Infectious diseases and immune system in infants
Risk factors and consequences

The Generation R Study

Liesbeth Duijts

Acknowledgements

The Generation R Study is conducted by the Erasmus Medical Center, Rotterdam in close collaboration with the School of Law and Faculty of Social Sciences of the Erasmus University Rotterdam, the Municipal Health Service Rotterdam area, the Rotterdam Homecare Foundation and the Stichting Trombosedienst & Artsenlaboratorium Rijnmond (STAR), Rotterdam. We gratefully acknowledge the contribution of general practitioners, hospitals, midwives and pharmacies in Rotterdam. The first phase of the Generation R Study was made possible by financial support from the Erasmus Medical Center, Rotterdam, the Erasmus University Rotterdam and the Netherlands Organization for Health Research and Development (Zon Mw). The studies described in this thesis were additionally supported by a grant from Stichting W.H. Kröger, Rotterdam (Grant No. 00-048)
The Generation R Study Group, Erasmus University Rotterdam, GlaxoSmithKline, Pfizer and Becton Dickinson financially supported the publication of this thesis.

ISBN 978-90-8559-382-9

Cover design: L. Duijts, 'Desiderius Erasmus'
Lay-out: Optima Grafische Communicatie, Rotterdam (www.ogc.nl)
Print: Optima Grafische Communicatie, Rotterdam (www.ogc.nl)

Infectious Diseases and Immune System in Infants
Risk factors and consequences
The Generation R Study

Infectieziekten en immuun systeem bij kinderen
Risicofactoren en consequenties
Het Generation R onderzoek

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College van Promoties.
De openbare verdediging zal plaatsvinden op
woensdag 11 juni om 9.45 uur

door

Liesbeth Duijts
geboren te Haarlem

Promotiecommissie

Promotoren:	Prof. dr. H.A. Moll
	Prof. dr. R. de Groot
Overige leden:	Prof. dr. A. Hofman
	Prof. dr. H. Hooijkaas
	Prof. dr. J.C. de Jongste

Contents

Manuscripts based on this thesis

Chapter 2.1
L Duijts, GIJG Rours, HA Moll, R de Groot, VWV Jaddoe, A Hofman, EAP Steegers, JP Mackenbach, A Ott, HFM Willemse, EAE van der Zwaan, HA Verbrugh, RP Verkooyen. Pregnancy outcomes in women infected with Chlamydia trachomatis. The Generation R Study. *Submitted.*

Chapter 2.2
L Duijts, VWV Jaddoe, A Hofman, EAP Steegers, HA Moll. Urogenital symptoms in different periods of pregnancy and adverse birth outcomes. The Generation R Study. *Submitted.*

Chapter 3.1
L Duijts, MK Ramadhani, HA Moll. Breastfeeding protects against infectious diseases during infancy in industrialized countries. A systematic review. *Submitted.*

Chapter 3.2
L Duijts, VWV Jaddoe, A Hofman, HA Moll. Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy. The Generation R Study. *Submitted.*

Chapter 3.3
L Duijts, VWV Jaddoe, A Hofman, EAP Steegers, JP Mackenbach, JC de Jongste, HA Moll. Maternal smoking in prenatal and early postnatal life and the risk of respiratory tract infections in infancy. The Generation R Study. *Eur J Epidemiol, 2008; In press.*

Chapter 3.4
JAM Labout, L Duijts, VWV Jaddoe, A Hofman, R de Groot, PWM Hermans, HA Moll. Determinants of nasopharyngeal carriage of *Streptococcus Pneumoniae* in infants. The Generation R Study. *Submitted.*

Chapter 4.1
L Duijts, LE Bakker-Jonges, VWV Jaddoe, A Hofman, RS Jugooa, JHAM Kuijpers-Entrup, M AW Smits-Te Nijenhuis, IJ Bronmans-Smit, AX de Jong, JJM van Dongen, HA Moll, H Hooijkaas. Maturation of lymphocyte subsets from birth until the age of 2 years. The Generation R Study.

Chapter 4.2
L Duijts, LE Bakker-Jonges, JAM Labout, VWV Jaddoe, A Hofman, JJM van Dongen, H Hooijkaas, HA Moll. Fetal growth influences lymphocyte subset counts at birth. The Generation R Study. *Neonatology, 2008; In press.*

Chapter 4.3
L Duijts, LE Bakker-Jonges, JAM Labout, VWV Jaddoe, A Hofman, JJM van Dongen, H Hooijkaas, HA Moll. Perinatal stress influences lymphocyte subset counts in neonates. The Generation R Study. *Pediatr Res, 2008 Mar;63 (3):292-298*

Chapter 4.4
L Duijts, LE Bakker-Jonges, DO Mook-Kanamori, JAM Labout, A Hofman, CM van Duijn, JJM van Dongen, H Hooijkaas, HA Moll, VWV Jaddoe. Variants of the insulin-like growth factor-I gene and lymphocyte subset counts in neonates. The Generation R Study. *Clin Endocrinol, 2008; In press.*

1

Background

Infectious diseases in pregnancy

Preterm birth and low birth weight are considered as important public health concerns since both are important causes of perinatal morbidity and mortality (1-3). Furthermore, these adverse birth outcomes seem to have long term consequences. Preterm birth infants are at risk for neurodevelopmental problems (4). Low birth weight infants are at risk for the development of coronary heart disease and diabetes mellitus (5). Several determinants of preterm birth and low birth weight have been identified, including biological, genetic and socio-demographic determinants (6-8).

Of the biological determinants, *Chlamydia trachomatis* during pregnancy may lead to low birth weight, preterm birth, premature rupture of membranes and other adverse pregnancy outcomes (9-11). However, the literature regarding the adverse effects of these infections yields conflicting results mainly due to differences in study design and population and microbiological tests employed (12-22). Laboratory testing on *C. trachomatis* or other urogenital infections during pregnancy is not routinely performed. Specific antenatal attention for urogenital symptoms, indicating a possible underlying urogenital tract infection, may be helpful in identifying women at increased risk for delivering preterm or low birth weight infants. Not much is known of the effects of urogenital symptoms in different periods of pregnancy with pregnancy outcomes. This may be relevant for identifying critical periods during pregnancy that could be used for targeting preventive strategies.

Infectious diseases in infancy

World wide, respiratory infections and gastrointestinal tract infections are the leading cause of morbidity in children (23, 24). In industrialized countries, prospective cohort studies in the last decade revealed prevalence's of 3.4% to 32.1% for respiratory tract diseases and 1.2% to 26.3% for gastrointestinal diseases in the first year of life of infants. The wide range in prevalence's are mainly due to the different definitions (25-30). Respiratory tract diseases refer to hospitalized or non-hospitalized upper respiratory tract infections, lower respiratory tract infections, wheezing or a combination of these diagnoses (25-30). Gastrointestinal diseases usually comprises infants with diarrhea including those who are hospitalized (27, 30).

Various risk factors for upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI) and gastrointestinal infections (GI) have been identified, including gestational age, birth weight, socio-economic status, ethnicity, number of siblings, daycare attendance, maternal smoking and breastfeeding (25-46). Of these, maternal smoking and breastfeeding seem to be the most modifiable influencing factors. The disadvantages of maternal smoking and the advantages of breastfeeding on infectious

diseases in infants seem to depend on the dose and duration of these factors at which infants are exposed. The independent effects of maternal smoking of different duration in pregnancy and in the postnatal period on the risk or respiratory tract infections are not yet clear. This may be relevant for identifying critical periods for the effect of exposure of maternal smoking on airway and lung development and respiratory tract infections in infants. Breastfeeding is assumed to protect infants from respiratory and gastrointestinal infections. The World Health Organization (WHO) recommended in 2001 to exclusively breastfeed all children for 6 months instead of 4 months. However, they called for more research regarding the benefits of 6 versus 4 months of exclusive breastfeeding (47).

Respiratory tract illnesses and gastrointestinal illnesses in infancy are mostly caused by various viruses and to a lesser extent by bacteria or parasites. *Streptococcus pneumoniae* is one of most important bacterial pathogens that causes respiratory tract illnesses with serious morbidity in infants, including sinusitis, otitis media, bronchitis and pneumonia (4). Nasopharyngeal carriage of *S. pneumoniae* is common and is mostly asymptomatic. However, nasopharyngeal carriage of *S. pneumoniae* might lead to serious pneumococcal disease when the host-pathogen balance is disturbed (48-51). Several risk factors of nasopharyngeal carriage of *S. pneumoniae* have been identified, including age, day care attendance, smoking exposure, and large households (52, 53). However, population-based prospective cohort studies in infants regarding environmental factors, dynamics and interference of *S. pneumoniae* with other pathogens are lacking.

Immune system in infancy

Major reasons for the high rates of recurrent infections in infants are excessive exposure to infectious agents in group settings, such as day care attendance, but also their relative immature immune system. In the nineties, the first immunophenotyping studies on lymphocytes in healthy neonates, infants and children were performed (54-65). Various lymphocyte subsets were identified with the use of flow cytometry immunophenotyping. Compared with adults, several technical problems had to be managed, including the limited available amount of blood, the presence of normoblasts and the lysis-resistant erythrocytes in neonatal cord blood and the variability in staining patterns of some fluorochrome-conjugated antibodies combinations in neonates (61, 62, 66). After addressing the technical difficulties, it was found that neonates and infants had higher absolute lymphocyte counts than adults, which made comparative studies between neonates, infants and adults less informative when using relative lymphocyte counts (61). Furthermore, it seemed that at birth a large poole of naïve, untriggered, lymphocytes are stand-by for primary immune responses. In the first two years of life, absolute numbers of the various lymphocyte subsets change as result from maturation processes. After birth, several studies observed an increase of absolute numbers of $CD3^+$ T lymphocytes until the age of 6 months and a decrease from that age onwards until adult ranges are attained

(62, 63, 65). Within the T lymphocyte population, absolute numbers of $CD4^+$ $CD3^+$ helper T lymphocytes followed the same pattern as the total $CD3^+$ T lymphocytes. $CD8^+$ $CD3^+$ cytotoxic T lymphocytes remain stable after birth up to 2 years of age, followed by a gradual decrease towards adult levels. Absolute numbers of $CD19^+$ B lymphocytes increase immediately after birth and gradually decline after the age of 6 months. In the first 2 months of life, absolute numbers of $CD16.56^+$ NK lymphocytes decrease and remain stable thereafter. In infancy, the maturation process of the various lymphocyte subsets is suggested to be triggered by infectious diseases, nutrition and vaccinations. However, before assessing how and to what extent these parameters affect the maturation of the various lymphocyte subsets, the different maturation phases of the lymphocyte subsets themselves need to be established in more detail.

At birth, the large poole of naïve, untriggered, lymphocytes found in neonates seemed smaller in preterm than in term born neonates (61). These findings suggest a prenatal development of the immune system. Since not only preterm birth but also fetal growth restriction or low birth weight represents suboptimal fetal growth and development, an adverse development of lymphocytes in those infants might be present. It is not well known whether other perinatal factors associated with fetal well-being, stress and hypoxia, including mode of delivery, Apgar scores or umbilical cord blood pH, influence the various lymphocyte subset counts at birth (67-76). Since insulin-like growth factor (IGF-I) seem to stimulate growth, development and function of lymphocytes (9, 77-85), it is of interest to study whether functional variants of the *IGF-I* gene are associated with absolute lymphocyte subset counts in neonates. Large population-based studies assessing the associations of influencing pre-and perinatal factors with absolute numbers of lymphocyte subsets are lacking.

Aims

The overall aims of this thesis are to study the risk factors and consequences of infectious diseases in pregnancy and infancy and to study the developing immune system in infancy in a population-based prospective cohort study. The specific aims are to assess:

1. The associations of *C. trachomatis* infection and symptoms of subclinical urogenital infections in pregnancy with preterm birth and low birth weight.

2. The protective effect of breastfeeding in industrialized countries against infectious diseases in infancy in a review, the associations of duration and exclusiveness of breastfeeding and maternal smoking habits with respiratory tract or gastrointestinal

infections in infancy, and the prevalence and determinants of pneumococcal carriage during the first 14 months of life.

3. The different maturation phases of the lymphocyte subsets during the first 24 months of life, and the associations of fetal growth and development, stress-related perinatal factors and an *IGF-1* promoter polymorphism with absolute numbers of T, B and NK lymphocytes and T lymphocyte subsets (helper, cytotoxic, naïve and memory) in cord blood of neonates.

Outline thesis

In **chapter 2** of this thesis, consequences of infectious diseases in pregnancy are described. Prevalence and risk factors of *C. trachomatis* infection in pregnant women are presented (**chapter 2.1**). Additionally, associations of *C. trachomatis* infection and urogenital symptoms in different periods of pregnancy with adverse pregnancy outcomes are shown (**chapter 2.2**).

Risk factors for infectious diseases in infancy are presented in **chapter 3**. The protective effect of breastfeeding on infectious diseases in infants is explored in a systematic review of studies published in the last two decades (**chapter 3.1**). The associations of the duration of exclusive breastfeeding and the risk of respiratory infections and gastrointestinal tract infections in infants in the first year of life are examined (**chapter 3.2**). Special interest was in the beneficial protective effects of 3 and 6 months of exclusive breastfeeding, compared with infants who were never breastfed. In **chapter 3.3**, the independent effects of maternal smoking in different periods of pregnancy and in early postnatal life on respiratory tract infections at the age of 6 months are assessed. **Chapter 3.4** describes the prevalence of nasopharyngeal carriage of *Streptococcus pneumoniae* in infants, prospectively followed until the age of 14 months. Furthermore, risk factors for nasopharyngeal carriage of *S. pneumoniae* at different ages (6 weeks, 6 months and 14 months) are examined.

Chapter 4 presents the development of absolute numbers of lymphocytes in infants and its influencing factors. Maturation of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory) at birth and at the age of 6, 14 and 24 months are explored (**chapter 4.1**). In **chapter 4.2**, the associations of gestational age, fetal growth and birth weight with absolute numbers of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory) in cord blood of neonates are assessed. Associations of stress-related perinatal factors, including mode of delivery, 1- and 5-minute Apgar scores and umbilical cord blood pH, with the various types of lymphocytes are described in **chapter 4.3**. In **chapter 4.4**, we studied the hypothesis

whether variants of the *IGFI* gene promotor region are related to changes in absolute numbers of lymphocyte subsets.

In **chapter 5**, the main findings, methodological considerations and future perspectives of the studies described in this thesis are discussed.

References

1. Wilcox AJ, Skjaerven R. Birth weight and perinatal mortality: the effect of gestational age. Am J Public Health. 1992 Mar;82(3):378-82.
2. Alexander GR, Kogan M, Bader D, Carlo W, Allen M, Mor J. US birth weight/gestational age-specific neonatal mortality: 1995-1997 rates for whites, hispanics, and blacks. Pediatrics. 2003 Jan;111(1):e61-6.
3. Wen SW, Smith G, Yang Q, Walker M. Epidemiology of preterm birth and neonatal outcome. Semin Fetal Neonatal Med. 2004 Dec;9(6):429-35.
4. Barker DJ. Fetal nutrition and cardiovascular disease in later life. Br Med Bull. 1997 Jan;53(1):96-108.
5. Marlow N, Wolke D, Bracewell MA, Samara M. Neurologic and developmental disability at six years of age after extremely preterm birth. N Engl J Med. 2005 Jan 6;352(1):9-19.
6. Kramer MS, Olivier M, McLean FH, Dougherty GE, Willis DM, Usher RH. Determinants of fetal growth and body proportionality. Pediatrics. 1990 Jul;86(1):18-26.
7. Kramer MS. Intrauterine growth and gestational duration determinants. Pediatrics. 1987 Oct;80(4):502-11.
8. Kramer MS. Determinants of low birth weight: methodological assessment and meta-analysis. Bull World Health Organ. 1987;65(5):663-737.
9. Goldenberg RL, Culhane JF, Johnson DC. Maternal infection and adverse fetal and neonatal outcomes. Clin Perinatol. 2005 Sep;32(3):523-59.
10. Pararas MV, Skevaki CL, Kafetzis DA. Preterm birth due to maternal infection: Causative pathogens and modes of prevention. Eur J Clin Microbiol Infect Dis. 2006 Sep;25(9):562-9.
11. Nelson DB, Macones G. Bacterial vaginosis in pregnancy: current findings and future directions. Epidemiol Rev. 2002;24(2):102-8.
12. Association of Chlamydia trachomatis and Mycoplasma hominis with intrauterine growth retardation and preterm delivery. The John Hopkins Study of Cervicitis and Adverse Pregnancy Outcome. Am J Epidemiol. 1989 Jun;129(6):1247-57.
13. Alger LS, Lovchik JC, Hebel JR, Blackmon LR, Crenshaw MC. The association of Chlamydia trachomatis, Neisseria gonorrhoeae, and group B streptococci with preterm rupture of the membranes and pregnancy outcome. Am J Obstet Gynecol. 1988 Aug;159(2):397-404.
14. Andrews WW, Goldenberg RL, Mercer B, Iams J, Meis P, Moawad A, et al. The Preterm Prediction Study: association of second-trimester genitourinary chlamydia infection with subsequent spontaneous preterm birth. Am J Obstet Gynecol. 2000 Sep;183(3):662-8.
15. Andrews WW, Klebanoff MA, Thom EA, Hauth JC, Carey JC, Meis PJ, et al. Midpregnancy genitourinary tract infection with Chlamydia trachomatis: association with subsequent preterm delivery in women with bacterial vaginosis and Trichomonas vaginalis. Am J Obstet Gynecol. 2006 Feb;194(2):493-500.
16. Claman P, Toye B, Peeling RW, Jessamine P, Belcher J. Serologic evidence of Chlamydia trachomatis infection and risk of preterm birth. Cmaj. 1995 Aug 1;153(3):259-62.
17. Gencay M, Koskiniemi M, Ammala P, Fellman V, Narvanen A, Wahlstrom T, et al. Chlamydia trachomatis seropositivity is associated both with stillbirth and preterm delivery. Apmis. 2000 Sep;108(9):584-8.
18. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and Chlamydia trachomatis infection with adverse pregnancy outcome. Jama. 1986 Oct 10;256(14):1899-903.

19. Harrison HR, Alexander ER, Weinstein L, Lewis M, Nash M, Sim DA. Cervical Chlamydia trachomatis and mycoplasmal infections in pregnancy. Epidemiology and outcomes. Jama. 1983 Oct 7;250(13):1721-7.
20. Martin DH, Koutsky L, Eschenbach DA, Daling JR, Alexander ER, Benedetti JK, et al. Prematurity and perinatal mortality in pregnancies complicated by maternal Chlamydia trachomatis infections. Jama. 1982 Mar 19;247(11):1585-8.
21. Martius J, Krohn MA, Hillier SL, Stamm WE, Holmes KK, Eschenbach DA. Relationships of vaginal Lactobacillus species, cervical Chlamydia trachomatis, and bacterial vaginosis to preterm birth. Obstet Gynecol. 1988 Jan;71(1):89-95.
22. Sweet RL, Landers DV, Walker C, Schachter J. Chlamydia trachomatis infection and pregnancy outcome. Am J Obstet Gynecol. 1987 Apr;156(4):824-33.
23. World Health Organization. Major causes of death among children under 5 years of age and neonates in the world, 2000-2003. http://www.who.int/child-adolescent-health/OVERVIEW/CHILD_HEALTH/map_00-03_world.jpg: The World Health Organization; 2003.
24. Chretien J, Holland W, Macklem P, Murray J, Woolcock A. Acute respiratory infections in children. A global public-health problem. N Engl J Med. 1984 Apr 12;310(15):982-4.
25. Wright AL, Taussig LM, Ray CG, Harrison HR, Holberg CJ. The Tucson Children's Respiratory Study. II. Lower respiratory tract illness in the first year of life. Am J Epidemiol. 1989 Jun;129(6):1232-46.
26. Neuspiel DR, Rush D, Butler NR, Golding J, Bijur PE, Kurzon M. Parental smoking and post-infancy wheezing in children: a prospective cohort study. Am J Public Health. 1989 Feb;79(2):168-71.
27. Baker D, Taylor H, Henderson J. Inequality in infant morbidity: causes and consequences in England in the 1990s. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. J Epidemiol Community Health. 1998 Jul;52(7):451-8.
28. Brunekreef B, Leaderer BP, van Strien R, Oldenwening M, Smit HA, Koopman L, et al. Using nicotine measurements and parental reports to assess indoor air: the PIAMA birth cohort study. Prevention and Incidence of Asthma and Mite Allergy. Epidemiology. 2000 May;11(3):350-2.
29. Panico L, Bartley M, Marmot M, Nazroo JY, Sacker A, Kelly YJ. Ethnic variation in childhood asthma and wheezing illnesses: findings from the Millennium Cohort Study. Int J Epidemiol. 2007 May 24.
30. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. Pediatrics. 2007 Apr;119(4):e837-42.
31. Celedon JC, Litonjua AA, Weiss ST, Gold DR. Day care attendance in the first year of life and illnesses of the upper and lower respiratory tract in children with a familial history of atopy. Pediatrics. 1999 Sep;104(3 Pt 1):495-500.
32. Marbury MC, Maldonado G, Waller L. Lower respiratory illness, recurrent wheezing, and day care attendance. Am J Respir Crit Care Med. 1997 Jan;155(1):156-61.
33. Leeder SR, Corkhill R, Irwig LM, Holland WW, Colley JR. Influence of family factors on the incidence of lower respiratory illness during the first year of life. Br J Prev Soc Med. 1976 Dec;30(4):203-12.
34. Fergusson DM, Horwood LJ, Shannon FT, Taylor B. Parental smoking and lower respiratory illness in the first three years of life. J Epidemiol Community Health. 1981 Sep;35(3):180-4.
35. Martinez FD, Wright AL, Holberg CJ, Morgan WJ, Taussig LM. Maternal age as a risk factor for wheezing lower respiratory illnesses in the first year of life. Am J Epidemiol. 1992 Nov 15;136(10):1258-68.
36. Paradise JL, Rockette HE, Colborn DK, Bernard BS, Smith CG, Kurs-Lasky M, et al. Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. Pediatrics. 1997 Mar;99(3):318-33.
37. Margolis PA, Keyes LL, Greenberg RA, Bauman KE, LaVange LM. Urinary cotinine and parent history (questionnaire) as indicators of passive smoking and predictors of lower respiratory illness in infants. Pediatr Pulmonol. 1997 Jun;23(6):417-23.
38. Greenough A, Giffin FJ, Yuksel B. Respiratory morbidity in preschool children born prematurely. Relationship to adverse neonatal events. Acta Paediatr. 1996 Jul;85(7):772-7.

39. Rylander E, Pershagen G, Eriksson M, Bermann G. Parental smoking, urinary cotinine, and wheezing bronchitis in children. Epidemiology. 1995 May;6(3):289-93.
40. Ponsonby AL, Couper D, Dwyer T, Carmichael A, Kemp A. Relationship between early life respiratory illness, family size over time, and the development of asthma and hay fever: a seven year follow up study. Thorax. 1999 Aug;54(8):664-9.
41. Nafstad P, Hagen JA, Oie L, Magnus P, Jaakkola JJ. Day care centers and respiratory health. Pediatrics. 1999 Apr;103(4 Pt 1):753-8.
42. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breast-feeding for at least 4 months protects against otitis media. Pediatrics. 1993 May;91(5):867-72.
43. Caudri D, Wijga A, Gehring U, Smit HA, Brunekreef B, Kerkhof M, et al. Respiratory symptoms in the first 7 years of life and birth weight at term: the PIAMA Birth Cohort. Am J Respir Crit Care Med. 2007 May 15;175(10):1078-85.
44. Lux AL, Henderson AJ, Pocock SJ. Wheeze associated with prenatal tobacco smoke exposure: a prospective, longitudinal study. ALSPAC Study Team. Arch Dis Child. 2000 Oct;83(4):307-12.
45. Sherriff A, Peters TJ, Henderson J, Strachan D. Risk factor associations with wheezing patterns in children followed longitudinally from birth to 3(1/2) years. Int J Epidemiol. 2001 Dec;30(6):1473-84.
46. Kamper-Jorgensen M, Wohlfahrt J, Simonsen J, Thrane N, Benn CS. Temporal trend in paediatric infections in Denmark. Arch Dis Child. 2006 May;91(5):401-4.
47. World Health Organization, Department of Nutrition for Health and Development. The optimal duration of exclusive breastfeeding: report of an expert consultation. Geneva, Switzerland: World Health Organization; 2001.
48. Hament JM, Kimpen JL, Fleer A, Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. FEMS Immunol Med Microbiol. 1999 Dec;26(3-4):189-95.
49. Mulholland K. Strategies for the control of pneumococcal diseases. Vaccine. 1999 Jul 30;17 Suppl 1:S79-84.
50. Plotkowski MC, Puchelle E, Beck G, Jacquot J, Hannoun C. Adherence of type I *Streptococcus pneumoniae* to tracheal epithelium of mice infected with influenza A/PR8 virus. Am Rev Respir Dis. 1986 Nov;134(5):1040-4.
51. Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis. 2004 Mar;4(3):144-54.
52. Ghaffar F, Friedland IR, McCracken GH, Jr. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. Pediatr Infect Dis J. 1999 Jul;18(7):638-46.
53. Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rumke HC, et al. Colonisation by *Streptococcus pneumoniae* and Staphylococcus aureus in healthy children. Lancet. 2004 Jun 5;363(9424):1871-2.
54. de Vries E, de Groot R, de Bruin-Versteeg S, Comans-Bitter WM, van Dongen JJ. Analysing the developing lymphocyte system of neonates and infants. Eur J Pediatr. 1999 Aug;158(8):611-7.
55. Heldrup J, Kalm O, Prellner K. Blood T and B lymphocyte subpopulations in healthy infants and children. Acta Paediatr. 1992 Feb;81(2):125-32.
56. Melaranci C, Ciaffi P, Zerella A, Martinelli V, Cutrera R, Plebani A, et al. T-cell subpopulations in pediatric healthy children: age-normal values. J Clin Lab Immunol. 1992;38(3):143-9.
57. Denny T, Yogev R, Gelman R, Skuza C, Oleske J, Chadwick E, et al. Lymphocyte subsets in healthy children during the first 5 years of life. Jama. 1992 Mar 18;267(11):1484-8.
58. Kotylo PK, Fineberg NS, Freeman KS, Redmond NL, Charland C. Reference ranges for lymphocyte subsets in pediatric patients. Am J Clin Pathol. 1993 Aug;100(2):111-5.
59. Raes M, Alliet P, Gillis P, Zimmermann A, Kortleven J, Magerman K, et al. Lymphocyte subpopulations in healthy newborn infants: comparison of cord blood values with values five days after birth. J Pediatr. 1993 Sep;123(3):465-7.

60. D'Arena G, Musto P, Cascavilla N, Di Giorgio G, Fusilli S, Zendoli F, et al. Flow cytometric characterization of human umbilical cord blood lymphocytes: immunophenotypic features. Haematologica. 1998 Mar;83(3):197-203.
61. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Hop WC, Boerma GJ, et al. Neonatal blood lymphocyte subpopulations: a different perspective when using absolute counts. Biol Neonate. 2000 May;77(4):230-5.
62. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr. 1997 Mar;130(3):388-93.
63. Shahabuddin S, Al-Ayed I, Gad El-Rab MO, Qureshi MI. Age-related changes in blood lymphocyte subsets of Saudi Arabian healthy children. Clin Diagn Lab Immunol. 1998 Sep;5(5):632-5.
64. Tsegaye A, Wolday D, Otto S, Petros B, Assefa T, Alebachew T, et al. Immunophenotyping of blood lymphocytes at birth, during childhood, and during adulthood in HIV-1-uninfected Ethiopians. Clin Immunol. 2003 Dec;109(3):338-46.
65. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003 Nov;112(5):973-80.
66. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Boerma GJ, Lotgering FK, et al. Correction for erythroid cell contamination in microassay for immunophenotyping of neonatal lymphocytes. Arch Dis Child Fetal Neonatal Ed. 1999 May;80(3):F226-9.
67. Cairo MS, Wagner EL, Fraser J, Cohen G, van de Ven C, Carter SL, et al. Characterization of banked umbilical cord blood hematopoietic progenitor cells and lymphocyte subsets and correlation with ethnicity, birth weight, sex, and type of delivery: a Cord Blood Transplantation (COBLT) Study report. Transfusion. 2005 Jun;45(6):856-66.
68. Kotiranta-Ainamo A, Apajasalo M, Pohjavuori M, Rautonen N, Rautonen J. Mononuclear cell subpopulations in preterm and full-term neonates: independent effects of gestational age, neonatal infection, maternal pre-eclampsia, maternal betamethason therapy, and mode of delivery. Clin Exp Immunol. 1999 Feb;115(2):309-14.
69. Nikischin W, Peter M, Oldigs HD. The influence of mode of delivery on hematologic values in the umbilical vein. Gynecol Obstet Invest. 1997;43(2):104-7.
70. Pittard WB, 3rd, Schleich DM, Geddes KM, Sorensen RU. Newborn lymphocyte subpopulations: the influence of labor. Am J Obstet Gynecol. 1989 Jan;160(1):151-4.
71. Lim FT, van Winsen L, Willemze R, Kanhai HH, Falkenburg JH. Influence of delivery on numbers of leukocytes, leukocyte subpopulations, and hematopoietic progenitor cells in human umbilical cord blood. Blood Cells. 1994;20(2-3):547-58; discussion 58-9.
72. Samelson R, Larkey DM, Amankwah KS, McConnachie P. Effect of labor on lymphocyte subsets in full-term neonates. Am J Reprod Immunol. 1992 Sep;28(2):71-3.
73. Thilaganathan B, Meher-Homji N, Nicolaides KH. Labor: an immunologically beneficial process for the neonate. Am J Obstet Gynecol. 1994 Nov;171(5):1271-2.
74. Thornton CA, Capristo CC, Power LL, Holloway JA, Popplewell EJ, Diaper ND, et al. The effect of labor on neonatal T-cell phenotype and function. Pediatr Res. 2003 Jul;54(1):120-4.
75. Gasparoni A, Maccario R, Chirico G, Belloni C, Mingrat G, De Amici D, et al. Neonatal B lymphocyte subpopulations and method of delivery. Biol Neonate. 1992;61(3):137-41.
76. Chirico G, Gasparoni A, Ciardelli L, Martinotti L, Rondini G. Leukocyte counts in relation to the method of delivery during the first five days of life. Biol Neonate. 1999 May;75(5):294-9.
77. Foster M, Montecino-Rodriguez E, Clark R, Dorshkind K. Regulation of B and T cell development by anterior pituitary hormones. Cell Mol Life Sci. 1998 Oct;54(10):1076-82.
78. Clark R, Strasser J, McCabe S, Robbins K, Jardieu P. Insulin-like growth factor-1 stimulation of lymphopoiesis. J Clin Invest. 1993 Aug;92(2):540-8.

79. Dorshkind K, Horseman ND. The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. Endocr Rev. 2000 Jun;21(3):292-312.
80. LeRoith D, Yanowski J, Kaldjian EP, Jaffe ES, LeRoith T, Purdue K, et al. The effects of growth hormone and insulin-like growth factor I on the immune system of aged female monkeys. Endocrinology. 1996 Mar;137(3):1071-9.
81. de Mello Coelho V, Villa-Verde DM, Farias-de-Oliveira DA, de Brito JM, Dardenne M, Savino W. Functional insulin-like growth factor-1/insulin-like growth factor-1 receptor-mediated circuit in human and murine thymic epithelial cells. Neuroendocrinology. 2002 Feb;75(2):139-50.
82. Tarantal AF, Hunter MK, Gargosky SE. Direct administration of insulin-like growth factor to fetal rhesus monkeys (Macaca mulatta). Endocrinology. 1997 Aug;138(8):3349-58.
83. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. Blood. 1992 Sep 1;80(5):1207-12.
84. Tu W, Cheung PT, Lau YL. Insulin-like growth factor 1 promotes cord blood T cell maturation and inhibits its spontaneous and phytohemagglutinin-induced apoptosis through different mechanisms. J Immunol. 2000 Aug 1;165(3):1331-6.
85. Tu W, Cheung PT, Lau YL. IGF-I increases interferon-gamma and IL-6 mRNA expression and protein production in neonatal mononuclear cells. Pediatr Res. 1999 Dec;46(6):748-54.

Infectious diseases in pregnancy

2

Pregnancy outcomes in women infected with Chlamydia trachomatis

2.1

Abstract

Context: Chlamydia trachomatis infection is the most prevalent sexually transmitted infection and may influence pregnancy outcome.

Objective: To assess the effect of *C. trachomatis* infection during pregnancy on pregnancy outcomes.

Design and Setting: A population-based prospective cohort study of women presenting for antenatal care to the midwifery practices and antenatal clinics in Rotterdam, the Netherlands. Women completed a self-administered questionnaire and submitted a urine sample. *C. trachomatis* was detected by PCR. Pregnancy outcomes were obtained from midwives and hospital registries.

Participants: This study was embedded in the Generation R Study. Women, who attended one of the participating centres between February 2003 and January 2005 and who were expected to deliver in Rotterdam, were eligible for the study. In total 5,167 pregnant women were enrolled. From 4,055 women urine samples and questionnaires were available for analysis.

Main outcome measures: Unfavourable pregnancy outcomes including abortion, stillbirth, perinatal death, prematurity and low birth weight.

Results: The prevalence of *C. trachomatis* infection was 3.9% and was highest in women with young age, Antillean, Cape Verdian or Surinamese ethnicity, single marital status, multiple sexual partners or a history of a sexually transmitted infection, and with partners with similar risk factors. *C. trachomatis* infection was associated with preterm delivery, especially with early prematurity before 32 weeks gestation (OR 6.14 [95% CI 1.69, 22.30]), but not with low birth weight. Of all preterm deliveries before 32 weeks gestation in this region 15.8 % was attributable to *C. trachomatis* infection.

Conclusion: C. trachomatis infection in pregnant women is an important risk factor for early premature delivery. *C. trachomatis* infection in pregnancy should be considered a public health problem, especially for young women in certain socio-economic groups at increased risk of *C. trachomatis* infection.

Introduction

Chlamydia trachomatis is an important cause of sexually transmitted infections (STIs) in women, which may lead to tubal infertility, ectopic pregnancy, pelvic inflammatory disease, and chronic abdominal pain. (1-3) Screening for *C. trachomatis* in pregnant women has revealed prevalence rates varying from 0% to 37%. (4-6) In the Netherlands, a *C. trachomatis* prevalence of 2.5% was reported in women, (7) but data on pregnant women are lacking. Various risk factors have been associated with chlamydial infection including in particular age and socio-economic status. (6, 8)

C. trachomatis infection during pregnancy may influence pregnancy outcomes leading to prematurity, premature rupture of membranes, low birth weight and perinatal mortality. The literature regarding these latter detrimental effects of *C. trachomatis* infection yields conflicting results that seem primarily due to differences in study design, population and microbiological tests employed. (9-19) The objective of this study was to assess the prevalence of *C. trachomatis* infection in pregnant women and to investigate the association of chlamydial infection with pregnancy outcomes in a large population-based prospective study in Rotterdam, the Netherlands.

Methods

Design

This *C. trachomatis* study was embedded in the Generation R Study, which is a population-based prospective cohort study designed to identify early environmental and genetic determinants of growth, development and health of children, starting from foetal life until adolescence, as previously described. (20, 21) Pregnant women, who attended one of the participating midwifery practices or antenatal clinics and who were expected to deliver in Rotterdam, were eligible for the study. Regular health care workers (midwives, obstetricians) informed the women about the study. Most women spoke Dutch, otherwise the study was explained and questionnaires provided in their native language. Enrolment was scheduled in early pregnancy (gestational age < 18 weeks) at the first routine foetal ultrasound examination, but was allowed until delivery. Women who were scheduled for an induced abortion and women with a pregnancy resulting in foetal death or miscarriage prior to or at the first ultrasound were not included in the study. All children were born between April 2002 and January 2006. (21) Inclusion for the current *C. trachomatis* sub-study was between February 2003 and January 2005.

Risk factors

Data were obtained using confidentially administered standardized questionnaires at the time of inclusion. Maternal age was defined as age at enrolment of the study. Ethnicity has been associated with chlamydial infection and was self-classified in this study. Most women were Dutch (49%), Surinamese (9%), Turkish (9%) or Moroccan (7%). Educational levels of participating women were defined in groups by highest attained education (primary school, secondary school, higher education). Marital status was defined as married if formally married or living together in partnership. Further information was obtained regarding gravidity, number of sexual partners in the year prior to pregnancy, history of an STI, and the use of cigarettes, alcohol and drugs. Information about partners was collected indirectly via maternal questionnaires and included age, ethnicity, number of sexual partners in the year prior to pregnancy, history of an STI and the use of cigarettes, alcohol and drugs. The partner was defined as the biological father of the current pregnancy.

Microbiological diagnosis

Women provided a first-void urine specimen to test for *C. trachomatis* at enrolment. Urines were stored at 4°C, transported the same or following working day, and processed within 24 hours of receipt by the laboratory. DNA was isolated from pooled urine specimens using the MagNA Pure LC Bacterial DNA isolation Kit III (Roche Molecular Systems, Inc, Alameda, CA, USA) and amplified by polymerase chain reaction (PCR) (Cobas Amplicor, Roche Molecular Diagnostics, Branchburg USA) as previously described. (22) In brief, pools were made of five individual urines by adding 200 µl of each of the urines into one tube. From each pool the full 1000 µl were taken, and centrifuged for 10 minutes. Subsequently 900 µl were removed and the pellet was resuspended in 100 µl of the remaining supernatant, mixed with 130 µl lysis buffer and 20 µl proteinase K, incubated for 10 minutes and thereafter denatured for 10 minutes. Finally, DNA was isolated in the automated MagNA Pure LC using a sample volume of 250 µl and an elution volume of 100 µl. Then, 25 µl was used for PCR. Urines from positive pools were individually re-tested and reported as negative or positive.

Pregnancy outcomes

Information about pregnancy outcomes (abortion, stillbirth, perinatal death, gestational age, birth weight) was obtained postnatally from midwives and hospital registries. Gestational age was established by foetal ultrasound examination. Preterm birth was defined as delivery at a gestational age of less than 37 weeks with subgroups that had gestational ages of less than 32 weeks and less than 35 weeks. Birth weight measurements were converted into gestational age adjusted standard deviation scores (SDSs). (23)

Ethical aspects

Within the framework of the Generation R Study several sub-studies are embedded, as was our *C. trachomatis* study. Both studies were approved by the Medical Ethical Committee for Research on Human Subjects of the Erasmus University Medical Centre, Rotterdam. Written informed consent was obtained from all participants. Generation R provided data anonymously to protect the privacy of participants; we had no access to names or addresses. (20, 21) We did not treat *C. trachomatis* positive women nor refer women to regular health care providers. The study did not interfere with current Dutch standard medical practice. Standard medical practice was, and still is at the time, not to screen for *C. trachomatis* antenatally and only to treat Chlamydia in pregnancy if women are identified as being at risk of infection due to clinical signs or via contact tracing from a Chlamydia-positive partner.

Data analysis

The associations of *C. trachomatis* infection during pregnancy with socio-economic and life style risk factors of women and partners were assessed using multiple logistic regression models. Unequivocal confounders were selected based on previous studies. In addition, we included variables that, in our study cohort, were associated both with Chlamydia infection and adverse pregnancy outcomes as possible confounders. Associations were studied for each risk factor while adjusting for all other risk factors. Associations of socio-economic and life style risk factors of partners with *C. trachomatis* infection of women were additionally adjusted for maternal risk factors (maternal age, ethnicity, multiple partners the year prior to pregnancy, history of an STI).

For the analysis of birth weight as a continuous variable, multiple linear regression models were used. For the analysis of low birth weight and preterm birth multiple logistic regression models were employed. The latter regressions were adjusted for known determinants of low birth weight and preterm birth (maternal age, ethnicity, gravidity) and for socio-economic status and life style related variables (education, smoking). Measures of association are presented with 95% confidence intervals (CI). The Kaplan-Meier procedure was used to illustrate the proportion of women who delivered at given gestational ages.

The proportion of preterm delivery attributable to *C. trachomatis* infection in women in the total population was assessed using the population attributable risk (PAR). (24) The PAR was calculated on the basis of the observed relative risk (RR) and the population fraction with chlamydial infection (PF), using the formula:

$$PAR = [(RR - 1)PF] / [1 + (RR - 1)PF].$$

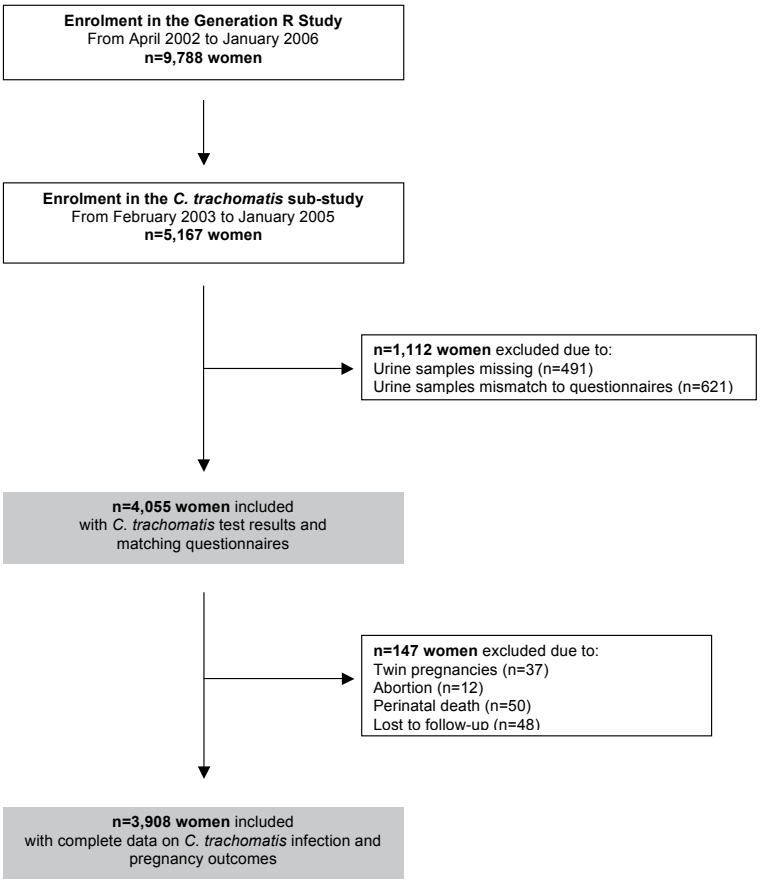
Statistical analysis was performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Population for analysis

In total 5,167 pregnant women were enrolled in the study (Figure 1). Women with incomplete data sets (N = 1,112) were excluded: from 491 women no urine sample was obtained or urine was lost during transport to the laboratory, and from 621 women the urines could not be matched to their respective questionnaires in the data base. From

Figure 1. Profile of the Generation R sub-study on *Chlamydia trachomatis*.



4,055 (78%) women urine samples and data on socio-economic and life style risk factors were available, which were used for the analyses of the prevalence and risk factors for

Table 1. Socio-economic and life style risk factors of *Chlamydia trachomatis* infection in pregnant women.

	Chlamydia trachomatis infection		
	No n=3898	**Yes n=157**	**Adjusted OR (95% CI)#**
Age groups			
< 21	217	34	3.89 (1.85, 8.21)**
21-25	741	53	2.93 (1.54, 5.56)**
26-30	1194	41	2.04 (1.13, 3.69)**
> 30	1746	29	1.00
Ethnicity			
Dutch	1701	33	1.00
Cape Verdian	137	15	1.56 (0.70, 3.50)
Antillean	105	19	2.74 (1.28, 5.85)**
Surinamese	298	30	2.06 (1.10, 3.84)*
Moroccan/Turkish	563	14	0.71 (0.30, (1.67)
Other (non-) western	630	19	0.98 (0.51, 1.89)
Education			
Primary school	396	27	1.29 (0.61, 2.73)
Secondary school	1421	69	1.09 (0.62, 1.92)
Higher education	1474	27	1.00
Marital status			
Not married	452	64	3.55 (2.13, 5.91)**
Married	2846	65	1.00
Gravidity ≥ 1			
No	1652	73	1.00
Yes	2083	73	0.78 (0.51, 1.19)
Multiple sexual partners in year prior to pregnancy			
No	2918	99	1.00
Yes	251	21	1.19 (0.67, 2.11)
History of STI			
No	2778	100	1.00
Yes	370	25	1.06 (0.59, 1.88)
Do not know	38	2	1.06 (0.24, 4.78)

Values are frequencies and odds ratios (95% CI)

*P-value < 0.05, **p-value < 0.01

[$]Data missing for ethnicity (n=491), education (n=641), marital status (n=628), gravidity (n=174), number of sexual partners (n=766), STI (n=742)

[#]Adjusted for all other risk factors, drug use, alcohol use and smoking

chlamydial infection. Half the women were included within the first 14 weeks of their pregnancy, 95% within 22 weeks.

Prevalence

C. trachomatis infection was detected in 157 of 4,055 (3.9%) women. Age-specific prevalences were 13.5% in women age 20 years or less, 6.7% between 21 and 25 years, 3.3% between 26 and 30 years and 1.6% in women over 30 years. The prevalence was highest in Antillean (15.3%), Cape Verdian (9.8%) or Surinamese (9.1%) women, and in women with low education (6.4%), single marital status (12.4%), first pregnancies (4.2%), multiple sexual partners in the past year (7.7%) or a history of an STI (6.3%) (Table 1). Chlamydia prevalence was also high in women whose partner was less than 21 years (18.8%), or between 21 and 25 years of age (7.3%), Antillean (17.1%), Cape Verdian (13.4%), or Surinamese (9.5%), or who had more than two partners in the past year (9.8%). *C. trachomatis* prevalence rates were also high among women who did not know whether their partner had multiple sexual contacts in the past year or a history of an STI (data on partners not shown in Table 1).

