with untreated MEE and children with no middle ear disease.

Our study supports the previous findings of an effect of MEE on concurrent early language development. Except for the 1-year language scores, the children with VT had lower estimates compared to children with MEE or healthy children. We cannot conclude whether treatment with VT had any beneficial neurological effects, but we demonstrated no long-term positive differences between treated and untreated children.

In our cohort 29% of the children received VT before the age of 3^{38} , which is a very high

prevalence for a treatment with no solid evidence of long-term beneficial effects. A more conservative approach could be considered in children with MEE, if the purpose of the VT insertion was to prevent delayed development.

Conclusion

Children with MEE at 1 year had slightly lower language scores at 1-year of age, but they were not affected in any other neurological endpoints to age 3 years. Furthermore, our data do not support a beneficial neurological effect of treatment with VT. This warrants caution when considering VT insertion as a mean to prevent delayed development.

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Authors Contributions:

The guarantor of the study is HB, from conception and design to conduct of the study and acquisition of data, data analysis, and interpretation of data. TMP was responsible for the acquisition, analysis, interpretation of the data and writing of the manuscript. JS, JT, ARCMJ and EB have contributed substantially to the analyses and interpretation of the data, and have provided important intellectual input. All authors have agreed that the accuracy and integrity of any part of the work has been appropriately investigated and resolved and all have approved the final version of the manuscript. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

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Conflict of interest:

The authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript.

Governance:

We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU's Directive on Good Clinical Practice, the International Conference on Harmonisation's (ICH) good clinical practice guidelines and the Helsinki Declaration. We follow national and international rules on the processing of personal data, including the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

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FIGURE 1. Flowchart



FIGURE 2. PCA biplot of the neurological development scores of each child and loadings of the neurological variables. Ellipses illustrate the scores (95% CI) of children with middle ear effusion, treatment with ventilation tubes and no disease.



Figure legend:

Gross motor milestones (sit alone, stand with help, stand alone, walk with help, walk alone) language development at 1 and 2 years, cognitive score at 2.5 years and the ASQ-3 scores (problem solving, communication, gross motor, fine motor) at 3 years comparing children with middle ear effusion, ventilation tubes and children without middle ear disease.

	No middle ear disease ≤ 3 y N=146	Middle ear effusion ≤ 3 y n=312	P value	Ventilation tubes ≤ 3 y n=192	P value
Pregnancy and birth					
Maternal age at birth, mean (SD)	31.7 (4.2)	32.5 (4.3)	0.07	32.3 (4.5)	0.21
Delivery, vaginal, % (n)	81.5 (119)	80.5 (251)	0.14	79.7 (153)	0.14
Length in cm at birth, mean (SD)	52.2 (2.1)	52.1 (2.2)	0.43	52.2 (2.0)	0.90
Birth weight in kg, mean (SD)	3.6 (0.5)	3.6 (0.5)	0.44	3.6 (0.5)	0.68
Head circumference in cm at birth, mean (SD)	35.2 (1.3)	35.0 (1.7)	0.31	35.2 (1.6)	0.86
Sex, boys, % (n)	41.8 (61)	52.6 (164)	0.03	55.7 (107)	0.01
Race, caucasians, % (n)	94.5 (138)	96.2 (300)	0.43	97.4 (187)	0.17
Season of birth, % (n)	22.2 (24)	26.5(114)	<0.001	26.0 (50)	0.11
Spring	23.3 (34) 21.2 (31)	29.2 (91)		26.6 (50)	
Summer	34.9 (51)	14.4 (45)		22.9 (44)	
fall	20.6 (30)	19.9 (62)		24.5 (47)	
Exposures					
Smoking during pregnancy, % (n)	6.9 (10)	6.7 (21)	0.96	6.8 (13)	0.98
Older siblings, % (n)	50.0 (73)	57.1 (178)	0.16	67.2 (129)	0.001
Duration of solely breastfeeding in days, mean (SD)	104.3 (60.8)	103.4 (57.1)	0.83	104.6 (60.6)	0.90
Age at start in daycare in months, mean (SD)	11.3 (3.5)	10.9 (3.0)	0.22	10.6 (2.9)	0.05
Social circumstances PCA score ^a	- 0.05 (1.0)	0.08 (1.0)	0.19	- 0.09 (1.0)	0.71

TABLE 1. Baseline characteristics of the COPSAC₂₀₁₀ cohort: the children with middle ear effusion (MEE), ventilation tubes (VT) or children without middle ear disease before the age of 3.