Women with missing data on marital status, gravidity, multiple sexual partners or a history of an STI had similar rates of *C. trachomatis* infection when compared to women with these data recorded. Women with missing data on ethnicity and education had somewhat higher rates of *C. trachomatis* infection, 5.5 % and 5.3%, respectively, (p=0.05 and p=0.04).

Risk factors

In the adjusted analysis age below 21 years, or between 21-25 years, and between 26-30 years remained risk factors for *C. trachomatis* infection, as did Antillean or Surinamese ethnicity and single marital status. Other maternal factors were not independently associated with *C. trachomatis* infection.

Adjusted risk factors of partners showed that if partners were Cape Verdian, Surinamese or Moroccan/Turkish, the risk of *C. trachomatis* infection for women was higher. If the partner had multiple sexual partners the year prior to pregnancy or the woman did not know whether her partner had had multiple sexual contacts, women also had a significantly higher risk of *C. trachomatis* infection. Drugs use by women or their partners were, in the adjusted analysis, not associated with *C. trachomatis* infection of women nor were alcohol use and smoking (data not shown).

Pregnancy outcomes

Of the 4,055 pregnancies 3,908 resulted in live singleton births and 37 in the birth of live twins. Women who gave birth to twins were more often Chlamydia positive than women who had singletons, which difference was borderline significant (Table 2). Adverse preg-

Table 2. Pregnancy outcomes of women and their association with *Chlamydia trachomatis* infection.

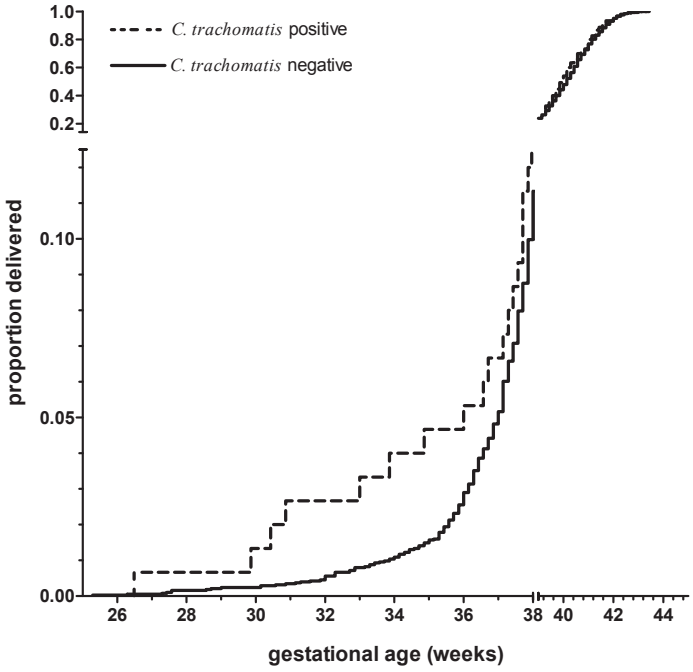
	Chlamydia trachomatis infection			
Outcomes	**No**	**Yes**	**Percentage positive (95% CI)**	**P-value ***
All outcomes				
all women enrolled	3898	157	3.9	
Live pregnancy outcomes				
live singleton birth	3758	150	3.8	
live twin birth	33	4	10.8 (4.2 – 25.1)	0.05
lost to follow up	45	3	6.3 (2.1 – 17.1)	0.43
Fetal death				
abortion	12	0	0 (0 – 24.4)	1.00
stillbirth	33	0	0 (0 – 10.5)	0.64
perinatal death	17	0	0 (0 – 18.6)	1.00
*Gestational age***				
term, ≥ 37 weeks	3577	140	3.8	
prematurity, < 37 weeks	181	10	5.2 (2.8 – 9.6)	0.33
prematurity, < 35 weeks	56	7	11.1 (5.4 – 21.7)	0.01
prematurity, < 32 weeks	17	4	19.0 (7.6 – 40.8)	0.01
*Birth weight***				
≥ 2500 gram	3585	140	3.8	
< 2500 gram	173	10	5.5 (2.9 – 10.0)	0.24

* P-value (Fishers exact test) for comparison of Chlamydia prevalence with respective outcome categories of live singleton birth, gestational age ≥ 37 weeks, and birth weight ≥ 2500 gram
** Including live singleton birth outcomes only

nancy outcomes leading to loss of fetal or neonatal live, including abortion, stillbirth and perinatal death, occurred in 62 (1.5%) of the pregnancies. These adverse events were not associated with Chlamydia infection during pregnancy. Preterm delivery occurred in 191 women (4.9%) of which prematurity before gestational ages of 32 weeks and of 35 weeks was significantly associated with Chlamydia infection during pregnancy (Table 2). Regarding birth weights, no significant association was observed between low birth weight and Chlamydia infection during pregnancy. The effect of Chlamydia infection on gestational age and birth weight were further analysed. For these analyses women who were lost to follow up, who had twin pregnancies, or who had an abortion, stillbirth or perinatal death were excluded. Thus, 3908 women and neonates remained to analyse the effect of Chlamydia infection on gestational age and birth weight.

Gestational age. The distribution of gestational ages of neonates according to the chlamydial status of women is shown in a Kaplan-Meier plot (Figure 2). *C. trachomatis*

Figure 2. Kaplan-Meier analysis of duration of gestation at delivery in women with and without *Chlamydia trachomatis* infection



infected women had a significantly higher rate of delivery at 35 weeks gestation or earlier.

Unadjusted analysis (Table 3) showed that *C. trachomatis* infected women had a significantly higher risk of preterm delivery before 32 weeks compared to delivery at term (OR 6.01 [95% CI 2.00, 18.10]). This six-fold increased risk (OR 6.14 [95% CI 1.69, 22.30]) remained after adjustment for gestational age and the potential confounders maternal age, ethnicity, education, gravidity, and smoking. *C trachomatis* infected women also had a significantly higher risk of preterm delivery before 35 weeks compared to delivery at term (OR 3.19 [95% CI 1.43, 7.13]). Although this risk increase was half of that observed for preterm delivery at 32 weeks, it also remained significant after adjustment for the confounders stated above (OR 2.82 [95% CI 1.10, 7.22]). *C. trachomatis* infected women did not have an increased risk of preterm delivery before 37 weeks. Alcohol, drug use and marital status did not meet our criteria of potential confounders. Indeed, including alcohol and/or drug use in our regression model did not have a significant effect on the calculated ORs. Adjustment for marital status somewhat reduced the calculated ORs (4.46 [95% CI 1.33, 14.97] for preterm delivery before 32 weeks, and 2.72 [95% CI 1.15, 6.40] for preterm delivery before 35 weeks), but the risk remained significantly elevated.The frac-

Table 3. Risk for preterm delivery among women with *Chlamydia trachomatis* infection.

	Risk for preterm delivery		
	< 32 weeks n=21	**< 35 weeks** n=63	**< 37 weeks** n=191
	Unadjusted odds ratio		
***C. trachomatis* infection**	6.01 (2.00,18.10)**	3.19 (1.43, 7.13)**	1.41 (0.73, 2.73)
	Adjusted odds ratio#		
***C. trachomatis* infection**	6.14 (1.69, 22.30) #**	2.82 (1.10, 7.22) #*	1.00 (0.44, 2.26) #

[a]Values are odds ratios (95% confidence interval)
Analyses are done versus delivery ≥ 37 weeks (n=3717)
[#]Adjusted for maternal age, ethnicity, education, gravidity and smoking
*P-value < 0.05, **p-value < 0.01

Figure 3. Birth weight standard deviation scores (SDS) versus gestational ages of neonates born to women with and without *Chlamydia trachomatis* infection

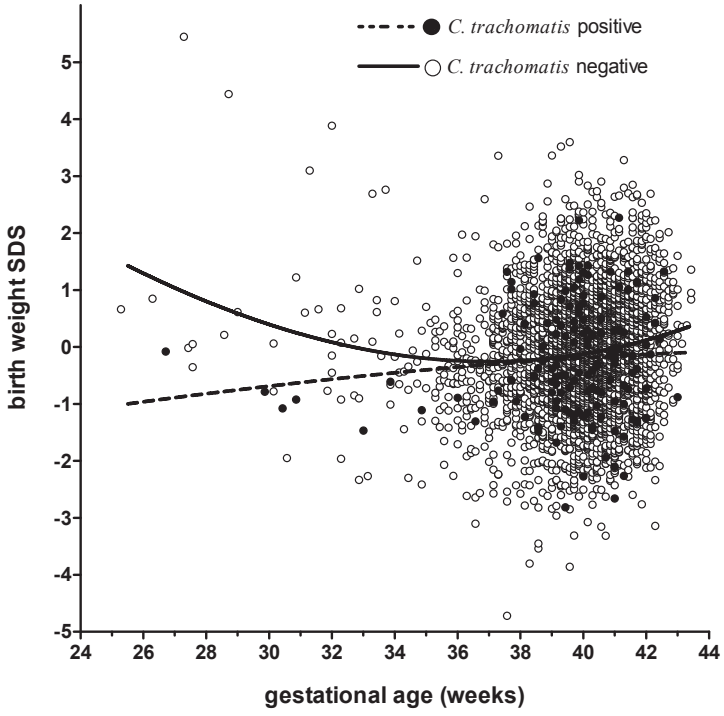


Table 4. Differences in birth weight between neonates born to women with and without *Chlamydia trachomatis* infection.

	Number (%)	Mean birth weight SDS	Difference in birth weight SDS *	
			Unadjusted	Adjusted #
Gestational age				
< 32 weeks (n=21)				
C. trachomatis –	17 (81)	0.83		
C. trachomatis +	4 (19)	–0.72	–1.54 (–3.56, 0.47)	–2.66 (–6.93, 1.61)
< 35 weeks (n=63)				
C. trachomatis –	56 (89)	0.13		
C. trachomatis +	7 (11)	–0.87	–0.99 (–2.20, 0.22)	–1.18 (–2.69, 0.33)
< 37 weeks (n=191)				
C. trachomatis –	181 (95)	–0.27		
C. trachomatis +	10 (5)	–0.85	–0.59 (–1.34, 0.17)	–0.43 (–1.38, 0.52)
≥ 37 weeks (n=3717)				
C. trachomatis –	3577 (96)	–0.08		
C. trachomatis +	140 (4)	–0.19	–0.10 (–0.27, 0.07)	–0.04 (–0.23, 0.16)

* SDS, standard deviation score. Difference and 95% confidence interval calculated with linear regression analysis
# Adjusted for maternal age, ethnicity, education, gravidity and smoking

tion of all premature deliveries before 32 weeks gestation attributable to *C. trachomatis* infection in women was 15.8% (95% CI 4.9, 41.0) in this study cohort. The population attributable risk of *C. trachomatis* infection for preterm delivery before 35 weeks gestation was 7.5% (95% CI 2.5, 20.4).

Birth weight. Crude analysis of the difference in birth weight (in grams) between neonates born to Chlamydia positive and Chlamydia negative women showed a significant difference (-113 grams [95% CI -204, -22]), which disappeared after adjustment for the potential confounders gestational age, maternal age, ethnicity, education, gravidity and smoking (-24 grams [95% CI -107, 59]). A comparison of the presence of low birth weight (n=183), defined as birth weight below 2500 grams, among neonates born to Chlamydia positive and Chlamydia negative women also showed no significant difference, neither in the unadjusted analysis (OR 1.48 [95% CI 0.77, 2.86]) nor in the adjusted analysis (OR 0.52, [0.12, 2.23]). Analysis of the correlation between birth weights of neonates, expressed in SDSs, and their gestational ages according to the Chlamydia status of the women during pregnancy is demonstrated in Figure 3. This figure suggests that neonates born to *C. trachomatis* infected women had, on average, a lower birth weight SDS, especially when prematurely born. However, this difference in birth weight SDSs between neonates born to Chlamydia positive women and Chlamydia negative women did not reach statistical significance, also not after stratification by gestational ages (Table 4).

Discussion

This population-based prospective cohort study provides strong evidence that *C. trachomatis* infection is associated with preterm delivery, but not with low birth weight. In this study region young age, Antillean and Surinamese ethnicity and single marital status were independent risk factors for *C. trachomatis* infection in pregnant women as were Cape Verdian, Surinamese and Moroccan/Turkish ethnicity and promiscuity of their partners.

The strength of this study is its population-based, non-interventional and prospective design with a large number of well-described participants, adjustments for many potential confounders, use of a highly sensitive microbiological test method and near perfect follow-up until delivery (97%).

However, several potential weaknesses should be mentioned. The Generation R Study cohort is slightly skewed towards a relatively affluent and healthy study population (21). We do not expect this to affect our results since the present study was designed to assess the effects of *C. trachomatis* infection on pregnancy outcomes in a non-hospital based, low risk population. A weakness could be that 21% of initially enrolled women could not be tested for *C. trachomatis*. However, all risk factors were similarly distributed among tested and untested women and no differences were found in the median gestational age and mean birth weight (40.1 weeks versus 40.1 weeks; p=0.33, 3419 grams versus 3418 grams; p=0.99). Also, no differences were found in the proportion of *C. trachomatis* infection between women included and not included in the analyses of gestational age and birth weight (3.6% versus 4.5%); p=0.57). These selection mechanisms might lead to bias if the associations of *C. trachomatis* infection with gestational age and birth weight would differ between the women included and not included in the present analyses. This seems unlikely. One more limitation could have been the missing information on risk factors (on average 16%; range 4.3 – 18.9%), but the distribution of *C. trachomatis* infection was similar in women with and without information about risk factors. Finally our numbers were too small to properly assess the association of *C. trachomatis* infection with abortion, stillbirth or perinatal death. Of all women enrolled in the study and with information about *C. trachomatis* infection, 62 (1.5%) had an abortion, stillbirth or perinatal death. Data on gestational age and birth weight were not available for women with the latter adverse pregnancy outcomes, but it is likely that these women delivered prematurely relatively more frequently. None of these women had a *C. trachomatis* infection. Theoretically, our effect estimates for the associations of chlamydial infection with gestational age and birth weight could be biased and exaggerated when these associations would differ between all fetuses and fetal `survivors`. This would be the case when *C. trachomatis* infection has a `protective` effect on early fetal death. However, this is most unlikely. (9,17)

Previous studies of associations between maternal chlamydial infection and subsequent spontaneous preterm delivery produced mixed results. Early studies were often based on serology, which does not reliably distinguish current from past infection. (10, 12, 16, 25) One case-control study found an association between the presence of IgM anti-chlamydial antibody, but not IgG antibody, and preterm delivery. (12) Studies using cervical culture for *C. trachomatis* at that time also yielded conflicting results. (10, 11, 15, 25) More recently, sensitive DNA amplification techniques have been used to screen pregnant women for *C. trachomatis*. One case-control study, nested in a large USA study among 2,929 pregnant women, used this methodology and reported a two- to three-fold increased risk of preterm delivery before 35 weeks gestation. (18) Interestingly, the same study group reported more recently that *C. trachomatis* infection in midterm pregnancy was not associated with an increased risk of preterm delivery among 2,470 women enrolled in an antibiotic treatment trial for bacterial vaginosis or *Trichomonas vaginalis* infection. (19) Since all women were infected and bacterial vaginosis itself may induce preterm delivery, (11, 13) the findings of the latter study cannot be extrapolated to the population at large. Another population-based retrospective cohort study in the USA recently found that Chlamydia infected pregnant women (cases, n=851) had a relative risk of preterm delivery before 37 weeks gestation of only 1.50 compared to non-infected women (controls, n=3,404). (26) However, the true effect of Chlamydia infection on pregnancy outcomes cannot be ascertained from that study since all women were screened for Chlamydia in the first trimester of their pregnancy, and, when positive, treated. In contrast, our study is prospective non-interventional and population-based (n = 4,055), which makes that our findings better estimate the effect of Chlamydia on pregnancy and are more predictive for the population as a whole.

Preterm birth represents a major problem for obstetrics and neonatology due to its increasing frequency and accompanying socio-economic impact. We found *C. trachomatis* infection to be associated with preterm delivery before 32 weeks as well as before 35 weeks gestation. The association was much stronger for 32 weeks than for 35 weeks gestation indicating that *C. trachomatis* infection contributes relatively more to early prematurity compared to late prematurity.

For such an important issue, a six-fold increased risk of preterm delivery before 32 weeks gestation implies that a considerable proportion (15.8% in our cohort) of preterm deliveries before 32 weeks gestation is attributable to chlamydial infection in pregnancy. Although there is a considerable confidence interval around this estimate this would classify *C. trachomatis* among the important infective risk factors of early prematurity. For preterm delivery before 35 weeks the attributable fraction remains significant (7.5%). It should be kept in mind, however, that the number and proportion of early and late premature deliveries attributable to chlamydial infection highly depends upon the *C. trachomatis* prevalence in a given population, and that some confounding as a result of

co-infection by other genital pathogens cannot be excluded. We also did not correct for a previous history of induced abortion in these women since this potential confounder was only recently established (27). Finally, we did not screen the women for the use of macrolide antibiotics during pregnancy, but suggest that such use would mitigate the observed effect of Chlamydia infection of pregnancy outcome rather than exaggerate it.

Extrapolation of our findings to the Netherlands, where approximately 3,000 neonates are born before 32 weeks gestation annually, (28) showed that *C. trachomatis* infection in pregnancy contributes approximately 475 cases to this burden; for deliveries before 35 weeks gestation these numbers are 7,100 and 530, respectively.

Interestingly, Chlamydia infection during pregnancy was more prevalent among women who had twins compared to women who had singletons, a finding not reported before. This observation warrants further study into the medical histories of women with twins versus women with singletons as pregnancy outcomes.

Our finding of an association between *C. trachomatis* infection with premature delivery may be useful in a cost-benefit analysis of screening for *C. trachomatis* infection during pregnancy, especially among women with increased risk for infection.

The major risk factors we found are amongst the many previously described including young age, urban residence, low socio-economic class, specific ethnic groups, single marital status and recent changes in sexual partnerships or sexual promiscuity. (6, 9, 10, 29) Young age is one of the most influential risk factors. (8) Adolescent women are at highest risk, which was also observed in this study. Urban residence has been described to be of importance, which effect we could not evaluate since all participating women resided in Rotterdam. The risk factors we describe are similar to those reported in another recent Dutch community-based study. (7, 30) Importantly, on global scale *C. trachomatis* prevalences vary widely, (6) which will directly affect the incidence of complications attributable to this infection, including preterm delivery.

In conclusion, *C. trachomatis* urogenital infection in pregnant women increases the risk of preterm delivery, especially early prematurity, such that a significant proportion of preterm deliveries can be attributed to this infection. Therefore, *C. trachomatis* infection in pregnancy should be regarded as a serious public health problem, especially for young women belonging to socio-economic groups at increased risk for *C. trachomatis* infection.

References

1. Wiesenfeld HC, Hillier SL, Krohn MA, Amortegui AJ, Heine RP, Landers DV, et al. Lower genital tract infection and endometritis: insight into subclinical pelvic inflammatory disease. *Obstet Gynecol* 2002;100(3):456-63.

2. Brunham RC, Peeling R, Maclean I, Kosseim ML, Paraskevas M. *Chlamydia trachomatis*-associated ectopic pregnancy: serologic and histologic correlates. *J Infect Dis* 1992;165(6):1076-81.
3. Marais NF, Wessels PH, Smith MS, Gericke A, Richter A. *Chlamydia trachomatis, Mycoplasma hominis* and *Ureaplasma urealyticum* infections in women. Prevalence, risks and management at a South African infertility clinic. *J Reprod Med* 1991;36(3):161-4.
4. Ross JM, Furr PM, Taylor-Robinson D, Altman DG, Coid CR. The effect of genital mycoplasmas on human fetal growth. *Br J Obstet Gynaecol* 1981;88(7):749-55.
5. Hardy PH, Hardy JB, Nell EE, Graham DA, Spence MR, Rosenbaum RC. Prevalence of six sexually transmitted disease agents among pregnant inner-city adolescents and pregnancy outcome. *Lancet* 1984;2(8398):333-7.
6. Smith JR, Taylor-Robinson D. Infection due to *Chlamydia trachomatis* in pregnancy and the newborn. *Baillieres Clin Obstet Gynaecol* 1993;7(1):237-55.
7. van Bergen J, Gotz HM, Richardus JH, Hoebe CJ, Broer J, Coenen AJ. Prevalence of urogenital *Chlamydia trachomatis* increases significantly with level of urbanisation and suggests targeted screening approaches: results from the first national population based study in the Netherlands. *Sex Transm Infect* 2005;81(1):17-23.
8. Adams EJ, Charlett A, Edmunds WJ, Hughes G. *Chlamydia trachomatis* in the United Kingdom: a systematic review and analysis of prevalence studies. *Sex Transm Infect* 2004;80(5):354-62.
9. Martin DH, Koutsky L, Eschenbach DA, Daling JR, Alexander ER, Benedetti JK, et al. Prematurity and perinatal mortality in pregnancies complicated by maternal *Chlamydia trachomatis* infections. *Jama* 1982;247(11):1585-8.
10. Harrison HR, Alexander ER, Weinstein L, Lewis M, Nash M, Sim DA. Cervical *Chlamydia trachomatis* and mycoplasmal infections in pregnancy. Epidemiology and outcomes. *Jama* 1983;250(13):1721-7.
11. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and *Chlamydia trachomatis* infection with adverse pregnancy outcome. *Jama* 1986;256(14):1899-903.
12. Sweet RL, Landers DV, Walker C, Schachter J. *Chlamydia trachomatis* infection and pregnancy outcome. *Am J Obstet Gynecol* 1987;156(4):824-33.
13. Martius J, Krohn MA, Hillier SL, Stamm WE, Holmes KK, Eschenbach DA. Relationships of vaginal Lactobacillus species, cervical *Chlamydia trachomatis*, and bacterial vaginosis to preterm birth. *Obstet Gynecol* 1988;71(1):89-95.
14. Alger LS, Lovchik JC, Hebel JR, Blackmon LR, Crenshaw MC. The association of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and group B streptococci with preterm rupture of the membranes and pregnancy outcome. *Am J Obstet Gynecol* 1988;159(2):397-404.
15. Association of *Chlamydia trachomatis* and *Mycoplasma hominis* with intrauterine growth retardation and preterm delivery. The John Hopkins Study of Cervicitis and Adverse Pregnancy Outcome. *Am J Epidemiol* 1989;129(6):1247-57.
16. Claman P, Toye B, Peeling RW, Jessamine P, Belcher J. Serologic evidence of *Chlamydia trachomatis* infection and risk of preterm birth. *Cmaj* 1995;153(3):259-62.
17. Gencay M, Koskiniemi M, Ammala P, Fellman V, Narvanen A, Wahlstrom T, et al. *Chlamydia trachomatis* seropositivity is associated both with stillbirth and preterm delivery. *APMIS* 2000;108(9):584-8.
18. Andrews WW, Goldenberg RL, Mercer B, Iams J, Meis P, Moawad A, et al. The Preterm Prediction Study: association of second-trimester genitourinary chlamydia infection with subsequent spontaneous preterm birth. *Am J Obstet Gynecol* 2000;183(3):662-8.
19. Andrews WW, Klebanoff MA, Thom EA, Hauth JC, Carey JC, Meis PJ, et al. Midpregnancy genitourinary tract infection with *Chlamydia trachomatis*: association with subsequent preterm delivery in women with bacterial vaginosis and *Trichomonas vaginalis*. *Am J Obstet Gynecol* 2006;194(2):493-500.
20. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. *Paediatr Perinat Epidemiol* 2004;18(1):61-72.

21. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. *Eur J Epidemiol* 2006;21(6):475-84.
22. Rours GI, Verkooyen RP, Willemse HF, van der Zwaan EA, van Belkum A, de Groot R, et al. Use of Pooled Urine Samples and Automated DNA Isolation To Achieve Improved Sensitivity and Cost-Effectiveness of Large-Scale Testing for *Chlamydia trachomatis* in Pregnant Women. *J Clin Microbiol* 2005;43(9):4684-90.
23. Niklasson A EA, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). *Acta Paediatr Scand* 1991;80(8-9):756-62.
24. Rothman K.J. GS. *Modern epidemiology*. second ed: Lippincott-Raven; 1998.
25. Ngassa PC, Egbe JA. Maternal genital *Chlamydia trachomatis* infection and the risk of preterm labor. Int J Gynaecol Obstet 1994;47(3):241-6.
26. Blas MM CF, Alva IE, Hawes S. Pregnancy outcome in women infected with *Chlamydia trachomatis*: a population-based cohort study in Washington State. Sexually Transmitted Infections 2007;published online 7 Mar 2007.
27. Moreau C, Kaminski M, Ancel PY, Bouyer J, Escande B, Thiriez G, Boulot P, Fresson J, Arnaud C, Subtil D, Marpeau L, Rozé JC, Maillard F, Larroque B. Previous induced abortions and the risk of very preterm delivery: results of the EPIPAGE study. BJOG 2005;112:430-7.
28. Nederland SPR. Perinatal Care in the Netherlands 2003. Bilthoven: Stichting Perinatale Registratie; 2006.
29. Thomas AG, Brodine SK, Shaffer R, Shafer MA, Boyer CB, Putnam S, et al. Chlamydial infection and unplanned pregnancy in women with ready access to health care. Obstet Gynecol 2001;98(6):1117-23.
30. Gotz HM, van Bergen JE, Veldhuijzen IK, Broer J, Hoebe CJ, Steyerberg EW, et al. A prediction rule for selective screening of *Chlamydia trachomatis* infection. Sex Transm Infect 2005;81(1):24-30.

Urogenital symptoms in different periods of pregnancy and adverse birth outcomes

2.2

Abstract

Objective: The aim of this study was to assess the association of urogenital symptoms in different periods of pregnancy with birth weight and gestational age.
Study Design: This study was embedded in the Generation R Study, a population-based prospective cohort study from early fetal life. Urogenital symptoms, including vaginal bleeding, other vaginal symptoms (vaginal discharge, itching vagina) and urinary tract symptoms (frequent need to urinate, pain or burning feeling during urination), were assessed by questionnaires in early, mid- and late pregnancy. Pregnancy outcomes were obtained from midwife and hospital registries. The present analyses were based on 8,169 pregnant women with complete records.
Results: Vaginal bleeding in late pregnancy was associated with preterm birth (adjusted odds ratio (aOR) 1.94 (95% confidence interval: 1.05, 3.59), but not with birth weight. Associations of other vaginal symptoms with adverse pregnancy outcomes were not found. Urinary tract symptoms in early or in multiple periods of pregnancy were associated with an increased risk of low birth weight, adjusted for gestational age (aOR 2.39 (1.21, 4.72) and 1.71 (1.02, 2.88)), not of preterm birth.
Conclusions: Vaginal bleeding in late pregnancy is associated with an increased risk of preterm birth and urinary tract symptoms in early or present in multiple periods of pregnancy with an increased risk of low birth weight.

Introduction

The incidence of low birth weight and premature birth varies from 4% to 25% and 5 to15%, respectively, depending on geographical and demographical features of the population studied [1-3]. Delivery of low birth weight and premature infants is recognized as a major public health problem, accounting for the majority of neonatal death and morbidity in developed countries. In the last decades, there seems to be an upward trend in the incidence of premature births in Western societies [4-6].

Presence of urogenital tract infections during pregnancy are associated with low birth weight, preterm delivery, premature rupture of membranes and other adverse pregnancy outcomes [7-9]. Laboratory testing on urogenital infections during pregnancy is not routinely performed. Specific antenatal attention for urogenital symptoms, indicating a possible underlying urogenital tract infection, may be helpful in identifying women at increased risk of delivering low birth weight or preterm infants. Symptoms mostly associated with common urogenital infections include vaginal bleeding, change in vaginal discharge, frequent need to urinate or lower abdominal pain [10]. These symptoms result from ascending reproductive tract infections leading to (sub)clinical cervicitis, endometritis, deciduitis or chorioamnionitis which can adversely effect implantation, placental development, fetal growth and gestational duration [11, 12]. Not much is known of the effects of urogenital symptoms in different periods of pregnancy with pregnancy outcomes. This may be relevant for identifying critical time-periods that could be used for preventive strategies.

Therefore, we examined in a population-based cohort study of pregnant women and their children the associations of urogenital symptoms in different periods of pregnancy with the risk of low birth weight and preterm birth.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development and health and has been described previously in detail [13, 14]. Briefly, the cohort includes 9,778 mothers and their children living in Rotterdam, the Netherlands. Enrolment of mothers was aimed in early pregnancy (gestational age < 18 weeks) but was possible until birth of the child. All children were born between April 2002 and January 2006 and form a prenatally enrolled birth-cohort that is currently followed until young adulthood. Of all eligible children in the study area, 61% participated at birth in the study [14]. The

Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Urogenital symptoms

Information about urogenital symptoms was prospectively obtained by postal questionnaires in early (gestational age < 18 weeks), mid- (gestational age18 - 25 weeks) and late (gestational age ≥ 25 weeks) pregnancy. Response rates for the questionnaires were 91% (early pregnancy), 81% (mid- pregnancy) and 77% (late pregnancy). Women were asked whether they experienced the following symptoms in the previous two or three months (no, yes): vaginal bleeding, postcoital bleeding, frequent need to urinate, pain during urination, burning feeling or itching on urethra, vaginal discharge, burning feeling or itching vagina. These urogenital symptoms were subsequently categorized in three groups: 1. vaginal bleeding (vaginal bleeding, postcoital bleeding); 2. other vaginal symptoms (vaginal discharge, burning feeling or itching vagina) and 3. urinary tract symptoms (frequent need to urinate, pain or burning feeling during urination or itching on urethra).

Pregnancy outcomes

Gestational age was established by the first fetal ultrasound examination at enrolment [15]. Crown-rump length was used for pregnancy dating during the first 12 weeks of gestational age and the biparietal diameter for pregnancy dating thereafter. Information about date of birth and birth weight was obtained from community midwives and hospital registries. Preterm birth was defined as a gestational age of less than 37 weeks at birth. Birth weight measurements were converted into gestational age adjusted standard deviation scores (sds) [16]. Low birth weight was defined as birth weight below the 10^{th} percentile (< -1.39 sds) in the study cohort.

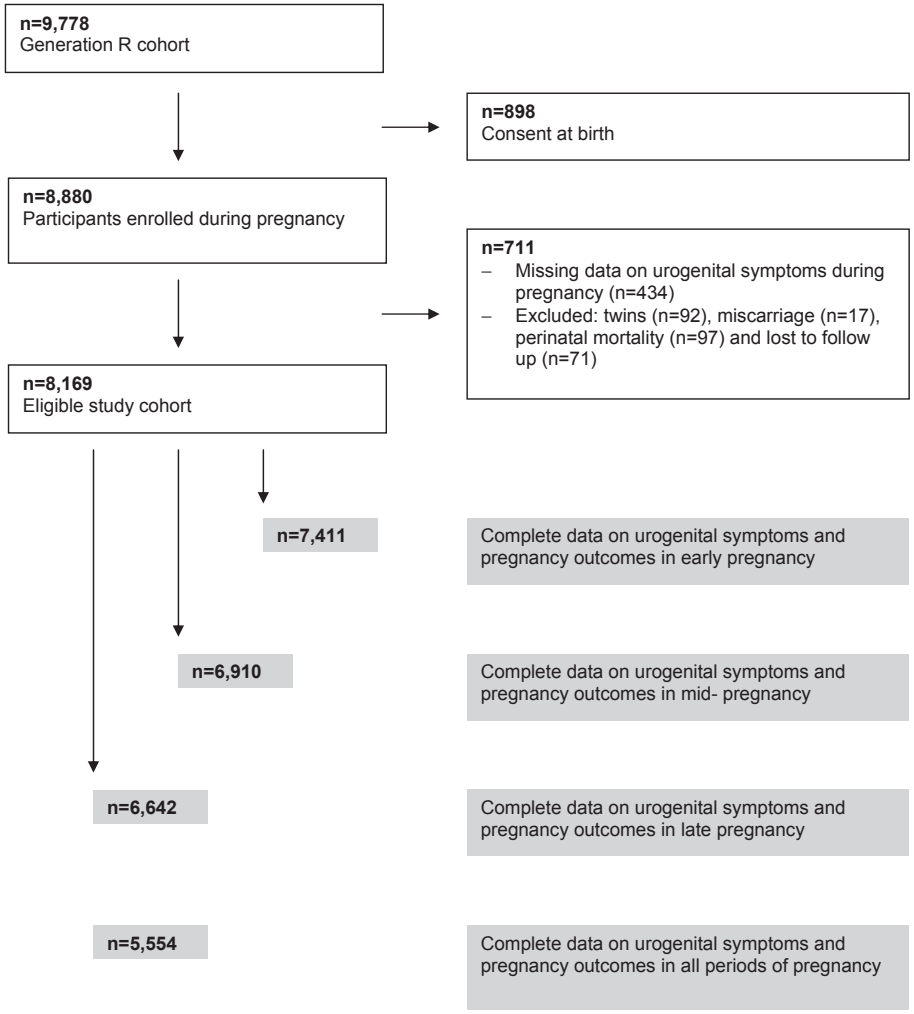
Covariates

Maternal age was recorded at enrolment. Information about educational level, ethnicity, smoking and parity was obtained by the first questionnaire at enrolment in the study. Ethnicity and educational level of the mother were defined according to the classification of Statistics Netherlands [17, 18]. Maternal smoking was categorized into 'non-smoking', 'stopped smoking when pregnancy was known' and 'continued smoking during pregnancy' groups. Parity was defined as nulliparity and multiparity.

Population for analysis

Of the total of 9,778 mothers, 8,880 (91%) and 898 (9%) were enrolled in pregnancy and at delivery of their child, respectively [14]. The present analysis was restricted to mothers who were enrolled in pregnancy (n=8,880). Mothers with no information about urogenital symptoms in any period of pregnancy were excluded (n=434) (Figure 1). Furthermore,

Figure 1. Flow chart participants



those with twin pregnancies (n=92), miscarriage (n=17), perinatal mortality (n=97) or missing pregnancy outcomes (n=71) were excluded. Of the remaining 8,169 mothers, 6.0% was registered with a second (n=479) or third (n=8) pregnancy within the same study period [14]. Since there were no differences in results after exclusion of these subjects, they were included in the analyses presented. Complete data about urogenital symptoms and pregnancy outcomes were available on 7,411 mothers (early pregnancy), 6,910 mothers (mid-pregnancy) and 6,642 mothers (late pregnancy).

Data analysis

The associations between urogenital symptoms and birth weight were analyzed using multiple linear regression analysis. For the associations with low birth weight or preterm birth as the outcome, logistic regression analysis was used. After the crude analyses, potential confounders, including maternal age, education, ethnicity, smoking and parity, were added to the model. First, the associations of urogenital symptoms with pregnancy outcomes were analyzed in each period of pregnancy, not taking urogenital symptoms in the other periods of pregnancy into account. Secondly, urogenital symptoms were reclassified in having symptoms in only early, only mid-, only late or in multiple periods of pregnancy. This was performed to examine whether associations with pregnancy outcomes are explained by having urogenital symptoms in one period only or in multiple periods during pregnancy. These analyses were assessed in mothers of whom data on urogenital symptoms was available in all three periods of pregnancy (n=5,554). The sta-

Table 1. Maternal and infant characteristics.

	Single live births*	
	Boys (n=4120)	**Girls (n=4049)**
Age (years)	29.8 (5.3)	29.7 (5.2)
Ethnicity (%)		
Dutch	50.0	50.7
Capeverdean	3.7	4.3
Antillean	3.4	3.5
Surinamese	9.3	8.6
Moroccan	6.8	6.4
Turkish	9.2	8.8
Other western	11.6	11.6
Other (non-) western	6.0	6.3
Education (%)		
Primary school	12.3	13.2
Secondary school	45.1	44.7
Higher education	42.7	42.1
Smoking (%)		
Never	74.3	76.4
Until pregnancy was known	7.2	7.7
Continued	18.6	15.9
Nulliparae (%)	55.9	57.6
Birth weight (grams)	3476 (635)	3353 (538)
Gestational age (weeks)†	40.1 (36.9-42.1)	40.1 (37.0-42.0)

Values are means (standard deviation) or percentages. †Median (5-95% range).
*Data were missing on ethnicity (n=589), education (n=830), smoking (n=44) and parity (n=226).

tistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Subject characteristics

Characteristics of the mothers and their infants are presented in Table 1. The age of mothers at enrolment ranged from 15.3 to 46.3 years (mean 29.7 years). Of all mothers, those of Dutch, Surinamese, Moroccan and Turkish origin and those with a secondary or higher education were most present. Of the mothers, 17.3% continued smoking during pregnancy and 56.7% were nulliparae. Mean birth weight was higher in boys than girls (3476 versus 3353 grams). No differences in gestational age between boys and girls were observed.

Vaginal bleeding during pregnancy was present varying from 15.8% in early pregnancy to 6.1% in late pregnancy (Table 2). Other vaginal and urinary tract symptoms ranged from 64% to 73.5% and 75.5% to 80.2% in early and late pregnancy, respectively.

Maternal and infant characteristics and prevalence of urogenital symptoms did not differ between mothers with data in early pregnancy (n=7,411), mid-pregnancy (n=6,910), late pregnancy (n=6,642) and mothers of whom data were available in all three periods of pregnancy (n=5,554).

Table 2. Prevalences of urogenital symptoms in early, mid- and late pregnancy.

	Urogenital symptoms (%)*		
	Vaginal bleeding	**Other vaginal symptoms**	**Urinary tract symptoms**
Early pregnancy (n=7,411)	15.8	64.0	75.5
Mid- pregnancy (n=6,910)	7.5	68.4	77.6
Late pregnancy (n=6,642)	6.1	73.5	80.2

Values are percentages.
*Data on symptoms were missing in:
Early pregnancy: vaginal bleeding (n=33), other vaginal symptoms (n=121) and urinary tract symptoms (n=82).
Mid- pregnancy: vaginal bleeding (n=19), other vaginal symptoms (n=109) and urinary tract symptoms (n=86).Late pregnancy: vaginal bleeding (n=11), other vaginal symptoms (n=42) and urinary tract symptoms (n=18).

Urogenital symptoms and pregnancy outcomes

Mothers with vaginal bleeding, other vaginal or urinary tract symptoms in early pregnancy, did not show a difference in continuously measured birth weight or low birth weight, compared to mothers who did not had these symptoms (Table 3). However, mothers with vaginal bleeding in early pregnancy showed a higher risk of preterm birth, adjusted for age, education, ethnicity, smoking and parity (aOR 1.38 (95% confidence interval (95% CI) 1.06, 1.81)). In mid- pregnancy, vaginal bleeding was associated with low birth

Table 3. Urogenital symptoms in early, mid- and late pregnancy and pregnancy outcomes.

	Pregnancy outcomes		
	Difference in birth weight (grams (95% CI))	**Low birth weight (aOR (95% CI))**	**Preterm birth (aOR (95% CI))**
Early pregnancy			
Vaginal bleeding	-2 (-30, 26)	1.10 (0.89, 1.36)	1.38 (1.06, 1.81)*
Other vaginal symptoms	12 (-10, 33)	0.90 (0.76, 1.07)	0.84 (0.67, 1.05)
Urinary tract symptoms	-5 (-29, 19)	1.09 (0.89, 1.33)	1.00 (0.76, 1.29)
Mid- pregnancy			
Vaginal bleeding	-27 (-70, 15)	1.41 (1.05, 1.91)*	1.36 (0.90, 2.05)
Other vaginal symptoms	15 (-9, 39)	0.90 (0.74, 1.09)	0.92 (0.71, 1.20)
Urinary tract symptoms	-5 (-32, 22)	1.06 (0.84, 1.33)	1.28 (0.92, 1.77)
Late pregnancy			
Vaginal bleeding	-38 (-85, 9)	1.19 (0.82, 1.69)	1.49 (0.96, 2.33)
Other vaginal symptoms	-4 (-32, 25)	0.92 (0.75, 1.14)	1.24 (0.92, 1.69)
Urinary tract symptoms	16 (-10, 41)	1.06 (0.83, 1.36)	1.42 (0.99, 2.04)

Reference for each type of symptom is the group of pregnant women without that specific symptom.
Values are regression coefficients (95% confidence interval (CI)) or odds ratio's (OR (95% CI)), adjusted for maternal age, education, ethnicity, smoking and parity. Birth weight was additionally adjusted for gestational age.
*p-value <0.05.

weight (aOR 1.41 (95% CI: 1.05, 1.91)). No other urogenital symptoms in mid-pregnancy were associated with adverse pregnancy outcomes. In late pregnancy, trends were found for vaginal bleeding, other vaginal and urinary tract symptoms with preterm birth with an aOR 1.49 (95% CI: 0.96, 2.33), 1.24 (95% CI: 0.92, 1.69) and 1.42 (95% CI: 0.99, 2.04), respectively. No associations of urogenital symptoms in late pregnancy with a difference in continuously measured birth weight or low birth weight were found.

As for urogenital symptoms in only one period of pregnancy, mothers with urinary tract symptoms in only early pregnancy showed an association with low birth weight (aOR 2.39 (95% CI: 1.21, 4.72)) (Table 4). Mothers with vaginal bleeding only in mid-pregnancy were associated with continuously measured birth weight (-93 grams (95% CI: -172, -14)). Vaginal bleeding only in late pregnancy increased the risk of preterm birth (aOR 1.94 (95% CI: 1.05, 3.59)). Other associations of urogenital symptoms in only one period of pregnancy with adverse pregnancy outcomes were not observed.

As for mothers with urogenital symptoms in multiple periods of pregnancy, vaginal bleeding or other vaginal symptoms in ≥ 2 periods of pregnancy were not related to adverse pregnancy outcomes. Mothers with urinary tract symptoms in ≥ 2 periods of pregnancy had a higher risk of low birth weight infants compared to mothers without urinary tract symptoms in ≥ 2 periods of pregnancy (aOR 1.71 (95% CI: 1.02, 2.88)).

Table 4. Urogenital symptoms present in only one period or in multiple periods of pregnancy and pregnancy outcomes.

	Pregnancy outcomes		
	Difference in birth weight (grams (95% CI))	**Low birth weight (aOR (95% CI))**	**Preterm birth (aOR (95% CI))**
Early pregnancy only			
Vaginal bleeding	4 (-33, 41)	0.92 (0.68, 1.26)	1.10 (0.73, 1.65)
Other vaginal symptoms	3 (-65, 70)	1.12 (0.67, 1.89)	0.63 (0.26, 1.52)
Urinary tract symptoms	-70 (-142, 3)	2.39 (1.21, 4.72)*	0.81 (0.32, 2.05)
Mid- pregnancy only			
Vaginal bleeding	-93 (-172, -14)*	1.64 (0.96, 2.81)	0.96 (0.38, 2.38)
Other vaginal symptoms	12 (-69, 92)	0.89 (0.45, 1.75)	0.67 (0.23, 1.93)
Urinary tract symptoms	-27 (-111, 58)	1.86 (0.85, 4.09)	1.05 (0.39, 2.81)
Late pregnancy only			
Vaginal bleeding	-55 (-127, 16)	0.93 (0.53, 1.65)	1.94 (1.05, 3.59)*
Other vaginal symptoms	33 (-24, 89)	1.05 (0.67, 1.17)	1.43 (0.81, 2.51)
Urinary tract symptoms	-46 (-114, 21)	1.69 (0.86, 3.33)	0.62 (0.25, 1.56)
≥ 2 periods of pregnancy			
Vaginal bleeding	-10 (-61, 42)	1.15 (0.77, 1.73)	1.25 (0.73, 2.16)
Other vaginal symptoms	25 (-9, 59)	0.99 (0.67, 1.17)	0.95 (0.6, 1.40)
Urinary tract symptoms	-29 (-75, 17)	1.71 (1.02, 2.88)*	1.07 (0.61, 1.88)

Reference for each type of symptom is the group of pregnant women without that specific symptom.
Values are regression coefficients (95% confidence interval (CI)) or odds ratio's (OR (95% CI)), adjusted for maternal age, education, ethnicity, smoking and parity. Birth weight was additionally adjusted for gestational age.
*p-value <0.05.

Discussion

This prenatally enrolled birth-cohort study showed that mothers with vaginal bleeding in late pregnancy have an increased risk of preterm birth. Other vaginal symptoms of mother in any period of pregnancy did not affect birth weight or gestational age of the infants. Mothers with urinary tract symptoms in early or in multiple periods of pregnancy had an increased risk of delivering a low birth weight infant. Having urinary tract symptoms in mid- or late pregnancy was weakly associated with low birth weight.

The strength of this study is the population-based cohort with a large number of subjects with data on urogenital symptoms in different periods of pregnancy, information about a large number of potential confounders and a high follow-rate until birth (97%). Because of the large sample size and the high prevalence of the diverse urogenital symptoms, we were able to study the association of these symptoms with pregnancy outcomes in single or multiple periods of pregnancy separately.

Accounting for having vaginal bleeding in multiple periods of pregnancy, the overall prevalence of vaginal bleeding during pregnancy in our study (21.8%) was similar to that

found in other cohort studies (15-24.4%) [19-21]. According to the association of vaginal bleeding and adverse pregnancy outcomes, former studies found relative risk estimates between 0.3 to 6.4 for vaginal bleeding and preterm birth and risk estimates between 1.6 to 3.0 for vaginal bleeding and low birth weight [22-26]. However, in these studies neither different periods of bleeding in pregnancy independent from each other nor low birth weight adjusted for gestational age were studied. One similar study assessed the separate effects of vaginal bleeding in different trimesters and showed that vaginal bleeding in first trimester only led to a higher risk of 1.6 for preterm birth < 34 weeks, but not of preterm birth <37 weeks [19]. Our study replicated the latter conclusion and additionally, we found that vaginal bleeding in only late pregnancy was associated with preterm birth. Risk estimates of vaginal bleeding and low birth weight found in earlier studies probably reflect the underlying effect of gestational age, since in our study no effect of vaginal bleeding in any period of pregnancy was seen for gestational age adjusted low birth weight.

Not finding an association of other vaginal symptoms with adverse pregnancy outcomes could be accounted to the fact that not all of the assessed symptoms are specific enough for infections like vaginal candidiasis, bacterial vaginosis or vaginal trichomoniasis which increase the risk of preterm birth or low birth weight [27]. Furthermore, vaginal symptoms could also imply normal, physiological pregnancy induced changes of the vagina.

Studies that examined urinary tract infections or asymptomatic bacteriuria in pregnancy, showed a high rate of low birth weight and preterm birth [28, 29]. Screening and treating of bacteruria in early pregnancy has been recommended to prevent adverse pregnancy outcomes [30, 31]. Our lack of associations between urinary tract symptoms, a proxy for urinary tract infection, with preterm birth may be explained by treatment of underlying tract infections. However, associations of urinary tract symptoms in only early pregnancy and present in multiple periods of pregnancy with low birth weight were observed. A pathophysiological mechanism could be that urinary tract infections lead to irreversible placental changes leading to inadequate supply of nutrients to the fetus. This would subsequently lead to fetal growth retardation and low birth weight. This hypothesis is supported by animal models that demonstrated that urinary tract infected mice (*E. Coli* Dr^+) had a reduction of fetal birth weight and massive bacterial infiltration of the placenta, causing increased inflammation with a reduction of the placental villi [32]. This implies a different mechanism than that leading to preterm birth, including cytokine induced prostaglandin synthesis leading to uterine contractions, cervical dilatation and greater entry of microbes into the uterine cavity [33].

Some methodological issues ought to be considered. Data on urogenital symptoms in any period during pregnancy was missing in 5% of the participating mothers. No difference in prevalence of low birth weight or preterm birth was present between those

with and without data on urogenital symptoms during pregnancy (low birth weight 10.1% and 11.7% (χ^2-test, p-value 0.317); preterm birth 5.1% and 6.2% (χ^2-test, p-value 0.316), respectively). Furthermore, infants of whom data on birth weight and gestational age was missing were as much from mothers who had urogenital symptoms as from mothers who had no urogenital symptoms (3.2% and 4% (χ^2-test, p-value 0.390), respectively). Therefore it is unlikely that the observed association of urogenital symptoms and pregnancy outcomes were a result of selection bias.

Information about urogenital symptoms of mother in different periods of pregnancy was prospectively collected by questionnaires without reference to the pregnancy outcomes. Using self-reported urogenital symptoms may have introduced misclassification because of over- or underreporting of urogenital symptoms in pregnancy. Previous studies suggested for example that early vaginal bleeding could be unintentionally reported as last menstrual period bleeding, consequently leading to a lower estimated gestational age and an increased association of vaginal bleeding and preterm birth [20, 26]. Since in our study gestational age in all women was established by ultrasound examination at enrolment, misclassification of preterm birth seems unlikely. In late pregnancy, vaginal bleeding might be caused as well by infections as by structural placental abnormalities which are also associated with preterm birth [34]. Therefore, the reported association between vaginal bleeding in late pregnancy and preterm birth could probably partly be explained by pregnancies complicated with these placental abnormalities.

Our findings suggest that vaginal bleeding in late pregnancy increases the risk of preterm birth and urinary tract symptoms in early or present in multiple periods of pregnancy increase the risk of low birth weight. The exact underlying pathogens or other non-infectious causes regarding the association of vaginal bleeding and urinary tract symptoms with adverse pregnancy outcomes need to be explored. Screening on these urogenital symptoms might contribute to primary prevention of these adverse pregnancy outcomes.

References

1. Steer P. The epidemiology of preterm labor--a global perspective. J Perinat Med. 2005;33(4):273-6.
2. Slattery MM, Morrison JJ. Preterm delivery. Lancet. 2002 Nov 9;360(9344):1489-97.
3. Kramer MS. The epidemiology of adverse pregnancy outcomes: an overview. J Nutr. 2003 May;133(5 Suppl 2):1592S-6S.
4. Langhoff-Roos J, Kesmodel U, Jacobsson B, Rasmussen S, Vogel I. Spontaneous preterm delivery in primiparous women at low risk in Denmark: population based study. Bmj. 2006 Apr 22;332(7547):937-9.
5. Stoelhorst GM, Rijken M, Martens SE, Brand R, den Ouden AL, Wit JM, et al. Changes in neonatology: comparison of two cohorts of very preterm infants (gestational age <32 weeks): the Project On Preterm and Small for Gestational Age Infants 1983 and the Leiden Follow-Up Project on Prematurity 1996-1997. Pediatrics. 2005 Feb;115(2):396-405.

6. Davidoff MJ, Dias T, Damus K, Russell R, Bettegowda VR, Dolan S, et al. Changes in the gestational age distribution among U.S. singleton births: impact on rates of late preterm birth, 1992 to 2002. Semin Perinatol. 2006 Feb;30(1):8-15.
7. Goldenberg RL, Culhane JF, Johnson DC. Maternal infection and adverse fetal and neonatal outcomes. Clin Perinatol. 2005 Sep;32(3):523-59.
8. Pararas MV, Skevaki CL, Kafetzis DA. Preterm birth due to maternal infection: Causative pathogens and modes of prevention. Eur J Clin Microbiol Infect Dis. 2006 Sep;25(9):562-9.
9. Nelson DB, Macones G. Bacterial vaginosis in pregnancy: current findings and future directions. Epidemiol Rev. 2002;24(2):102-8.
10. French JI, McGregor JA, Draper D, Parker R, McFee J. Gestational bleeding, bacterial vaginosis, and common reproductive tract infections: risk for preterm birth and benefit of treatment. Obstet Gynecol. 1999 May;93(5 Pt 1):715-24.
11. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med. 2000 May 18;342(20):1500-7.
12. Witkin S, McGregor JA. Infection-induced activation of cell-mediated immunity: possible mechanism for preterm birth. Clin Obstet Gynecol. 1991 Mar;34(1):112-21.
13. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol. 2004 Jan;18(1):61-72.
14. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21(6):475-84.
15. Tunon K, Eik-Nes SH, Grottum P. A comparison between ultrasound and a reliable last menstrual period as predictors of the day of delivery in 15,000 examinations. Ultrasound Obstet Gynecol. 1996 Sep;8(3):178-85.
16. Niklasson A, Ericson A, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). Acta Paediatr Scand. 1991 Aug-Sep;80(8-9):756-62.
17. Standaard onderwijsindeling 2003. Voorburg/Heerlen: Statistics Netherlands; 2004.
18. Allochtonen in Nederland 2004. Voorburg/Heerlen: Statistics Netherlands; 2004.
19. Yang J, Hartmann KE, Savitz DA, Herring AH, Dole N, Olshan AF, et al. Vaginal bleeding during pregnancy and preterm birth. Am J Epidemiol. 2004 Jul 15;160(2):118-25.
20. Axelsen SM, Henriksen TB, Hedegaard M, Secher NJ. Characteristics of vaginal bleeding during pregnancy. Eur J Obstet Gynecol Reprod Biol. 1995 Dec;63(2):131-4.
21. Ananth CV, Savitz DA. Vaginal bleeding and adverse reproductive outcomes: a meta-analysis. Paediatr Perinat Epidemiol. 1994 Jan;8(1):62-78.
22. Funderburk SJ, Guthrie D, Meldrum D. Outcome of pregnancies complicated by early vaginal bleeding. Br J Obstet Gynaecol. 1980 Feb;87(2):100-5.
23. Berkowitz GS, Harlap S, Beck GJ, Freeman DH, Baras M. Early gestational bleeding and pregnancy outcome: a multivariable analysis. Int J Epidemiol. 1983 Jun;12(2):165-73.
24. Strobino B, Pantel-Silverman J. Gestational vaginal bleeding and pregnancy outcome. Am J Epidemiol. 1989 Apr;129(4):806-15.
25. Harger JH, Hsing AW, Tuomala RE, Gibbs RS, Mead PB, Eschenbach DA, et al. Risk factors for preterm premature rupture of fetal membranes: a multicenter case-control study. Am J Obstet Gynecol. 1990 Jul;163(1 Pt 1):130-7.
26. Yang J, Savitz DA. The effect of vaginal bleeding during pregnancy on preterm and small-for-gestational-age births: US National Maternal and Infant Health Survey, 1988. Paediatr Perinat Epidemiol. 2001 Jan;15(1):34-9.
27. Anderson MR, Klink K, Cohrssen A. Evaluation of vaginal complaints. Jama. 2004 Mar 17;291(11):1368-79.

28. Romero R, Oyarzun E, Mazor M, Sirtori M, Hobbins JC, Bracken M. Meta-analysis of the relationship between asymptomatic bacteriuria and preterm delivery/low birth weight. Obstet Gynecol. 1989 Apr;73(4):576-82.
29. McGrady GA, Daling JR, Peterson DR. Maternal urinary tract infection and adverse fetal outcomes. Am J Epidemiol. 1985 Mar;121(3):377-81.
30. Smaill F. Antibiotics for asymptomatic bacteriuria in pregnancy. Cochrane Database Syst Rev. 2001(2):CD000490.
31. Vazquez JC, Villar J. Treatments for symptomatic urinary tract infections during pregnancy. Cochrane Database Syst Rev. 2003(4):CD002256.
32. Kaul AK, Khan S, Martens MG, Crosson JT, Lupo VR, Kaul R. Experimental gestational pyelonephritis induces preterm births and low birth weights in C3H/HeJ mice. Infect Immun. 1999 Nov;67(11):5958-66.
33. Romero R, Sirtori M, Oyarzun E, Avila C, Mazor M, Callahan R, et al. Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. Am J Obstet Gynecol. 1989 Sep;161(3):817-24.
34. Salafia CM, Lopez-Zeno JA, Sherer DM, Whittington SS, Minior VK, Vintzileos AM. Histologic evidence of old intrauterine bleeding is more frequent in prematurity. Am J Obstet Gynecol. 1995 Oct;173(4):1065-70.

3

Breastfeeding protects against infectious diseases during infancy in industrialized countries. A systematic review.

3.1

Abstract

Objective: This review was performed to assess the effect of breastfeeding against infectious diseases during infancy in industrialized countries. Secondly, the effect of duration and exclusiveness of breastfeeding were explored.

Method: Studies were identified using Medline, Cochrane Library, Science Citation Index, and by a manual search from bibliographies of articles from August 1986 until January 2008. Follow-up and case control studies performed in an industrialized country, published in English, with overall infections, gastrointestinal or respiratory tract infections as a major outcome, and at least 40 subjects in the study were included. Using Bauchner's criteria published in a review in 1986, two reviewers and a peer reviewer assessed the internal validity of those studies.

Results: Twenty-one studies that met the inclusion and internal validity criteria were included. These included five case controls and sixteen follow-up studies. Four out of five studies observed decreased effects on overall infections in breastfed infants. With regard to gastrointestinal infections, five out of seven studies suggested that breastfeeding had a protective effect. Thirteen out of fifteen studies concluded that breastfeeding protects infants against respiratory tract infections. Five studies combined duration and exclusiveness of breastfeeding. All studies observed a protective dose/duration-response effect on gastrointestinal or respiratory tract infections.

Conclusions: These studies strongly suggest that breastfeeding protects infants against overall infections, gastrointestinal and respiratory tract infections in industrialized countries. The optimal duration of exclusive breastfeeding for protection against infectious diseases needs to be studied in more detail.

Introduction

The World Health Organization recommends mothers to breastfeed their infants, due to an accumulating evidence of the breastfeeding's benefits in growth, development and health (2). There are several mechanisms explaining how human milk can protect infants from infectious diseases. Human milk has secretory IgA antibodies which react on microbes in the infant's mucosa by inducing an inflammatory response consequently preventing infection, tissue engagement and energy loss (3, 4). Human milk also contains other protective factors like lactoferrin and oligosaccharides, functioning as the analogues for microbial receptors and thus preventing from mucosa attachment (3-5). Furthermore, the transfer of numerous cytokines and growth factors via milk may add to an active stimulation of the infant's immune system (3). Consequently, the risks of infections like gastrointestinal infections, otitis media, other respiratory tract infections, and infection-induced wheezing may be reduced for several years after the termination of breastfeeding (3).

In developing or low income countries, it has been repeatedly shown that breastfeeding has a protective effect especially against two major infectious diseases in infancy, gastrointestinal and respiratory tract infections (1). On the contrary, research results were often conflicting in developed or industrialized countries, where overall sanitation and health care are better (6, 7). These conflicting result may be due to methodological flaws suggested by Bauchner et al. in 1986 and include failure to prevent from detection bias, failure to control for confounding variables, non-blind assessment, failure to control for reverse causality, small samples sizes, and lack of precision in specifying the categories of feeding and health outcomes (7). In Europe and the United States, the incidences of breastfeeding showed an increasing trend after the eighties (8-10). However, more than half of the mothers who initially start breastfeeding stop before the infants reach the age of six months (9-12).

This review was performed to assess the effect of breastfeeding on the risks of overall infections, gastrointestinal and respiratory tract infections during infancy in industrialized countries, using studies started after the publication of the article of Bauchner et al. Secondly, the duration and exclusiveness of breastfeeding were taken into account.

Methods

Articles were searched using medical databases, namely Medline, Cochrane Library, and Science Citation Index. Secondary references were scanned from the bibliographies of each article on breastfeeding and infection and relevant studies were obtained. The keywords used were: breastfeeding, breast milk; infant; gastrointestinal infection, respira-

tory tract infection, infectious diseases; developed, western or industrialized countries; and combinations of those terms. The last search was performed in January 2008.

Inclusion criteria were as follows: the article had to be written in English and published after 1986, the year in which the review of Bauchner et al was published; the study design had to be case control or follow-up study and the study had to be performed in an industrialized country. An industrialized country was defined as a country that the World Bank considered to have a high income. In January 2008 this income applied to 60 countries, mostly in Western and Northern Europe, Northern America, Australia, and some Asian countries (www.worldbank.org/data/countryclass/classgroups.htm#High_income). Furthermore, breastfeeding had to be at least one of the determinants and overall infections, gastrointestinal or respiratory tract infections had to be one of the major outcomes. The study also had to have a total of at least 40 subjects, since studies with smaller sample sizes are likely to have inadequate statistical power (7). Two reviewers screened the abstracts on the above criteria. For further analysis, both reviewers independently read the articles obtained by all methods. Differences in opinion between the reviewers were proposed to a third reviewer and discussed to get consensus.

Based on the search, twenty-six studies met the inclusion criteria for further analysis. After inclusion, the internal validity of those studies were assessed using Bauchner's criteria (7): (1) avoidance of detection bias, (2) adjustment for confounding variables, such as socioeconomic status, size of the family, smoking by the mother, and the mother's level of education, (3) a definition of outcome events, and (4) a definition of infant feeding (7). Studies that met at least three criteria were included and twenty-one articles matched those pre-specified criteria. From those valid studies, five were case control and sixteen were follow-up studies. We categorized those studies according to the outcome measured (Table 1). There were eleven studies which met four criteria, and ten studies which met three of the internal validity criteria.

Results

Breastfeeding and overall infections

A limited number of articles that studied overall infectious morbidity as the outcome was found.

Leventhal et al (13) found that breastfeeding protects infants from hospitalization rather than from infections (Table 1). The protective effect of breastfeeding in their study was substantially diminished when the data were stratified according to the severity of infections, in order to minimize the potential surveillance bias. They ended up with an odds ratio of 0.79 (95% confidence interval: 0.47 to 1.32) for infants with serious infections and an odds ratio 0.17 (0.03, 0.44) for infants with mild infections. Another study

came from Beaudry et al (14) which found that the Incidence Density Ratio (IDR) for all infections in infants from 0-6 months of age was 0.67 (0.54,0.82). However, they did not control for potential confounders for this relation.

Recent studies that adjusted for confounders showed that infants with a shorter breastfeeding duration had higher risks of infections or illnesses that required a doctor or hospital admission (15-17).

Breastfeeding and gastrointestinal infections

Seven studies had gastrointestinal infections (e.g. gastroenteritis, diarrhea, vomiting) as the outcome measured (Table 1). From those studies, five studies suggested a protective effect of breastfeeding, which varied according to the duration and exclusiveness of breastfeeding. Howie et al (18) found that babies breastfed for 13 weeks or more had significantly less gastrointestinal infections at the age of 0 to 13 weeks ($p<0.01$; reduction in incidence 6.6%-16.8%), at 14 to 26 weeks ($p<0.05$), at 27 to 39 weeks ($p<0.05$) and at 40 to 52 weeks ($p<0.05$), than those who were bottle fed from birth. Infants who were breastfed less than 13 weeks had a similar rate of gastrointestinal infections as bottle fed infants. Dewey et al (19) found that during the first year of life, the incidence of gastrointestinal infections among formula fed infants was approximately twice that of breastfed infants, even after controlling for day care use and number of siblings. Scariati et al (20) concluded that, after adjusting for potential confounders, the risk of gastrointestinal infections remained significant between infants who received no breast milk compared with those who received only breast milk (OR 1.8, $p<0,05$). Compared with infants exclusively breastfed for 3 months, Kramer et al (21) showed a reduced risk for gastrointestinal infections at age 3 to 6 months in infants exclusively breastfed for 6 months (IDR 0.35 (0.13, 0.96). This reduced risk was not observed for gastrointestinal infections at the age of 6 to 12 months. Quigley et al (22) found a protective effect for diarrhea in the first 8 months of life in infants exclusively breastfed (OR 0.37 (0.18, 0.78)).

Two studies did not observe a protective effect of breastfeeding were from Beaudry et al and Rubin et al (14, 23). The first study concluded that breastfeeding was protective against gastrointestinal infections during the first 6 months of life (Incidence Density Ratio (IDR) 0.53 (95% CI 0.27-1.04). However, after adjusting per potential confounders (socioeconomic status, infant's age, mother's age or maternal cigarette consumption) the IDR's mostly became non significant. The latter study observed no substantial protective effect of breastfeeding against gastrointestinal infections, with an adjusted IDR of 1.07 (0.98, 1.23).

Table 1. Characteristics of studies included in the review categorized by outcomes.
BF = breastfeeding; RTI = respiratory tract infection/illness, URTI = upper respiratory tract infection/illness, LRTI = lower respiratory tract infection/illness and OM = otitis media.
OR = odds ratio; RR = relative risk; HR = hazard ratio and IDR = incidence density ratio.
*IDR calculated as the rate of infections among breastfed infants divided by the rate of same type infections among bottle fed infants.
**At 2 months RR calculated as percentage of infections among those with risk factors divided by percentage of infections among those without risk factors.
Recalculated OR from original proportions.

Outcome	Study	Country	Design	Number of subjects	Age included	Result (95%CI)
Overall infections	Leventhal, 1986 (13)	US	Case control	146 cases 151 controls	< 90 days	OR 0.79 (0.47,1.32) for hospitalized serious illness if BF OR 0.17 (0.03, 0.44) for mild illness if BF Reference: bottle feeding
	Beaudry, 1995 (14)	Canada	Retrospective cohort	776	0-6 months	IDR 0.67 (0.54, 0.82) for respiratory illness*
	Pettigrew, 2003, (16)	US	Prospective cohort	674	0-6 months	For firstborn children, per week longer BF, 4% ($p < 0.01$) increase of illness requiring a health care provider
	Pardo-Crespo, 2004 (15)	Spain	Case control	336 cases and controls	0-24 months	Shorter BF duration in children admitted for all causes (40.6 ± 5.4 vs. 99.5 ± 5.4 days; $p < 0.01$)) and fever of unknown origin ≤ 6 months (40.8 ± 12.4 vs. 91.7 ± 12.4 days; $p < 0.01$), not ≥ 6 months.
	Paricio Talayero, 2006 (17)	Spain	Prospective cohort	1,385	0-12 months	HR 2.45 (1.28, 4.66) for hospitalization for infections if exclusive BF < 4 months Reference: exclusive BF ≥ 4 months
Gastrointestinal infections	Howie, 1990 (18)	UK	Prospective cohort	750	0-24 months	Significant differences in rate GI ($p<0.01$ at ages 0-13 weeks and 14-26 weeks; $p<0.05$ at ages 27-39 weeks and 40-52 weeks) OR 0.17 (0.05, 0.59) if BF > 13 weeks# Reference: never BF
	Rubin, 1990 (23)	Denmark	Prospective cohort	500	0-12 months	IDR 1.067 (0.98, 1.23) for gastroenteritis*
	Beaudry, 1995 (14)	Canada	Retrospective cohort	776	0-6 months	IDR 0.53 (0.27, 1.04) for gastrointestinal illness*
	Dewey, 1995 (19)	US	Prospective cohort	144	0-24 months	Incidence 0.31 vs. 0.15 episodes/100 days at risk (significant using log-linear analysis) for diarrheal illness 0-12 months if bottle feeding Reference: BF

Table 1 continued.

Outcome	Study	Country	Design	Number of subjects	Age included	Result (95%CI)
	Scariati, 1997 (20)	US	Prospective cohort	2,615	0-12 months	OR 1.8 ($p < 0.05$) for diarrhea if formula feeding only Reference: BF only
	Kramer, 2003 (21)	Belarus	Nested observational cohort	3,483	0-12 months	IDR 0.35 (0.13, 0.96) for GI at age 3-6 months if exclusive BF 6 months IDR 0.90 (0.46, 1.78) for GI 6-12 months if exclusive BF 6 months Reference: exclusive BF < 3 months
	Quigley, 2007 (22)	UK	Prospective cohort	15,980	0-8 months	OR 0.37 (0.18, 0.78) for diarrhea if exclusive BF OR 1.98 (1.32, 2.96) for diarrhea 1-4 months; per month cessation of BF OR 1.28 (1.01, 1.61) for diarrhea 5-7 months; per month cessation of BF Reference: no BF
Respiratory tract infections	Howie, 1990 (18)	UK	Prospective cohort	750	0-24 months	OR 0.37 (0.15, 0.96) for RTI if BF > 13 weeks[#] Reference: never BF
	Rubin, 1990 (23)	Denmark	Prospective cohort	500	0-12 months	IDR 0.98 (0.88, 1.10) for URTI*
	Holberg, 1991 (24)	US	Prospective cohort	1,179	0-12 months	RR 4.0 (1.1, 14.1) for RTI if formula feeding** RR 2.2 (0.5, 10.9) for RTI if mixed feeding** Non-significant at any older age Reference: exclusive BF
	Duncan, 1993 (25)	US	Retrospective cohort	1,120	0-12 months	Significant difference in mean number of episodes of acute and recurrent OM ($p<0.01$) if exclusive BF for 4 and 6 months, respectively. Reference: never BF and exclusive BF < 4 months, respectively
	Aniansson, 1994 (26)	Sweden	Prospective cohort	448	0-12 months	Significant lower frequencies of acute OM if BF ($p<0.05$) Reference: no BF
	Pisacane, 1994 (27)	Italy	Case Control	74 cases 88 controls	0-6 months	OR 0.22 (0.09, 0.55) for acute LRTI if BF on admission Reference: never BF
	Beaudry, 1995 (14)	Canada	Retrospective cohort	776	0-6 months	IDR 0.78 (0.61, 1.00) for RTI*

Table 1 continued.

Outcome	Study	Country	Design	Number of subjects	Age included	Result (95%CI)
	Dewey, 1995 (19)	US	Prospective cohort	144	0-24 month	No significant difference in incidence of RTI if BF Incidence 0.53 vs. 0.45 episodes/100 days at risk (significant using log-linear analysis) for OM 0-12 months if bottle feeding Reference: breastfeeding
	Cushing, 1998 (28)	US	Prospective cohort	1202	0-6 months	OR 1.10 (0.98, 1.24) for URTI if full BF OR 0.79 (0.67, 0.91) for LRTI if full BF Reference: no BF
	Koch, 2003 (29)	Greenland	Prospective cohort	288	0-24 months	RR 2.98 (0.91, 9.71) for LRTI if partly BF RR 3.66 (1.06, 12.6) for LRTI if stopped BF Reference group: exclusive BF
	Oddy, 2003 (30)	Australia	Prospective cohort	2,602	0-12 months	OR 1.43 (1.02, 2.01) for URTI if predominant BF < 2 months Reference group: predominant BF > 2 months
	Sinha, 2003 (31)	US	Nested Case Control	12,420	0-30 days	OR 1.1 (0.64, 2.0) for neonatal RTI in boys if exclusive BF OR 1.4 (0.78, 2.4) for neonatal RTI in boys if mixed feeding OR 0.5 (0.29, 0.79) for neonatal RTI in girls if exclusive BF OR 0.6 (0.34, 0.93) for neonatal RTI in girls if mixed feeding Reference group: exclusive bottle-fed
	Kramer, 2003 (21)	Belarus	Nested observational cohort	3,483	0-12 months	No significant differences for ≥ 2 episodes of any RTI, URTI, hospitalization for RTI or ≥ 1 episode of OM if exclusive BF 3 months Reference group: exclusive BF ≥ 6 months
	Chantry, 2006 (32)	US	Cross-sectional	2,277	6-24 months	OR 4.27 (1.27, 14.35) for pneumonia if full BF 4-6 months OR 1.95 (1.06, 3.59) for recurrent otitis media if full BF 4-6 months Reference group: full BF ≥ 6 months
	Quigley, 2007 (22)	UK	Prospective cohort	15,980	0-8 months	OR 0.66 (0.47, 0.91) for LRTI if exclusive BF OR 1.46 (1.19, 1.80) for LRTI 1-4 months; per month cessation of BF OR 1.12 (1.00, 1.27) for LRTI 5-7 months; per month cessation of BF Reference group: no BF

Breastfeeding and respiratory tract infections

Fifteen studies were included with respiratory tract infections or illnesses, including upper respiratory tract infections, lower respiratory tract infections and otitis media, as the outcomes (Table 1).

Howie et al (18) discovered a significant reduction of respiratory tract infections at ages 0 to 13 weeks and 40 to 52 weeks if the infants were breastfed for 13 weeks or more, compared to infants never breastfed. In this study, they did not differentiate between upper or lower respiratory tract infection. Holberg et al (24) studied 1,179 infants in the US. They observed that the relative risk of having Respiratory Syncytial Virus-lower respiratory tract infections (RSV) between 1 and 3 months old infants increased from 5.6 (2.00, 15.30) to 8.0 (2.80, 22.80). The increase was observed when minimal breastfeeding and other confounders (≥ 2 people sharing the same room with the child, maternal education ≤ 12 years) were included in the model. At older ages, breastfeeding did not significantly affect the relative risk for RSV infections. Furthermore in the US, Duncan et al (25), concluded that exclusive breastfeeding of 4 or more months protected infants from single and recurrent episodes of otitis media. In their studies, infants exclusively breastfed for 4 or more months had half of the mean number of acute otitis media episodes than those who were not breastfed at all. Furthermore, those infants had 40% less acute otitis media than those infants whose diets were supplemented with other foods before 4 months. Aniansson et al (26) studied 448 children and observed that the frequency of acute otitis media was significantly lower in breastfed infants than in non-breastfed infants in each age group. The first acute otitis media episode occurred significantly earlier in children who were weaned before 6 months of age than in the remaining groups.

In Italy, Pisacane et al (27) studied 74 cases and 88 controls and found that infants with a pneumonia or bronchiolitis were less likely to have been breastfed until admission (OR 0,22 (0.09, 0.55)). Severely ill infants were less breastfed than those with mild illnesses. Nevertheless, in the pertussis group, they found no protective effect of breastfeeding. They concluded that breastfeeding has a strong protective effect against acute lower respiratory tract infections in industrialized countries. As one of the outcome in their study, Beaudry et al (14) studied respiratory tract infections (including upper respiratory tract infections, lower respiratory tract infections and otitis media) and found that after adjustment for age of the infant, socioeconomic status, maternal age, and cigarette consumption, the incidence density ratio was 0.78 (0.61, 1.00). It is borderline significant, but we may suggest that there is a protective effect of breastfeeding against respiratory tract infections. In the USA, Dewey et al (19) concluded that in the first year of life, the percentage of otitis media was 19% lower and the prolonged episodes of otitis media (>10 days) was 80% lower in breastfed compared with formula fed infants. Except for otitis media, there were no significant differences in rates of respiratory tract infections. In their study, Cushing et al (28) found that, after adjustment for important confounders, breastfeeding

did not have a significant effect on the risk of total respiratory tract infections or upper respiratory tract infections. Full breastfeeding was associated with a significant reduction in risk of lower respiratory tract infections, OR 0.79 (0.67, 0.91). In addition, breastfeeding significantly reduced the duration of respiratory tract infections.

In Greenland, Koch et al (29), studied several risk factors for respiratory tract infections in children less than 2 years of age. Compared with children who were exclusively breastfed, partly or never breastfed children had a higher risk of lower respiratory tract infections, OR 2.98 (0.91, 9.71) and 3.66 (1.06, 12.6), respectively. Although a tendency of protective effect for lower respiratory tract infections was seen, the association for partly breastfeeding was not significant in the final model. Oddy et al (30) concluded that predominant breastfeeding for at least 6 months and partial breastfeeding for up to one year might reduce the prevalence and subsequent morbidity of respiratory tract infections in infancy. They found that hospital, doctor, or clinic visits for four or more upper respiratory tract infections were significantly higher if predominant breastfeeding was stopped before 2 months or partial breastfeeding was stopped before 6 months. Compared with neonates only fed with formula, Sinha et al (31) observed that exclusive breastfeeding was inversely associated with neonatal respiratory tract infections among neonatal girls (OR 0.5, 95% CI 0.29-0.79) but not among neonatal boys (OR 1.1, 95% CI 0.64-2.00), after adjustment for confounders. Mixed feeding also showed a protective effect on neonatal respiratory tract infections, again only in neonatal girls (OR 0.6, 95% CI 0.34-0.93). Infants fully breastfed for 4 to 6 months had higher risks for pneumonia or recurrent OM, compared with infants fully breastfed ≥ 6 months (OR 4.27 (1.27, 14.35) and OR 1.95 (1.06, 3.59)), according to Chantry et al (32). Associations of breastfeeding with recurrent upper respiratory tract infections or wheezing were not found. Quigley et al (22) found a protective effect of exclusive breastfeeding on lower respiratory tract infections (OR 0.66 (0.47, 0.91). Furthermore, per month shorter breastfeeding, an OR of 1.46 (1.19, 1.80) was observed for lower respiratory tract infections at the age of 1 to 4 months, and an OR of 1.12 (1.00, 1.27) for lower respiratory tract infections at the age of 5 to 7 months.

In line with the other findings in their study, Rubin et al (23) did not find any significant relation between breastfeeding and upper respiratory tract illnesses. The adjusted incidence density ratio was 0.98 (95% CI 0.88-1.10). Kramer et al found (21) no associations between episodes of respiratory tract infections, upper respiratory tract infections, hospitalisation for respiratory tract infections or otitis media and duration of exclusive breastfeeding (< 3 months versus ≥ 6 months).

Duration and exclusiveness of breastfeeding and infections

Of all studies included in our study, several assessed the effects of exclusiveness, others the duration and some the combination of duration and exclusiveness of breastfeeding on infectious diseases (Table 1).

Compared with exclusive breastfeeding, Holberg and Koch observed higher risks of RSV-lower respiratory tract infections and respiratory tract infections, respectively, if infants who were not breastfed (24, 29). They found no difference in these outcomes in infants who were partially breastfed. Scariati et al (20) found a dose-response effect when each feeding group (breast milk only, high mixed, middle mixed, low mixed) was compared with infants who received breast milk only (reference group), even after adjusting for confounders. There was a small, but steady increase in the risk of developing gastrointestinal infections as the amount of breast milk that an infant received decreased. They also observed the similar phenomenon when assessing the effect of feeding on otitis media. Compared with never breastfed infants, exclusively breastfed infants had lower risks of lower respiratory tract infections (28), neonatal respiratory tract infections (only girls (31)), respiratory tract infections and gastrointestinal infections (22). Except in the study of Sinha et al (31), no protective effects of partial breastfeeding on the major infectious diseases were seen.

According to the duration of breastfeeding, Pettigrew et al (16) observed a 4% increase of illnesses requiring a health care provider, per week longer breastfeeding of first born children. Infants admitted for all sort of causes or for fever of unknown origin had been breastfed for a shorter period, as shown by a study of Pardo-Crespo et al (15). Howie et al (18) found a significant protective effect against gastrointestinal and respiratory tract infections if the infants were breastfed for more than 13 months (full or partial) compared to those who were bottle fed since birth. Per month shorter breastfeeding, the risk of diarrhea and lower respiratory tract infections at the age of 1 to 4 months increased, as did the risk of lower respiratory tract infections at the age of 5 to 7 months, observed by Quigley et al (22).

Some studies combined the exclusiveness and duration of breastfeeding, like Duncan et al (25), who used the categories of no breastfeeding, less than 4 months, 4 to 6 months, and longer than 6 months. From both birth to 6 months of age and from 6 months to 12 months, the mean number of episodes of acute otitis media decreased significantly with increasing duration and exclusivity of breastfeeding. Oddy et al found that predominantly breastfeeding less than 2 months increased the risk of upper respiratory tract infections with 1.43 (1.02, 2.01), compared with infants predominantly breastfed for > 2 months. If infants were exclusively breastfed less than 4 months, they had an increased risk of hospitalization for an infections, compared with infants exclusively breastfed for ≥ 4 months, according to Paricio-Talayero et al (17). Compared with infants exclusively or fully breastfed for ≥ 6 months, risks for respiratory tract infections were

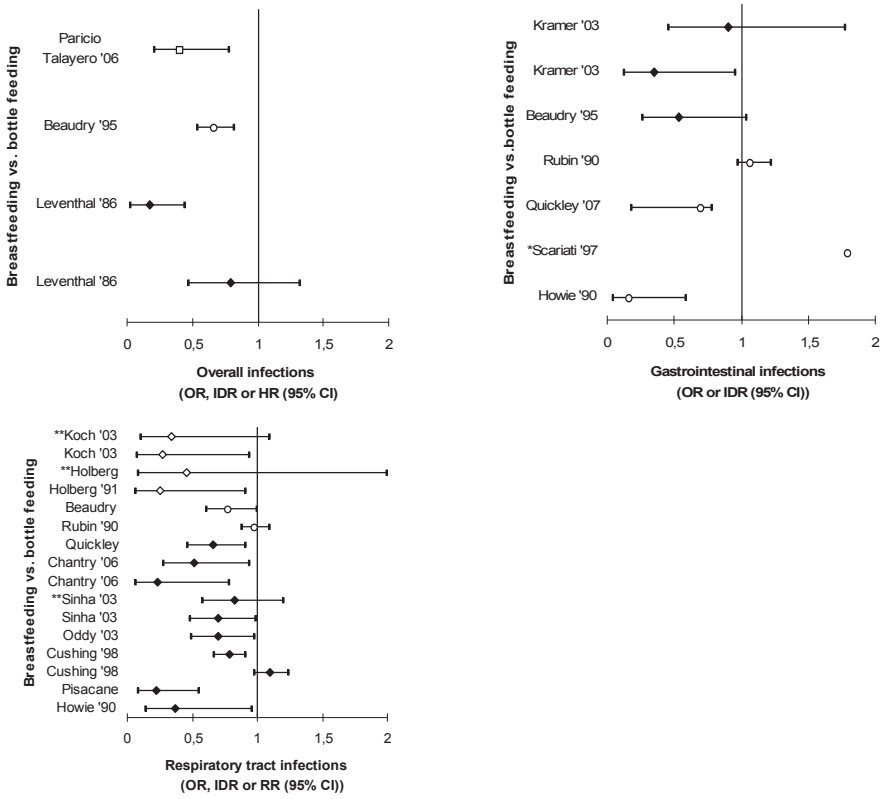


Figure 1. Associations of different types of feeding with overall, gastrointestinal or respiratory tract infections in industrialized countries.
Odds ratio (OR ♦), Incidence Density Ratio (IDR ○), Hazard Ratio (HR □), or Relative Risk (RR ◊) with their 95% confidence intervals (95% CI).
If breastfeeding was de reference group, 1/OR, 1/RR or 1/HR was used. Studies using differences in means or frequencies as effect estimates are not presented.
*No 95% confidence interval was given. ** Mixed feeding versus bottle feeding.

not different (Kramer et al (21)) or increased (Chantry et al (32)) in infants exclusively breastfed for 3 months or fully breastfed for 4 to 6 months, respectively.

Discussion

With the improvement of the general quality of recent studies on breastfeeding and infectious diseases in industrialized countries, the positive protective effects of breastfeeding seem to be clear. It is has been consistently shown that breastfeeding is protective

against infections, gastrointestinal and respiratory tract infections, even in industrialized countries (Figure 1).

Important reviews about the methodological flaws in studies on breastfeeding were done by Kovar et al and Bauchner et al (7, 33). Their criteria are adequate for assessing validity, even though there are more aspects for assessing internal validity such as the presence of reverse causality, selection bias, and other types of information bias like non-blind assessment (33). They concluded in their article that studies with important methodological standards and controlling for confounding variables suggest that breastfeeding has at least a minimal protective effect in industrialized countries (7). Our review, of studies taking their criteria into account, confirms this suggestion, and are in line with former reviews around that time (6, 8, 30, 33-35)

The methods used for the study collection in this review led to a selection of only the high quality studies with minimal methodological imperfections. The use of Bauchner's criteria, to assess the study quality, limited the numbers of studies, but assured the high quality of all studies included. However, there are some limitations, including no representation of industrialized Asian countries in this review. One study from Asia was found, but did not meet our inclusion criteria for the validity of the study design (34). The use of only English literature also limited the range of studies included, and allowed for publication bias. In their review, Bauchner et al (7) found two studies that met all four of their criteria and four that met three criteria. In our review, eleven studies that met all four criteria and ten that met three criteria were found. All 15 studies controlled for at least one major confounder, such as socioeconomic status. From all twenty-one studies included, five did not meet the first criterion for active surveillance because they used mailed questionnaires that were only once being filled in (14, 16, 22, 30, 32). But, as others stated in their article (14, 32), detection bias, which would modify their results, would probably underestimate the protective effect of breastfeeding. One study validated the questionnaires with hospital case notes in a subsample and concluded that 99% of the parental recall was valid (30). Furthermore, assessing infectious diseases by questionnaires is currently widely accepted in epidemiological studies, and reliably reflect the true incidence of those infections (35). The outcomes of several studies included, used terminology that compose a mix of causes of the disease, such as gastrointestinal or respiratory illnesses that can consist of infectious or non-infectious etiology. This could dilute or exaggerate the real protective effect of breastfeeding against infections and should be prevented.

The protection of human breast milk against gastrointestinal infections or illnesses was first established in developing countries (36). It was suggested that the minimal morbidity due to gastrointestinal infectious diseases in infants in industrialized countries was not caused by the breastfeeding itself, but mostly because of the overall better quality of water, infant feeding, sanitation, and health care system (6, 37). From the studies

with gastrointestinal infections as the outcome five out of seven studies suggested that breastfeeding had a protective effect against gastrointestinal infections. The qualities of those studies had a similar quality, carefully designed to avoid biases and consequently leading to inappropriate conclusions. One of the studies that did not support the others had an extensive definition of doctor diagnosed-gastroenteritis, but relatively small number of subjects (23). Another study did observe an association of breastfeeding and gastrointestinal infections in the unadjusted, but not in the adjusted analyses (14). Overall, based on these studies we may conclude that human breast milk protects infants from gastrointestinal infections.

Thirteen out of fifteen studies concluded that breastfeeding protected infants against respiratory tract infections. However, not all studies separated infectious from non-infectious respiratory tract illnesses as their outcome since it is difficult to distinguish between the infectious and non-infectious origin of respiratory tract illnesses during infancy. The protection seems to be different against upper respiratory tract infections and lower respiratory tract infections. Therefore, it is necessary to differentiate between upper respiratory tract infections, lower respiratory tract infections and acute otitis media, instead of analyzing them under one entity (respiratory tract infections). Our findings are in line with a meta-analysis, which observed on overall protective effect of breastfeeding and lower respiratory disease hospitalization in the first two years of life (RR 0.28 (0.14, 0.54)), adjusted for smoking and socio-economic status (12). They did not differentiate between lower respiratory tract infections and asthma.

For the association between dose and duration of breastfeeding and its protection against infections, a positive correlation of the duration, the exclusiveness and the combination of duration and exclusiveness of breastfeeding with gastrointestinal and respiratory diseases were observed. This suggests a dose-response effect whereby a longer and more exclusive breastfeeding period leads to a better protection against infection diseases. However, the exact minimal duration of exclusive breastfeeding that still protects infants in industrialized countries against infectious diseases need to be further explored (8, 38, 39). The benefits and general applicability of 4 versus 6 months of exclusively breastfeeding are not completely clarified, although the World Health Organization recommended in 2001 to exclusively breastfeed infants for 6 months in all countries (40).

We found an established protective association of breastfeeding with gastrointestinal and respiratory tract infections. These findings might be used to support the health policy in industrialized countries in order to promote breastfeeding as well for increasing the incidence and the duration of exclusive breastfeeding. However, the optimal duration of exclusive breastfeeding (4 or 6 months) for protection against infectious diseases needs to be further studied.

References

1. Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. WHO Collaborative Study Team on the Role of Breastfeeding on the Prevention of Infant Mortality. Lancet. 2000 Feb 5;355(9202):451-5.
2. Global strategy for infant and young child feeding. The optimal duration for exclusive Breastfeeding. World Health Organization. Fifty-fourth World Health Assembly World Health Assembly 542; 2001 1 May 2001; 2001.
3. Hanson LA, Korotkova M, Haversen L, Mattsby-Baltzer I, Hahn-Zoric M, Silfverdal SA, et al. Breastfeeding, a complex support system for the offspring. Pediatr Int. 2002 Aug;44(4):347-52.
4. Lawrence RM, Pane CA. Human breast milk: current concepts of immunology and infectious diseases. Curr Probl Pediatr Adolesc Health Care. 2007 Jan;37(1):7-36.
5. Newburg DS, Peterson JA, Ruiz-Palacios GM, Matson DO, Morrow AL, Shults J, et al. Role of human-milk lactadherin in protection against symptomatic rotavirus infection. Lancet. 1998 Apr 18;351(9110):1160-4.
6. Cunningham AS, Jelliffe DB, Jelliffe EF. Breastfeeding and health in the 1980s: a global epidemiologic review. J Pediatr. 1991 May;118(5):659-66.
7. Bauchner H, Leventhal JM, Shapiro ED. Studies of breastfeeding and infections. How good is the evidence? Jama. 1986 Aug 15;256(7):887-92.
8. Yngve A, Sjostrom M. Breastfeeding in countries of the European Union and EFTA: current and proposed recommendations, rationale, prevalence, duration and trends. Public Health Nutr. 2001 Apr;4(2B):631-45.
9. Bolling K, Grant C, Hamlyn B, A. T. The Infant Feeding Survey 2005. Leeds, UK: The Information Centre; 2005.
10. Breastfeeding Practices: Results From the 2005 national Immunization Survey. Atlanta, USA: Centers for Disease Control and Prevention; 2005.
11. Breastfeeding and the use of human milk. American Academy of Pediatrics. Pediatrics. 1997 Dec;100(6):1035-9.
12. Bachrach VR, Schwarz E, Bachrach LR. Breastfeeding and the risk of hospitalization for respiratory disease in infancy: a meta-analysis. Arch Pediatr Adolesc Med. 2003 Mar;157(3):237-43.
13. Leventhal JM, Shapiro ED, Aten CB, Berg AT, Egerter SA. Does breastfeeding protect against infections in infants less than 3 months of age? Pediatrics. 1986 Nov;78(5):896-903.
14. Beaudry M, Dufour R, S M. Relation between infant feeding and infections during the first six months of life. J Pediatr. 1995;126:191-7.
15. Pardo-Crespo R, Perez-Iglesias R, Llorca J, Alvarez-Granda L, Garcia-Fuentes M, Martinez-Gonzalez MA, et al. Breastfeeding and risk of hospitalization for all causes and fever of unknown origin. Eur J Public Health. 2004 Sep;14(3):230-4.
16. Pettigrew MM, Khodaee M, Gillespie B, Schwartz K, Bobo JK, Foxman B. Duration of breastfeeding, daycare, and physician visits among infants 6 months and younger. Ann Epidemiol. 2003 Jul;13(6):431-5.
17. Paricio Talayero JM, Lizan-Garcia M, Otero Puime A, Benlloch Muncharaz MJ, Beseler Soto B, Sanchez-Palomares M, et al. Full breastfeeding and hospitalization as a result of infections in the first year of life. Pediatrics. 2006 Jul;118(1):e92-9.
18. Howie PW, Forsyth JS, Ogston SA, Clark A, Florey CD. Protective effect of breast feeding against infection. Bmj. 1990 Jan 6;300(6716):11-6.
19. Dewey KG, Heinig MJ, Nommsen-Rivers LA. Differences in morbidity between breastfed and formula-fed infants. J Pediatr. 1995 May;126(5 Pt 1):696-702.
20. Scariati PD, Grummer-Strawn LM, Fein SB. A longitudinal analysis of infant morbidity and the extent of breastfeeding in the United States. Pediatrics. 1997 Jun;99(6):E5.