^a PCA component consist of household income, maternal age and maternal educational level at the age of 2

Gross Motor Milestones PPCA °									
PC1 ^d	N=499	Estimate	95% CI	P value					
$VT \le 1$ year	34	0.31	[(-0.38)-1.00]	0.37					
MEE at 1 year	253	(-0.04)	[(-0.39)-0.31]	0.82					
No VT, no MEE ≤ 1 year	212	reference	-	-					
Word production at 1 year									
	N=307	Median	IQR	P value					
$VT \le 1$ year	21	6	[2-9]	0.73					
MEE at 1 year	161	3	[1-7]	0.02					
No VT, no MEE ≤ 1 year	125	5	[2-8]	reference					
Word comprehension at 1 year									
	N=307	Median	IQR	P value					
$VT \le 1$ year	21	45	[21-77]	0.99					
MEE at 1 year	161	37	[22-65]	0.03					
No VT, no MEE ≤ 1 year	125	48	[28-85]	reference					
Word production at 2 years									
	N=496	Median	IQR	P value					
$VT \le 2$ years	130	226	[86-362]	0.16					
MEE at 1 or 2 years	173	238	[114-345]	0.25					
No VT, no MEE ≤ 2 years	193	269	[153-365]	reference					
Cognitive test at 2.5 years									
	N=584	Estimate	95% CI	P value					
$VT \le 2.5$ years	169	(-2.04)	[(-4.09)-0.00]	0.05					
MEE at 1 or 2 years	258	(-0.10)	[(-1.97)-1.76]	0.91					
No VT, no MEE \leq 2.5 years	157	reference							
ASQ-3 PCA at 3 years									
PC1	N=437	Estimate	95% CI	P value					
$VT \le 3$ years	124	(-0.96)	[(-3.89)-1.97]	0.52					
MEE ever (at 1, 2 or 3 years)	219	(-1.49)	[(-4.14)-1.16]	0.27					
No VT, No MEE \leq 3 years	94	reference	-	-					
Combined PCA of all neurological assessments									
PC1	N=530	Estimate	95% CI	P value					
$VT \le 1$ year	35	0.41	[(-0.29)-1.10]	0.25					
MEE at 1 year	270	(-0.16)	[(-0.50)-0.18]	0.36					
No VT, No MEE ≤ 1 year	225	reference	-	-					
PC2	N=530	Estimate	95% CI	P value					
$VT \le 1$ year	35	(-0.06)	[(-0.52)-(-0.40)]	0.80					
MEE at 1 year	270	(-0.25)	[(-0.48)-(-0.02)]	0.04					
No VT, No MEE ≤ 1 year	225	reference	-	-					

TABLE 2. Associations between middle ear effusion (MEE ^a) or treatment with ventilation tubes (VT ^b) and neurological development

All analyses are adjusted for sex.

^a MEE = Middle ear effusion

^b VT = Ventilation tubes

^c PPCA = Proportional Principal Component Analysis ^d PC = Principal Component Supplementary material Paper III

Middle Ear Effusion and Ventilation Tubes and the effect on the Neurological development in early childhood

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eFIGURE 1. PPCA biplot of the gross motor milestone scores of each child and loadings of the neurological variables. Ellipses illustrate the scores (95% CI) of children with middle ear effusion, treatment with ventilation tubes and no disease.



Figure legend:

The PC1 explains 64.9%. The figure shows that there is no difference between children with middle ear effusion, ventilation tubes and no disease in the age of achieving the gross motor milestones.

eFIGURE 2. PCA biplot of the ASQ-3 scores of each child and loadings of the neurological variables. Ellipses illustrate the scores (95% CI) of children with middle ear effusion, treatment with ventilation tubes and children with no disease.



Figure legend:

The ASQ-3 consists of 5 categories; fine motor development, gross motor development, personal-social skills, communication and problem solving. When the scores are analyzed together as one measure of the child's development it results in a PC1 score, which explains 48.1% of the variation.

eFIGURE 3. Prevalence of middle ear effusion measured by tympanometry at 1, 2 and 3 years of age in the COPSAC₂₀₁₀ cohort.




eFIGURE 4. Kaplan-Meier curve illustrating time to first ventilation tube insertion in the COPSAC₂₀₁₀ cohort.