21. Kramer MS, Guo T, Platt RW, Sevkovskaya Z, Dzikovich I, Collet JP, et al. Infant growth and health outcomes associated with 3 compared with 6 mo of exclusive breastfeeding. Am J Clin Nutr. 2003 Aug;78(2):291-5.
22. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. Pediatrics. 2007 Apr;119(4):e837-42.
23. Rubin DH, Leventhal JM, Krasilnikoff PA, Kuo HS, Jekel JF, Weile B, et al. Relationship between infant feeding and infectious illness: a prospective study of infants during the first year of life. Pediatrics. 1990 Apr;85(4):464-71.
24. Holberg CJ, Wright AL, Martinez FD, Ray CG, Taussig LM, Lebowitz MD. Risk factors for respiratory syncytial virus-associated lower respiratory illnesses in the first year of life. Am J Epidemiol. 1991 Jun 1;133(11):1135-51.
25. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breastfeeding for at least 4 months protects against otitis media. Pediatrics. 1993 May;91(5):867-72.
26. Aniansson G, Alm B, Andersson B, Hakansson A, Larsson P, Nylen O, et al. A prospective cohort study on breastfeeding and otitis media in Swedish infants. Pediatr Infect Dis J. 1994 Mar;13(3):183-8.
27. Pisacane A, Graziano L, Zona G, Granata G, Dolezalova H, Cafiero M, et al. Breast feeding and acute lower respiratory infection. Acta Paediatr. 1994 Jul;83(7):714-8.
28. Cushing AH, Samet JM, Lambert WE, Skipper BJ, Hunt WC, Young SA, et al. Breastfeeding reduces risk of respiratory illness in infants. Am J Epidemiol. 1998 May 1;147(9):863-70.
29. Koch A, Molbak K, Homoe P, Sorensen P, Hjuler T, Olesen ME, et al. Risk factors for acute respiratory tract infections in young Greenlandic children. Am J Epidemiol. 2003 Aug 15;158(4):374-84.
30. Oddy WH, Sly PD, de Klerk NH, Landau LI, Kendall GE, Holt PG, et al. Breast feeding and respiratory morbidity in infancy: a birth cohort study. Arch Dis Child. 2003 Mar;88(3):224-8.
31. Sinha A, Madden J, Ross-Degnan D, Soumerai S, Platt R. Reduced risk of neonatal respiratory infections among breastfed girls but not boys. Pediatrics. 2003 Oct;112(4):e303.
32. Chantry CJ, Howard CR, Auinger P. Full breastfeeding duration and associated decrease in respiratory tract infection in US children. Pediatrics. 2006 Feb;117(2):425-32.
33. Kovar MG, Serdula MK, Marks JS, Fraser DW. Review of the epidemiologic evidence for an association between infant feeding and infant health. Pediatrics. 1984 Oct;74(4 Pt 2):615-38.
34. al-Ali FM, Hossain MM, Pugh RN. The associations between feeding modes and diarrhoea among urban children in a newly developed country. Public Health. 1997 Jul;111(4):239-43.
35. Kalter HD, Gray RH, Black RE, Gultiano SA. Validation of the diagnosis of childhood morbidity using maternal health interviews. Int J Epidemiol. 1991 Mar;20(1):193-8.
36. Brown KH, Black RE, Lopez de Romana G, Creed de Kanashiro H. Infant-feeding practices and their relationship with diarrheal and other diseases in Huascar (Lima), Peru. Pediatrics. 1989 Jan;83(1):31-40.
37. Habicht JP, DaVanzo J, Butz WP. Does breastfeeding really save lives, or are apparent benefits due to biases? Am J Epidemiol. 1986 Feb;123(2):279-90.
38. Fewtrell MS, Morgan JB, Duggan C, Gunnlaugsson G, Hibberd PL, Lucas A, et al. Optimal duration of exclusive breastfeeding: what is the evidence to support current recommendations? Am J Clin Nutr. 2007 Feb;85(2):635S-8S.
39. Kramer MS, Kakuma R. The optimal duration of exclusive breastfeeding: a systematic review. Adv Exp Med Biol. 2004;554:63-77.
40. World Health Organization, Department of Nutrition for Health and Development. The optimal duration of exclusive breastfeeding: report of an expert consultation. Geneva, Switzerland: World Health Organization; 2001.

Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy

3.2

Abstract

Objective: To examine the associations of duration of exclusive breastfeeding with upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI) and gastrointestinal tract infections (GI) in infancy.

Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards in the Netherlands. Breastfeeding during the first 6 months (never; partial < 4 months, not thereafter; partial 4-6 months; exclusive 4 months, not thereafter; exclusive 4 months, partial thereafter; exclusive 6 months) and the occurrence of doctor-attended URTI, LRTI and GI until the age was 12 months were assessed by questionnaires and available in 4,164 subjects.

Results: Prolonged and exclusively breastfed infants had lower risks of URTI, LRTI and GI during the first 6 months, and a lower risk of LRTI between the ages of 7 and 12 months (p-values for trend < 0.01). Compared to never breastfed infants, those who were breastfed exclusively until 4 months and partially thereafter, had lower risks of URTI, LRTI and GI until the age of 6 months (adjusted odds ratio (aOR) 0.65 (95% confidence interval: 0.51 to 0.83), aOR 0.50 (0.32, 0.79) and aOR 0.41 (0.26, 0.64), respectively) and of LRTI between 7 and 12 months (aOR 0.46 (0.31, 0.69)). Similar tendencies were observed for infants exclusively breastfed ≥ 6 months.

Conclusions: Prolonged duration of exclusiveness of breastfeeding is associated with lower risks of common infectious diseases in the first 6 months life. These findings support current health policy strategies to promote breastfeeding in industrialized countries.

Introduction

Respiratory and gastrointestinal tract infections are the leading cause of morbidity in children (1, 2). Prospective cohort studies in industrialized countries, revealed prevalences of 3.4% to 32.1% for respiratory tract infectious diseases and 1.2% to 26.3% for gastrointestinal infectious diseases in infancy (3-8). The risks of these infectious diseases are affected by several factors including birth weight, gestational age, socio-economic status, ethnicity, number of siblings, daycare attendance and parental smoking (3, 5, 6, 8-20).

Breastfeeding has been suggested as a modifiable influencing factor. When given exclusively, breastfeeding reduces the risk of infectious diseases in infants in developing countries (21, 22). In industrialized countries, exclusive breastfeeding during the first 6 months seems to decrease the risk of gastrointestinal tract infections, compared to exclusive breastfeeding during only the first 3 to 4 months (23, 24). Based on these and other reports, the World Health Organization (WHO) recommended in 2001 to exclusively breastfeed all children for 6 months instead of 4 months. However, they also called for more research regarding the benefits of 6 versus 4 months of exclusive breastfeeding (25). Thus far, several studies in industrialized countries showed that a shorter duration of breastfeeding increases the risk of common infectious diseases, such as respiratory and gastrointestinal tract infections (8, 19, 24, 26-32). However, in these studies various definitions of the 'relative amount' of breastfeeding were used (24, 27, 28, 30) or the combination of duration and exclusiveness of breastfeeding was not taken into account (8, 31).

We examined in a population-based prospective cohort study in the city of Rotterdam, the Netherlands, the associations of the duration of exclusive breastfeeding with upper and lower respiratory and gastrointestinal tract infections during the first year of life.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood, in the city of Rotterdam, the Netherlands. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development and health and has been described previously in detail (33, 34). Enrolment of mothers was aimed in early pregnancy (gestational age < 18 weeks) but was possible until birth of the child. All children were born between April 2002 and January 2006 and form a prenatally enrolled birth-cohort that is currently followed

until young adulthood. Response rate for the Generation R Study is 61%. In total, 7893 parents gave informed consent for participation of their infants in the postnatal phase of the study (33). The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Duration and exclusiveness of breastfeeding

Information about breastfeeding was obtained by postal questionnaires at the ages of 6 and 12 months (33). Mothers were asked whether they ever breastfed their infant (no, yes). Duration of breastfeeding was assessed by asking at what age (months) the infant received breast milk for the last time. Breastfeeding was categorized into three groups of infants who received breastfeeding for less than 4 months, 4 to 6 months or 6 months or longer. Duration of exclusive breastfeeding was defined using information about at what age other types of milk and/or solids were introduced in the first 6 months of life. The information about duration and exclusiveness of breastfeeding was combined and categorized into the following six breastfeeding categories: 1) never; 2) partial < 4 months, not thereafter; 3) partial 4-6 months; 4) exclusive 4 months, not thereafter; 5) exclusive 4 months, partial thereafter; 6) exclusive 6 months. Never indicates infants who have never been breastfed. Partial indicates infants receiving both breastfeeding and formula feeding or solids in this period. Exclusive indicates infants who have been breastfed, without any other milk, solids or fluids. After the age of 6 months, all infants received other milk, fluids and/or solids.

Infectious diseases

Information about infectious diseases was obtained by questionnaires at the ages of 6 and 12 months. Parents were asked whether their infant has had a serious cold, ear or throat infection, pneumonia, bronchitis, bronchiolitis or gastrointestinal tract infection ('no or yes, not visited a doctor'; 'yes, visited a doctor'). The respiratory tract infections were combined into doctor-attended and no doctor-attended upper (serious cold, ear infection, throat infection) and lower respiratory tract infections (pneumonia, bronchitis and bronchiolitis).

Covariates

Information about ethnicity and educational level of the mother was obtained at enrolment in the study. Ethnicity and educational level of the mother were defined according to the classification of Statistics Netherlands (35). Birth weight and date of birth were obtained from midwife and hospital registries. Gestational age was established by fetal ultrasound examination (37). Information about siblings, day-care attendance and maternal smoking (no, yes) was obtained by questionnaire at the age of 6 months of the infant.

Cohort for analysis

Of the 7,893 participants of the postnatal cohort, 7,295 (92%) gave consent for receiving questionnaires. Twins (n=179) were excluded from the present analyses, since these were correlated with each other. Of the remaining 7,116 infants, 60% and 69% responded to the 6 months- and 12 months questionnaire, respectively. Information about breastfeeding in infancy was available in 4,618 infants (65%). Of those, information about infectious diseases at the ages of 6 and 12 months was available in 4,164 and 3,962 infants, respectively.

Data analysis

First, the associations of duration of breastfeeding with upper and lower respiratory and gastrointestinal tract infections in infants at the ages of 6 and 12 months were analyzed using multiple logistic regression analysis. Second, to examine whether the effects of duration of breastfeeding were due to exclusive breastfeeding, the associations of duration of exclusive breastfeeding with upper and lower respiratory and gastrointestinal tract infections were examined. All models were adjusted for potential confounders including maternal education, ethnicity, smoking, gestational age, birth weight, siblings and day-care attendance. Tests for trend were performed by including the breastfeeding categories as continuous variables in the regression models. The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

The median infant's ages at which parents filled in the 6 and 12 months questionnaire were 6.3 (90% range 5.7 to 8.8) months and 11.9 (90% range 11.6 to 13.6) months, respectively. Most mothers were high educated (55%) and of Dutch ethnicity (61%) (Table 1). Median gestational age at birth was 40.1 weeks (90% range 37.1 to 42.1 weeks) and mean birth weight was 3456 grams (SD 547 grams). Of all mothers, 88% initiated breastfeeding. Of all infants, 29% received breastfeeding for less than 4 months, 25% for 4 to 6 months and 34% for 6 months or more. When duration of breastfeeding was taken into account, most mothers partially breastfed their infants for less than 4 months or for 4 to 6 months (29% and 29%) or breastfed their infants exclusively until the age of 4 months and partially thereafter (26%). Exclusive breastfeeding for 6 months was given to only 1.4% of all infants. In the first 6 months of life, 40% of all infants had an upper respiratory tract infection and 8% had a lower respiratory tract infection and a gastrointestinal tract infection (Table 1). Between 7 and 12 months of life, upper and lower respiratory and gastrointestinal tract infections were present in

27%, 10% and 9% of all infants. Table 2 shows that mothers who were older than 30 years, higher educated and non-smoking tended to breastfed their infants longer and more exclusively.

Duration of breastfeeding

Infants who were breastfed for 4 months or 4 to 6 months did not have lower risks of upper and lower respiratory or gastrointestinal tract infections in the first 6 months compared to never breastfed infants (all p-values > 0.05) (Figure 1). Compared to never breastfed infants, those who were breastfed for 6 months or more had lower risks of upper respiratory tract infections (adjusted odds ratio (aOR) 0.62 (95% confidence interval (CI): 0.49, 0.78)), lower respiratory tract infections (aOR 0.60 (95% CI: 0.40, 0.91)) and gastrointestinal tract infections (aOR 0.46. (95% CI: 0.30, 0.70)). Between 7 and 12 months, the risks of upper and lower respiratory and gastrointestinal tract infections were similar in infants who were breastfed until 4 months and who were never breastfed. Infants breastfed for 4 to 6 months or 6 months or more had lower risks of lower respiratory tract infections (aOR 0.54 (95% CI: 0.37, 0.81) and aOR 0.54 (95% CI: 0.37, 0.78), respectively). No associations were found with upper respiratory and gastrointestinal tract infections.

Duration and exclusiveness of breastfeeding

Compared to never breastfed infants, infants who were breastfed partially until 4 months or for 4 to 6 months and infants who were breastfed exclusively until 4 months but not thereafter, did not have lower risks of upper and lower respiratory and gastrointestinal tract infections until the age of 6 months (all p-values > 0.05) (Table 3). Infants who were breastfed exclusively until 4 months and partially thereafter, had lower risks of upper and lower respiratory and gastrointestinal tract infections in the first 6 months after birth (aOR 0.65 (95% CI: 0.51, 0.83), 0.50 (95% CI: 0.32, 0.79) and 0.41 (95% CI: 0.26, 0.67), respectively). Infants who were breastfed exclusively for 6 months had a lower risk of upper respiratory tract infections (aOR 0.37 (95% CI: 0.18, 0.74)), but no associations were found with lower respiratory tract or gastrointestinal tract infections. Between 7 and 12 months, lower respiratory tract infections were less present in infants who were breastfed partially until 4 to 6 months and exclusively until 4 months and partially thereafter, compared to never breastfed infants (aOR 0.68 (95% CI: 0.47, 0.99) and aOR 0.46 (95% CI: 0.31, 0.69)). No other associations of the various breastfeeding categories with upper and lower respiratory and gastrointestinal tract infections between the age of 7 to 12 months were observed. All tests for trend for the associations of prolonged exclusive breastfeeding with upper and lower respiratory and gastrointestinal tract infections until the age of 6 months, and lower respiratory tract infections between the age of 7 to 12 months were significant (p-values for trend < 0.01).

Table 1. Maternal and infant characteristics of the study population.

	Infants (n=4,164)
Mother	
Maternal age (years)	31.1 (4.8)
Education (%)	
Low	6.2 (246)
Intermediate	38.7 (1,538)
High	55.2 (2,195)
Non-Dutch ethnicity (%)	38.9 (1,574)
Smoking (%)	16.2 (647)
Infant	
Girl	50.6 (2,107)
Gestational age (weeks)*	40.1 (37.1-42.1)
Birth weight (grams)	3,456 (547)
Siblings ≥ 1 (%)	40.2 (1,604)
Daycare attendance (%)	49.7 (1,949)
Duration of breastfeeding (%)	
Never	12.5 (519)
< 4 months	29.1 (1,203)
4-6 months	24.5 (1,1012)
≥ 6 months	33.9 (1,404)
Duration of exclusive breastfeeding (%)	
Never	12.8 (519)
Partial < 4 months, not thereafter	29.2 (1,182)
Partial 4-6 months	28.8 (1,166)
Exclusive 4 months, not thereafter	2.0 (80)
Exclusive 4 months, partial thereafter	25.7 (1,037)
Exclusive 6 months	1.4 (58)
Doctor-attended infectious diseases ≤ 6 months (%)	
Upper respiratory tract infection (URTI)	39.5 (1,644)
Lower respiratory tract infection (LRTI)	7.8 (323)
Gastrointestinal tract infection (GI)	7.6 (309)
Doctor-attended infectious diseases 7-12 months (%)	
Upper respiratory tract infection (URTI)	26.6 (912)
Lower respiratory tract infection (LRTI)	10.2 (360)
Gastrointestinal tract infection (GI)	8.7 (295)

Values are means (standard deviation) or percentages (absolute numbers).
*Median (95% range).
Data were missing on education (n=185), ethnicity (n=121), smoking (n=170), birth weight (n=3), siblings (n=171), daycare attendance (n=240), duration of breastfeeding (n=26), duration of exclusive breastfeeding (n=122); at age 6 months: URTI (n=2), LRTI (n=36) and GI (n=92); at age 12 months: URTI (n=737), LRTI (n=644) and GI (n=772).

Table 2. Maternal characteristics and breastfeeding habits.

	Duration of exclusive breastfeeding						
	Never	Partial < 4 months, not thereafter	Partial 4-6 months	Exclusive 4 months, not thereafter	Exclusive 4 months, partial thereafter	Exclusive 6 months	P-value
Maternal age							
< 26	76 (12.8)	264 (44.4)	151 (25.4)	6 (1.0)	95 (16.0)	3 (0.5)	
26-30	184 (14.9)	369 (29.8)	360 (29.1)	21 (1.7)	289 (23.1)	17 (1.4)	
>30	256 (11.6)	549 (24.9)	655 (29.7)	53 (2.4)	656 (29.7)	38 (1.7)	*< 0.01*
Education							
Low	32 (13.9)	80 (34.8)	74 (32.2)	3 (1.3)	39 (17.0)	2 (0.9)	
Intermediate	272 (18.2)	555 (37.2)	366 (24.5)	29 (1.9)	263 (17.6)	8 (0.5)	
High	183 (8.6)	490 (22.9)	672 (31.4)	45 (2.1)	704 (32.9)	46 (2.1)	*< 0.01*
Ethnicity							
Dutch	342 (14.1)	668 (27.6)	647 (26.8)	51 (2.1)	666 (27.6)	43 (1.8)	
Non-Dutch	155 (10.3)	474 (31.4)	490 (32.5)	26 (1.7)	350 (23.2)	13 (0.9)	*< 0.01*
Smoking							
No	360 (11.1)	882 (27.1)	985 (30.3)	68 (2.1)	906 (27.9)	52 (1.6)	
Yes	127 (20.4)	249 (39.9)	140 (22.4)	9 (1.4)	95 (15.2)	4 (0.6)	*< 0.01*

Values are absolute numbers (percentages).
Data were missing on education (n=185), ethnicity (n=121), smoking (n=170), siblings (n=171), daycare attendance (n=240), duration of breastfeeding (n=26) and duration of exclusive breastfeeding (n=122). Differences between groups were tested by Chi-square. **P-value < 0.01.

Discussion

The main findings of this population-based prospective cohort study were that breastfeeding for 6 months seems to have protective effects for development of respiratory and gastrointestinal tract infections during the first 6 months, and lower respiratory tract infections between the age of 7 and 12 months. When taking the exclusiveness of breastfeeding into account, infants who were breastfed exclusively until 4 months and partially thereafter, had lower risks of respiratory and gastrointestinal tract infections until the age of 6 months and lower respiratory tract infections between the age of 7 and 12 months.

Several studies showed that a shorter period of breastfeeding increases the risks of physician visits for illness, lower respiratory tract infections and gastrointestinal symptoms (8, 28, 32). Studies that were able to take the 'relative amount ' of breastfeeding into account, showed that exclusive, followed by partial breastfeeding, or predominantly breastfeeding during 6 months or more was associated with a lower risk of gastrointestinal tract infection compared to less than 3 months breastfeeding (24). Infants who were breastfed for less than 4 months, showed a higher risk of hospitalization for infectious diseases (hazard ratio 2.45 (95% CI 1.28, 4.66)) compared to those who were breastfed for more than 4 months. Furthermore, infants who were breastfed for 4 to 6 months showed

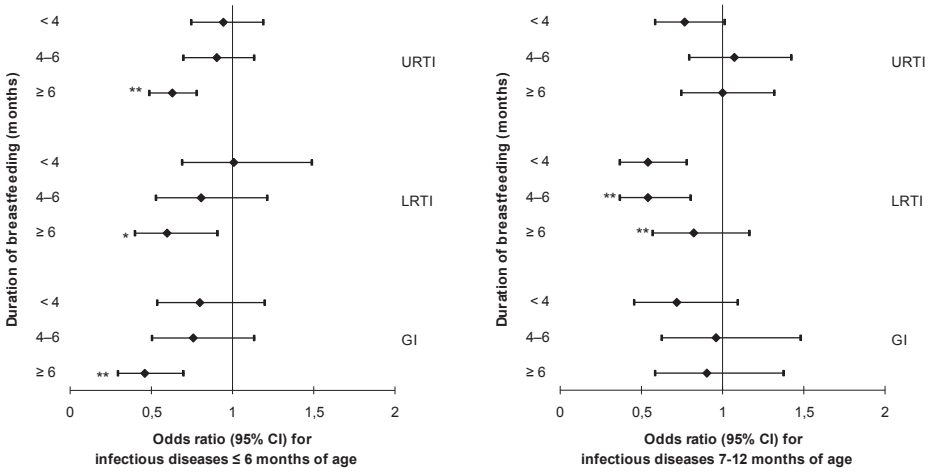


Figure 1. Duration of breastfeeding and risk of infectious diseases in the first year of life.
URTI = upper respiratory tract infections, LRTI = lower respiratory tract infections and GI = gastrointestinal tract infections.
Values are odds ratios (95% confidence interval), adjusted for maternal education, ethnicity, smoking, gestational age, birth weight, siblings and daycare attendance. *P-value < 0.05, ** p-value < 0.01.

higher risks of both pneumonia (OR 4.27 (95% CI 1.27, 14.35) and recurrent otitis media (OR 1.95 (95% CI 1.06, 3.59) compared to those who were breastfed 6 or more months (29, 30). One study observed a protective effect of predominant breastfeeding for at least 6 months on doctor visits for ≥ 4 upper respiratory tract infections or ≥ 2 wheezing episodes, compared to breastfeeding for less than 6 months (27). Our results are difficult to compare with these studies, since different breastfeeding categories and various definitions of the breastfeeding categories (predominantly or exclusive) and the outcomes (self-reported or doctor-diagnosed infections) were used.

We observed protective effects of breastfeeding on infectious diseases mainly in the first 6 months of life. Most studies reported protective effects of breastfeeding on common infections in the first 8 to12 months of life (8, 27, 29, 30). One study, that distinguished between infectious diseases until and from the age of 6 months, found similar results as in our study (24). Although they used exclusive breastfeeding for 3 months as the reference group, exclusive breastfeeding for 6 months reduced the risk of gastrointestinal tract infections between the age of 3 to 6 months, but not between the age of 6 to 12 months (24). Breastfeeding might have prolonged protective effect effects by influencing the severity (hospital admission) and frequency of common infectious diseases between the age of 6 and 12 months. However, immunological evidence of prolonged protective effect of increased dose and duration of breastfeeding is not well established. Short-term protective effects are due to several factors in human breast milk. Epidermal

Table 3. Duration of exclusive breastfeeding and risk of infectious diseases in the first year of life.

	≤ 6 months			7-12 months		
	URTI	**LRTI**	**GI**	**URTI**	**LRTI**	**GI**
Duration of exclusive breastfeeding						
Never	1.00	1.00	1.00	1.00	1.00	1.00
Partial < 4 months, not thereafter	0,96 (0.76, 1.21)	1.01 (0.68, 1.50)	0.77 (0.52, 1.15)	1.02 (0.78, 1.34)	0.81 (0.57, 1.17)	0.95 (0.68, 1.31)
Partial 4-6 months	0,85 (0.67, 1.07	0.89 (0.60, 1.34)	0.72 (0.48, 1.09)	0.96 (0.73, 1.27)	0.68 (0.47, 0.99)*	0.97 (0.70, 1.35)
Exclusive 4 months, not thereafter	0.70 (0.41, 1.20)	0.39 (0.12, 0.31)	1.01 (0.44, 2.38)	1.75 (0.99, 3.14)	0.45 (0.17, 1.19)	1.16 (0.60, 2.27)
Exclusive 4 months, partial thereafter	0.65 (0.51, 0.83)**	0.50 (0.32, 0.79)**	0.41 (0.26, 0.64)**	0.88 (0.66, 1.16)	0.46 (0.31, 0.69)**	1.07 (0.77, 1.49)
Exclusive breastfeeding 6 months	0.37 (0.18, 0.74)**	0.33 (0.08, 1.40)	0.46 (0.14, 1.59)	0.63 (0.30, 1.33)	0.54 (0.18, 1.58)	0.93 (0.42, 2.06)
P_{trend}	*< 0.01*	*< 0.01*	*< 0.01*	*NS*	*< 0.01*	*NS*

URTI = upper respiratory tract infections, LRTI = lower respiratory tract infections and GI = gastrointestinal tract infections.

Values are odds ratios (95% confidence interval), adjusted for maternal education, ethnicity, smoking, gestational age, birth weight, siblings and daycare attendance. *P-value < 0.05, ** p-value < 0.01.

growth factor helps to induce maturation of the intestinal epithelium, IgA and oligosaccharides prevent attachment of pathogens and lactoferrin has broad antimicrobial properties, including disruption of the bacterial outer membrane (38, 39).

The strength of this study is its prospective population-based cohort design with a large number of subjects and the possibility to adjust for all major confounders. Furthermore, we were able to categorize the various breastfeeding habits in combination with the duration of breastfeeding. Some methodological considerations need to be considered. Of all postnatal eligible participants of the Generation R Study, questionnaires with breastfeeding data were available in 65%. Infectious diseases until the age of 6 months were more present in those without information (50%) than those with information about breastfeeding (44%). This difference might have led to an underestimation of our effect estimates of infectious diseases. There were no differences in prevalences of infectious diseases from the age of 7 through 12 months between those with (35%) and without (36%) breastfeeding data. Of all participants with breastfeeding data, 10% and 14% had missing data on infectious diseases at the ages of 6 and 12 months, respectively. Among those infants without data on infectious diseases, on average 28% were exclusive breastfed for 4 months and 1.7% were exclusively breastfed for 6 months. Since these frequencies did not differ from the frequencies in our cohort for analysis (27% and 1.4%, respectively), biased estimates due to selective loss to follow-up seems unlikely.

Information about breastfeeding was prospectively collected by questionnaires without direct reference to upper and lower respiratory and gastrointestinal tract infections. Although assessing breastfeeding by questionnaires seems to be a valid method, misclassification may occur. A recent review of studies performed between 1966 and 2003 showed that maternal report of breastfeeding is reliable through the age of 3 years (40). The main outcomes in our study were self-reported upper and lower respiratory and gastrointestinal tract infections. This method is widely accepted in epidemiological studies and reliably reflect the true incidence of those infections (41).

Our findings support previous studies suggesting that prolonged and exclusive breastfeeding protects against respiratory and gastrointestinal tract infections in the first year of life. Exclusive breastfeeding until 4 months followed by partial breastfeeding, lead to a significant reduction of respiratory and gastrointestinal morbidity in infants. These findings support current health policy strategies to promote breastfeeding in industrialized countries. Biological, cultural and social constraints related to breastfeeding habits need to be studied more extensively. The effects of prolonged and exclusive breastfeeding on infectious diseases at older ages in industrialized countries remains to be studied.

References

1. World Health Organization. Major causes of death among children under 5 years of age and neonates in the world, 2000-2003. http://www.who.int/child-adolescent-health/overview/child_helath/map_00-03_world.jpg: The World Health Organization; 2003.
2. Chretien J, Holland W, Macklem P, Murray J, Woolcock A. Acute respiratory infections in children. A global public-health problem. N Engl J Med. 1984 Apr 12;310(15):982-4.
3. Wright AL, Taussig LM, Ray CG, Harrison HR, Holberg CJ. The Tucson Children's Respiratory Study. II. Lower respiratory tract illness in the first year of life. Am J Epidemiol. 1989 Jun;129(6):1232-46.
4. Neuspiel DR, Rush D, Butler NR, Golding J, Bijur PE, Kurzon M. Parental smoking and post-infancy wheezing in children: a prospective cohort study. Am J Public Health. 1989 Feb;79(2):168-71.
5. Baker D, Taylor H, Henderson J. Inequality in infant morbidity: causes and consequences in England in the 1990s. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. J Epidemiol Community Health. 1998 Jul;52(7):451-8.
6. Koopman LP, Smit HA, Heijnen ML, Wijga A, van Strien RT, Kerkhof M, et al. Respiratory infections in infants: interaction of parental allergy, child care, and siblings-- The PIAMA study. Pediatrics. 2001 Oct;108(4):943-8.
7. Panico L, Bartley M, Marmot M, Nazroo JY, Sacker A, Kelly YJ. Ethnic variation in childhood asthma and wheezing illnesses: findings from the Millennium Cohort Study. Int J Epidemiol. 2007 May 24.
8. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. Pediatrics. 2007 Apr;119(4):e837-42.
9. Holberg CJ, Wright AL, Martinez FD, Morgan WJ, Taussig LM. Child day care, smoking by caregivers, and lower respiratory tract illness in the first 3 years of life. Group Health Medical Associates. Pediatrics. 1993 May;91(5):885-92.

10. Paradise JL, Rockette HE, Colborn DK, Bernard BS, Smith CG, Kurs-Lasky M, et al. Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. Pediatrics. 1997 Mar;99(3):318-33.
11. Cushing AH, Samet JM, Lambert WE, Skipper BJ, Hunt WC, Young SA, et al. Breastfeeding reduces risk of respiratory illness in infants. Am J Epidemiol. 1998 May 1;147(9):863-70.
12. Koch A, Molbak K, Homoe P, Sorensen P, Hjuler T, Olesen ME, et al. Risk factors for acute respiratory tract infections in young Greenlandic children. Am J Epidemiol. 2003 Aug 15;158(4):374-84.
13. Nafstad P, Jaakkola JJ, Hagen JA, Botten G, Kongerud J. Breastfeeding, maternal smoking and lower respiratory tract infections. Eur Respir J. 1996 Dec;9(12):2623-9.
14. Celedon JC, Litonjua AA, Weiss ST, Gold DR. Day care attendance in the first year of life and illnesses of the upper and lower respiratory tract in children with a familial history of atopy. Pediatrics. 1999 Sep;104(3 Pt 1):495-500.
15. Marbury MC, Maldonado G, Waller L. Lower respiratory illness, recurrent wheezing, and day care attendance. Am J Respir Crit Care Med. 1997 Jan;155(1):156-61.
16. Leeder SR, Corkhill R, Irwig LM, Holland WW, Colley JR. Influence of family factors on the incidence of lower respiratory illness during the first year of life. Br J Prev Soc Med. 1976 Dec;30(4):203-12.
17. Margolis PA, Greenberg RA, Keyes LL, LaVange LM, Chapman RS, Denny FW, et al. Lower respiratory illness in infants and low socioeconomic status. Am J Public Health. 1992 Aug;82(8):1119-26.
18. Rylander E, Eriksson M, Pershagen G, Nordvall L, Ehrnst A, Ziegler T. Wheezing bronchitis in children. Incidence, viral infections, and other risk factors in a defined population. Pediatr Allergy Immunol. 1996 Feb;7(1):6-11.
19. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breast-feeding for at least 4 months protects against otitis media. Pediatrics. 1993 May;91(5):867-72.
20. Caudri D, Wijga A, Gehring U, Smit HA, Brunekreef B, Kerkhof M, et al. Respiratory symptoms in the first 7 years of life and birth weight at term: the PIAMA Birth Cohort. Am J Respir Crit Care Med. 2007 May 15;175(10):1078-85.
21. Effect of breastfeeding on infant and child mortality due to infectious diseases in less developed countries: a pooled analysis. WHO Collaborative Study Team on the Role of Breastfeeding on the Prevention of Infant Mortality. Lancet. 2000 Feb 5;355(9202):451-5.
22. Bahl R, Frost C, Kirkwood BR, Edmond K, Martines J, Bhandari N, et al. Infant feeding patterns and risks of death and hospitalization in the first half of infancy: multicentre cohort study. Bull World Health Organ. 2005 Jun;83(6):418-26.
23. Kramer MS, Kakuma R. Optimal duration of exclusive breastfeeding. Cochrane Database Syst Rev. 2002(1):CD003517.
24. Kramer MS, Guo T, Platt RW, Sevkovskaya Z, Dzikovich I, Collet JP, et al. Infant growth and health outcomes associated with 3 compared with 6 mo of exclusive breastfeeding. Am J Clin Nutr. 2003 Aug;78(2):291-5.
25. World Health Organization, Department of Nutrition for Health and Development. The optimal duration of exclusive breastfeeding: report of an expert consultation. Geneva, Switzerland: World Health Organization; 2001.
26. Bachrach VR, Schwarz E, Bachrach LR. Breastfeeding and the risk of hospitalization for respiratory disease in infancy: a meta-analysis. Arch Pediatr Adolesc Med. 2003 Mar;157(3):237-43.
27. Oddy WH, Sly PD, de Klerk NH, Landau LI, Kendall GE, Holt PG, et al. Breast feeding and respiratory morbidity in infancy: a birth cohort study. Arch Dis Child. 2003 Mar;88(3):224-8.
28. Pettigrew MM, Khodaee M, Gillespie B, Schwartz K, Bobo JK, Foxman B. Duration of breastfeeding, daycare, and physician visits among infants 6 months and younger. Ann Epidemiol. 2003 Jul;13(6):431-5.
29. Paricio Talayero JM, Lizan-Garcia M, Otero Puime A, Benlloch Muncharaz MJ, Beseler Soto B, Sanchez-Palomares M, et al. Full breastfeeding and hospitalization as a result of infections in the first year of life. Pediatrics. 2006 Jul;118(1):e92-9.

30. Chantry CJ, Howard CR, Auinger P. Full breastfeeding duration and associated decrease in respiratory tract infection in US children. Pediatrics. 2006 Feb;117(2):425-32.
31. Pardo-Crespo R, Perez-Iglesias R, Llorca J, Alvarez-Granda L, Garcia-Fuentes M, Martinez-Gonzalez MA, et al. Breast-feeding and risk of hospitalization for all causes and fever of unknown origin. Eur J Public Health. 2004 Sep;14(3):230-4.
32. Howie PW, Forsyth JS, Ogston SA, Clark A, Florey CD. Protective effect of breast feeding against infection. Bmj. 1990 Jan 6;300(6716):11-6.
33. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21(6):475-84.
34. Jaddoe VW, Bakker R, van Duijn CM, van der Heijden AJ, Lindemans J, Mackenbach JP, et al. The Generation R Study Biobank: a resource for epidemiological studies in children and their parents. Eur J Epidemiol. 2007;22(12):917-23.
35. Allochtonen in Nederland 2004. Voorburg/Heerlen: Statistics Netherlands; 2004.
36. Hanson LA. Protective effects of breastfeeding against urinary tract infection. Acta Paediatr. 2004 Feb;93(2):154-6.
37. New charts for ultrasound dating of pregnancy and assessment of fetal growth: longitudinal data from a population-based cohort study. Verburg BO, Steegers EA, De Ridder M, Snijders RJ, Smith E, Hofman A, Moll HA, Jaddoe VW, Witteman JC. Ultrasound Obstet Gynecol.2008;DOI 10.1002/uog.5225
38. Hanson LA, Korotkova M, Haversen L, Mattsby-Baltzer I, Hahn-Zoric M, Silfverdal SA, et al. Breastfeeding, a complex support system for the offspring. Pediatr Int. 2002 Aug;44(4):347-52.
39. Lawrence RM, Pane CA. Human breast milk: current concepts of immunology and infectious diseases. Curr Probl Pediatr Adolesc Health Care. 2007 Jan;37(1):7-36.
40. Li R, Scanlon KS, Serdula MK. The validity and reliability of maternal recall of breastfeeding practice. Nutr Rev. 2005 Apr;63(4):103-10.
41. Kalter HD, Gray RH, Black RE, Gultiano SA. Validation of the diagnosis of childhood morbidity using maternal health interviews. Int J Epidemiol. 1991 Mar;20(1):193-8.

Maternal smoking in prenatal and early postnatal life and the risk of respiratory tract infections in infancy

3.3

Abstract

Objective: To assess the associations of maternal smoking during pregnancy and in the postnatal period with respiratory tract infections in young infants.
Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards. All data were assessed by questionnaires. Maternal smoking was assessed in pregnancy (no, stopped when pregnancy was known, continued during pregnancy) and at 6 months postnatally. Doctor-attended respiratory tract infections were recorded at the age of 6 months. The present analyses were based on 3,418 subjects.
Results: Continued maternal smoking during pregnancy was not associated with respiratory tract infections in young infants. Maternal smoking in the postnatal period showed a tendency for an increased risk of lower respiratory tract infections in infants (adjusted odds ratio (aOR) 1.61 (95% confidence interval: 0.99, 2.63)). Dose-response effects for maternal smoking during pregnancy or in the postnatal period on the risk of respiratory tract infections were not observed. In infants of mothers who smoked neither during pregnancy nor in the postnatal period, environmental smoking during pregnancy and in the postnatal period together was associated with upper respiratory tract infections (aOR 1.58 (95% CI: 1.07, 2.35)).
Conclusions: No effect of maternal smoking during pregnancy with respiratory tract infections was observed. Weak evidence for the association between maternal smoking in the postnatal period and lower respiratory tract infections were found. Exposure to non-maternal environmental smoking during pregnancy and in the postnatal period together increases the risk of upper respiratory tract infections in young infants.

Introduction

Upper and lower respiratory tract infections are the most common cause of morbidity and mortality in infancy (1-3). On average an infant has 3-12 respiratory tract infections per year (4). The risk of respiratory morbidity is influenced by several factors including birth weight, gestational age, socio-economic status, ethnicity, number of siblings, attending a daycare centre and breastfeeding (4-20). Exposure to maternal smoking during pregnancy and in the postnatal period has been proposed as an additional and modifiable risk factor in early life (21-28). Most of these studies were not able to assess the separate effects of the timing of smoking since most mothers who smoked during pregnancy also smoked in the postnatal period.

During pregnancy, maternal smoking and fetal exposure to nicotine cause growth retardation and a reduction in fetal breathing movements, which can lead to abnormal growth and maturation of the airways and lungs (29). In the postnatal period, exposure to smoke may increase the susceptibility to pathogens due to impaired defence mechanisms of the airways and bronchial tree, such as mucociliary clearance (30). The independent effects of maternal smoking of different duration in pregnancy and in the postnatal period on the risk of respiratory tract infections in young infants are not yet clear, and may be relevant for identifying critical periods for the effect of exposure to maternal smoking on airway and lung development and respiratory tract infections in infants.

We examined in a population-based prospective cohort study the associations of maternal smoking habits of different duration in pregnancy and in the postnatal period with respiratory tract infections in the infants.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development and health and has been described previously in detail (31, 32). Briefly, the cohort includes 9,778 mothers and their children living in Rotterdam, the Netherlands. Enrolment of mothers was aimed in early pregnancy (gestational age < 18 weeks) but was possible until birth of the child. All children were born between April 2002 and January 2006 and form a prenatally enrolled birth-cohort that is currently followed until young adulthood. Of all eligible children in the study area, 61% participated at birth in the study (32). The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Exposure to maternal smoking in pregnancy and the postnatal period

Information about maternal smoking was obtained by postal questionnaires in early (gestational age < 18 weeks), mid- (gestational age 18 - 25 weeks) and late (gestational age ≥ 25 weeks) pregnancy and in the postnatal period at the age of 6 months (32). Maternal smoking at enrolment was assessed in the first questionnaire by asking whether the mother smoked in pregnancy (no, yes-but stopped when pregnancy was known, yes-continued during pregnancy). This questionnaire was send to all mothers, independent of their gestational age at enrolment. In the second and third questionnaire, the mothers were asked whether they smoked in the past 2 months in mid- and late pregnancy (no, yes), respectively. Because of our interest in duration of exposure to maternal smoking in prenatal life, the 3 smoking categories in the first questionnaire were combined with the smoking categories in mid- and late pregnancy: mothers who reported in the first questionnaire to have stopped smoking when pregnancy was known but still reported to smoke in the second or third questionnaire (n=92), were reclassified into the 'continued smoking during pregnancy' category. The same strategy was used for mothers who reported not to smoke in the first questionnaire but smoked in the second or third questionnaire (n=31). In the fourth questionnaire used for this study, mothers were asked whether they smoked (no, yes) when their child was 6 months old. These categories were used for exposure to maternal smoking in early postnatal life.

Among the smoking mothers, the number of cigarettes was asked for in the following categories: less than 1 per day; 1-2 per day; 3-4 per day; 5-9 per day; 10-19 per day; and 20 or more per day. To increase the exposure groups, these categories were combined and reclassified into the following categories: non-smoking; less than 5 cigarettes per day; 5-9 cigarettes per day and 10 or more cigarettes per day.

Non-maternal environmental smoking during pregnancy was obtained in early, mid- and late pregnancy as the number of hours per day that mothers were exposed to environmental smoke at home and at work. For both exposure to smoke at home and at work, categories were combined and reclassified into the following categories: non- or occasionally exposed; and exposed (less than one hour per day, one to three hours per day or more than three hours per day). If mothers reported in the first questionnaire to be non-exposed to smoke at home or at work, but reported to be exposed in the second or third questionnaire, they were reclassified into the exposed category (n=87). Environmental smoking in the postnatal period was obtained by the fourth questionnaire at the infants' age of 6 months. Parents were asked whether their infants were exposed to environmental smoke at home or at other places (no, yes). These questions were combined and reclassified into the categories 'non-exposed' and 'exposed'.

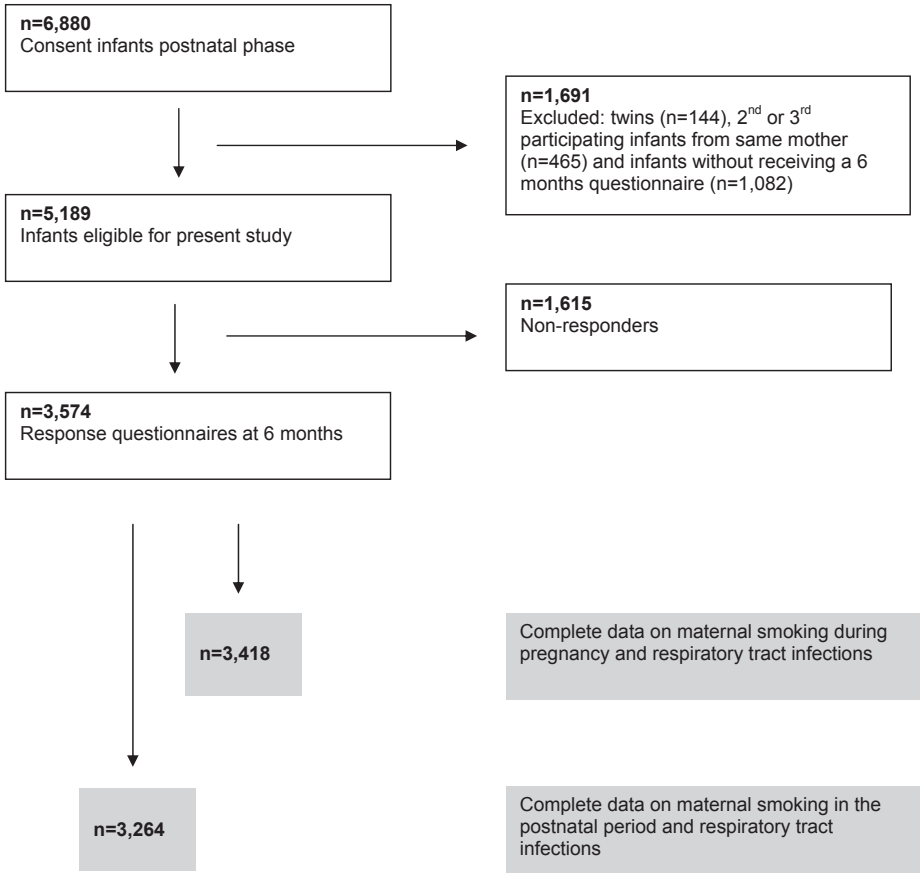
Respiratory tract infections

Information about respiratory tract infections was obtained by a postal questionnaire at the age of 6 months. Parents were asked whether their infant has had an upper respiratory tract infection (serious cold, ear infection, throat infection) and/or a lower respiratory tract infection (pneumonia, bronchitis or bronchiolitis) for which they attended a doctor in the previous 6 months.

Covariates

Information about ethnicity and educational level of the mother was obtained by the first questionnaire at enrolment in the study. Ethnicity and educational level of the mother was defined according to the classification of Statistics Netherlands (33, 34). Gestational age was established by fetal ultrasound examination (32). Birth weight and date of birth were obtained from midwife and hospital registries. Information about siblings, daycare

Figure 1. Flow chart of participants in the study on exposure to maternal smoking during pregnancy and in the postnatal period and the risk of respiratory tract infections in infancy.



centre and breastfeeding for at least one month (no, yes) was asked in the fourth questionnaire at the age of 6 months.

Cohort for analysis

Of the total of 9,778 mothers, 8,880 (91%) were enrolled in pregnancy (32). Of these 8,880 mothers, 6,880 gave informed consent for participation in the postnatal phase of the study (32) (Figure 1). Second or third participating infants of the same mother (n=465) and twins (n=144) were excluded from the present analyses to prevent potential bias due to correlation. Of the remaining 6,271 infants, a 6 months-questionnaire was not send to 1,082 parents since part of the parents did not give informed consent for postnatal participation of their children before the age of 6 months and because this questionnaire was started to sent out from a later stage in the study (June 2003). Of the 5,189 parents the questionnaire was sent to, 3,574 (69%) responded. Complete data for the analyses on the associations of maternal smoking during pregnancy and in the postnatal period were available in 3,418 and 3,264 mothers and infants, respectively.

Data analysis

The associations of maternal smoking of different duration in pregnancy and in the postnatal period with reported respiratory tract infections in the infants were analyzed using multiple logistic regression analysis. After the crude analysis (model A), potential confounders including birth weight, gestational age, education, ethnicity, siblings, daycare centre and breastfeeding were added to the model (model B). In the third model (model C), maternal smoking in the postnatal period was added as a confounder to the models focused on the associations of maternal smoking during pregnancy with respiratory tract infections. Maternal smoking during pregnancy was added as a confounder to the models focused on the association between maternal smoking in the postnatal period and respiratory tract infections. The associations of the reported number of cigarettes smoked by mother with the risk of respiratory tract infections were analyzed using multiple logistic regression models. These models were adjusted for all potential confounders mentioned above and the exposure to maternal smoking habits in other periods. Furthermore, tests for trends were performed by treating each categorized variable as a continuous term and entering the variable in to the fully adjusted logistic regression model. Adjusted analyses of exposure to environmental smoking were performed in mothers who neither smoked during pregnancy nor in the postnatal period. Four strata of exposure to environmental smoking were used: non-exposed; during pregnancy but not in the postnatal period; not during pregnancy but in the postnatal period; and both during pregnancy as in the postnatal period. The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Characteristics of the mothers and their infants according to maternal smoking habits during pregnancy are presented in Table 1. Of all mothers, 8.5% stopped smoking when pregnancy was known and 13.7% continued smoking during pregnancy. Of mothers who smoked until pregnancy was known, 30.9% started smoking again in the postnatal period and of mothers who continued smoking in pregnancy, 76.1% still smoked in the postnatal period. Of mothers who did not smoke during pregnancy, 19.7% were exposed

Table 1. Maternal and infant characteristics according to smoking habits of mother during pregnancy.

	Maternal smoking during pregnancy		
	Non-smoking (n=2,659)	**Stopped when pregnancy was known (n=291)**	**Continued during pregnancy (n=468)**
Mothers			
Age (years)	31.0 (4.6)	30.3 (4.9)	29.8 (5.5)
Education (%)			
Primary school	5.5 (132)	4.9 (14)	10.9 (44)
Secondary school	35.4 (852)	49.9 (115)	62.9 (254)
Higher education	59.1 (1,420)	55.2 (159)	26.2 (106)
Non-Dutch ethnicity (%)	38.8 (964)	38.5 (112)	45.6 (193)
Smoking in the postnatal period (%)			
Non-smoking	92.0 (2,445)	66.3 (193)	19.7 (92)
Smoking	3.3 (88)	30.9 (90)	76.1 (356)
Unknown	4.7 (126)	2.7 (8)	4.3 (20)
Non-maternal environmental smoking during pregnancy (%)	19.7 (523)	33.7 (98)	72.2 (338)
Non-maternal environmental smoking in the postnatal period (%)	15.0 (380)	18.6 (52)	32.1 (143)
Infants			
Siblings ≥ 1 (%)	32.6 (828)	27.0 (76)	39.1 (174)
Daycare centre (%)	80.7 (2,145)	75.9 (221)	74.8 (350)
Breastfeeding ≥ 1 month (%)	80.3 (2,114)	81.2 (233)	63.7 (295)
Birth weight (grams)	3463 (540)	3512 (512)	3256 (540)
Gestational age (weeks)*	40.1 (37.1-42.1)	40.1 (37.4-42.1)	40.0 (36.7-42.1)
Upper respiratory tract infection (%)	37.9 (1006)	39.5 (115)	44.2 (207)
Lower respiratory tract infection (%)	7.3 (194)	6.9 (20)	8.5 (40)

Values are means (standard deviation) or percentages (absolute numbers). *Median (95% range).
Data were missing on education (n=322), ethnicity (n=224), environmental smoking during pregnancy (n=2), environmental smoking in the postnatal period (n=160), siblings (n=153), breastfeeding (n=34) and birth weight (n=2).

Table 2. Exposure to maternal smoking during pregnancy and the risk of respiratory tract infections in infants.

	Upper respiratory tract infections OR (95%CI)		
Maternal smoking	**Model A**	**Model B**	**Model C**
Non-smoking (n=2,659)	Reference	Reference	Reference
Stopped when pregnancy was known (n=291)	1.07 (0.84, 1.37)	1.09 (0.84, 1.41)	1.06 (0.81, 1.40)
Continued during pregnancy (n=468)	1.30 (1.07, 1.59)**	1.25 (0.99, 1.57)	1.18 (0.86, 1.61)
	Lower respiratory tract infections OR (95%CI)		
Maternal smoking	**Model A**	**Model B**	**Model C**
Non-smoking (n=2,659)	Reference	Reference	Reference
Stopped when pregnancy was known (n=291)	0.94 (0.58, 1.51)	0.97 (0.58, 1.61)	0.84 (0.49, 1.44)
Continued during pregnancy (n=468)	1.19 (0.83, 1.69)	1.16 (0.77, 1.75)	0.81 (0.45, 1.45)

Values are odds ratios (95% confidence interval), *P-value < 0.05.
Model A: unadjusted.
Model B: adjusted for birth weight, gestational age, education and ethnicity mother, siblings, daycare centre and breastfeeding.
Model C: model B + adjusted for maternal smoking in the postnatal period.

Table 3. Exposure to maternal smoking in the postnatal period and the risk of respiratory tract infections in infants.

	Upper respiratory tract infections OR (95%CI)		
Maternal smoking	**Model A**	**Model B**	**Model C**
Non-smoking (n=2,730)	Reference	Reference	Reference
Postnatal (n=534)	1.23 (1.02, 1.49)*	1.21 (0.98, 1.50)	1.13 (0.86, 1.47)
	Lower respiratory tract infections OR (95%CI)		
Maternal smoking	**Model A**	**Model B**	**Model C**
Non-smoking (n=2,730)	Reference	Reference	Reference
Postnatal (n=534)	1.34 (0.97, 1.87)	1.41 (0.98, 2.04)	1.61 (0.99, 2.63)

Values are odds ratios (95% confidence interval), *P-value < 0.05.
Data were missing on maternal smoking in the postnatal period (n=154).
Model A: unadjusted.
Model B: adjusted for birth weight, gestational age, education and ethnicity mother, siblings, daycare centre and breastfeeding.
Model C: model B + adjusted for maternal smoking 'stopped when pregnancy was known' or 'continued during pregnancy'.

to environmental smoke during pregnancy and 15.0% were exposed to environmental smoke in the postnatal period.

At 6 months, upper and lower respiratory tract infections were reported for 38.8% and 7.4% of all infants, respectively. Infants whose mother stopped smoking when pregnancy was known did not have more upper and lower respiratory infections than infants with non-smoking mothers (Table 2). In the unadjusted analyses (model A), continued smoking during pregnancy was associated with more upper respiratory tract infections

Table 4. Number of cigarettes smoked by mother and the risk of respiratory tract infections in infants.

Smoking stopped when pregnancy was known	**Upper respiratory tract infections aOR (95%CI)**	**Lower respiratory tract infections aOR (95% CI)**
Non-smoking (n=2,659)	Reference	Reference
< 5 cigarettes per day (n=171)	0.77 (0.54, 1.11)	0.75 (0.36, 1.53)
5-9 cigarettes per day (n=56)	1.88 (1.07, 3.32)*	1.02 (0.35, 2.95)
≥ 10 cigarettes per day (n=57)	1.29 (0.74, 2.24)	1.14 (0.40, 3.26)
P-value for trend	*0.23*	*0.93*
Continued smoking during pregnancy	**Upper respiratory tract infections aOR (95%CI)**	**Lower respiratory tract infections aOR (95% CI)**
Non-smoking (n=2,659)	Reference	Reference
< 5 cigarettes per day (n=184)	1.39 (0.93, 2.09)	1.10 (0.53, 2.29)
5-9 cigarettes per day (n=112)	1.29 (0.76, 2.18)	1.04 (0.42, 2.59)
≥ 10 cigarettes per day (n=71)	1.42 (0.78, 2.57)	0.65 (0.20, 2.10)
P-value for trend	*0.21*	*0.59*
Postnatal smoking	**Upper respiratory tract infections aOR (95%CI)**	**Lower respiratory tract infections aOR (95% CI)**
Non-smoking (n=2,730)	Reference	Reference
< 5 cigarettes per day (n=210)	1.11 (0.79, 1.56)	1.92 (1.09, 3.39)*
5-9 cigarettes per day (n=163)	1.01 (0.68, 1.51)	1.26 (0.58, 2.71)
≥ 10 cigarettes per day (n=149)	1.12 (0.73, 1.70)	1.55 (0.73, 3.28)
P-value for trend	*0.65*	*0.22*

Values are odds ratios (95% confidence interval). *P-value < 0.05.
Data were missing on numbers of cigarettes smoked by mother during pregnancy (n=108) and numbers of cigarettes smoked by mother in the postnatal period (n=12).
Values are adjusted for birth weight, gestational age, education and ethnicity mother, siblings, daycare centre and breastfeeding. Also adjusted for maternal smoking in the postnatal period (in analyses on maternal smoking 'stopped when pregnancy was known' or 'continued during pregnancy' and respiratory tract infections) or for maternal smoking 'stopped when pregnancy was known' or 'continued during pregnancy' (in analysis on maternal smoking in the postnatal period and respiratory tract infections).

but not with lower respiratory tract infections (OR 1.30 (95% CI: 1.07, 1.59) and OR 1.19 (95% CI: 0.83, 1.69)). After adjusting for potential confounders and maternal smoking in the postnatal period the effect of continued smoking during pregnancy on upper respiratory tract infections decreased (aOR 1.18 (95% CI: 0.86, 1.61)). Maternal smoking in the postnatal period was associated with more upper respiratory tract infections in the unadjusted but not in the adjusted analysis (OR 1.23 (95% CI: 1.02, 1.49) and aOR 1.13 (95% CI: 0.86, 1.47)) (Table 3). The aOR for lower respiratory tract infection in infants exposed to maternal smoking in the postnatal period was 1.61 (95% CI: 0.99, 2.63). Table 4 shows that there were no significant trends for the associations of number of cigarettes smoked by mother during pregnancy or in the postnatal period with upper or lower respiratory tract infections in infancy (all p-values for trend > 0.05).

Table 5. Exposure to environmental smoking and the risk of respiratory tract infections in infants of mothers who smoked neither during pregnancy nor in the postnatal period.

	Upper respiratory tract infections OR (95%CI)	
Environmental smoking	**Model A**	**Model B**
Non-exposed (n=1,735)	Reference	Reference
During pregnancy +, in postnatal period - (n=231)	0.87 (0.65, 1.16)	0.80 (0.58, 1.09)
During pregnancy -, in postnatal period + (n=329)	0.99 (0.78, 1.26)	0.92 (0.71, 1.20)
During pregnancy +, in postnatal period + (n=133)	1.65 (1.16, 2.36)**	1.58 (1.07, 2.35)*
	Lower respiratory tract infections OR (95%CI)	
Environmental smoking	**Model A**	**Model B**
Non-exposed (n=1,735)	Reference	Reference
During pregnancy +, in postnatal period - (n=231)	0.59 (0.31, 1.10)	0.59 (0.29, 1.18)
During pregnancy -, in postnatal period + (n=329)	0.76 (0.47, 1.24)	0.82 (0.48, 1.41)
During pregnancy +, in postnatal period + (n=133)	0.65 (0.30, 1.43)	0.92 (0.41, 2.04)

Values are odds ratios (95% confidence interval), *P-value < 0.05.
Analyses were performed in mothers who neither smoked during pregnancy nor in the postnatal period (n=2,445).
Data were missing on environmental smoking during pregnancy and/or in the postnatal period (n=17).
Model A: unadjusted.
Model B: adjusted for birth weight, gestational age, education and ethnicity mother, siblings, daycare centre and breastfeeding.

Of the mothers who neither smoked during pregnancy nor in the postnatal period (n=2,445), exposure to environmental smoking during pregnancy and in the postnatal period together was associated with upper respiratory tract infections (aOR 1.58 (95% CI: 1.07, 2.35) but not with lower respiratory tract infections (aOR 0.92 (95% CI: 0.41, 2.04)). Associations of the other strata of exposure to non-maternal environmental smoking with respiratory tract infections were not observed (Table 5).

Discussion

This prenatally enrolled birth-cohort study showed weak evidence for an association of maternal smoking in the early postnatal period with lower respiratory tract infections in infants in the first 6 months of life. No effect of maternal smoking of different duration in pregnancy on respiratory tract infections in infants was found. We did not observe dose-response effects for maternal smoking in different periods on respiratory tract infections. Exposure to non-maternal environmental smoking both during pregnancy as in the postnatal period was associated with upper respiratory tract infections.

No previous large population-based prospective cohort study has assessed the separate effects of different duration of maternal smoking exposure in pregnancy and in

the postnatal period on both upper and lower respiratory tract infections, until recently. Håberg et al observed that maternal smoking during pregnancy and postnatal paternal smoking were independent risk factors for lower respiratory tract infections until the age of 18 months (35). Other previous studies found risk estimates of 0.77 and 1.23 for the associations of prenatal maternal smoking and upper respiratory tract infections and lower respiratory tract symptoms, respectively (21, 24). Risk estimates between 1.25 and 1.74 were found for the associations of maternal smoking in the postnatal period and upper and acute respiratory tract infections (24-26, 28). These studies were not performed independent of the timing of smoking relative to pregnancy since most mothers who smoked during pregnancy also smoked in the postnatal period. Two previous studies showed an enhancing effect of prenatal maternal smoking in the associations of postnatal smoke exposure with acute respiratory tract infection and bronchitis, respectively (25, 28). One similar study assessed the separate effects of prenatal and postnatal smoke exposure and showed that maternal smoking at the time of the first antenatal care clinic visit in pregnancy led to a higher risk of at least 1.6 for acute ear infections in children at the age of 5 years whereas maternal smoking in the third trimester of pregnancy or in the postnatal period did not (27). In our study, we report follow up for respiratory tract infections until the age of 6 months. This limited time span may underestimate the total effect of smoke exposure, since assessing respiratory tract infections at older ages might show stronger effects. After 6 months, there will be less protection of circulating maternal antibodies and higher pathogen exposure. The lack of association of maternal smoking until early pregnancy with respiratory tract infections might be explained by the short fetal exposure time of smoking since it is likely that most women were aware of their pregnancy in an early stage. Thus, our findings suggest that exposure to maternal smoking limited to early pregnancy has no important influence on postnatal risk of respiratory tract infections in the first 6 months.

The initial response in the Generation R Study is 61%. In our study, non-participation would lead to selection bias if the associations of maternal smoking with respiratory tract infections differ between those with and without complete data. This seems unlikely as far as non-response on maternal smoking questions in pregnancy is concerned. Information about smoking in pregnancy at enrolment was missing in 2.9% of all mothers and no difference in prevalence of upper and lower respiratory tract infections was present between those with and without data on maternal smoking habits during pregnancy (upper respiratory tract infections 34.5% and 39.3%; lower respiratory tract infections 7.4% and 7.4%, respectively). Selection bias due to loss to follow-up could be present in our study since infants lost to follow-up more often had smoking mothers than non-smoking mothers (48.4% and 41.9%, respectively). As a result of this selective loss to follow up, a truly existing effect of maternal smoking with respiratory tract infection could be missed in our study, subsequently leading to an underestimation of the tendencies

we found. The odds ratio's for the associations of the numbers of cigarettes continuously smoked by mother during pregnancy with upper respiratory tract infections (Table 4; aOR 1.39, aOR 1.29 and aOR 1.42) all exceeds that of the odds ratio of continued smoking with upper respiratory tract infections (Table 2; aOR 1.18). Of the mothers who continued smoking during pregnancy (n=468), 101 did not report the numbers of cigarettes smoked. Infants of these mothers had less upper respiratory tract infections than infants of mothers with reported number of cigarettes (41.6% vs. 45%). Bias might have occurred leading to overestimated tendencies of the associations of numbers of cigarettes smoked by mother during pregnancy with upper respiratory tract infections.

Information about maternal smoking in pregnancy and in the postnatal period was prospectively collected by questionnaires without direct reference to respiratory tract infections. Although assessing smoking habits by questionnaires seem to be a valid method, misclassification may occur, with underreporting of maternal smoking during pregnancy and in the postnatal period (36, 37). This could lead to underestimating the risk of respiratory tract infections in infants of smoking mothers, especially if smoking mothers also tend to underreport respiratory symptoms of their infants. To overcome the limitation of underreporting, other studies have used biomarkers of tobacco exposure including cotinine in maternal urine, saliva or blood samples or nicotine in indoor air (37-40). However, so far these studies have demonstrated that these biomarkers are not superior to self-report when studying the effect of maternal smoking on respiratory tract infections of their offspring (36, 40-44). The main outcomes in our study were self-reported upper and lower respiratory tract infections, and it could be argued that self-report may be inaccurate. However, assessing these diagnoses by questionnaires is widely accepted in epidemiological studies and reliably reflect the true incidence of respiratory infections (45).

Respiratory tract infections are a major cause of hospitalizations for infants and maternal smoking seems to be a risk factor that can be modified. According to the tendencies found in this study, prevention strategies for maternal smoking should be focused on early postnatal life. Exposure to environmental smoking during pregnancy and in the postnatal period should be limited. Furthermore, studies on respiratory tract infections in infants at a later age are necessary to examine the long-term effects of maternal smoking during and after pregnancy.

References

1. Graham NM. The epidemiology of acute respiratory infections in children and adults: a global perspective. Epidemiol Rev. 1990;12:149-78.
2. Chretien J, Holland W, Macklem P, Murray J, Woolcock A. Acute respiratory infections in children. A global public-health problem. N Engl J Med. 1984 Apr 12;310(15):982-4.

3. Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Dis. 2002 Jan;2(1):25-32.
4. Wright AL, Taussig LM, Ray CG, Harrison HR, Holberg CJ. The Tucson Children's Respiratory Study. II. Lower respiratory tract illness in the first year of life. Am J Epidemiol. 1989 Jun;129(6):1232-46.
5. Holberg CJ, Wright AL, Martinez FD, Morgan WJ, Taussig LM. Child day care, smoking by caregivers, and lower respiratory tract illness in the first 3 years of life. Group Health Medical Associates. Pediatrics. 1993 May;91(5):885-92.
6. Koopman LP, Smit HA, Heijnen ML, Wijga A, van Strien RT, Kerkhof M, et al. Respiratory infections in infants: interaction of parental allergy, child care, and siblings-- The PIAMA study. Pediatrics. 2001 Oct;108(4):943-8.
7. Paradise JL, Rockette HE, Colborn DK, Bernard BS, Smith CG, Kurs-Lasky M, et al. Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. Pediatrics. 1997 Mar;99(3):318-33.
8. Cushing AH, Samet JM, Lambert WE, Skipper BJ, Hunt WC, Young SA, et al. Breastfeeding reduces risk of respiratory illness in infants. Am J Epidemiol. 1998 May 1;147(9):863-70.
9. Koch A, Molbak K, Homoe P, Sorensen P, Hjuler T, Olesen ME, et al. Risk factors for acute respiratory tract infections in young Greenlandic children. Am J Epidemiol. 2003 Aug 15;158(4):374-84.
10. Nafstad P, Hagen JA, Oie L, Magnus P, Jaakkola JJ. Day care centers and respiratory health. Pediatrics. 1999 Apr;103(4 Pt 1):753-8.
11. Baker D, Taylor H, Henderson J. Inequality in infant morbidity: causes and consequences in England in the 1990s. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. J Epidemiol Community Health. 1998 Jul;52(7):451-8.
12. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. Pediatrics. 2007 Apr;119(4):e837-42.
13. Celedon JC, Litonjua AA, Weiss ST, Gold DR. Day care attendance in the first year of life and illnesses of the upper and lower respiratory tract in children with a familial history of atopy. Pediatrics. 1999 Sep;104(3 Pt 1):495-500.
14. Marbury MC, Maldonado G, Waller L. Lower respiratory illness, recurrent wheezing, and day care attendance. Am J Respir Crit Care Med. 1997 Jan;155(1):156-61.
15. Leeder SR, Corkhill R, Irwig LM, Holland WW, Colley JR. Influence of family factors on the incidence of lower respiratory illness during the first year of life. Br J Prev Soc Med. 1976 Dec;30(4):203-12.
16. Margolis PA, Greenberg RA, Keyes LL, LaVange LM, Chapman RS, Denny FW, et al. Lower respiratory illness in infants and low socioeconomic status. Am J Public Health. 1992 Aug;82(8):1119-26.
17. Rylander E, Eriksson M, Pershagen G, Nordvall L, Ehrnst A, Ziegler T. Wheezing bronchitis in children. Incidence, viral infections, and other risk factors in a defined population. Pediatr Allergy Immunol. 1996 Feb;7(1):6-11.
18. Nafstad P, Jaakkola JJ, Hagen JA, Botten G, Kongerud J. Breastfeeding, maternal smoking and lower respiratory tract infections. Eur Respir J. 1996 Dec;9(12):2623-9.
19. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breastfeeding for at least 4 months protects against otitis media. Pediatrics. 1993 May;91(5):867-72.
20. Caudri D, Wijga A, Gehring U, Smit HA, Brunekreef B, Kerkhof M, et al. Respiratory symptoms in the first 7 years of life and birth weight at term: the PIAMA Birth Cohort. Am J Respir Crit Care Med. 2007 May 15;175(10):1078-85.
21. Latzin P, Frey U, Roiha HL, Baldwin DN, Regamey N, Strippoli MP, et al. Prospectively assessed incidence, severity, and determinants of respiratory symptoms in the first year of life. Pediatr Pulmonol. 2007 Jan;42(1):41-50.
22. DiFranza JR, Aligne CA, Weitzman M. Prenatal and postnatal environmental tobacco smoke exposure and children's health. Pediatrics. 2004 Apr;113(4 Suppl):1007-15.
23. Hofhuis W, de Jongste JC, Merkus PJ. Adverse health effects of prenatal and postnatal tobacco smoke exposure on children. Arch Dis Child. 2003 Dec;88(12):1086-90.

24. Jaakkola JJ, Kosheleva AA, Katsnelson BA, Kuzmin SV, Privalova LI, Spengler JD. Prenatal and postnatal tobacco smoke exposure and respiratory health in Russian children. Respir Res. 2006;7:48.
25. Jedrychowski W, Flak E. Maternal smoking during pregnancy and postnatal exposure to environmental tobacco smoke as predisposition factors to acute respiratory infections. Environ Health Perspect. 1997 Mar;105(3):302-6.
26. Li JS, Peat JK, Xuan W, Berry G. Meta-analysis on the association between environmental tobacco smoke (ETS) exposure and the prevalence of lower respiratory tract infection in early childhood. Pediatr Pulmonol. 1999 Jan;27(1):5-13.
27. Stathis SL, O'Callaghan DM, Williams GM, Najman JM, Andersen MJ, Bor W. Maternal cigarette smoking during pregnancy is an independent predictor for symptoms of middle ear disease at five years' postdelivery. Pediatrics. 1999 Aug;104(2):e16.
28. Zlotkowska R, Zejda JE. Fetal and postnatal exposure to tobacco smoke and respiratory health in children. Eur J Epidemiol. 2005;20(8):719-27.
29. Manning FA, Feyerabend C. Cigarette smoking and fetal breathing movements. Br J Obstet Gynaecol. 1976 Apr;83(4):262-70.
30. Koenig JQ. Pulmonary reaction to environmental pollutants. J Allergy Clin Immunol. 1987 Jun;79(6):833-43.
31. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol. 2004 Jan;18(1):61-72.
32. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21(6):475-84.
33. Allochtonen in Nederland 2004. Voorburg/Heerlen: Statistics Netherlands; 2004.
34. Standaard onderwijsindeling 2003. Voorburg/Heerlen: Statistics Netherlands; 2004.
35. Haberg SE, Stigum H, Nystad W, Nafstad P. Effects of pre- and postnatal exposure to parental smoking on early childhood respiratory health. Am J Epidemiol. 2007 Sep 15;166(6):679-86.
36. Klebanoff MA, Levine RJ, Morris CD, Hauth JC, Sibai BM, Ben Curet L, et al. Accuracy of self-reported cigarette smoking among pregnant women in the 1990s. Paediatr Perinat Epidemiol. 2001 Apr;15(2):140-3.
37. Patrick DL, Cheadle A, Thompson DC, Diehr P, Koepsell T, Kinne S. The validity of self-reported smoking: a review and meta-analysis. Am J Public Health. 1994 Jul;84(7):1086-93.
38. Wang X, Tager IB, Van Vunakis H, Speizer FE, Hanrahan JP. Maternal smoking during pregnancy, urine cotinine concentrations, and birth outcomes. A prospective cohort study. Int J Epidemiol. 1997 Oct;26(5):978-88.
39. Hebel JR, Fox NL, Sexton M. Dose-response of birth weight to various measures of maternal smoking during pregnancy. J Clin Epidemiol. 1988;41(5):483-9.
40. Brunekreef B, Leaderer BP, van Strien R, Oldenwening M, Smit HA, Koopman L, et al. Using nicotine measurements and parental reports to assess indoor air: the PIAMA birth cohort study. Prevention and Incidence of Asthma and Mite Allergy. Epidemiology. 2000 May;11(3):350-2.
41. Rylander E, Pershagen G, Eriksson M, Bermann G. Parental smoking, urinary cotinine, and wheezing bronchitis in children. Epidemiology. 1995 May;6(3):289-93.
42. Margolis PA, Keyes LL, Greenberg RA, Bauman KE, LaVange LM. Urinary cotinine and parent history (questionnaire) as indicators of passive smoking and predictors of lower respiratory illness in infants. Pediatr Pulmonol. 1997 Jun;23(6):417-23.
43. O'Connor TZ, Holford TR, Leaderer BP, Hammond SK, Bracken MB. Measurement of exposure to environmental tobacco smoke in pregnant women. Am J Epidemiol. 1995 Dec 15;142(12):1315-21.
44. Gehring U, Leaderer BP, Heinrich J, Oldenwening M, Giovannangelo ME, Nordling E, et al. Comparison of parental reports of smoking and residential air nicotine concentrations in children. Occup Environ Med. 2006 Nov;63(11):766-72.
45. Kalter HD, Gray RH, Black RE, Gultiano SA. Validation of the diagnosis of childhood morbidity using maternal health interviews. Int J Epidemiol. 1991 Mar;20(1):193-8.

Determinants of nasopharyngeal carriage of *Streptococcus Pneumoniae* in infants

3.4

Abstract

Objective: To study the prevalence, risk factors and dynamics of pneumococcal carriage in infancy.

Study design: In a population-based prospective cohort study in Rotterdam, the Netherlands (June 2003 - November 2006), nasopharyngeal swabs were obtained at the age of 1.5, 6 and 14 months. Data on risk factors were obtained by midwives, hospital registries and by postal questionnaires.

Results: Prevalence of pneumococcal carriage increased from 8.3% to 31.3% to 44.5% at the ages of 1.5 (n=627), 6 (n=832) and 14 months (n=757), respectively. The prevalence of serotypes covered by the 7-valent conjugate increased from 3.0% to 16.2% and 27.7%, at the different ages. Having siblings (aOR 4.33, CI 1.22-15.35) and day care attendance (aOR 3.05, CI 1.88-4.95 at 6 months and aOR 2.78, CI 1.70-4.55 at 14 months) were associated with pneumococcal carriage. Pneumococcal carriage at 6 months was associated with pneumococcal carriage at 14 months (aOR 2.43, CI 1.50-3.94). Pneumococcal carriage was not associated with gender, maternal smoking, educational level mother and breastfeeding.

Conclusions: The prevalence of serotypes covered by the 7-valent conjugate vaccine increases in the first year of life. Siblings, day care attendance and previous pneumococcal carriage are independent risk factors for pneumococcal carriage.

Introduction

Streptococcus pneumoniae (pneumococcus) is one of the major causes of bacterial infections worldwide. Pneumococcal infections range from otitis media to life threatening invasive infections like sepsis, meningitis and pneumonia (1-3). Young children, elderly people, and patients with immunodeficiencies have an increased risk for pneumococcal infections (4). Pneumococci are frequently carried in the nasopharynx, especially in children. Although usually asymptomatic, pneumococcal colonization is the first step in the pathogenic route towards an invasive disease. Besides, carriage is the source for horizontal spread in the community (5).

Numerous studies have shown that acquisition of pneumococci in the nasopharynx occurs early in life (6). Prevalence of pneumococcal carriage increases in the first years of life. Crowding, defined by family size and day care attendance, is a well-established risk factor for pneumococcal carriage (7, 8). For other risk factors like gender, socioeconomic status, smoking and breastfeeding the association with pneumococcal carriage is inconclusive. Coles et al. (9) found an association between pneumococcal carriage and gender and passive smoking. Other could not confirm this association (10, 11). Greenberg et al.(12) found an association between passive smoking and pneumococcal carriage in infants, in contrast to other studies (9,13). Breastfeeding is protective against respiratory tract infections(14-16) and invasive pneumococcal disease (17). However, the association between breastfeeding and pneumococcal carriage in human and in vitro studies is largely inconclusive (18-22).

Large longitudinal studies on dynamics and risk factors of pneumococcal carriage in the first year of life are lacking. Our aim was to study in a population-based prospective cohort the prevalence, dynamics and risk factors for pneumococcal carriage during the first 14 months of life.

Material and methods

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood (23, 24). Detailed assessments of fetal and postnatal growth and development were conducted 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study (23, 24). The study was conducted in Rotterdam, the Netherlands from June 2003 till November 2006. The children were planned to visit the research centre at the ages of 1.5 month, 6 months and 14 months. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

To detect pneumococcal carriage, trained research assistants obtained a single nasopharyngeal swab at each visit. Nasopharyngeal samples were taken with rayon tipped dacron pernasal swabs. The flexible swab was inserted into the anterior nares, gently rubbed on the posterior nasopharyngeal wall, removed, and stored in Amies transport medium at room temperature. Swabs were plated within 6 hours of sampling on a blood agar plate with 5% sheep blood to isolate *S. pneumoniae*. Infants, who used antibiotics 48 hours preceding the visit, were excluded. All pneumococci were serotyped by capsular swelling method (Quellung reaction) with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark), according to the instructions of the manufacturer. These procedures are in line with the standard method as described by O'Brien et al.6 and applied by our lab in a previous study (25). All children were born before the start of the national pneumococcal vaccination program in the Netherlands. The registry of the baby well clinic showed that none of the infants of the cohort was vaccinated. In the currently used conjugate vaccine in children under the age of 2 years 7 serotypes are included (4, 6B, 9V, 14, 18C, 19F and 23F). Based on these 7 serotypes, we divided the pneumococci into vaccine-types (VT) and non-vaccine-types (NVT). The number of swabs were taken as denominator for the prevalence rate of pneumococcal carriage.

Data on gender, parity, birth weight and gestational age were obtained by midwives and hospital registries. Information about siblings, breastfeeding, educational level of mother, smoking and day care attendance was obtained by postal questionnaires at the infants' age of 6 and 12 months. Mothers were asked whether they ever breastfed their infant (no, yes). The duration of breastfeeding was categorized into the groups of never breastfeeding, less than 3 months, 3 to 6 months and 6 months or longer. The duration of exclusive breastfeeding was estimated by combining the question of duration of breastfeeding and the questions at what age formula feeding, other types of milk and solid food were started in the first 6 months of life. This resulted in three categories: 1. never breastfed; 2. partially breastfed (breastfeeding, other milk and/or solid food); and 3. exclusively breastfed for at least 3 months ('exclusive' indicates only breastfeeding, no other milk, solid food, or fluids other than water). The socio-economic status of the mother was defined as highest followed education according to the classification of Statistics Netherlands and categorized in low, medium and high (26).

Differences of infant characteristics between infants with and without nasopharyngeal samples were assessed by the independent sample t-test for continuous normal distributed variables, non-parametric Mann-Whitney test for continuous non-normal distributed variables and the chi-square test for categorical variables. Differences of pneumococcal carriage between groups with missing risk factors and complete data were assessed in the same way. For the longitudinal analyses, the proportion of children with pneumococcal carriage was calculated at the age of 1.5, 6 and 14 months. Because

repeated measurements within subjects are correlated, a Generalized estimating equations (GEE) model adjusting for this correlation was applied. Additionally, these regression models were adjusted for the potential confounders birth weight, parity gestational age, gender, siblings, educational level of mother, day care attendance, smoking and duration of breastfeeding. Associations of risk factors with pneumococcal carriage are presented with odds ratios (OR) with their 95% confidence interval (CI). All tests were carried out using a two-sided alpha level of 5%. The repeated measurement analyses were performed using SAS statistical software, version 9.1 (SAS Institute, Cary, NC, USA). The other statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Of the 1,232 pregnant women enrolled in the Generation R Focus Study 3 mothers had a stillborn infant. The remaining mothers gave birth to 1,244 live born infants. Of 138 of these children the consent was withdrawn after birth. Twins (n=27) were excluded from the present analyses since they are correlated. Our cohort for analysis consisted of 1,079

Table 1. General and infant characteristics (n=1079).

Birth weight (grams)	3509 (538)
Gestational age (weeks)	40.3 (27.6-43.4)
Parity ≥ 1 (%)	38.0 (420)
Gender girl (%)	48.3 (521)
Maternal smoking (%)	12.7 (85)
Educational level mother (%)	
Primary school	2.0 (21)
Secondary school	33.9 (361)
Higher education	64.1 (683)
Siblings ≥ 1 (%)	36.7 (256)
Day care (%)	
At 6 months	63.7 (611)
At 12 months	69.0 (666)
Breastfeeding (%)	
Never	12.7 (98)
< 3 months	25.4 (196)
3-6 months	22.3 (172)
> 6 months	39.6 (306)

Values of birth weight is mean (standard deviation), gestational age is median (range). Other values are percentages (absolute numbers). Data were missing on parity (n=2), maternal smoking (n=411), educational level of mother (n=14), siblings (n=381), day care attendance at 6 and 12 months of age (n=413 and n=193, respectively) and breastfeeding (n=307).

infants. The response rates of the visits were 81.8% (n=883) at 1,5 months, 81.6% (n=881) at 6 months and 80.0% (n=863) at 14 months of age. Seventy percent of the infants came

Table 2. Risk factors for pneumococcal carriage at different ages of the infant.

	S. pneumoniae 1.5 months		S. pneumoniae 6 months		S. pneumoniae 14 months	
	OR	**aOR**	**OR**	**aOR**	**OR**	**aOR**
Birth weight	2.69 (1.51-4.79)***	2.67 (1.22-5.86)*	1.41 (1.07-1.85)*	1.30 (0.84-2.01)	1.34 (1.02-1.75)*	1.09 (0.70-1.71)
Gestational age	1.08 (0.99-1.17)	1.07 (0.86-1.34)	1.10 (1.01-1.20)*	1.05 (0.91-1.21)	1.13 (0.96-1.33)	0.96 (0.84-1.10)
Parity	7.98 (3.91, 16.26)***	6.85 (1.96, 23.92)**	1.96 (1.45, 2.64)***	1.87 (0.70-5.00)	1.74 (1.29, 2.33)***	1.19 (0.43-3.26)
Gender	1.10 (0.62-1.93)	1.27 (0.62-2.61)	1.12 (0.84-1.50)	1.18 (0.78-1.78)	1.11 (0.84-1.48)	1.04 (0.68-1.58)
Educational level mother	1.51 (0.84-2.70)	1.35 (0.62-2.97)	1.77 (1.31-2.39)***	1.31 (0.82-2.08)	1.58 (1.18-2.12)**	0.95 (0.59-1.53)
Siblings	9.58 (3.87-23.71)***	4.33 (1.22-15.35)***	2.45 (1.68-3.58)***	1.37 (0.50-3.73)**	1.69 (1.16-2.44)**	0.81 (0.29-2.25)
Day care attendance	n.a.	n.a.	3.22 (2.05-5.06)***	3.05 (1.88-4.95)***	3.14 (2.17-4.53)***	2.78 (1.70-4.55)***
Duration of breastfeeding						
Continuous variable	n.a.	n.a.	1.33** (1.12-1.57)	1.14 (0.93-1.40)	1.24** (1.06-1.46)	1.11 (0.91-1.37)
Never (reference)						
<3 months	n.a.	n.a.	1.15 (0.59-2.25)	1.42 (0.63-3.20)	0.86 (0.47-1.61)	0.50 (0.21-1.19)
3-6 months	n.a.	n.a.	1.71 (0.88-3.32)	1.82 (0.82-4.04)	1.50 (0.81-2.78)	1.10 (0.47-2.55)
>6 months	n.a.	n.a.	2.16 (1.18-3.98)	1.46 (0.67-3.20)	1.59 (0.91-2.81)	1.23 (0.55-2.74)
Exclusive breastfeeding						
Formula fed only (reference)						
Partially breastfed	n.a.	n.a.	1.63 (0.89-2.99)	1.76 (0.84-3.68)	1.31 (0.75-2.29)	0.91 (0.42-1.98)
Exclusive breastfed > 3 months	n.a.	n.a.	1.48 (0.77-2.81)	1.19 (0.54-2.65)	1.31 (0.72-2.37)	0.95 (0.42-2.14)
Pneumococcus at 1.5 months	n.a.	n.a.	2.01* (1.08-3.73)	1.26 (0.50-3.14)	1.02 (0.53-1.95)	0.55 (0.23-1.30)
Pneumococcus at 6 months	n.a.	n.a.	N.A.	N.A.	2.42*** (1.73-3.39)	2.43*** (1.50-3.94)

Values are crude odds ratios (OR) and adjusted odds ratios (aOR), assessed using a GEE model. Birth weight, gestational age and educational level of mother were included in the models as continuous variables (Table 1). Breastfeeding was included as continuous variable and as categorical variable. Other variables are binary.

* p<0.05, ** p<0.01, ***p<0.001.

to all three visits. The first postnatal measurements in the Generation R Focus Study started June 2003, data collection on pneumococcal carriage started November 2003, so data on pneumococcal carriage in the first 224 participants at 1.5 months of age are missing. Therefore, the number of swabs taken was 627 at 1.5 months, 832 at 6 months and 757 at 14 months of age. The number of swabs were taken as denominator for the prevalence rate of pneumococcal carriage. Characteristics of the infants are presented in Table 1.

Prevalence of pneumococcal carriage increased significantly ($p<0.001$) from 8.3% (52/627) to 31.3% (260/832) to 44.5% (337/757) at the ages of 1.5, 6 and 14 months, respectively. The prevalence of serotypes covered by the 7-valent conjugate vaccine (VT) increased from 3.0% (19/627) to 16.2% (135/832) and 27.2% (206/757), at the different ages. VT-related serotype 6A was found in 1.0% (6/627), 3.4% (28/832) and 5.8% (44/757) infants at the different ages. Differences in prevalence of VT and NVT carriage at different ages were statistically significant ($p<0.05$). Of the 21 children who carried pneumococci at both 1.5 and 6 months of age, five (23.8%) pneumococci were of the same serotype. Of the 116 children who carried pneumococci at both 6 and 14 months of age, 17 (14.6%) pneumococci were of the same serotype. The distribution of serotypes in the infants carrying pneumococci at two ages was comparable to the serotype distribution in the infants with one positive pneumococci sample. Pneumococcal carriage at three sample time points was found in 6 infants. None of these were the same serotype.

Risk factors for pneumococcal carriage at different ages are shown in Table 2. Analyses of missing nasapharyngeal samples showed that infants without nasapharyngeal samples at the age of 1.5 months more often were breastfed than infants with nasapharyngeal samples (94.0% and 86.8%; p-value<0.001). Infants without nasapharyngeal samples at the age of 14 months had a lower birth weight than infants with nasopharyngeal samples (3451 grams and 3533 grams; p-value=0.02). All other risk factors showed no significant differences between children with and without nasopharyngeal samples at different ages. Analyses of missing risk factors showed that infants with missing data on siblings more often carried pneumococci ($p< 0.05$) Analyses of all other missing risk factors showed no differences in pneumococcal carriage between children with non-missing and missing risk factors.

Discussion

In our study pneumococcal carriage increased during the first year of life from 8.3% to 31.3% to 44.5% at the ages of 1,5, 6 and 14 months of age. This is in line with other studies of comparable populations (8, 10, 25, 27-29). Interestingly, the prevalence of serotypes that are present in the 7-valent conjugate vaccine was low 3.0% at 1.5 months of age, and increases to 27.2% at 14 months of age. At 1.5 months of age the prevalence as well

as the vaccine-type versus non-vaccine-type distribution mirrors that of the adults (25, 30), while at 6 and 14 months of age carriage rates and vaccine-type non-vaccine-type distribution showed a similar pattern as found in children (25). The reason for the difference in carriage rates among adults and children is not yet known. Regev-Yochay et al. (31) speculate that the presence of antibodies to pneumococci in adults might be the explanation. Circulating maternal antibodies in young infants might explain the adult pneumococcal carriage pattern in infants at 1.5 months of age.

Pneumococcal carriage is determined by environmental and host factors. The most important environmental factors in our study were having siblings and day care attendance. This is in line with previous studies (5). No association was found between pneumococcal carriage and maternal smoking. However, number of mothers who smoke is rather low. The cohort represents a rather healthy population (24). Detailed data of exposure to passive smoking are missing. We found no association between pneumococcal carriage and duration of breastfeeding or exclusive breastfeeding. The duration of breastfeeding showed a tendency to be a risk factor for pneumococcal carriage in the univariate analysis, but the confounding factors siblings, day care attendance and educational level of mother explained this effect. In vitro studies have shown that human milk and colostrums inhibits the attachment of pneumococci to epithelial cells (18, 19, 31). On the other hand, Rosen et al. (22) showed that pneumococcal capsular antibodies in human milk do not protect against carriage. Lower rates of carriage in exclusively breastfed children were described once, while another study concluded no association between breastfeeding and pneumococcal carriage (20, 21). One study has demonstrated that colostrum-fed infants were more likely to carry pneumococci at 2 months of age than infants from whom colostrum was withheld (9). We expected breastfeeding to be protective against pneumococcal carriage because it is shown to protect against otitis media, other respiratory tract infections (14-16), and invasive pneumococcal disease (17, 32). An explanation that breastfeeding does not protect against pneumococcal carriage but does protect against mucosal infections is given by Finn et al. (33). They showed that secretory IgA-associated killing by phagocytes is complement-dependent, but levels of complement at mucosal surfaces are low. It is speculated that in the presence of active mucosal infection, sufficient amounts of complement may exude from plasma into the mucosal site to support killing of pneumococci.

In our study, high birth weight was associated with pneumococcal carriage at 1.5 months of age. To our knowledge this has never been reported before. One might speculate that infants with higher birth weight are exposed to crowding environment more easily, because infants with low birth weight are treated differently by their parents.

We found an independent association between pneumococcal carriage at 6 months of age and carriage at 14 months of age. This was not explained by any of the risk factors or by lengthy carriage of the same serotype. Only 17 (14.6%) out of the 116 pneumococci

carried at both 6 and 14 months of age were of the same serotype. The distribution of serotypes in the group carrying pneumococci at two sample times was comparable to the serotype distribution in the infants carrying pneumococci once. This finding implies that repeated pneumococcal carriage is not restricted to specific serotypes. Our findings suggest that, besides the studied environmental factors, other unknown environmental (exposure) factors not evaluated in our cohort or (genetic) host characteristics might influence the risk of being colonized at 14 months of age.

To appreciate the results some methodological issues should be considered. The response in the Generation R Focus Study is above 80%. None of the participating infants had used antibiotics in the previous 48 hours. This suggests that children with active infectious diseases were not attending at the scheduled visit and their visits to the research centre were delayed by the parents. Excluding participants with missing data would lead to selection bias if the associations of the risk factors with pneumococcal carriage differ between infants with and without missing data. No difference in the distribution of the risk factors siblings, day care attendance and breastfeeding was found between those with and without data on pneumococcal carriage at any of the ages. Complete data were available for birth weight, gestational age and gender. Infants with missing data on siblings more often carried pneumococci than infants with data on siblings. Due to selective missing data on siblings the observed association of siblings with pneumococcal carriage might be underestimated. No other differences in carriage rates were found between those with and without missing data on all other risk factors.

In summary, having siblings and day care attendance are independent risk factors for pneumococcal carriage, as well as previous pneumococcal carriage. No association between duration and exclusive breastfeeding and pneumococcal carriage was found. In infants 1.5 months of age the prevalence of VT's is significantly lower than in infants of age 6 and 14 months. Besides the environmental factors, additional research is justified to clarify the role of circulating maternal antibodies in infants, mucosal antibody response and genetic predispositions of the infants in pneumococcal carriage.

References

1. Douglas RM. Respiratory tract infections as a public health challenge. Clin Infect Dis. 1999 Feb;28:192-4.
2. Peltola H. Burden of meningitis and other severe bacterial infections of children in africa: implications for prevention. Clin Infect Dis. 2001 Jan;32:64-75.
3. Shann F. The management of pneumonia in children in developing countries. Clin Infect Dis. 1995 Dec;21:S218-25.
4. Prevention of pneumococcal disease: recomendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep. 1997;46:1-24.

5. Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis. 2004 Mar;4:144-54.
6. O'Brien KL, Nohynek H, World Health Organization Pneumococcal Vaccine Trials Carriage Working G. Report from a WHO Working Group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*. Pediatr Infect Dis J. 2003 Feb;22:e1-11.
7. Ghaffar F, Friedland IR, McCracken GH, Jr. Dynamics of nasopharyngeal colonization by *Streptococcus pneumoniae*. Pediatr Infect Dis J. 1999 Jul;18:638-46.
8. Regev-Yochay G, Dagan R, Raz M, Carmeli Y, Shainberg B, Derazne E, et al. Association between carriage of *Streptococcus pneumoniae* and Staphylococcus aureus in Children. Jama. 2004 Aug 11;292:716-20.
9. Coles CL, Kanungo R, Rahmathullah L, Thulasiraj RD, Katz J, Santosham M, et al. Pneumococcal nasopharyngeal colonization in young South Indian infants. Pediatr Infect Dis J. 2001 Mar;20:289-95.
10. Syrjanen RK, Kilpi TM, Kaijalainen TH, Herva EE, Takala AK. Nasopharyngeal carriage of *Streptococcus pneumoniae* in Finnish children younger than 2 years old. J Infect Dis. 2001 Aug 15;184:451-9.
11. Watson K, Carville K, Bowman J, Jacoby P, Riley TV, Leach AJ, et al. Upper respiratory tract bacterial carriage in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia. Pediatr Infect Dis J. 2006 Sep;25:782-90.
12. Greenberg D, Givon-Lavi N, Broides A, Blancovich I, Peled N, Dagan R. The contribution of smoking and exposure to tobacco smoke to *Streptococcus pneumoniae* and Haemophilus influenzae carriage in children and their mothers. Clin Infect Dis. 2006 Apr 1;42:897-903.
13. Principi N, Marchisio P, Schito GC, Mannelli S. Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. Pediatr Infect Dis J. 1999 Jun;18:517-23.
14. Chantry CJ, Howard CR, Auinger P. Full breastfeeding duration and associated decrease in respiratory tract infection in US children. Pediatrics. 2006 Feb;117:425-32.
15. Paricio Talayero JM, Lizan-Garcia M, Otero Puime A, Benlloch Muncharaz MJ, Beseler Soto B, Sanchez-Palomares M, et al. Full breastfeeding and hospitalization as a result of infections in the first year of life. Pediatrics. 2006 Jul;118:e92-9.
16. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. Pediatrics. 2007 Apr;119:e837-42.
17. Levine OS, Farley M, Harrison LH, Lefkowitz L, McGeer A, Schwartz B. Risk factors for invasive pneumococcal disease in children: a population-based case-control study in North America. Pediatrics. 1999 Mar;103:E28.
18. Andersson B, Porras O, Hanson LA, Lagergard T, Svanborg-Eden C. Inhibition of attachment of *Streptococcus pneumoniae* and Haemophilus influenzae by human milk and receptor oligosaccharides. J Infect Dis. 1986 Feb;153:232-7.
19. Aniansson G, Andersson B, Lindstedt R, Svanborg C. Anti-adhesive activity of human casein against *Streptococcus pneumoniae* and Haemophilus influenzae. Microb Pathog. 1990 May;8:315-23.
20. Duffy LC, Faden H, Wasielewski R, Wolf J, Krystofik D. Exclusive breastfeeding protects against bacterial colonization and day care exposure to otitis media. Pediatrics. 1997 Oct;100:E7.
21. Kaleida PH, Nativio DG, Chao HP, Cowden SN. Prevalence of bacterial respiratory pathogens in the nasopharynx in breast-fed versus formula-fed infants. J Clin Microbiol. 1993 Oct;31:2674-8.
22. Rosen IA, Hakansson A, Aniansson G, Hansson C, Andersson B, Nylen O, et al. Antibodies to pneumococcal polysaccharides in human milk: lack of relationship to colonization and acute otitis media. Pediatr Infect Dis J. 1996 Jun;15:498-507.
23. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol. 2004 Jan;18:61-72.
24. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21:475-84.

25. Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rumke HC, et al. Colonisation by *Streptococcus pneumoniae* and Staphylococcus aureus in healthy children. Lancet. 2004 Jun 5;363:1871-2.
26. Standaard onderwijsindeling 2003. Voorburg/Heerlen. Statistics Netherlands. 2004.
27. Bogaert D, Engelen MN, Timmers-Reker AJ, Elzenaar KP, Peerbooms PG, Coutinho RA, et al. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. J Clin Microbiol. 2001 Sep;39:3316-20.
28. Faden H, Duffy L, Wasielewski R, Wolf J, Krystofik D, Tung Y. Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J Infect Dis. 1997 Jun;175:1440-5.
29. Syrogiannopoulos GA, Katopodis GD, Grivea IN, Beratis NG. Antimicrobial use and serotype distribution of nasopharyngeal *Streptococcus pneumoniae* isolates recovered from Greek children younger than 2 years old. Clin Infect Dis. 2002 Nov 15;35:1174-82.
30. Regev-Yochay G, Raz M, Dagan R, Porat N, Shainberg B, Pinco E, et al. Nasopharyngeal carriage of *Streptococcus pneumoniae* by adults and children in community and family settings. Clin Infect Dis. 2004 Mar 1;38:632-9.
31. Deubzer HE, Obaro SK, Newman VO, Adegbola RA, Greenwood BM, Henderson DC. Colostrum obtained from women vaccinated with pneumococcal vaccine during pregnancy inhibits epithelial adhesion of *Streptococcus pneumoniae*. J Infect Dis. 2004 Nov 15;190:1758-61.
32. Gessner BD, Ussery XT, Parkinson AJ, Breiman RF. Risk factors for invasive disease caused by *Streptococcus pneumoniae* among Alaska native children younger than two years of age. Pediatr Infect Dis J. 1995 Feb;14:123-8.
33. Finn A, Zhang Q, Seymour L, Fasching C, Pettitt E, Janoff EN. Induction of functional secretory IgA responses in breast milk, by pneumococcal capsular polysaccharides. J Infect Dis. 2002 Nov 15;186:1422-9.

Immune system in infancy

4

Maturation of lymphocyte subsets from birth until the age of 2 years

4.1

Abstract

Objective: To examine the maturation of T, B and NK lymphocytes in children from birth until the age of 24 months.

Methods: This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards. Lymphocytes (T, B and NK) and their different maturation phases were immunophenotyped in (cord) blood samples at birth (n=570), 6 months (n=384), 14 months (n=251) and 24 months (n=200) by 6-color flow cytometry.

Results: A large pool of naïve lymphocyte subsets was present at all ages. Within the T lymphocytes, both the subsets of memory D and effector $CD8^+$ T lymphocytes increased (0.1% to 1.5%) from birth until 24 months. Memory $CD19^+$ B lymphocytes increased from 0.5% to 3.0%. Within this subset, IgM decreased (65% to 36%), IgA remained stable (20%) and IgG increased (11% to 19%). The amount of effector $CD19^+$ B lymphocytes did not materially change (4%). The percentage of 'memory' $CD19^+$ B lymphocytes decreased from birth until 6 months (6% to 4%) and increased thereafter (6%). Of the $CD56^+$ NK lymphocytes, both the immature $CD56^{bright}$ subsets increased from birth until 6 months (2% to 6% and 7% to 13%, respectively). Immature $CD56^{dim}$ decreased 1.5-fold in the first 6 months of age and remained stable thereafter (50%). A 1.7-fold increase from birth until 6 months was observed for effector $CD56^+$ NK lymphocytes (18% to 31%).

Conclusions: Our study showed that in the first postnatal years the largest change in maturation phases of lymphocytes subsets occurs in the first 6 months of life.

Introduction

Lymphocytes have an important role in the adaptive human immune response. The lymphocyte compartment in the peripheral blood consists of three major subsets; T, B and NK lymphocytes. It is known that the amount of T, B and NK lymphocytes changes during childhood (1, 2). Within each of these lymphocyte populations several subsets might be identified and related to their different function or maturation state.

T lymphocytes: in the peripheral blood, T lymphocytes can be divided into two major subsets based on the expression of CD4 or CD8. While $CD4^+$ T lymphocytes are determined as 'helper' T lymphocytes, $CD8^+$ T lymphocytes are cytotoxic lymphocytes which might react upon viral infections in the host. They recognize viral peptides in the context with MHC class I and lyse cells bearing these complexes. $CD8^+$ T lymphocytes can be divided into different subsets according to their maturation stage (3, 4). Naïve $CD8^+$ T lymphocytes are lymphocytes that have not been exposed to antigens yet. During a viral infection, naïve $CD8^+$ T lymphocytes become activated and either differentiate into effector $CD8^+$ T lymphocytes or convert into memory-type $CD8^+$ T lymphocytes. Memory $CD8^+$ T lymphocytes await re-challenge with antigens and than become effector lymphocytes. Memory $CD8^+$ T lymphocytes can be further subdivided based on the frequency of antigen-stimulation and proliferation capacity (5). The effector $CD8^+$ T lymphocytes are lymphocytes that are capable of lysis of target (virus infected) lymphocytes.

Based on the expression of CD45RO, CD45RA, CD27, CD28 and CD197, the different $CD8^+$ T lymphocyte subsets can be discriminated in naïve, memory A to D and effector cells (3, 4). It could be expected that with increasing age, $CD8^+$ T lymphocytes have more often been exposed to antigens so that more 'mature' memory and effector T lymphocytes will arise.

B lymphocytes: B lymphocytes appear in peripheral blood and in secondary lymphoid tissues after they have differentiated from precursor B lymphocytes in the bone marrow. Multiple B lymphocyte subsets have been identified in the peripheral blood, either naïve or already exposed to antigens (6-8). The transitional B lymphocytes are the most immature lymphocytes that appear in the peripheral blood and can be identified due to expressing $CD38^+$/ $CD24^+$/ IgD^+. They form a relatively small subset compared with $CD38^+$/IgM^+/IgD^+/$CD27^-$ naïve mature B lymphocytes (9, 10). A substantial fraction of the naïve B lymphocytes express the CD5 antigen and is capable of producing polyreactive antibodies (11). The natural effector B lymphocytes in blood are the counterparts of splenic marginal zone B lymphocytes expressing IgM, IgD and CD27. The last B lymphocyte subset that can be identified in peripheral blood are the memory B lymphocytes (IgD^-/$CD27^+$), which can be further subdivided into IgM, IgG and IgA expressing memory B lymphocytes.

NK lymphocytes: NK lymphocytes represent approximately 10-15% of the circulating lymphocytes in the peripheral blood. These lymphocytes are critical for host immunity because of their ability to quickly mediate cytotoxicity against pathogen infected or malignantly transformed lymphocytes. They also produce a wide variety of cytokines and chemokines that influence other lymphocytes of the immune system. The phenotype of NK lymphocytes is based on the absence of CD3 and expression of CD56. Based on their CD56 expression, NK lymphocytes can be divided into two subsets: the major part mildly expressing this antigen ($CD56^{dim}$) and a small part (approximately 2-5% of the total $CD3^-$/$CD56^+$ population) with a very bright expression of this antigen ($CD56^{bright}$) (12, 13). The $CD56^{dim}$ and $CD56^{bright}$ lymphocytes differently express several other antigens. In adults, at least 48 subsets within the NK lymphocyte population can be identified in the peripheral blood (14). For example CD16, CD94, CD2 and CD25 are all heterogeneously expressed on NK lymphocytes, but with different intensity within the $CD56^{dim}$ and $CD56^{bright}$ subsets. It has been postulated that these subsets of NK lymphocytes represent different functions, which also might explain the differences in marker expression of adhesion molecules and killer inhibitory receptors (KIR) (15). It has been suggested that $CD56^{dim}$ lymphocytes are granular cytotoxic lymphocytes and that $CD56^{bright}$ lymphocytes are modulators by producing cytokines (16). The general idea is that the $CD56^{bright}$ are immature lymphocytes which can give rise to $CD56^{dim}$ lymphocytes (17, 18). This maturation step of NK lymphocytes is based on the differential expression of CD94 and CD16.

During childhood, absolute and relative numbers of T, B and NK lymphocytes change (1, 2). However, detailed information of the normal distribution and maturation of the subsets of these lymphocytes are lacking. Therefore, our aim was to examine the different maturation phases of T, B and NK lymphocytes in peripheral blood samples of children from birth until 24 months in a population-based prospective cohort study.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development and health and has been described previously in detail (19, 20). Detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of Dutch pregnant women and their children, referred to as the Generation R Focus Study (19, 20). This subgroup is ethnic homogeneous to exclude possible confounding or effect modification by ethnicity. Of all approached Dutch pregnant women and their partners, 1,232 (79%) participated in the Generation R Focus Study. Their children were born between

February 2003 and August 2005. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants. Sampling of (cord) blood was performed at birth and at the ages of 6, 14 and 24 months.

Immunophenotyping of lymphocyte subsets

At birth, venous cord blood was sampled in heparinized tubes by midwifes and obstetricians immediately after delivery and transported at room temperature to the Laboratory of Immunology of the Erasmus Medical Center within 24 hours. Umbilical cord blood samples not received within 24 hours (weekend days) were excluded since flow cytometric analyses of those samples showed no reliable results in the pilot phase of the study. At the ages of 6, 14 and 24 months, venous blood was sampled in tubes with EDTA as anticoagulant. Flow cytometric immunophenotyping was performed to determine absolute numbers of lymphocytes (T, B, NK, $CD4^+$, $CD8^+$ and $TCR^{\gamma\delta}$). Furthermore, relative numbers of the subsets within the T, B and NK lymphocyte population were detected. For this, the several monoclonal antibodies were conjugated with the labels fluorescein isothiocyanate (FITC[1]), peridin chlorophyll protein (PerCP[1]), peridin chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5[1]), allophycocyanin (APC[1]), phycoerythrin (PE[1,3]), phycoerythrin-cyanin dye (PE-cy7[1]), allophycocyanin-cyanin dye (APC-cy7[1]) and rhodamine (RD1[2]) ([1]Becton Dickinson,[2]Beckman Coulter and [3]Dako) (Table 1). Absolute numbers of T, B and NK lymphocytes were determined with the lysed whole blood technique (50 µl of whole blood per sample) using the routine standardized single platform method with BD TruCOUNT Tubes of Becton Dickinson (BD). With this, lymphocytes were gated on the basis of CD45 and FSC to prevent contamination of unlysed erythrocytes (21, 22). The samples

Table 1. Flow cytometric monoclonal antibodies.

	Monoclonal antibody labels					
	FITC	**PE**	**PerCPCy5.5**	**PE-Cy7**	**APC**	**APC-Cy7**
1	CD3	CD16.56	CD45	CD4	CD19	CD8
2	CD27	CD45RA	CD3	CD4	CD45RO	CD8
3	CD2	CD56	CD3	CD7	CD5	CD8
4	CD28	CD197	CD3	CD8	CD45RO	CD27
5	TCRAB	TCRGD	CD3	CD4	CD8	-
6	CD94	CD16	CD3	CD56	CD8	CD2
7	SmIgK	SmIgL	CD19	CD21	CD22	CD81
8	SmIgD	CD23	CD19	CD21	SmIgM	CD5
9	SmIgA	SmIgG	CD19	CD40	SmIgM	CD27
10	CD57	CD38	CD3	CD56	HLA-DR	CD8

FITC=fluorescein isothiocyanate, PE=phycoerythrin, PerCPCy5.5=peridin chlorophyll protein, PE-cy7=phycoerythrin-cyanin dye, APC=allophycocyanin and APC-cy7=allophycocyanin-cyanin dye.

were measured on a FACS Calibur flow cytometer (BD) and analyzed with BD LymphocyteQuest™ software as indicated by the manufacturer. This flow cytometer is routinely used in the diagnostic laboratory and every day reference calibration beads are used. Subsets within the T, B and NK lymphocytes were determined using 6-colour staining and detected on a BD™ LSR II flow cytometer (Becton Dickinson, San Jose, California, US). Therefore, erythrocytes of 1 ml whole blood were lysed using 50 ml ammoniumchloride. After centrifugation, leucocytes were washed twice and suspended in 900 µl PBS/1% BSA/0.1% NaAz. Of this suspension (5-10*10^6/ml), 50 µl was incubated for 10 minutes at room temperature with combinations of the optimally titrated labeled monoclonal antibodies. After incubation the cells were washed and subsequently identified by flow cytometry. Using a BD™ LSR II flow cytometer that had been calibrated with rainbow beads, 10,000 lymphocytes were measured.

Subsets of within the T, B and NK populations were analyzed as percentages of the total population using BD FacsDIVA™. For the helper T, cytotoxic T and TCR$^{\gamma\delta}$ lymphocytes, absolute counts could not be obtained directly from the TruCOUNT method. Therefore, the absolute counts for these subsets were calculated as follows: the relative counts of helper T, cytotoxic T and TCR$^{\gamma\delta}$ lymphocytes were expressed as the percentage within the total T lymphocyte population and, if possible, calculated by the average of 2-4 independent incubations for each subset. The absolute counts of these T lymphocyte subsets were subsequently calculated from the absolute T lymphocyte counts as obtained by the lysed whole blood technique. The absolute counts of the total T lymphocytes determined with the single platform method were compared with the recalculated counts of the total T lymphocytes (% of lymphocytes within the leukocyte count, determined by a lymphocyte counter). There was no significant difference between the two methods (23).

Statistical analysis

Absolute numbers and percentages of lymphocyte subsets are presented as medians with their 5 to 95% range, since these variables were not normally distributed. The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Cohort for analysis

In total, 1,232 women were enrolled in the Generation R Focus Study. Of these women, 901 (73%), 882 (72%) and 856 (70%) children participated on the postnatal examinations at the age of 6, 14 and 24 months, respectively. In June 2007, blood samples for

immunophenotyping were available from 571 (birth), 384 (6 months), 251 (14 months) and 200 (24 months) children. Main reasons for missing cord blood sample data were due to logistic constraints at delivery (27%) and non-heparinized cord blood samples (26%). Missing blood sample data at the postnatal visits were mainly due to no consent of the parents (approximately 55% per visit) and technical failure (approximately 10% per visit). Of the children with available blood sample data 2% were twins and 10% were siblings since mothers were allowed to participate with second pregnancies. These children were included in the present study since there were no differences in results between twins and siblings mutually or between twins or siblings and the other participating children. At birth, 301 (53%) children were boys and 207 (47%) were girls. Median ages (90% range) of the children at the timing of blood sampling were gestational age 40.4 (37.7-42.1) weeks, 6.2 (5.5-7.8) months, 14.4 (13.5-16.3) months and 25.2 (23.8-27.5) months.

T, B and NK lymphocytes

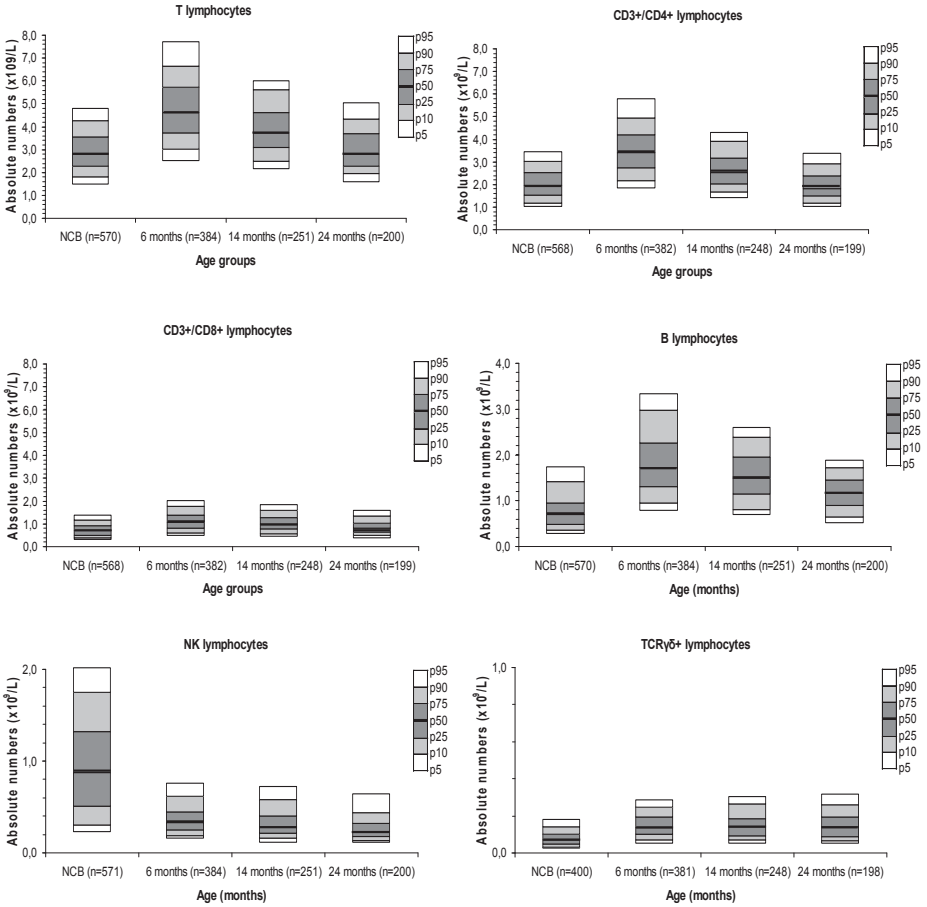
Absolute numbers of T lymphocytes increased 1.6-fold from birth (median 2.81 (90% range: 1.51, 4.79) x 10^9/L) until 6 months (4.61 (2.51, 7.73) x 10^9/L) (Figure 1). Thereafter, absolute numbers gradually decreased to a median of 2.82 (1.59, 5.04) x 10^9/L at 24 months. For B lymphocytes, a 2.5-fold increase from birth (0.70 (0.29, 1.73) x 10^9/L) until 6 months (1.72 (0.79, 3.34) x 10^9/L), followed by a decrease to 1.17 (0.52, 1.89) x 10^9/L at 24 months was observed. The medians of absolute numbers of NK lymphocytes were 0.88 (0.23, 2.02) x 10^9/L at birth gradually decreasing to 0.23 (0.12, 0.61) x 10^9/L at 24 months. The absolute numbers of $TCR^{\gamma\delta+}$ showed a 2-fold increase from birth (0.07 (0.03, 0.18) x 10^9/L) until 6 months (0.14 (0.06, 0.29) x 10^9/L) and remained stable thereafter.

Maturation of lymphocyte subsets

T lymphocytes

Absolute numbers of $CD3^+/CD4^+$ helper T lymphocytes and $CD3^+/CD8^+$ cytotoxic T lymphocytes both followed the trend of absolute numbers of total T lymphocytes with a 1.6-fold increase from birth until 6 months and a gradual decrease thereafter (figure 1). Of the $CD3^+/CD8^+$ cytotoxic T lymphocytes, the percentage of naïve $CD8^+$ lymphocytes increased from 66% to 75% from birth until 24 months (Figure 2). From birth until 6 months, memory A and C $CD8^+$ lymphocytes decreased 2-fold and memory B $CD8^+$ lymphocytes increased 2-fold. Thereafter, all showed a gradual decrease until 24 months. Memory D and effector $CD8^+$ lymphocytes increased from 0.1% to 1.5% and 0.1 to 1.2%, respectively, from birth until 24 months.

Figure 1. Absolute numbers (x 10^9/L) of the main lymphocyte subsets in children from birth until 24 months. Age groups and the number of individuals per age group are indicated. NCB, neonatal cord blood.



B lymphocytes

The percentage of naïve CD19$^+$B lymphocytes remained stable at all ages (Figure 3). An increase from 0.5% to 3% of absolute numbers of memory CD19$^+$B lymphocytes was observed from birth until 24 months. Within this subset, Ig's redistributed largely. IgM decreased from 65% to 36%, IgA remained stable around 20% and IgG increased from 11% to 19%. The percentage of effector CD19$^+$B lymphocytes did not materially change (approximately 4%). The percentage of 'memory' CD19$^+$B lymphocytes decreased from birth until 6 months (6% to 4%) but increased again to 6% thereafter. The SmIgκ/SmIgλ-ratio increased from birth until 6 months (1 to 1.5) and remained stable thereafter.

Figure 2. Maturation of $CD3^+/CD8^+$ cytotoxic T lymphocytes in children from birth until 24 months.
A. Scheme of the maturational stages of $CD3^+/CD8^+$ cytotoxic T lymphocytes based on the differential expression of CD27, CD28, CD45RO and CD197 (3, 4). **B.** Percentage of each $CD3^+/CD8^+$ cytotoxic T lymphocyte subset presented as median (90% range).

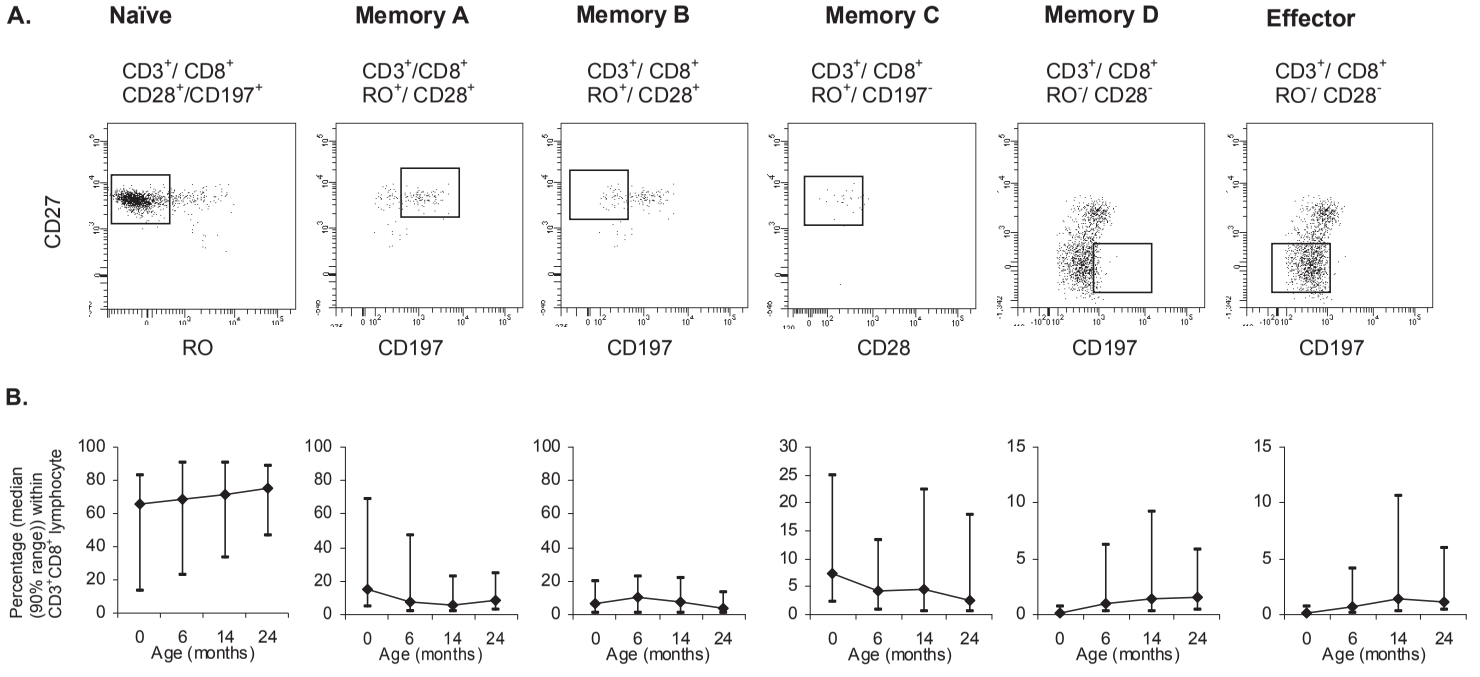


Figure 3. Maturation of CD19$^+$ B lymphocytes in children from birth until 24 months.
A. Scheme of the maturational stages of CD19$^+$ B lymphocytes based on the differential expression of CD27 and IgD (9, 10). **B.** Percentage of each CD19$^+$ B lymphocyte subset presented as median (90% range). **C.** Distribution of IgM, IgA and IgG within memory CD19$^+$ B lymphocytes. **D.** SmIgκ/SmIgλ-ratio of CD19$^+$ B lymphocytes.

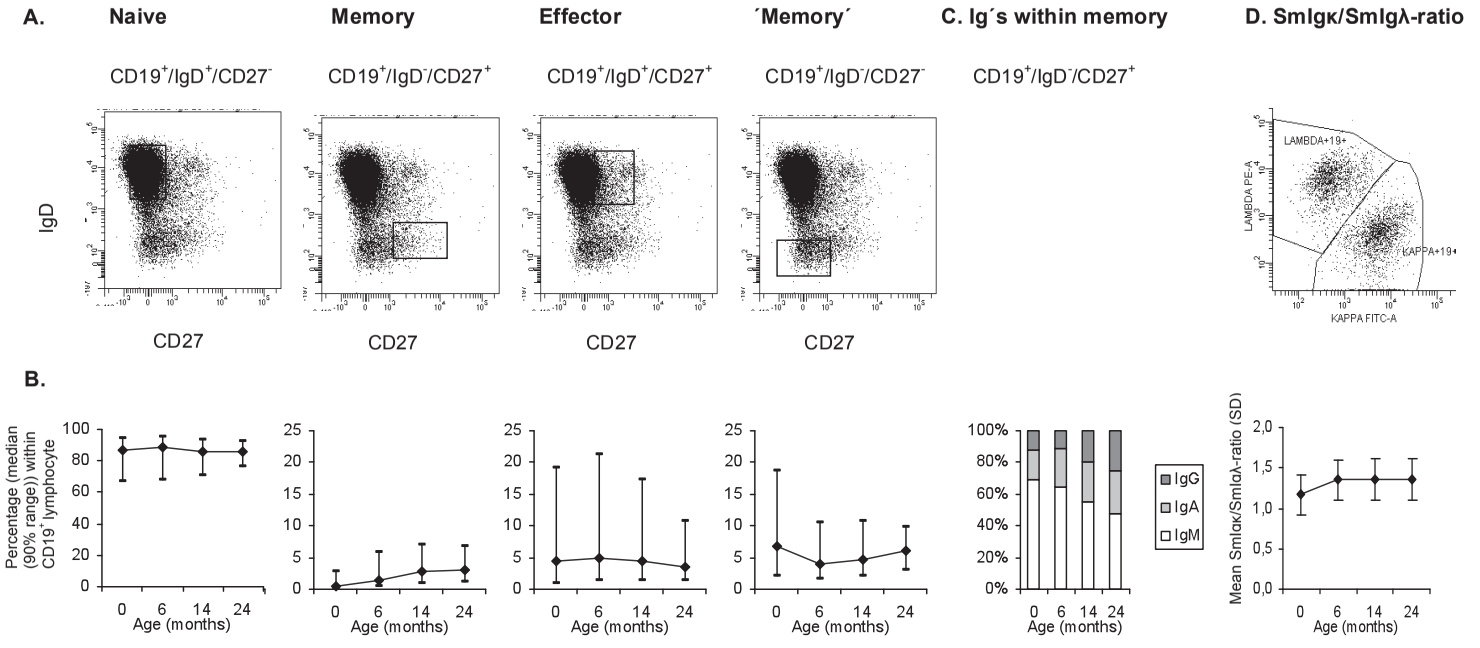
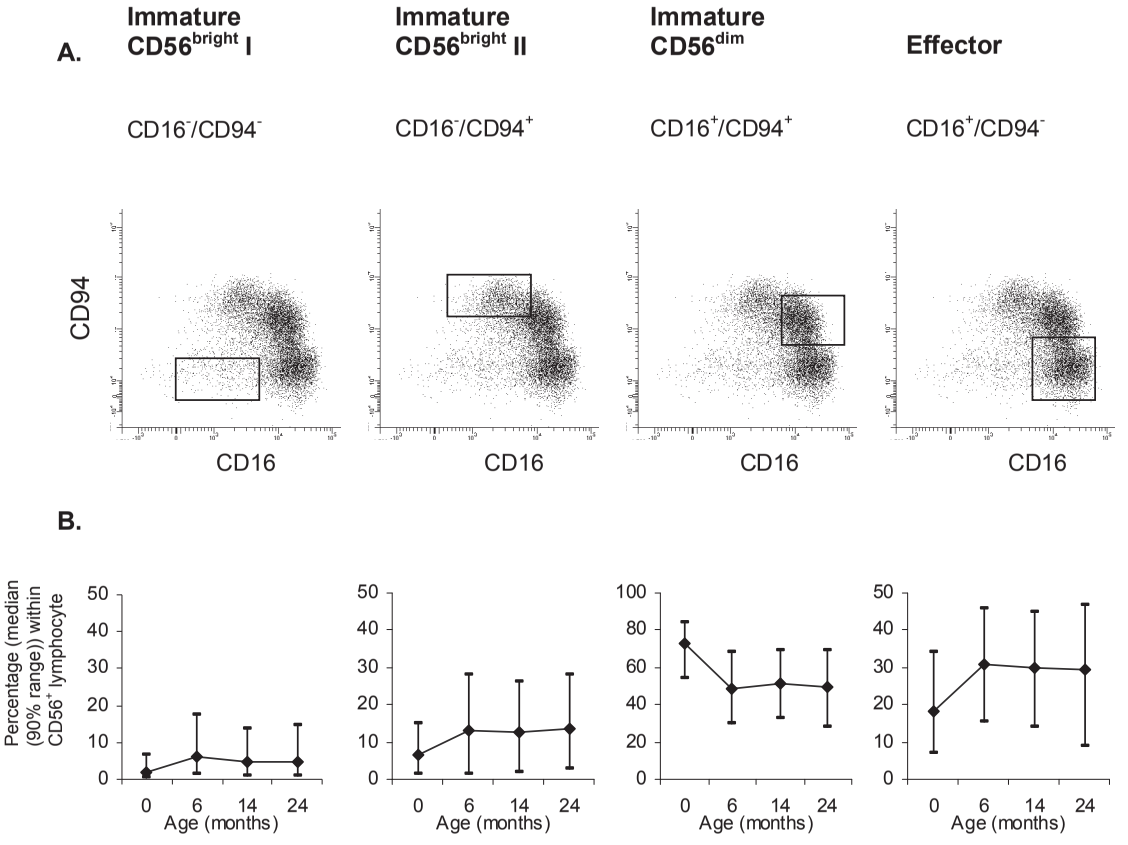


Figure 4. Maturation of CD56$^+$ NK lymphocytes in children from birth until 24 month.
A. Scheme of the maturational stages of CD56$^+$ NK lymphocytes based on the differential expression of CD56, CD94 and CD16 (12-14). **B.** Percentage of each CD56$^+$ NK lymphocyte subset presented as median (90% range).



NK lymphocytes

NK lymphocyte subsets determination was based on the expression of CD56, CD16 and CD94, which might reflect different maturation stages. Of the $CD56^+$ NK lymphocytes, the percentage of both immature $CD56^{bright}/CD94^-/CD16^-$ (immature I) and immature $CD56^{bright}/CD94^+/CD16^-$ (immature II) lymphocytes increased from birth until 6 months (2% to 6% and 7% to 13%, respectively) and did not materially change thereafter (Figure 4). Immature $CD56^{dim}/CD94^-/CD16^-$) decreased 1.5-fold in the first 6 months but remained stable thereafter (50%). A 1.7-fold increase from birth until 6 months was observed for effector $CD56^+$ NK lymphocytes (18% to 31%).

Discussion

This large prospective cohort study from birth until the age of 24 months showed major changes in absolute numbers of lymphocyte subsets mostly during the first 6 months after birth. These results are in line with previously published, mostly smaller sample sized, studies (1, 2, 24). A large pool of naïve lymphocytes of all subsets was present at all the measured age points (birth, 6, 14 and 24 months). The maturation phases of the different lymphocyte subsets largely changed between birth and 6 months. It seems that the adapted immune system at birth quickly matures and probably responses after being exposed to antigens for the first time. At the time of a viral infection, $CD8^+$ T lymphocytes become activated and differentiate into effector lymphocytes or convert into memory-type lymphocytes. Viral infections occur mostly in the first years of life and are the leading cause of morbidity in infancy (25, 26). In line with this, our study showed that with increasing age more 'mature' memory and effector $CD8^+$ T lymphocytes arise. Whether there are direct associations between the number and severity of infections and the maturation of the $CD8^+$ T lymphocytes in children remains to be studied.

From birth until 24 months, the percentage memory $CD19^+$ B lymphocytes increased with a decreased expression of IgM and increased expression of IgG. This class switch is in line with the maturation of B lymphocytes. Which and to whether extent these Ig receptors recognize and respond to antigens needs to be studied in further detail. We observed a switch of $CD27^+$ memory B lymphocytes into $CD27^-$ B lymphocytes in our cohort. $CD27^-$ B lymphocytes recently showed to be post germinal center cells belonging to the memory compartment (27). In our cohort, these new 'memory' B lymphocytes comprised 4 to 6% of the $CD19^+$ B lymphocytes and approximately 80% of the IgG^+ B lymphocytes. In adults, the percentage of this specific 'memory' B lymphocytes is only approximately 1% of the $CD19^+$ B lymphocytes and 25% of the IgG^+ B lymphocytes (27-29). The reason for the percentual difference of this B lymphocyte subset between children and adults needs to be investigated.

Median absolute numbers of $CD56^+$ NK lymphocytes were high at birth (0.9 $x10^9$/L) after which they decreased and remained stable over time (0.3 $x10^9$/L). The large pool of absolute numbers of $CD56^+$ NK lymphocytes at birth might be explained by mode of delivery related stress and hypoxia during delivery (30). Of the $CD56^+$ NK lymphocytes, an increase of both immature $CD56^{bright}$ subsets (I and II) and effector $CD56^+$ NK lymphocytes was observed. The distribution of the different NK lymphocyte subsets at the age of 6 months was already similar as in adults (14). Whether these changes are part of the maturation process or related to the NK lymphocyte function remains to be studied.

Some methodological issues need to be considered. Analyses of missing umbilical cord blood samples showed that neonates without cord blood samples more often had a lower median gestational age and lower mean birth weight than neonates with cord blood samples (median gestational age: 40.1 and 40.4 weeks (Mann-Whitney test, $p<0.01$); mean birth weight: 3454 and 3562 grams (independent sample t-test, $p<0.01$); respectively). Missing postnatal blood samples were mainly due to refusal to participate by the parents (55%), although we minimized the pain by using local anaesthetics like lidocain/prilocain (Emla ©). Unfortunately, we did not have information on possible underlying diseases of the children. It could be expected that parents whose children are 'sick' and regularly need blood examinations more often refuse to participate. Therefore, it might be that our cohort is selected towards a more healthy study population. However, for presenting our data as 'normal' maturation of lymphocyte subsets this potential selection to a 'healthy' group may be advantageous.

In conclusion, our extensive study on more than 200 blood samples per age group provides reliable insight into maturation phases of the different subsets of lymphocytes from birth until the age of 24 months of age. The largest changes in the maturation phases of these lymphocytes seem to occur in the first 6 months after birth probably due to contact with antigens for the first time. These immunophenotyping data might be used as reference values. However, when interpreting the values of the different lymphocyte subsets other potential influencing factors like growth, perinatal stress or hypoxia and infectious diseases that induce changes in the maturation phases of these lymphocytes need to be kept in mind.

References

1. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, Hooijkaas H, van Dongen JJ 1997 Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 130:388-393.
2. de Vries E, de Groot R, de Bruin-Versteeg S, Comans-Bitter WM, van Dongen JJ 1999 Analysing the developing lymphocyte system of neonates and infants. Eur J Pediatr 158:611-617.
3. Hamann D, Roos MT, van Lier RA 1999 Faces and phases of human CD8 T-cell development. Immunol Today 20:177-180.

4. van Baarle D, Kostense S, van Oers MH, Hamann D, Miedema F 2002 Failing immune control as a result of impaired CD8+ T-cell maturation: CD27 might provide a clue. Trends Immunol 23:586-591.
5. Appay V, Dunbar PR, Callan M, Klenerman P, Gillespie GM, Papagno L, Ogg GS, King A, Lechner F, Spina CA, Little S, Havlir DV, Richman DD, Gruener N, Pape G, Waters A, Easterbrook P, Salio M, Cerundolo V, McMichael AJ, Rowland-Jones SL 2002 Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 8:379-385.
6. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ 2007 Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med 204:645-655.
7. Ghia P, ten Boekel E, Rolink AG, Melchers F 1998 B-cell development: a comparison between mouse and man. Immunol Today 19:480-485.
8. Thomas MD, Srivastava B, Allman D 2006 Regulation of peripheral B cell maturation. Cell Immunol 239:92-102.
9. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE 2005 Identification and characterization of circulating human transitional B cells. Blood 105:4390-4398.
10. Cuss AK, Avery DT, Cannons JL, Yu LJ, Nichols KE, Shaw PJ, Tangye SG 2006 Expansion of functionally immature transitional B cells is associated with human-immunodeficient states characterized by impaired humoral immunity. J Immunol 176:1506-1516.
11. Casali P, Burastero SE, Nakamura M, Inghirami G, Notkins AL 1987 Human lymphocytes making rheumatoid factor and antibody to ssDNA belong to Leu-1+ B-cell subset. Science 236:77-81.
12. Ellis TM, Fisher RI 1989 Functional heterogeneity of Leu 19"bright"+ and Leu 19"dim"+ lymphokine-activated killer cells. J Immunol 142:2949-2954.
13. Sedlmayr P, Schallhammer L, Hammer A, Wilders-Truschnig M, Wintersteiger R, Dohr G 1996 Differential phenotypic properties of human peripheral blood CD56dim+ and CD56bright+ natural killer cell subpopulations. Int Arch Allergy Immunol 110:308-313.
14. Jonges LE, Albertsson P, van Vlierberghe RL, Ensink NG, Johansson BR, van de Velde CJ, Fleuren GJ, Nannmark U, Kuppen PJ 2001 The phenotypic heterogeneity of human natural killer cells: presence of at least 48 different subsets in the peripheral blood. Scand J Immunol 53:103-110.
15. Lanier LL 2003 Natural killer cell receptor signaling. Curr Opin Immunol 15:308-314.
16. Robertson MJ, Cameron C, Lazo S, Cochran KJ, Voss SD, Ritz J 1996 Costimulation of human natural killer cell proliferation: role of accessory cytokines and cell contact-dependent signals. Nat Immun 15:213-226.
17. Freud AG, Becknell B, Roychowdhury S, Mao HC, Ferketich AK, Nuovo GJ, Hughes TL, Marburger TB, Sung J, Baiocchi RA, Guimond M, Caligiuri MA 2005 A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. Immunity 22:295-304.
18. Freud AG, Caligiuri MA 2006 Human natural killer cell development. Immunol Rev 214:56-72.
19. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, Verhulst FC, Witteman JC, Buller HA 2004 Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol 18:61-72.
20. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, Witteman JC, Hofman A 2006 The Generation R Study: Design and cohort profile. Eur J Epidemiol 21:475-484.
21. Brando B, Gohde W, Jr., Scarpati B, D'Avanzo G, European Working Group on Clinical Cell A 2001 The "vanishing counting bead" phenomenon: effect on absolute CD34+ cell counting in phosphate-buffered saline-diluted leukapheresis samples. Cytometry 43:154-160.
22. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdaal NA, van Dongen JJ 2004 Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. Cytometry B Clin Cytom 60:1-13.
23. Cushing AH, Samet JM, Lambert WE, Skipper BJ, Hunt WC, Young SA, McLaren LC 1998 Breastfeeding reduces risk of respiratory illness in infants. Am J Epidemiol 147:863-870.

24. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, Wara DW, Douglas SD, Luzuriaga K, McFarland EJ, Yogev R, Rathore MH, Levy W, Graham BL, Spector SA 2003 Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol 112:973-980.
25. World Health Organization 2003 Major causes of death among children under 5 years of age and neonates in the world, 2000-2003. The World Health Organization, http://www.who.int/child-adolescent-health/OVERVIEW/CHILD_HEALTH/map_00-03_world.jpg.
26. Chretien J, Holland W, Macklem P, Murray J, Woolcock A 1984 Acute respiratory infections in children. A global public-health problem. N Engl J Med 310:982-984.
27. Fecteau JF, Cote G, Neron S 2006 A new memory CD27-IgG+ B cell population in peripheral blood expressing VH genes with low frequency of somatic mutation. J Immunol 177:3728-3736.
28. Wirths S, Lanzavecchia A 2005 ABCB1 transporter discriminates human resting naive B cells from cycling transitional and memory B cells. Eur J Immunol 35:3433-3441.
29. Ma CS, Pittaluga S, Avery DT, Hare NJ, Maric I, Klion AD, Nichols KE, Tangye SG 2006 Selective generation of functional somatically mutated IgM+CD27+, but not Ig isotype-switched, memory B cells in X-linked lymphoproliferative disease. J Clin Invest 116:322-333.
30. Duijts L, Bakker-Jonges LE, Labout JA, Jaddoe VW, Hofman A, Steegers EA, Van Dongen JJ, Hooijkaas H, Moll HA 2008 Perinatal stress influences lymphocyte subset counts in neonates. The generation R study. Pediatr Res 63:292-298.

Fetal growth influences lymphocyte subset counts at birth

4.2

Abstract

Background: Preterm born and low birth weight infants are at risk for severe infections in infancy. It has been suggested that these infants have an immature immune system.
Objective: To assess the associations of gestational age, birth weight and fetal growth with absolute lymphocyte subset counts at birth.
Methods: This study was conducted in 571 infants participating in the Generation R Study, a population-based prospective cohort study from fetal life onwards. Gestational age and birth weight were obtained from midwives and hospital registries. Fetal growth was defined as increase in weight between late pregnancy and birth. Lymphocytes and T lymphocyte subset counts in cord blood were determined by 6-color flowcytometry. Mulivariate linear regression models with adjustment for gender, maternal education, smoking, alcohol use, fever and mode of delivery were applied.
Results: Per week increase of gestational age, T, B and NK lymphocyte counts increased with 3%, 5% and 6%, respectively (p-values<0.05). Helper, cytotoxic and naïve T lymphocyte counts increased with 3%, 4% and 5%, respectively (p-values<0.05), but memory T lymphocyte counts did not. Increased birth weight and fetal growth were significantly associated with higher B lymphocyte counts, independent of gestational age, but not with the other lymphocyte subset counts.
Conclusions: Lymphocyte subset counts increase with prolonged gestation, suggesting an ongoing development of the immune system. Birth weight and fetal growth seem to influence only B lymphocyte counts.

Introduction

Preterm born or low birth weight infants are at risk for severe infections in infancy (1, 2). It has been suggested that these infants have an immature immune system. Decreased percentages or absolute counts of lymphocytes are described in infected preterm infants (3).

Previous studies, mostly small sample sized, presented lower percentage and absolute numbers of T lymphocytes and T lymphocyte subsets at an earlier gestational age, yet results on NK and B lymphocyte counts are inconclusive (4-11). A small number of studies on birth weight and the development of the immune system have been performed and showed lower absolute lymphocyte subset counts in low birth weight infants (9, 12, 13). However, most of these studies did not take gestational age into account. Birth weight is only a proxy for fetal growth since several fetal growth and development patterns may lead to the same birth weight. Fetal growth restriction may lead to normal birth weight if the fetus is actually supposed to grow on the upper percentiles based on the genetic growth potential. Fetal growth restriction with impaired fetal thymus and liver growth has been suggested to influence the development of the immune system (14-16). Therefore, longitudinally measured fetal growth may be stronger related to lymphocyte counts than birth weight per se.

We examined in the Generation R Focus Study, a population-based prospective cohort study from fetal life onwards, the associations of gestational age, birth weight and longitudinally measured fetal growth with absolute numbers of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory) at birth.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood in Rotterdam, The Netherlands. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development and health and has been described previously in detail (17, 18). Additional, more detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study (17, 18). This subgroup is ethnic homogeneous to exclude possible confounding or effect modification by ethnicity. Of all approached pregnant women and their partners, 79% participated in the Generation R Focus Study. Their children were born between February 2003 and August 2005. The

Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Gestational age, birth weight and fetal growth

Information about date of birth and birth weight was obtained from standardized midwife and hospital registries. Gestational age at birth was based on pregnancy dating by ultrasound in early pregnancy (19). Estimated fetal weight was determined in late pregnancy (>25 weeks) using the formula of Hadlock with the parameters abdominal circumference, head circumference and femur length measured by ultrasound (20). Fetal weight measurements were converted into gestational age adjusted standard deviation scores (SDS) (21). Fetal growth was defined as fetal weight gain (SDS) between late pregnancy and birth. For the latter analysis, gestational age adjusted standard deviation scores (SDS) were also constructed for birth weight measurements.

Immunophenotyping of lymphocyte subsets

Heparinized sampling of venous cord blood was carried out by midwifes and obstetricians immediately after delivery and transported to the Immunology laboratory of the Erasmus Medical Center within 24 hours. Umbilical cord blood samples not received within 24 hours (weekend days) were excluded since flow cytometric analyses of those samples showed no reliable results in the pilot phase of the study. Flow cytometric immunophenotyping was performed to determine absolute numbers of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory T). For this, the monoclonal antibodies CD3, CD19, CD16.CD56, CD4, CD8, CD45RA and CD45RO were conjugated with the labels fluorescein isothiocyanate (FITC[1]), peridin chlorophyll protein (PerCP[1]), peridin chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5[1]), allophycocyanin (APC[1]), phycoerythrin (PE[1,3]), phycoerythrin-cyanin dye (PE-cy7[1]), allophycocyanin-cyanin dye (APC-cy7[1]) and rhodamine (RD1[2]) ([1]Becton Dickinson,[2]Beckman Coulter and [3]Dako). Absolute numbers of T, B and NK lymphocytes were determined with the lysed whole blood technique (22). Subsets of the T lymphocytes were determined by 6-color flow cytometry. Therefore, erythrocytes of 1 ml whole blood were lysed using 50 ml ammoniumchloride. After centrifugation, leucocytes were washed twice and suspended in 900 µl PBS/1% BSA/0.1% NaAz. Of this cell suspension (5-10*10^6/ml), 50 µl was incubated for 10 minutes at room temperature with combinations of the optimally titrated labeled monoclonal antibodies. After incubation the cells were washed and subsequently identified by flow cytometry. Using a BD™ LSR II flow cytometer (Becton Dickinson, San Jose, California, US) that had been calibrated with rainbow beads, 10,000 lymphocytes were measured. The relative count of helper T lymphocytes, cytotoxic T lymphocytes, naïve T lymphocytes and memory T lymphocytes was expressed as the percentage within the total T lymphocyte population and calculated by the average of 2-4 independent

incubations for each subset. The absolute counts of the T lymphocyte subsets were subsequently calculated from the absolute T lymphocyte counts as obtained by the lysed whole blood technique.

Covariates

Information about maternal education, smoking and alcohol use was obtained by postal questionnaires in early, mid- and late pregnancy. Educational level was defined as highest followed education (lower or higher education) according to the classification of Statistics Netherlands (23). Maternal smoking and alcohol use during pregnancy were categorized into 'not during pregnancy' and ' during pregnancy'. Information about maternal fever (>38^0 C), indicating a possible underlying infection, was recorded in late pregnancy. Mode of delivery was registered by midwives and obstetricians.

Statistical analysis

Differences of maternal and infant characteristics between infants with and without umbilical cord blood samples were assessed by the independent sample t-test for continuous normal distributed variables, non-parametric Mann-Whitney test for continuous non-normal distributed variables and the chi-square test for categorical variables.

Data on all lymphocyte subsets were log-transformed to obtain normal distributed variables. Associations of continuously measured gestational age, birth weight and fetal growth in late pregnancy with absolute numbers of lymphocyte and T lymphocyte subsets were analyzed using linear regression models. Additionally, these models were adjusted for the potential confounders including gender, maternal education, smoking, alcohol use, fever and mode of delivery. Measures of associations are presented as log-transformed regression coefficients, interpreted as percentages after multiplying by 100 (24), or as back-transformed regression coefficients, with their 95% confidence interval (CI).

The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Cohort

In total, 1,232 women were enrolled in the Generation R Focus Study. Mothers with weekend deliveries (n=202), twin pregnancies (n=24) and pregnancies leading to perinatal death (n=2) were excluded from the present analysis. Of the remaining 1,004 singleton live births, cord blood was collected in 889 (88.5%) infants. Immunophenotyping of lymphocytes in cord blood was not possible in 318 infants, mainly due to non-heparinized

cord blood samples. Of the remaining 571 infants, 10.0% (n=57) were siblings since mothers were allowed to participate with second or more pregnancies in the defined

Table 1. Maternal and infant characteristics of the study population (n=571).

	Infants	
	Boys (n=301)	**Girls (n=270)**
Mother		
Maternal age (years)	31.5 (4.1)	31.9 (4.0)
Education (%)		
Lower	39.6	39.0
Higher	60.4	61.0
Smoking during pregnancy (%)		
No	87.4	88.5
Yes	12.6	11.5
Alcohol use during pregnancy (%)		
No	29.2	39.6
Yes	70.8	60.4
Fever (%)	7.2	4.1
Mode of delivery (%)		
Vaginal	72.1	73.3
Forceps or vacuum assisted	17.3	12.8
Caesarean section	10.6	14.0
Infant		
Gestational age (weeks)[#]	40.4 (34.6-43.3)	40.4 (34.1-43.0)
Birth weight (grams)	3613 (494)*	3507 (472)*
Late pregnancy estimated fetal weight (grams)	1638 (271)	1613 (263)
Lymphocyte subsets ($x10^9/L$)[#]		
Total lymphocytes	4.40 (1.15-10.91)	4.54 (0.96-12.40)
$CD3^+$ T lymphocytes	2.80 (0.86-7.15)	2.80 (0.69-5.79)
$CD19^+$ B lymphocytes	0.68 (0.01-2.40)	0.72 (0.0.7-2.85)
$CD16^-/CD56^+$ NK lymphocytes	0.85 (0.03-2.53)	0.89 (0.10- 2.50)
$CD3^+/CD4^+$ helper T lymphocytes	1.92 (1.03-3.38)	2.05 (1.01-3.47)
$CD3^+/CD8^+$ cytotoxic T lymphocytes	0.76 (0.33-1.45)*	0.65 (0.29-1.22)*
$CD3^+/CD45RA^+$ naïve T lymphocytes	2.21 (0.93-1.98)	2.19 (0.99-4.07)
$CD3^+/CD45RO^+$ memory T lymphocytes	0.18 (0-1.12)	0.21 (0-0.95)

Values are means (standard deviation) or percentages. [#]Median (range). Data were missing on education (n=35), maternal fever (n=44), mode of delivery (n=30), estimated fetal weight (n=10), total lymphocytes (n=2), T lymphocytes (n=1), B lymphocytes (n=2), $CD4^+$ helper T lymphocytes (n=3), $CD8^+$ cytotoxic T lymphocytes (n=3), $CD45RA^+$ naïve T lymphocytes (n=3) and $CD45RO^+$ memory T lymphocytes (n=5).
Differences were tested using the independent sample t-test, non-parametric Mann-Whitney test or Chi-square test. *P-value < 0.01.

study period of the Generation R Focus study. These infants were included in the present study since there were no differences in results after excluding them from the analyses.

Analyses of missing cord blood samples (n=433) showed that infants without cord blood samples had a shorter median gestational age (40.1 and 40.4 weeks, p-value<0.01), a lower mean birth weight (3454 and 3562 grams, p-value<0.01) and more often were born by caesarean delivery (20.3% and 12.2%, p-value < 0.05) than infants with cord blood samples.

Subject characteristics

Characteristics of the mothers and their infants are presented in Table 1. Of the study group, 52.7% (n=301) were boys. The age of mothers at enrolment ranged from 18.5 to 42.9 years (mean 31.7 years). In general, 60.6% of the mothers had a higher education, 12.1% smoked and 65.8% consumed alcohol throughout pregnancy. Of the mothers, 72.6% had a vaginal, 15.2% a vacuum or forceps assisted and 12.2% a cesarean section delivery. Median gestational age of the infants was 40.4 weeks (range 34.1 to 43.4 weeks) and was similar in boys and girls. Mean birth weight of the infants was 3563 grams (standard deviation 487 grams) and was lower in girls than boys. The medians of the lymphocyte subset counts for boys and girls are demonstrated in Table 1.

Gestational age, birth weight, fetal growth and lymphocyte subsets

Table 2 presents the unadjusted associations of gestational age, birth weight and fetal growth with absolute numbers of lymphocyte subsets at birth. Per week longer gestation, infants had a 3% increase of T lymphocyte counts (p=0.002), 5% increase of B lymphocyte counts (p=0.003) and 6% increase of NK lymphocyte counts (p=0.005). Of the T lymphocyte subsets, per week longer gestation an increase of 3%, 4% and 5% was observed

Table 2. Associations of gestational age, birth weight and fetal growth with absolute numbers of lymphocyte subsets at birth.

	Lymphocyte subsets			
	T	**B**	**NK**	
Gestational age (weeks)	3 (1, 6)**	5 (2, 9)**	6 (1, 10)*	
Birth weight (kilograms)	5 (-2, 12)	13 (2, 25)*	-5 (-19, 9)	
Fetal growth (sds)	1 (-20, 4)	6 (1, 11)*	-3 (-9, 3)	
	T lymphocyte subsets			
	Helper	**Cytotoxic**	**Naïve**	**Memory**
Gestational age (weeks)	3 (1, 5)**	4 (1, 7)**	5 (2, 8)**	-2 (-9, 4)
Birth weight (kilograms)	4 (-4, 11)	8 (-2, 17)	2 (-7, 11)	14 (-8, 35)
Fetal growth (sds)	1 (-2, 4)	2 (-3, 6)	1 (-3, 5)	3 (-6, 13)

Values are log-transformed regression coefficients (95% confidence interval) and reflect the increase of lymphocyte subsets (%) per week increase in gestational age, per kilogram increase in birth weight and per SDS increase in fetal growth between late pregnancy and birth. *P-value < 0.05, **p-value 0.01.

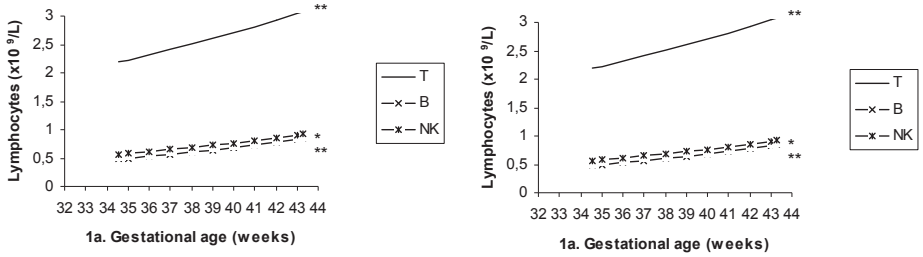


Figure 1. Absolute numbers of lymphocyte subsets in infants of different gestational ages.
The estimated back-transformed regression lines reflect the number of lymphocyte subsets per week increase of gestational age. All models were adjusted for gender, maternal education, smoking, alcohol use, fever and mode of delivery. *P-value < 0.05, **p-value 0.01.

for helper, cytotoxic and naïve T lymphocyte counts, respectively (all p-values<0.01). Gestational age was not associated with memory T lymphocyte counts (p=0.49). Per kilogram increase in birth weight, adjusted for gestational age, infants had 13% higher B lymphocyte counts (p=0.01). An increase of 6% of B lymphocyte counts was found per SDS increase in fetal growth between late pregnancy and birth (p=0.02). Total T and NK lymphocyte counts and T lymphocyte subset counts were not significantly associated with birth weight or fetal growth.

After adjusting for gender, maternal education, smoking, alcohol use, fever and mode of delivery the effect estimates of gestational age, birth weight and fetal growth on absolute T, B, NK and T lymphocyte subset counts did not materially change. The adjusted associations are graphically presented in Figures 1, 2 and 3.

Discussion

This prospective cohort study showed that with increasing gestational age at birth absolute numbers of T, helper, cytotoxic and naïve T lymphocytes, B and NK lymphocytes increase. Increased birth weight and fetal growth in late pregnancy were associated with higher absolute numbers of B lymphocytes, but not with T, NK and T lymphocyte subset counts.

To our knowledge, no large population-based prospective cohort studies have assessed the effects of gestational age, birth weight and fetal growth in late pregnancy on different lymphocyte subsets at birth. Previous studies of smaller sample sizes (50-120 subjects), showed that preterm birth infants had lower mean T lymphocyte counts and lower mean helper, cytotoxic and naïve T lymphocyte counts compared to infants born at term (5-7, 25, 26). Furthermore, it was shown that T lymphocyte subsets in fetal blood, obtained with cordocentesis during pregnancy, were lower in preterm than in term born

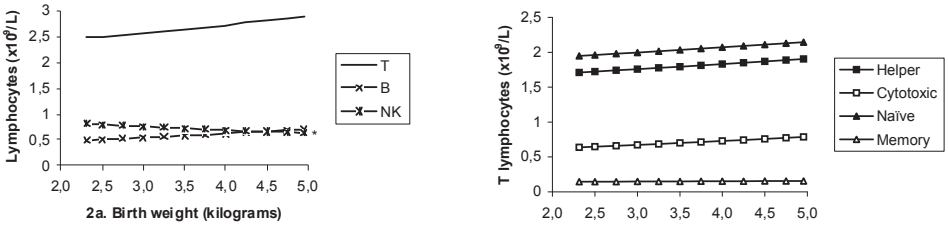


Figure 2. Absolute numbers of lymphocyte subsets in infants with different birth weights.
The estimated back-transformed regression lines reflect the number of lymphocyte subsets per kilogram increase in birth weight. All models were adjusted for gender, maternal education, smoking, alcohol use, fever and mode of delivery. *P-value < 0.05, **p-value 0.01.

infants (25, 27, 28). Our results, showing an influence of gestational age at birth on T lymphocyte subset counts, are in line with these previous studies and suggest an ongoing development of the immune system during pregnancy. For the association of gestational age with B and NK lymphocyte counts, previous studies showed inconsistent results (5-7, 9, 10, 25, 26). One large study of cord blood samples (more than 8000 cord blood units), collected at birth and banked for future umbilical cord blood transplantation, showed small positive partial correlations of gestational age at birth with NK, helper T and cytotoxic T lymphocyte counts. The exact amount of change in lymphocyte subset counts was not presented (9). Percentages or absolute counts of lymphocytes seem decreased in infected preterm infants or immunocompromised children with severe respiratory syncytial virus infections (3, 29). However, neonatal lymphocytes may have a near normal capacity to proliferate when needed (28). The precise functional role and consequences for morbidity of low lymphocyte counts in preterm infants remains to be studied.

Previous studies found that infants with a lower birth weight had lower or similar lymphocyte subset counts compared to term infants (9, 12, 13, 30). These studies did not examine birth weight independent of gestational age. One study assessed the effect of birth weight on numbers of different lymphocytes taking gestational age into account, and found that small for gestational age infants (SGA) had lower T and B lymphocyte counts and lower helper and cytotoxic T lymphocyte counts than appropriate for gestational age infants or preterm SGA infants (31). Studies assessing the association of fetal growth restriction and lymphocytes found a reduction of T lymphocyte counts, helper and cytotoxic T lymphocyte counts, neutrophils and monocytes in fetal growth restricted infants (16, 32). An explanation for the different results with our study could be that studies of low birth weight and fetal growth were performed in small sample sizes (19-104 subjects) with an other definition of fetal growth restriction (fetal abdominal circumference < 5th percentile for gestation and abnormal Doppler ultrasound measurements of the uterine or umbilical arteries) and without adjustment for potential confounders.

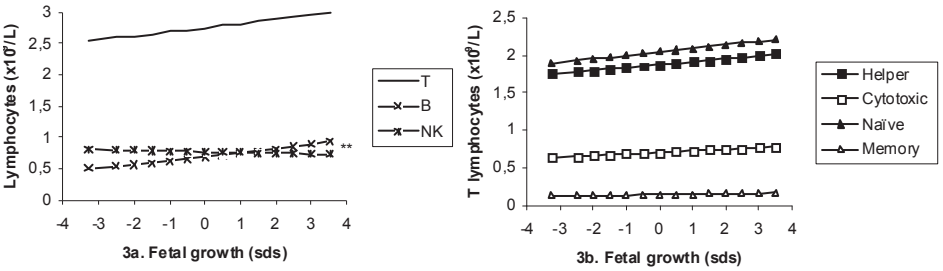


Figure 3. Absolute numbers of lymphocyte subsets in infants with different fetal growths.
The estimated back-transformed regression lines reflect the number of lymphocyte subsets per standard deviation increase in fetal growth. All models were adjusted for gender, maternal education, smoking, alcohol use, fever and mode of delivery. *P-value < 0.05, **p-value 0.01.

We found an association of reduced birth weight and fetal growth with lower B lymphocyte counts. A biological mechanism might be that low birth weight and fetal growth restricted infants have a reduced abdominal circumference, the so called 'brain sparing effect' (33). The reduced abdominal circumference can partly be explained by a reduced fetal liver size that may be caused by fetal malnutrition. Since B lymphocytes in fetal life are primarily produced by the fetal liver, a reduced fetal liver size might consequently lead to a decrease in B lymphocyte production. In line with this, we observed a 2% decrease of immature B lymphocytes (CD5+) per SDS decrease of fetal growth (p-value < 0.01). Similarly, an 8% decrease of immature B lymphocytes per kilogram decrease of birth weight was found (p-value < 0.01). However, the exact underlying pathophysiological mechanism of birth weight and fetal growth with different maturational stadia of B lymphocytes needs to be studied in more detail. Our study found no effect of birth weight or fetal growth on T lymphocyte counts. During pregnancy, T lymphocytes are mainly produced by the fetal thymus. Lower birth weight is associated with a reduced thymus size at birth, however, size of the fetal thymus relevant to its T lymphocyte production and function needs to be further explored (34, 35).

Some methodological issues should be considered. In our study, excluding participants with missing data would lead to selection biased results if the associations of gestational age, birth weight and fetal growth with lymphocyte subsets differ between participants with and without data. Of all single live births, information about gestational age and birth weight were not missing. Information about fetal growth was only missing in 1.5% of the infants. Participating infants without cord blood samples more often had a lower gestational age and lower birth weight, but not a lower estimated fetal weight in late pregnancy, than participating infants with cord blood samples. Therefore it is likely that due to these selective missing of cord blood samples the observed effects of gestational age and birth weight with lymphocyte subsets may be underestimated.

In summary, this population-based prospective cohort study implies that with increasing gestational age development of the various lymphocyte subsets progresses. Birth weight and fetal growth seem to only influence B lymphocyte counts. These results contribute to a better understanding of the early development of the immune system and to conditions that might affect this process. Long-term effects of gestational age, birth weight and fetal growth on lymphocyte subsets and their consequences remain to be studied.

References

1. Garra G, Cunningham SJ, Crain EF. Reappraisal of criteria used to predict serious bacterial illness in febrile infants less than 8 weeks of age. Acad Emerg Med. 2005 Oct;12(10):921-5.
2. Jaskiewicz JA, McCarthy CA, Richardson AC, White KC, Fisher DJ, Dagan R, et al. Febrile infants at low risk for serious bacterial infection--an appraisal of the Rochester criteria and implications for management. Febrile Infant Collaborative Study Group. Pediatrics. 1994 Sep;94(3):390-6.
3. Juretic E, Juretic A, Uzarevic B, Petrovecki M. Alterations in lymphocyte phenotype of infected preterm newborns. Biol Neonate. 2001;80(3):223-7.
4. Thilaganathan B, Nicolaides KH, Mansur CA, Levinsky RJ, Morgan G. Fetal B lymphocyte subpopulations in normal pregnancies. Fetal Diagn Ther. 1993 Jan-Feb;8(1):15-21.
5. Kotiranta-Ainamo A, Apajasalo M, Pohjavuori M, Rautonen N, Rautonen J. Mononuclear cell subpopulations in preterm and full-term neonates: independent effects of gestational age, neonatal infection, maternal pre-eclampsia, maternal betamethason therapy, and mode of delivery. Clin Exp Immunol. 1999 Feb;115(2):309-14.
6. Juretic E, Uzarevic B, Petrovecki M, Juretic A. Two-color flow cytometric analysis of preterm and term newborn lymphocytes. Immunobiology. 2000 Nov;202(5):421-8.
7. Peakman M, Buggins AG, Nicolaides KH, Layton DM, Vergani D. Analysis of lymphocyte phenotypes in cord blood from early gestation fetuses. Clin Exp Immunol. 1992 Nov;90(2):345-50.
8. Berry SM, Fine N, Bichalski JA, Cotton DB, Dombrowski MP, Kaplan J. Circulating lymphocyte subsets in second- and third-trimester fetuses: comparison with newborns and adults. Am J Obstet Gynecol. 1992 Oct;167(4 Pt 1):895-900.
9. Cairo MS, Wagner EL, Fraser J, Cohen G, van de Ven C, Carter SL, et al. Characterization of banked umbilical cord blood hematopoietic progenitor cells and lymphocyte subsets and correlation with ethnicity, birth weight, sex, and type of delivery: a Cord Blood Transplantation (COBLT) Study report. Transfusion. 2005 Jun;45(6):856-66.
10. Perez A, Gurbindo MD, Resino S, Aguaron A, Munoz-Fernandez MA. NK Cell Increase in Neonates from the Preterm to the Full-Term Period of Gestation. Neonatology. 2007 Apr 10;92(3):158-63.
11. Thilaganathan B, Mansur CA, Morgan G, Nicolaides KH. Fetal T-lymphocyte subpopulations in normal pregnancies. Fetal Diagn Ther. 1992;7(2):53-61.
12. Herrod HG, Cooke RJ, Valenski WR, Herman J, Dockter ME. Evaluation of lymphocyte phenotype and phytohemagglutinin response in healthy very low birth weight infants. Clin Immunol Immunopathol. 1991 Aug;60(2):268-77.
13. McDonald T, Sneed J, Valenski WR, Dockter M, Cooke R, Herrod HG. Natural killer cell activity in very low birth weight infants. Pediatr Res. 1992 Apr;31(4 Pt 1):376-80.
14. Chandra RK. Interactions between early nutrition and the immune system. Ciba Found Symp. 1991;156:77-89; discussion -92.

15. Moore SE. Nutrition, immunity and the fetal and infant origins of disease hypothesis in developing countries. Proc Nutr Soc. 1998 May;57(2):241-7.
16. Thilaganathan B, Plachouras N, Makrydimas G, Nicolaides KH. Fetal immunodeficiency: a consequence of placental insufficiency. Br J Obstet Gynaecol. 1993 Nov;100(11):1000-4.
17. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol. 2004 Jan;18(1):61-72.
18. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21(6):475-84.
19. Jaddoe VW, Verburg BO, de Ridder MA, Hofman A, Mackenbach JP, Moll HA, et al. Maternal smoking and fetal growth characteristics in different periods of pregnancy: the generation R study. Am J Epidemiol. 2007 May 15;165(10):1207-15.
20. Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of fetal weight with the use of head, body, and femur measurements--a prospective study. Am J Obstet Gynecol. 1985 Feb 1;151(3):333-7.
21. Niklasson A EA, Fryer JG, Karlberg J, Lawrence C, Karlberg P. An update of the Swedish reference standards for weight, length and head circumference at birth for given gestational age (1977-1981). Acta Paediatr Scand. 1991;80(8-9):756-62.
22. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Boerma GJ, Lotgering FK, et al. Correction for erythroid cell contamination in microassay for immunophenotyping of neonatal lymphocytes. Arch Dis Child Fetal Neonatal Ed. 1999 May;80(3):F226-9.
23. Standaard onderwijsindeling 2003. Voorburg/Heerlen: Statistics Netherlands; 2004.
24. Cole TJ. Sympercents: symmetric percentage differences on the 100 log(e) scale simplify the presentation of log transformed data. Stat Med. 2000 Nov 30;19(22):3109-25.
25. Schultz C, Reiss I, Bucsky P, Gopel W, Gembruch U, Ziesenitz S, et al. Maturational changes of lymphocyte surface antigens in human blood: comparison between fetuses, neonates and adults. Biol Neonate. 2000;78(2):77-82.
26. Berrington JE, Barge D, Fenton AC, Cant AJ, Spickett GP. Lymphocyte subsets in term and significantly preterm UK infants in the first year of life analysed by single platform flow cytometry. Clin Exp Immunol. 2005 May;140(2):289-92.
27. Strunk T, Temming P, Gembruch U, Reiss I, Bucsky P, Schultz C. Differential maturation of the innate immune response in human fetuses. Pediatr Res. 2004 Aug;56(2):219-26.
28. Zhao Y, Dai ZP, Lv P, Gao XM. Phenotypic and functional analysis of human T lymphocytes in early second- and third-trimester fetuses. Clin Exp Immunol. 2002 Aug;129(2):302-8.
29. El Saleeby CM, Somes GW, DeVincenzo JP, Gaur AH. Risk factors for severe respiratory syncytial virus disease in children with cancer: the importance of lymphopenia and young age. Pediatrics. 2008 Feb;121(2):235-43.
30. Baker DA, Hameed C, Tejani N, Thomas J, Dattwyler R. Lymphocyte subsets in the neonates of preeclamptic mothers. Am J Reprod Immunol Microbiol. 1987 Aug;14(4):107-9.
31. Thomas RM, Linch DC. Identification of lymphocyte subsets in the newborn using a variety of monoclonal antibodies. Arch Dis Child. 1983 Jan;58(1):34-8.
32. Davies N, Snijders R, Nicolaides KH. Intra-uterine starvation and fetal leucocyte count. Fetal Diagn Ther. 1991;6(3-4):107-12.
33. Crane JP, Kopta MM. Comparative newborn anthropometric data in symmetric versus asymmetric intrauterine growth retardation. Am J Obstet Gynecol. 1980 Nov 1;138(5):518-22.
34. Collinson AC, Moore SE, Cole TJ, Prentice AM. Birth season and environmental influences on patterns of thymic growth in rural Gambian infants. Acta Paediatr. 2003 Sep;92(9):1014-20.
35. Iscan A, Tarhan S, Guven H, Bilgi Y, Yuncu M. Sonographic measurement of the thymus in newborns: close association between thymus size and birth weight. Eur J Pediatr. 2000 Mar;159(3):223-4.

Perinatal stress influences lymphocyte subset counts in neonates

4.3

Abstract

In the general population, it is unknown whether stress-related perinatal factors influence lymphocyte subset counts in neonates. The aim of this study was to assess the associations of perinatal factors related to stress and hypoxia (mode of delivery, Apgar scores and umbilical cord blood pH) with absolute lymphocyte subset counts (T, B, NK, helper T, cytotoxic T, naïve, memory T) in cord blood of 571 neonates. This study was embedded in a population-based prospective cohort study from fetal life onwards. All models were adjusted for gestational age, birth weight, gender, maternal fever and each of the other perinatal stress-relating factors. Our results showed that increasing stress-related mode of delivery was positively associated with NK and memory T lymphocyte subset counts (all p-values < 0.01). Effects of Apgar scores on lymphocyte subsets were explained by umbilical cord blood pH. Lower umbilical cord blood pH was associated with higher B, NK and memory T lymphocyte counts (all p-values < 0.05). Effects of mode of delivery and umbilical cord blood pH on other lymphocyte subsets were not observed. We conclude that in the general population, lymphocyte subset counts in neonates increase with increasing stress- and hypoxia related perinatal factors.

Introduction

The immunologic status of the neonate is frequently established by assessing absolute numbers of various lymphocyte subsets in cord blood (1-4). However, such data at birth must be interpreted with care since the lymphocyte subset counts might be influenced by growth, stress and hypoxia related events that occur in late prenatal and early postnatal life. It has been suggested that the distribution of lymphocyte subsets is related to gestational age at birth and birth weight (5-8). It is not well known whether other perinatal factors associated with fetal well-being, stress and hypoxia, including mode of delivery, Apgar scores or umbilical cord blood pH, influence the various lymphocyte subset counts at birth.

So far, studies on mode of delivery and lymphocyte subsets remain inconclusive. This might be due to small study populations, the variability of lymphocyte subsets studied or not taking other possible influencing factors into account (5, 6, 9-16). Results of the majority of these studies suggested a tendency towards increased lymphocyte subset counts in neonates born by vaginal delivery compared to neonates born by caesarean section (9-11, 13-16). The endocrine-metabolic variations during a stressful delivery, particularly hypoxia and the increase of catecholamines and cortisol, were considered as the main cause of these effects (17-19). Additionally to this stress concept, adverse Apgar scores and umbilical cord blood pH, both results of perinatal hypoxia, are thought to influence lymphocyte subset counts at birth (9, 20-22).

We examined in 571 neonates participating in a population-based prospective cohort study, the associations of perinatal factors related to stress and hypoxia, including mode of delivery, Apgar scores and umbilical cord blood pH with absolute numbers of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory T) in cord blood of neonates.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development and health and has been described previously in detail (23, 24). More detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study (23, 24). Of all approached pregnant women and their partners, 79% participated in the Generation R Focus Study. Their children were born between February 2003 and August

2005. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Mode of delivery, Apgar scores and umbilical cord blood pH

Information about mode of delivery, 1- and 5- minute Apgar scores (assigning a score of 0 to 2 to heart rate, respiratory effort, muscle tone, reflex irritability and color) and umbilical cord blood pH were obtained from standardized delivery registrations of midwives and obstetricians (23-26). Heparinized cord blood samples were taken from an immediate clamped, isolated piece of the umbilical cord. Cord blood gases were analyzed on routine automatic gas check machines for pH, P02 and PC02, whereas base excess was computed. The delay between cord blood sampling and determination was less than 60 minutes. The blood gas analyzers were calibrated and standardized with reference gases and pH solutions.

Immunophenotyping of lymphocyte subsets

Venous cord blood was sampled in heparinized tubes by midwifes and obstetricians immediately after delivery and transported at room temperature to the Immunology laboratory of the Erasmus Medical Center within 24 hours. Umbilical cord blood samples not received within 24 hours (weekend days) were excluded since flow cytometric analyses of those samples showed no reliable results in the pilot phase of the study. Flow-cytometric immunophenotyping was performed to determine absolute numbers of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory T). For this, the monoclonal antibodies CD3, CD19, CD16.CD56, CD4, CD8, CD45RA and CD45RO were conjugated with the labels fluorescein isothiocyanate (FITC[1]), peridin chlorophyll protein (PerCP[1]), peridin chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5[1]), allophycocyanin (APC[1]), phycoerythrin (PE[1,3]), phycoerythrin-cyanin dye (PE-cy7[1]), allophycocyanin-cyanin dye (APC-cy7[1]) and rhodamine (RD1[2]) ([1]Becton Dickinson,[2]Beckman Coulter and [3]Dako)

Table 1. Flow cytometric reagents.

Lymphocyte subsets	Cluster of differentiation (CD)	Monoclonal antibodies labels
T lymphocyte	CD3	FITC (Sk7[1]) / PerCP (Sk7[1])
Helper T lymphocyte	CD4	PE-Cy7 (Sk3[1])
Cytotoxic T lymphocyte	CD8	APC (Sk11) / APC-Cy7 (Sk1[1])
Naïve T lymphocyte	CD45RA	RD1 (2H4[2])
Memory T lymphocyte	CD45RO	APC (UCHL-1[1])
B lymphocyte	CD19	PerCPCy5.5 (SJ25C1[1]) / APC (SJ25C1[1])
NK lymphocyte	CD16.CD56	PE (B73.1[1]) . PE (C5.9[3])

FITC=fluorescein isothiocyanate, PerCP=peridin chlorophyll protein, PE-cy7=phycoerythrin-cyanin dye, APC=allophycocyanin, APC-cy7=allophycocyanin-cyanin dye, RD1= rhodamine and PE=phycoerythrin. Company: Becton Dickinson[1], Beckman Coulter[2] and Dako[3]

(Table 1). Absolute numbers of T, B and NK lymphocytes were determined with the lysed whole blood technique (50 µl of whole blood per sample) using the routine standardized single platform method with BD TruCOUNT Tubes of Becton Dickinson (BD). With this, lymphocytes were gated on the basis of CD45 and FSC to prevent contamination of unlysed erythrocytes (27, 28, 29). The samples were measured on a Beckman Coulter flow cytometer (BD) and analyzed with BD CellQuest™ software as indicated by the manufacturer. Subsets of the T lymphocytes were determined using 6-colour staining and detected on a BD™ LSR II flow cytometer (Becton Dickinson, San Jose, California, US). This flow cytometer is routinely used in the diagnostic laboratory and every day reference calibration beads are used. The subsets of T lymphocytes were analyzed with BD FacsDIVA™. Therefore, erythrocytes of 1 ml whole blood were lysed using 50 ml ammoniumchloride. After centrifugation, leucocytes were washed twice and suspended in 900 µl PBS/1% BSA/0.1% NaAz. Of this cell suspension (5-10*10^6/ml), 50 µl was incubated for 10 minutes at room temperature with combinations of the optimally titrated labeled monoclonal antibodies. After incubation the cells were washed and subsequently identified by flow cytometry. Using a BD™ LSR II flow cytometer that had been calibrated with rainbow beads, 10,000 lymphocytes were measured. The relative count of helper T lymphocytes, cytotoxic T lymphocytes, naïve T lymphocytes and memory T lymphocytes was expressed as the percentage within the total T lymphocyte population and calculated by the average of 2-4 independent incubations for each subset. The absolute counts of the T lymphocyte subsets were subsequently calculated from the absolute T lymphocyte counts as obtained by the lysed whole blood technique. The absolute counts of the total T lymphocytes determined with the single platform method were compared with the recalculated counts of the total T lymphocytes (% of lymphocytes within the leukocyte count, determined by a cell counter). There was no significant difference between the two methods (30).

Covariates

Information about date of birth, birth weight and gender was obtained from midwives and hospital registries. Gestational age was established by the first fetal ultrasound examination after enrolment (31). Information about maternal fever (> 38^0 C), indicating a possible systemic underlying infection, was asked for in the questionnaire send to mothers > 30 weeks of gestation ('did you had a fever (>38^0 C) in the last two months?') (24).

Statistical analysis

Differences of maternal and neonatal characteristics between neonates with and without umbilical cord blood samples were assessed by the independent sample t-test for continuous normal distributed variables, non-parametric Mann-Whitney test for continuous

non-normal distributed variables and the chi-square test for categorical variables. Data on all lymphocyte subsets were log-transformed to obtain normally distributed variables. Associations of mode of delivery, continuously measured Apgar scores and umbilical cord blood pH with absolute numbers of lymphocyte subsets were analyzed using multiple linear regression models. Additionally, regression models were adjusted for the potential confounders gestational age, birth weight, gender and maternal fever. Finally, regression models were additionally adjusted for each of the stress-related perinatal factors to determine the independent effect of mode of delivery, Apgar scores and umbilical cord blood pH. Measures of associations are presented as log-transformed mean differences and regression coefficients, which can be interpreted as percentages after multiplying by 100 (32), or as geometric mean differences with their 95% confidence interval (CI). The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

In total, 1,232 women were enrolled in the Generation R Focus Study. Mothers with weekend deliveries (n=202), twin pregnancies (n=24) and pregnancies leading to perinatal death (n=2) were excluded from the present analysis. Of the remaining 1,004 singleton live births, cord blood was collected in 889 (88.5%) infants. Immunophenotyping of lymphocytes in cord blood was not possible in 318 infants, mainly due to non-heparinized cord blood samples. Of the remaining 571 infants, 10.0% (n=57) were siblings since mothers were allowed to participate with second or more pregnancies in the Generation R Focus study. These infants were included in the present study since there were no differences in results after excluding them from the analyses.

Analyses of missing immunophenotyped cord blood samples (n=433) showed that neonates without immunophenotyped cord blood samples more often were born by caesarean delivery (20.3% and 12.2%, p-value < 0.05), had a lower median gestational age (40.1 and 40.4 weeks, p-value < 0.01) and lower mean birth weight (3454 and 3562 grams, p-value < 0.01) than neonates with immunophenotyped cord blood samples.

Characteristics of the mothers and their neonates are presented in Table 2. Of the mothers, 72.6% had a vaginal, 15.2% a vacuum or forceps assisted and 12.2% a cesarean section delivery. Median Apgar scores of 1 and 5 minutes after birth of the neonates were 9 (range 2 to 10) and 10 (range 5 to 10), respectively. Mean umbilical cord blood pH was 7.26. The medians of the lymphocyte subset counts are demonstrated in Table 2.

Neonates born by a forceps or vacuum assisted delivery had 45% higher NK lymphocyte counts (p < 0.001) than neonates born by a vaginal delivery (Table 3). No differences were found for T and B lymphocyte counts. Within the T lymphocyte population, cyto-

Table 2. Maternal and neonatal characteristics of the study population (n=571).

	Single live births
Mothers	
Age (years)	31.7 (4.0)
Fever (%)	5.9 (31)
Mode of delivery (%)	
Vaginal	72.6 (393)
Forceps or vacuum assisted	15.2 (82)
Caesarean section	12.2 (66)
Neonates	
Boy (%)	52.7 (301)
Girl (%)	47.3 (270)
Gestational age (weeks)*	40.4 (34.1-43.4)
Birth weight (grams)	3563 (487)
Apgar scores*	
1-minute	9 (2-10)
5-minute	10 (5-10)
$pH_{cord\ blood}$	7.26 (0.09)
Lymphocytes ($x10^9$/L)*	
Total lymphocytes	4.49 (0.96-12.40)
$CD3^+$ T lymphocytes	2.80 (0.69-7.15)
$CD19^+$ B lymphocytes	0.70 (0-3.91)
CD16-/$CD56^+$ NK lymphocytes	0.87 (0.03-3.36)
$CD3^+/CD4^+$ helper T lymphocytes	1.97 (0.41-6.10)
$CD3^+/CD8^+$ cytotoxic T lymphocytes	0.70 (0.10-2.16)
$CD3^+/CD45RA^+$ naïve T lymphocytes	2.21 (0.03-6.01)
$CD3^+/CD45RO^+$ memory T lymphocytes	0.20 (0-2.36)

Values are means (standard deviation) or percentages (absolute numbers). *Median (range).
Data were missing on maternal fever (n=44), mode of delivery (n=30), 1-minute Apgar score (n=15), 5-minute Apgar score (n=11), $pH_{cord\ blood}$ (n=290), total lymphocytes (n=2), T lymphocytes (n=1), B lymphocytes (n=2), $CD4^+$ helper T lymphocytes (n=3), $CD8^+$ cytotoxic T lymphocytes (n=3), $CD45RA^+$ naïve T lymphocytes (n=3) and $CD45RO^+$ memory T lymphocytes (n=5).

toxic and memory T lymphocyte counts were increased. Neonates born by a caesarean section had 9% lower T ($p = 0.04$) and 40% lower NK lymphocyte counts ($p < 0.001$) than neonates born by a vaginal delivery. B lymphocyte counts did not differ. Within the T lymphocyte population, helper and naïve T lymphocyte counts were decreased in caesarean section delivered neonates.

Table 3. Associations of stress-related perinatal factors with absolute numbers of lymphocyte subsets in neonates.

	Lymphocyte subsets			
	T	**B**	**NK**	
Mode of delivery				
Vaginal	Reference	Reference	Reference	
Forceps or vacuum assisted	8 (0, 16)	13 (-1, 27)	45 (30, 61)**	
Caesarean section	-9 (-18, -0)*	-13 (-28, 3)	-40 (-57, -23)**	
Apgar scores				
1-minute	-2 (-5, 0)	-8 (-12, -3)**	-12 (-17, -6)**	
5-minute	-5 (-9, -1)**	-11 (-18, -5)**	-15 (-23, -7)**	
$pH_{cord\ blood}$	-5 (-10, -0.7)*	-22 (-29, -15)**	-30 (-38, -21)**	
	T lymphocyte subsets			
	Helper	**Cytotoxic**	**Naïve**	**Memory**
Mode of delivery				
Vaginal	Reference	Reference	Reference	Reference
Forceps or vacuum assisted	7 (-2, 16)	11 (0, 22)*	1 (-10, 12)	28 (2, 54)*
Caesarean section	-12 (-22, -3)*	-2 (-14, 10)	-12 (-24, -0)*	-4 (-33, 25)
Apgar scores				
1-minute	-2 (-5, 1)	-3 (-7, 1)	-2 (-6, 2)	2 (-6, 11)
5-minute	-4 (-8, 1)	-7 (-13, -2)**	-4 (-9, 2)	-4 (-17, 8)
$pH_{cord\ blood}$	-4 (-9, 10)	-6 (-12, 0.5)	-3 (-10, 4)	-14 (-29, 0.5)

Values are regression coefficients (95% confidence interval) and reflect the difference in lymphocyte subsets (%) of forceps or vacuum assisted and caesarean section delivered infants with vaginal delivered infants and the increase of lymphocyte subsets (%) per point increase in Apgar scores and umbilical cord blood pH.
*P-value < 0.05, **p-value 0.01.

Per point increase in 1-minute Apgar score, neonates had 8% lower B lymphocyte ($p = 0.001$) and 12% lower NK lymphocyte counts ($p < 0.001$). No significant changes in T lymphocytes and T lymphocyte subset counts were found for 1-minute Apgar score. Apgar scores at 5 minutes after birth revealed 5%, 11% and 15% lower T, B and NK lymphocyte counts, respectively, per point increase (all p-values < 0.01). Cytotoxic T lymphocyte counts were decreased with 7% ($p = 0.006$). No effect of the 5-minute Apgar score was seen on the other T lymphocyte subset counts.

Per 0.1-point increase in umbilical cord blood pH, 5%, 22% and 30% lower T, B and NK lymphocytes were found, respectively (all p-values < 0.05). Umbilical cord blood pH was not associated with the absolute numbers of T lymphocyte subsets.

The adjusted effect estimates of the various modes of delivery on T and B lymphocyte counts improved (p-values < 0.05), but on cytotoxic and naïve T lymphocyte counts declined (p-values > 0.05) (Figure 1). The associations of modes of delivery with the other absolute numbers of lymphocyte subsets did not materially change, nor did the associations of 1- and 5-minute Apgar scores and umbilical cord blood pH with absolute numbers of T, B and NK lymphocytes and T lymphocyte subsets (Figure 2 and 3).

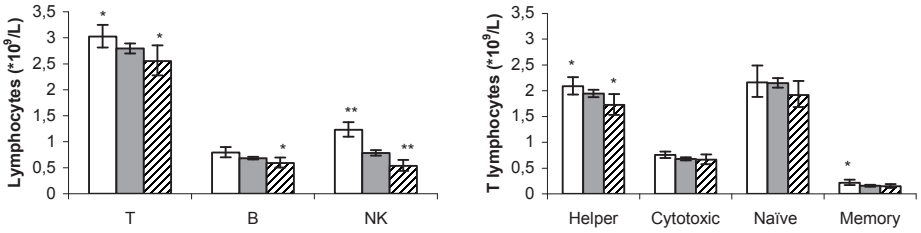


Figure 1. Absolute numbers of lymphocyte and T lymphocyte subsets in neonates born by various modes of deliveries.

All models were adjusted for gestational age, birth weight, gender and maternal fever.

□ = forceps or vacuum assisted delivery, ■ = vaginal delivery and ⊠ = caesarean section delivery.

Values are geometric means (95% confidence intervals); reference category is vaginal delivered neonates.

*P-value < 0.05, **p-value 0.01.

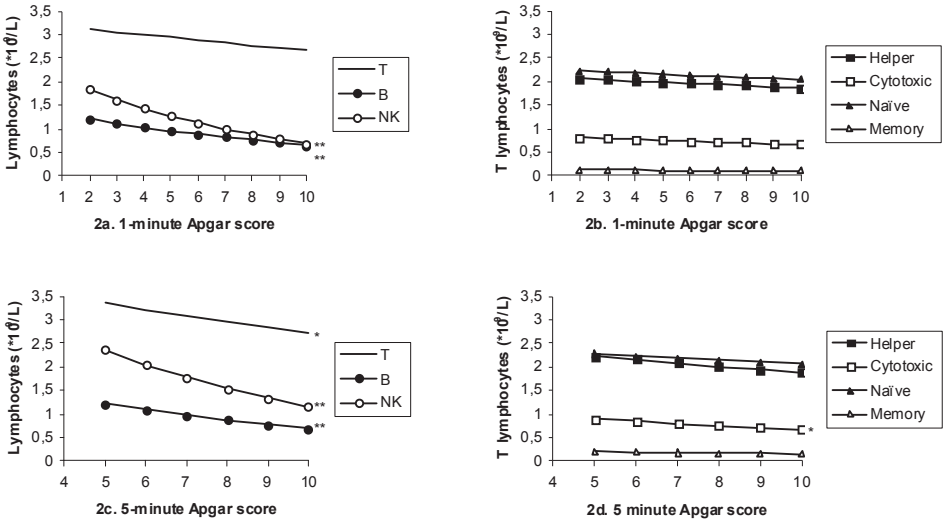


Figure 2. A and B. Absolute numbers of lymphocyte subsets in neonates with 1-minute Apgar scores at birth. All models were adjusted for gestational age, birth weight gender and maternal fever.

The estimated back-transformed regression lines reflect the number of lymphocyte subsets per point increase in 1-minute Apgar score. **C and D.** Absolute numbers of lymphocyte subsets in neonates with 5-minute Apgar scores at birth. All models were adjusted for gestational age, birth weight gender and maternal fever. The estimated back-transformed regression lines reflect the number of lymphocyte subsets per point increase in 5-minute Apgar score. Both panels (line, no symbol) T lymphocytes; ● B lymphocytes; ○ NK lymphocytes; ■ Helper T lymphocytes; □ cytotoxic T lymphocytes; ▲ naïve T lymphocytes; △ memory T lymphocytes.

*P-value < 0.05, **p-value 0.01.

An independent effect for mode of delivery on NK lymphocyte and memory T lymphocyte counts (p-values < 0.01) was found, but not for the other lymphocyte subset counts (Table 4). The effect of Apgar scores on all lymphocyte subset counts declined (p-values > 0.05). Umbilical cord blood pH showed an independent association with B, NK and memory T lymphocyte counts (p-value < 0.05), but not with the other T lymphocyte subset counts.

Discussion

This study showed that independent of the other perinatal stress-relating factors, increasing mode of delivery associated stress showed increasing NK and memory T lymphocyte counts and lower umbilical cord blood pH showed higher NK, B and memory T lymphocytes. We did not observe independent effects of 1- and 5-minute Apgar scores on lymphocyte subsets.

Previous studies on mode of delivery did not assess the effects on all lymphocyte and T lymphocyte subset counts (5, 9-13, 15, 16, 33). These studies showed inconclusive results, probably due to the small study populations (12 to 126 subjects). One large study of umbilical cord blood samples (more than 8000 cord blood units), banked for future umbilical cord blood transplantation, showed decreased percentages of helper T, cytotoxic T and B lymphocytes and increased percentages of NK lymphocytes in cord blood of caesarean section delivered neonates compared to vaginally delivered neonates (6). Differences between vaginal and forceps or vacuum deliveries, the exact amount of change in absolute numbers of lymphocyte subsets and the specific maternal and neonatal characteristics were not presented. Our study showed effects of mode of delivery mainly on NK lymphocytes. The precise functional role of these lymphocytes during labor remains to be studied. Forceps and vacuum assisted delivered neonates showed the highest lymphocyte subset counts and caesarean delivered neonates the lowest lymphocyte subset counts. This phenomenon suggests a continuous trend of increasing recruitment of lymphocyte subsets into neonatal blood with increasing mode of delivery-associated stress. Underlying biological mechanisms might be that neonates born by vaginal deliveries have higher levels of catecholamines and cortisol compared to neonates born by caesarean deliveries (16-19, 33-35). Vaginal delivered neonates with higher umbilical cord blood cortisol levels have higher lymphocyte counts (16, 19). It is has been suggested that caesarean section delivered infants have less recruitment of lymphocyte subsets due to maternal anaesthetics, which crosses the placenta and inhibit release of catecholamines and cortisol (33, 36, 37). A limitation in our study is that we could not made a distinction between a primary caesarean section delivery, usually

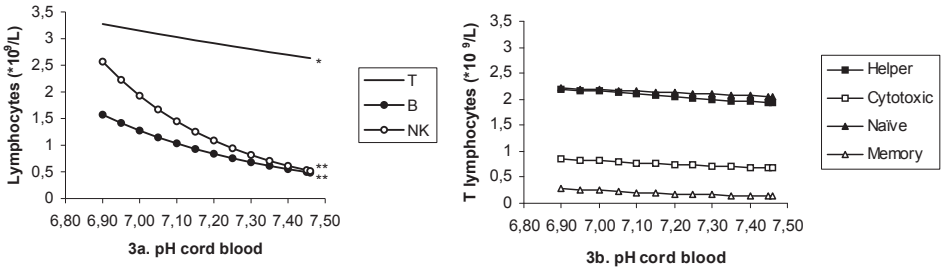


Figure 3. Absolute numbers of lymphocyte subsets in neonates with various umbilical cord blood pH at birth.
All models were adjusted for gestational age, birth weight gender and maternal fever.
The estimated back-transformed regression lines reflect the number of lymphocyte subsets per 0.1 point increase in umbilical cord blood pH.
(line, no symbol) T lymphocytes; ● B lymphocytes; ○ NK lymphocytes; ■ Helper T lymphocytes; □ cytotoxic T lymphocytes; ▲naïve T lymphocytes; △ memory T lymphocytes.
*P-value < 0.05, **p-value 0.01.

Table 4. Associations of independent stress-related perinatal factors with absolute numbers of lymphocyte subsets in neonates.

	Lymphocyte subsets			
	T	**B**	**NK**	
Mode of delivery				
Vaginal	Reference	Reference	Reference	
Forceps or vacuum assisted	7 (-4, 18)	12 (-4, 27)	41 (23, 59)**	
Caesarean section	-5 (-16, 8)	-14 (-32, 3)	-36 (-56, -16)**	
Apgar scores				
1-minute	0 (-5, 5)	-0 (-8, 8)	-4 (-15, 6)	
5-minute	-7 (-15, 1)	-9 (-21, 4)	4 (-12, 19)	
$pH_{cord\ blood}$	-1 (-7, 4)	-18 (-27, -10)**	-28 (-38, -17)**	
	T lymphocyte subsets			
	Helper	**Cytotoxic**	**Naïve**	**Memory**
Mode of delivery				
Vaginal	Reference	Reference	Reference	Reference
Forceps or vacuum assisted	6 (-5, 18)	9 (-5, 22)	-2 (-17, 14)	45 (13, 77)**
Caesarean section	-8 (-21, 5)	4 (-11, 20)	-5 (-22, 13)	1 (-4, 4)
Apgar scores				
1-minute	1 (-5, 7)	0 (-8, 7)	-2 (-10, 7)	16 (-2, 34)
5-minute	-8 (-17, 1)	-7 (-18, 4)	-4 (-16, 8)	-13 (-38, 13)
pHcord blood	0 (-6, 6)	1 (-9, 6)	1 (-7, 10)	-19 (-36, -1)*

Values are regression coefficients (95% confidence interval) and reflect the difference in lymphocyte subsets (%) of forceps or vacuum assisted and caesarean section delivered infants with vaginal delivered infants and the increase of lymphocyte subsets (%) per point increase in Apgar scores and umbilical cord blood pH. All models were adjusted for gestational age, birth weight gender, maternal fever and for each of the other stress-related perinatal factors.
*P-value < 0.05, **p-value 0.01.

planned due to previously known maternal indication, and a secondary caesarean section delivery because of acute fetal or maternal distress.

Both low Apgar scores and low umbilical cord blood pH can be seen as measures of hypoxia and stress of the neonate, implying that the labor-associated stress hypothesis could also be applied to these parameters. The effects of Apgar scores and umbilical cord blood pH were mainly found on the major lymphocyte subset counts, and to a lesser extent on T lymphocyte subset counts. The latter might be explained due to the fact that all T lymphocyte subsets contributed to an increase of the total T lymphocytes. Studies on Apgar scores and effects on lymphocyte subset counts are very limited and showed that low 1-minute Apgar scores and low 5-minute Apgar scores increase immunoglobulin secreting cell counts and proliferative responses of mononuclear cells in vitro (21, 22). We found larger effect estimates of 5-minute Apgar scores on lymphocyte subset counts than 1-minute Apgar scores. Apgar scores 5 minutes after birth probably better reflect the condition of neonates. This is in line with a previous study which observed associations of 5-minute Apgar scores and mortality rates (38). Apgar scores at 1 and 5 minutes after birth are subjective scoring systems for the condition of the neonate. Our fully adjusted regression models confirms this subjectivity of the Apgar scores since the independent effect of these scores declined severely and the effects of umbilical cord blood pH, an objective method for hypoxia, remained on most of the major lymphocyte subsets. A previous study found an inverse association between the number of leukocytes and umbilical cord blood pH (9). In this study, the effects of cord blood pH on the various leukocyte subtypes were not explored. One study assessed the relation between umbilical cord blood pH and distribution of major lymphocyte subsets and found decreasing percentages of T lymphocytes and increasing percentages of NK lymphocytes with decreasing umbilical cord blood pH (20). Differences with our study might be explained due to their different study population of only vaginal delivered infants with mothers frequently receiving labor-inducing medication, spasmolytics, neuroplegic and epidural analgesia (7 of 70 mothers received no medication). Unfortunately, data on perinatal medication were not available in our study. Another difference with our study might be that they used percentages of lymphocytes (T+B+NK=100%) instead of absolute values of lymphocyte subsets. A low percentage of T lymphocytes automatically provides a high percentage of B or NK lymphocytes. Umbilical cord blood pH therefore might lead to an opposite effect on those lymphocytes. The use of percentages instead of absolute counts of the major lymphocyte subsets in our cohort confirms this hypothesis. An increase of 0.1 point in umbilical cord blood pH showed an increase of percentual T lymphocytes (regression coefficient (95% CI): 5 (3, 6)), a decrease of percentual B lymphocytes (-1 (-2, -0.6)) and a decrease of NK lymphocytes (-3 (-4, -2)). For absolute counts of lymphocyte subsets, an increase of umbilical cord blood pH led to a decrease of all the major lymphocyte subsets (Table 3). Therefore, it is preferable to use absolute counts instead of

percentages of lymphocyte subsets to reflect actual changes of lymphocyte subsets due to various factors.

Some methodological issues should be considered. In our study, excluding participants with missing data would lead to selection biased results if the associations of stress-related perinatal factors with absolute numbers of lymphocyte subsets differ between participants with and without complete data. Of all participating singleton live births, information about mode of delivery was missing in only 5.3%. Of neonates with information of mode of delivery, neonates with missing data on lymphocyte subsets more often had a caesarean section delivery than neonates with data on lymphocyte subsets. Therefore it is likely that due to this selective group of missing outcomes the observed effects of mode of delivery with absolute numbers of lymphocyte subsets may be underestimated. However, caesarean section may be performed primary or secondary. Since the associations of primary and secondary caesarean deliveries with lymphocyte subsets counts are expected to be in the opposite direction, our effect estimates may be somewhat biased towards null. Our results are probably demonstrating the difference between primary caesarean delivery and vaginal delivery. We expect to have relatively more missing lymphocyte subset counts among neonates born after a secondary caesarean delivery due to logistical constraints in the acute setting.

Information about 1-minute Apgar scores, 5-minute Apgar scores and umbilical cord blood pH was missing in 2.6% 1.9% and 50.8% of the neonates, respectively. Measurements of umbilical cord blood pH in the Netherlands is not routinely performed, since approximately one-third of the deliveries in the Netherlands is performed at home and are at low risk for obstetric complications (39). In line with this, we found that neonates without umbilical cord blood pH values more often were vaginally delivered and had higher 1- and 5-minute Apgar scores. Neonates without umbilical cord blood pH are more frequently from uncomplicated pregnancies with fetal distress. Thus we expect that missing values in umbilical cord blood pH did not materially change the effect estimated but decreased the power of the study.

In our cohort study, information about maternal fever, indicating an underlying infection, was only available around the third trimester of gestation. It did not materially change the effect estimates in the associations of stress-related perinatal factors with lymphocyte subsets. However, due to the time of maternal fever in pregnancy its confounding effect is probably underestimated since an acute ongoing maternal infection during delivery might show more increasing effects on the neonatal lymphocyte subsets.

In conclusion, this population-based cohort study showed that with increasing mode of delivery-associated stress lymphocyte subset counts (NK, memory T) increase. Furthermore, lower umbilical cord blood pH is associated with an increase of lymphocyte subset counts (B, NK, memory T). Therefore, the clinical interpretation of absolute numbers of lymphocyte subsets at birth should take the effects of stress-and hypoxia related perinatal factors into account.

References

1. Motley D, Meyer MP, King RA, Naus GJ 1996 Determination of lymphocyte immunophenotypic values for normal full-term cord blood. Am J Clin Pathol 105:38-43
2. Rabian-Herzog C, Lesage S, Gluckman E 1992 Characterization of lymphocyte subpopulations in cord blood. Bone Marrow Transplant 9 Suppl 1:64-67
3. Raes M, Alliet P, Gillis P, Zimmermann A, Kortleven J, Magerman K, Peeters V, Rummens JL 1993 Lymphocyte subpopulations in healthy newborn infants: comparison of cord blood values with values five days after birth. J Pediatr 123:465-467
4. Stern DA, Hicks MJ, Martinez FD, Holberg CJ, Wright AL, Pinnas J, Halonen M, Taussig LM 1992 Lymphocyte subpopulation number and function in infancy. Dev Immunol 2:175-179
5. Kotiranta-Ainamo A, Apajasalo M, Pohjavuori M, Rautonen N, Rautonen J 1999 Mononuclear cell subpopulations in preterm and full-term neonates: independent effects of gestational age, neonatal infection, maternal pre-eclampsia, maternal betamethason therapy, and mode of delivery. Clin Exp Immunol 115:309-314
6. Cairo MS, Wagner EL, Fraser J, Cohen G, van de Ven C, Carter SL, Kernan NA, Kurtzberg J 2005 Characterization of banked umbilical cord blood hematopoietic progenitor cells and lymphocyte subsets and correlation with ethnicity, birth weight, sex, and type of delivery: a Cord Blood Transplantation (COBLT) Study report. Transfusion 45:856-866
7. Chandra RK 1991 Interactions between early nutrition and the immune system. Ciba Found Symp 156:77-89; discussion 89-92
8. Moore SE 1998 Nutrition, immunity and the fetal and infant origins of disease hypothesis in developing countries. Proc Nutr Soc 57:241-247
9. Nikischin W, Peter M, Oldigs HD 1997 The influence of mode of delivery on hematologic values in the umbilical vein. Gynecol Obstet Invest 43:104-107
10. Pittard WB, 3rd, Schleich DM, Geddes KM, Sorensen RU 1989 Newborn lymphocyte subpopulations: the influence of labor. Am J Obstet Gynecol 160:151-154
11. Lim FT, van Winsen L, Willemze R, Kanhai HH, Falkenburg JH 1994 Influence of delivery on numbers of leukocytes, leukocyte subpopulations, and hematopoietic progenitor cells in human umbilical cord blood. Blood Cells 20:547-558; discussion 558-549
12. Samelson R, Larkey DM, Amankwah KS, McConnachie P 1992 Effect of labor on lymphocyte subsets in full-term neonates. Am J Reprod Immunol 28:71-73
13. Thilaganathan B, Meher-Homji N, Nicolaides KH 1994 Labor: an immunologically beneficial process for the neonate. Am J Obstet Gynecol 171:1271-1272
14. Thornton CA, Capristo CC, Power LL, Holloway JA, Popplewell EJ, Diaper ND, Warner JO 2003 The effect of labor on neonatal T-cell phenotype and function. Pediatr Res 54:120-124
15. Gasparoni A, Maccario R, Chirico G, Belloni C, Mingrat G, De Amici D, Bonora MR, Rondini G 1992 Neonatal B lymphocyte subpopulations and method of delivery. Biol Neonate 61:137-141
16. Chirico G, Gasparoni A, Ciardelli L, Martinotti L, Rondini G 1999 Leukocyte counts in relation to the method of delivery during the first five days of life. Biol Neonate 75:294-299
17. Vogl SE, Worda C, Egarter C, Bieglmayer C, Szekeres T, Huber J, Husslein P 2006 Mode of delivery is associated with maternal and fetal endocrine stress response. Bjog 113:441-445
18. Ruth V, Hallman M, Laatikainen T 1993 Corticotropin-releasing hormone and cortisol in cord plasma in relation to gestational age, labor, and fetal distress. Am J Perinatol 10:115-118
19. De Amici D, Gasparoni A, Chirico G, Ceriana P, Bartoli A, Ramajoli I, Moretta A 1999 Natural killer cell activity and delivery: possible influence of cortisol and anesthetic agents. A study on newborn cord blood. Biol Neonate 76:348-354
20. Dostal M, Giguere Y, Fait T, Zivny J, Sram RJ 2001 The distribution of major lymphocyte subsets in cord blood is associated with its pH. Clin Biochem 34:119-124

21. Pittard WB, 3rd, Miller KM, Sorensen RU 1985 Perinatal influences on in vitro B lymphocyte differentiation in human neonates. Pediatr Res 19:655-658
22. Willwerth BM, Schaub B, Tantisira KG, Gold DR, Palmer LJ, Litonjua AA, Perkins DL, Schroeter C, Gibbons FK, Gillman MW, Weiss ST, Finn PW 2006 Prenatal, perinatal, and heritable influences on cord blood immune responses. Ann Allergy Asthma Immunol 96:445-453
23. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, Verhulst FC, Witteman JC, Buller HA 2004 Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol 18:61-72
24. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, Witteman JC, Hofman A 2006 The Generation R Study: Design and cohort profile. Eur J Epidemiol 21:475-484
25. Apgar V 1953 A proposal for a new method of evaluation of the newborn infant. Curr Res Anesth Analg 32:260-267
26. Apgar V, Holaday DA, James LS, Weisbrot IM, Berrien C 1958 Evaluation of the newborn infant; second report. J Am Med Assoc 168:1985-1988
27. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Boerma GJ, Lotgering FK, van Dongen JJ 1999 Correction for erythroid cell contamination in microassay for immunophenotyping of neonatal lymphocytes. Arch Dis Child - Fetal and Neonatal Edition 80:F226-229
28. Brando B, Gohde W, Jr, Scarpati B, D'Avanzo G, European Working Group on Clinical Cell Analysis 2001 The "vanishing counting bead" phenomenon: effect on absolute CD34+ cell counting in phosphate-buffered saline-diluted leukapheresis samples. Cytometry 43:154-160
29. van Lochem EG, van der Velden VH, Wind HK, te Marvelde JG, Westerdaal NA, van Dongen JJ 2004 Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts. Cytometry B Clin Cytom 60:1-13
30. Lambert C, Cristina I, Christian G 2006 Enumeration of peripheral lymphocyte subsets using 6 vs. 4 color staining: a clinical evaluation of a new flowcytometer. Cytometry B Clin Cytom 70:29-38
31. Jaddoe VW, Verburg BO, de Ridder MA, Hofman A, Mackenbach JP, Moll HA, Steegers EA, Witteman JC 2007 Maternal smoking and fetal growth characteristics in different periods of pregnancy: the generation R study. Am J Epidemiol 165:1207-1215
32. Cole TJ 2000 Sympercents: symmetric percentage differences on the 100 log(e) scale simplify the presentation of log transformed data. Stat Med 19:3109-3125
33. Irestedt L, Lagercrantz H, Hjemdahl P, Hagnevik K, Belfrage P 1982 Fetal and maternal plasma catecholamine levels at elective cesarean section under general or epidural anesthesia versus vaginal delivery. Am J Obstet Gynecol 142:1004-1010
34. Jones CM, 3rd, Greiss FC, Jr. 1982 The effect of labor on maternal and fetal circulating catecholamines. Am J Obstet Gynecol 144:149-153
35. Jouppila R, Puolakka J, Kauppila A, Vuori J 1984 Maternal and umbilical cord plasma noradrenaline concentrations during labour with and without segmental extradural analgesia, and during caesarean section. Br J Anaesth 56:251-255
36. Gasparoni A, Ciardelli L, De Amici D, Castellazzi AM, Autelli M, Bottino R, Polito E, Bartoli A, Rondini G, Chirico G 2002 Effect of general and epidural anaesthesia on thyroid hormones and immunity in neonates. Paediatr Anaesth 12:59-64
37. Marx GF 1995 Evaluation of stress during labor and parturition. Am J Obstet Gynecol 172:1948
38. Casey BM, McIntire DD, Leveno KJ 2001 The continuing value of the Apgar score for the assessment of newborn infants. N Engl J Med 344:467-471
39. Anthony S, Buitendijk SE, Offerhaus PM, Dommelen P, Pal-de Bruin KM 2005 Maternal factors and the probability of a planned home birth. Bjog 112:748-753

Variants of the insulin-like growth factor-I gene and lymphocyte subset counts in neonates

4.4

Abstract

Objective: Insulin-like growth factor (IGF-I) stimulates growth, development and function of lymphocytes. The aim of this study was to examine whether functional variants of the IGF-I gene are associated with absolute lymphocyte subset counts in neonates.

Design: This study was embedded in the Generation R Study, a prospective cohort study from fetal life onwards.

Measurements: IGF-I promoter region polymorphisms were determined in cord blood DNA. Lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory) in cord blood were immunophenotyped in 380 neonates by 6-color flow cytometry.

Results: In total, 39% of the neonates were homozygous for the 192-bp allele (wild-type), 48% were heterozygous and 13% were non-carrier. No differences in absolute lymphocyte and T lymphocyte subset counts were observed between the 192-bp allele heterozygous and homozygous groups. In non-carriers, we found 13% lower T lymphocyte (p=0.03), 21% lower B lymphocyte (p=0.04) and 11% lower NK lymphocyte counts (p=0.36) than in the 192-bp allele homozygous group. Analyses of T lymphocyte subsets showed 16% lower helper T lymphocyte counts (p=0.01) in non-carriers. No significant differences were found for cytotoxic, naïve and memory T lymphocyte counts. All associations were adjusted for gravidity, mode of delivery, gestational age, birth weight, gender, 1- and 5- minute Apgar scores.

Conclusions: Our study showed associations between the IGF-I promoter region polymorphisms and absolute lymphocyte subset counts in neonates. These results should be regarded as hypothesis generating until they have been replicated in other studies.

Introduction

Insulin-like growth factor I (IGF-I) is a single chain polypeptide that stimulates bone growth, metabolism and cell differentiation. IGF-I is synthesized by various tissues and has an endocrine, autocrine or paracrine function (1-4). Previous studies suggested that IGF-I also stimulates growth, development and function of lymphocytes. Studies in animals showed that IGF-I administration leads to an increase of relative and absolute counts of T lymphocytes, $CD4^+$ helper T and $CD8^+$ cytotoxic T lymphocytes in thymus and peripheral blood (5-10). IGF-I administration resulted also in increased relative and absolute B lymphocyte counts in bone marrow, spleen and blood (5, 6, 8, 10). Furthermore, animal studies showed that after allogeneic bone marrow transplantation or dexamethason treatment, which usually result in prolonged immunodeficiency, IGF-I administration enhanced lymphoid and myeloid reconstitution (11-15). IGF-I seems not only to lead to increased counts of lymphocytes, but also to increased differentiation of B lymphocytes in bone marrow, a progressed maturation of naïve into memory T lymphocytes and increased cytokine production by mononuclear cells in human cord blood (16-19).

Absence of the 192-bp allele in the IGF-I gene promoter region has shown to be associated with circulating IGF-I levels (20-22). Previous studies suggested that this functional polymorphism of the IGF-I gene is also associated with lower birth weight and reduced prenatal and postnatal linear growth (23, 24). We hypothesized that absence of the common 192-bp allele in the IGF-I gene promoter region is also related to absolute counts of lymphocyte subsets. Therefore, we examined in 380 neonates participating in a prospective cohort study from fetal life onwards the associations of this IGF-I promoter polymorphism with absolute counts of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory) in cord blood.

Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life onwards. The Generation R Study is designed to identify early environmental and genetic determinants of growth, development and health from fetal life until young adulthood and has been described previously in detail (25, 26). Detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study. This subgroup is ethnic homogeneous to exclude possible confounding or effect modification by ethnicity. Of all approached pregnant women and their partners, 79% participated in the Generation R Focus Study. The Medical Ethics Committee of the

Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Genotyping IGF-I gene

DNA was collected from cord blood samples of the children at birth. Polymerase chain reaction (PCR) was performed using oligonucleotide primers designed to amplify the polymorphic cytosine-adenine (CA) repeat 1 kb upstream of the human IGF-I gene (27). The reaction was carried out in a final volume of 10 ml containing 50 ng of genomic DNA obtained from peripheral blood cells, 0.5 nmol/l forward primer ('5-ACCACTCTGGGA-GAAGGGTA-3'), 0.5 nmol/l reverse primer ('5-GCTAGCCAGCTGGTGTTATT-3'), 0.25 mmol/l 2'-dNTP, 2.2 mmol/l MgCl2, 0.01% W1 (Gibco BRL), and 0.4 Taq DNA polymerase (Gibco BRL). PCR was performed in 384 well plates (94˚C 10 min; 35 PCR cycles of 30 s at 94˚C, 30 s on 55˚C, and 30 s on 72˚C; 72˚C 10 min; 4˚C hold). Forward primers were labelled with FAM, HEX or NED to determine the size of PCR products by autosequencer (ABI 3100, POP 4, filter set D, collecting time array 36 cm 7 s, peak-height between 100 and 2000, each lane containing three samples). The size of the PCR products was determined in comparison with internal ROX-size standard (Perkin Elmer). Seven different alleles of the IGF-I promoter region were identified (Table 1). The genotype frequency was similar to those found in previous studies and the frequency distribution did not deviate from the Hardy-Weinberg equilibrium (Chi-square = 0.005, P > 0.9) (22, 24). As in previous stud-

Table 1. IGF-I promoter polymorphisms and genotype distributions of the study population.

		Neonates (n = 380)
Polymorphism		
Allele (base pairs)	$(CA)_n$	
186	16	2 (0.3)
188	17	20 (2.6)
190	18	36 (4.7)
192 (wild-type)	19	481 (63.3)
194	20	158 (20.8)
196	21	53 (7.0)
198	22	10 (1.3)
Genotype		
Homozygous 192-bp		149 (39.2)
Heterozygous 192-bp		183 (48.2)
Non-carriers 192-bp		48 (12.6)

Values are absolute numbers (%). The allele distribution is based on 2 alleles per infant. (CA)n: number of cytosine-adenine repeats. Wild type allele refers to the most frequent allele in this population.

ies, IGF-I genotypes were categorized in the following categories based on their 192-bp allele: homozygous (wild-type), heterozygous and non-carrier (23, 24, 28, 29). In our study, serum IGF-I levels were not available due to logistic and financial constraints.

Immunophenotyping of lymphocyte subsets

Heparinized sampling of venous cord blood was carried out by midwifes and obstetricians immediately after delivery and transported to the Immunology laboratory of the Erasmus Medical Center within 24 hours. Cord blood samples not received within 24 hours (weekend days) were excluded since flow cytometric analyses of those samples showed no reliable results in the pilot phase of the study. Flow cytometric immunophenotyping was performed to determine absolute counts of lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory T). For this, the monoclonal antibodies CD3, CD19, CD16.CD56, CD4, CD8, CD45RA and CD45RO were conjugated with the labels fluorescein isothiocyanate (FITC[1]), peridin chlorophyll protein (PerCP[1]), peridin chlorophyll protein-cychrome 5.5 (PerCP-Cy5.5[1]), allophycocyanin (APC[1]), phycoerythrin (PE[1,3]), phycoerythrin-cyanin dye (PE-cy7[1]), allophycocyanin-cyanin dye (APC-cy7[1]) and rhodamine (RD1[2]) ([1]Becton Dickinson,[2]Beckman Coulter and [3]Dako). Absolute counts of T, B and NK lymphocytes were determined with the lysed whole blood technique (30). Subsets of the T lymphocytes were determined by 6-color flow cytometry. Therefore, erythrocytes of 1 ml whole blood were lysed using 50 ml ammoniumchloride. After centrifugation, leucocytes were washed twice and suspended in 900 µl PBS/1% BSA/0.1% NaAz. Of this cell suspension (5-10*10^6/ml), 50 µl was incubated for 10 minutes at room temperature with combinations of the optimally titrated labelled monoclonal antibodies. After incubation the cells were washed and subsequently identified by flow cytometry. Using a BD™ LSR II flow cytometer (Becton Dickinson, San Jose, California, US) that had been calibrated with rainbow beads, 10,000 lymphocytes were measured. The relative count of helper T lymphocytes, cytotoxic T lymphocytes, naïve T lymphocytes and memory T lymphocytes was expressed as the percentage within the total T lymphocyte population and calculated by the average of 2-4 independent incubations for each subset. The absolute counts of the T lymphocyte subsets were subsequently calculated from the absolute T lymphocyte counts as obtained by the lysed whole blood technique.

Covariates

Information about gravidity, mode of delivery (vaginal, forceps or vacuum assisted, caesarean section), date of birth, birth weight, gender, 1- and 5-minute Apgar scores (assigning a score of 0 to 2 to heart rate, respiratory effort, muscle tone, reflex irritability and colour) was obtained from standardized midwives and hospital registries. Gestational age was established by the first fetal ultrasound examination after enrolment (26).

Cohort for analysis

Of the total of 1,232 women who were enrolled in the Generation R Focus Study, those with weekend deliveries (n=202), twin pregnancies (n=24) and pregnancies leading to perinatal death (n=2) were excluded from the present analysis. Of the remaining singleton 1004 live births, IGF-I was successfully genotyped in DNA form cord blood in 604 (60%) neonates. Of those neonates, immunophenotyping of lymphocyte subsets was performed in 380 (63%). Missing data of immunophenotyped lymphocyte subsets was mainly due to constraints in the amount of blood and missing heparinized blood samples.

Data analysis

Differences of neonatal characteristics between the IGF-I genotype groups were assessed by the independent sample t-test for continuous normal distributed variables, non-parametric Mann-Whitney test for continuous non-normal distributed variables and the chi-square test for categorical variables.

Table 2. Delivery and neonatal characteristics according to the IGF-I genotype.

	IGF-I genotype groups		
	Homozygous 192-bp allele (n = 149)	Heterozygous 192-bp allele (n = 183)	No 192-bp allele (n = 48)
Gravidity (%)			
Primigravid	72 (48.3)	104 (57.5)	26 (54.2)
Multigravid	77(51.7)	77 (42.5)	22 (45.8)
Mode of delivery (%)			
Vaginal	104 (73.2)	126 (72.8)	36 (80.0)
Forceps or vacuum assisted	23 (16.2)	33 (19.1)	3 (6.7)
Caesarean section	15 (10.6)	14 (8.1)	6 (13.3)
Gender (%)			
Boy	82 (55.0)	82 (44.8)	28 (58.3)
Girl	67 (45.0)	101 (55.2)	20 (41.7)
Gestational age	40.3 (34.1-43.0)	40.4 (36.0-43.4)	40.5 (34.6-42.3)
Birth weight	3558 (472)	3588 (482)	3554 (467)
Apgar scores			
1-minute	9 (4-10)	9 (2-10)	9 (7-10)
5-minute	10 (8-10)	10 (5-10)	10 (8-10)

Values are proportions (%), medians (range) or means (sd).
Differences were tested using the independent sample t-test, non-parametric Mann-Whitney test or Chi-square test (all p-values > 0.05).
Of the total group, data were missing on gravidity (n=2), mode of delivery (n=20), 1-minute Apgar score (n=8) and 5-minute Apgar score (n=7).

Data on all lymphocyte subset counts were log-transformed to obtain normally distributed variables. Associations of the IGF-I gene with absolute counts of lymphocyte subsets were analyzed using multiple linear regression models. Additionally, regression

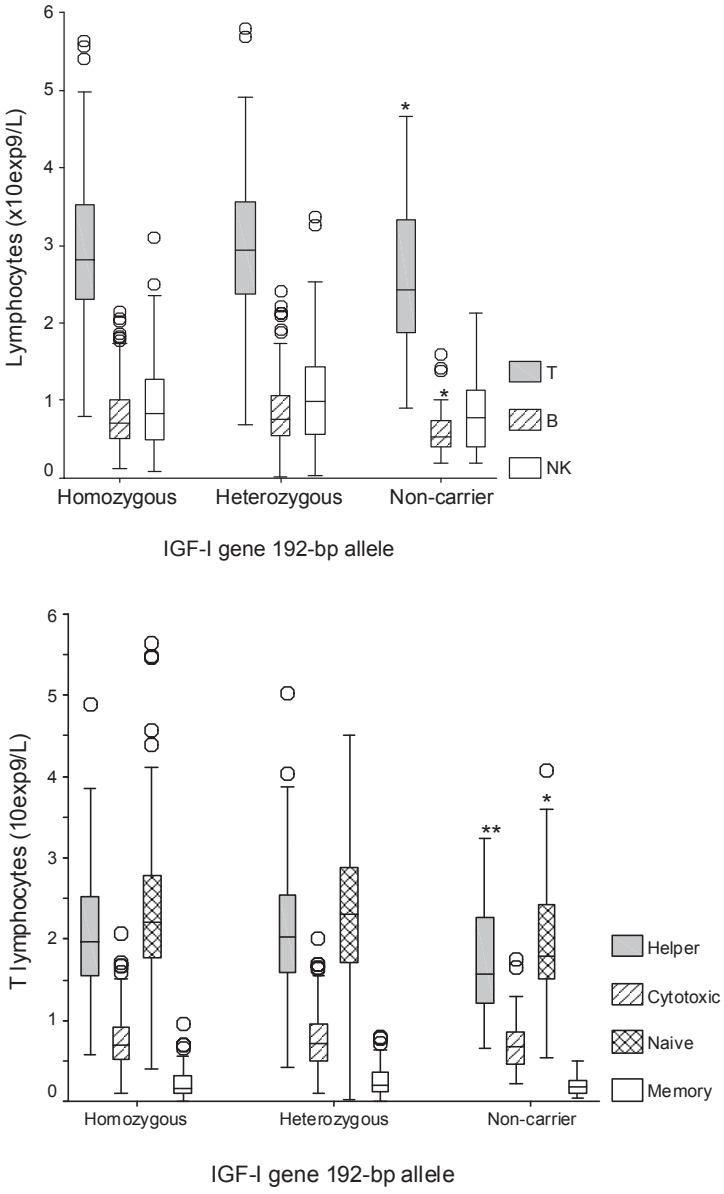


Figure 1. Associations between IGF-I genotype groups and absolute counts of lymphocyte subsets in neonates.
*P-value < 0.05, **p-value < 0.01 for difference with the homozygous 192-bp allele group.

models were adjusted for the potential confounders gravidity, mode of delivery, gestational age, birth weight, gender, 1- and 5- minute Apgar scores. Measures of associations are presented as geometric medians (range) or as log-transformed mean differences (95% confidence interval (CI)), which can be interpreted as percentages after multiplying by 100 (31). The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Birth characteristics for the three genotype groups are shown in Table 2. In total, 149 (39%) neonates were 192-bp homozygous, 183 (48%) were 192-bp heterozygous and 48 (13%) neonates were non-carriers. No differences in birth characteristics were observed between these groups.

Figure 1 shows the geometric medians of absolute counts of lymphocyte and T lymphocyte subsets of each IGF-I genotype group. No differences in absolute counts of lymphocyte and T lymphocyte subsets were observed between the 192-bp allele heterozygous and 192-bp allele homozygous groups. In non-carriers, we found 15% (95% CI: 4%, 27%) lower T lymphocyte counts, 22% (95% CI: 2%, 41%) lower B lymphocyte counts and 10% (95% CI: -13%, 33%) lower NK lymphocyte counts than in the 192-bp allele homozygous group, although the latter was not significant. Within the T lymphocyte population, the non-carriers had lower absolute counts of helper T (difference: -18% (95% CI: -30%, -6%)) and naïve T lymphocytes (difference: -16% (95% CI: -31%, 0%)) compared to the 192-bp allele homozygous group. Associations of the IGF-I genotype groups with cytotoxic and memory T lymphocyte counts were not observed.

Table 3. Associations between IGF-I genotype groups and absolute counts of lymphocyte subsets in neonates.

	Lymphocyte subsets			
192-bp allele:	T	B	NK	
Homozygous	Reference	Reference	Reference	
Heterozygous	-1 (-9, 7)	2 (-11, 16)	7 (-8, 22)	
Non-carrier	-13 (-25, -1)*	-21 (-40, -1)*	-11 (-33, 12)	
	T lymphocyte subsets			
192-bp allele:	Helper	Cytotoxic	Naïve	Memory
Homozygous	Reference	Reference	Reference	Reference
Heterozygous	0 (-9, 8)	-1 (-12, 9)	-3 (-15, 8)	10 (-16, 36)
Non-carrier	-16 (-28, -3)**	-7 (-22, 9)	-14 (-31, 2)	7 (-31, 45)

Values are regression coefficients (95% confidence interval) and reflect the percentual difference in absolute numbers of lymphocyte subsets with the homozygous 192-bp allele reference group (31). Values are adjusted for gravidity, mode of delivery, gestational age, birth weight, gender, 1- and 5-minute Apgar scores.
*P-value < 0.05, **p-value < 0.01.

Differences in absolute counts of lymphocyte subsets between the genotype groups, adjusted for gravidity, mode of delivery, gestational age, birth weight, gender, 1- and 5 minute Apgar scores, are given in Table 3. These adjustments by multiple regression models did not materially change the estimated differences, except for naïve T lymphocytes. No statistically significant difference in naïve T lymphocyte counts was found between the homozygous and non-carrier 192-bp allele group (difference: -14 (95% CI: -31, 2).

Discussion

This study showed that non-carriers of the 192-bp allele in IGF-I promoter region had lower absolute counts of T and B lymphocytes compared to 192-bp allele homozygous neonates. Within the T lymphocyte population, non-carriers had lower absolute counts of helper T lymphocytes. Of the other lymphocyte subsets, absolute counts of NK lymphocytes, cytotoxic and naïve T lymphocytes tended to be lower in non-carriers, but memory T lymphocytes did not. No differences in absolute counts of lymphocyte subsets and T lymphocyte subsets were observed between the 192-bp allele homozygous and 192-bp allele heterozygous groups.

To our knowledge, this is the first study that examined the associations between this IGF-I promoter region polymorphism and absolute counts of lymphocyte subsets in neonates. DNA for genotyping was available in 602 neonates (60%). Of all genotyped neonates, 63% had immunophenotyped lymphocyte subsets. Frequencies of the 192-bp allele homozygous group and non-carriers as well as birth characteristics did not significantly differ between those with and without data on counts of lymphocyte subsets. Therefore, we do not expect that missing counts of lymphocyte subsets led to biased effect estimates.

We observed different effects of the IGF-I promoter region polymorphisms on the various lymphocyte subset counts. Compared with 192-bp allele homozygous neonates, non-carriers had overall lower counts of lymphocytes and T lymphocyte subsets, although only a significant effect was observed on absolute counts of total T, helper T and B lymphocytes. IGF-I binds to the IGF-I receptor of all lymphocytes and stimulates growth and development of those cells. Expression of the IGF-I receptor is higher on helper T lymphocytes than on cytotoxic T lymphocytes. Since the expression of the IGF-I receptor is higher on helper T lymphocytes than on cytotoxic T, it seems plausible that the largest effects of the IGF-I genotype variants are found for those with the highest IGF-I receptor expression (32, 33).

The present study was initiated because previous studies suggested that IGF-I levels affect lymphocyte subset counts (5-19). However, studies in IGF-I knockout mice and in humans with deletions/mutations in the IGF-I gene or the IGF-I receptor gene did not

show deficiencies in counts of marrow myeloid lymphocytes or thymocyte subpopulations (34). This suggests that IGF-I is not an important thymopoietic or myelopoietic factor. Furthermore, no associations between IGF-I gene variants and immunodeficiency have been reported. Therefore, our results, suggesting an effect of the IGF-I promoter region polymorphism on lymphocyte subset counts, do not suggest that variants in the IGF-I gene are related to immunodeficiency syndromes. Observed differences between genotype groups in our study were all within a normal range and are unlikely to be of any clinical importance at birth. Whether and to what extend these differences in lymphocyte subset counts persist and have clinical consequences at older age should be examined in further follow up studies.

We have genotyped a highly polymorphic marker in the regulatory region of IGF-I. Due to limited counts of subjects, we were not able to test all alleles, but pooled the alleles based on the common allele. Our classification was based on earlier studies, which showed associations between IGF-I genotype with serum IGF-I levels as well as pathology in our earlier population-based studies (22, 23, 35). Most of these studies were focused on growth, anthropometrics or diabetes (22, 23, 29, 36-38). To our knowledge, no previous studies have studied the associations between our IGF-I promoter region polymorphism and lymphocyte subset counts. In animal studies, it has been suggested that the effect of IGF-I levels on lymphocyte subset counts is an indirect result of the effect of IGF-I on body or organ size (5). Due to lack of IGF-I, mice were smaller and counts of marrow myeloid cells and thymocyte subpopulations seemed normal when values were normalized to the weight of the mice (39, 40). In our study cohort, we did not observe an association of the IGF-I promoter region polymorphism with birth weight. Furthermore, we found associations between the IGF-I promoter region polymorphism and absolute counts of lymphocyte and T lymphocyte subsets that were independent of birth weight. These results suggest that the associations of variants in the IGF-I promoter region and absolute counts of lymphocyte subsets are not explained by body size.

In IGF-I treated mice, an increase of the absolute counts of marrow myeloid, B and T lymphocytes has been reported but the relative frequencies of these lineages seem to remain unaffected by IGF-I (5, 12, 39). We found differences in absolute counts, but no difference in relative counts of T (mean difference (95% CI): 0.3% (-3.0%, 3.7%)), B (-1.2% (-3.2%, 0.8%), helper T (-1.9% (-4.5%, 0.8%) or naïve T (0.6% (-1.1%, -0.1%) lymphocytes between the 192-bp allele homozygous group and non-carriers were observed. In humans, total lymphocyte counts per ml of blood are significantly higher in neonates than in adults (41). It has been suggested that the use of absolute counts instead of percentages of lymphocyte subsets is preferred to show actual changes of lymphocyte subsets in neonates (41).

A major limitation of the current study is that IGF-I levels were not available due to logistical and financial constraints. Previous studies showed this polymorphism to be

associated with IGF-I levels (20-22). Other IGF-I polymorphisms have also been shown to be associated with IGF-I levels and postnatal growth in infants born small size for gestational age (42). Whether these polymorphisms affect absolute counts of lymphocyte subsets remains to be studied. Several studies suggested that insulin-like growth factor II (IGF-II) affects growth, development and function of lymphocytes (19, 43). Therefore, it might be that polymorphisms of the IGF-II gene also influence absolute counts of lymphocyte subsets. Growth hormone (GH) or GH gene polymorphisms probably do not affect counts of lymphocyte subsets in cord blood of neonates. Growth hormone mainly affects postnatal growth and its effects are mostly mediated by IGF-I (7, 44).

It is found that higher cortisol levels in umbilical cord blood are associated with increased lymphocyte subset counts in neonates (45, 46). Unfortunately, for the present study, data of cortisol levels in umbilical cord blood were not available due to logistic and financial constraints. However, we adjusted our models for mode of delivery and for 1- and 5-minute Apgar scores. These variables are strongly associated with stressful events for the fetus. We expect that adjusting for mode of delivery and Apgar scores enabled us to take account of at least part of the effect of fetal stress.

In summary, this study shows an association between IGF-I promoter region polymorphisms and absolute counts of lymphocytes subsets in neonates. These results should be regarded as hypothesis generating rather than as clinically important. Since our study is the first showing these associations, replication studies are urgently needed before any conclusions or causal inference can be drawn focused on the role of this IGF-I polymorphism. These studies should also assess potential dose-response trends for relation between IGF-I promoter region polymorphisms, IGF-I levels and lymphocyte subset counts. Furthermore, genome-wide approaches may lead to identification of other, more specific IGF-I related genetic variants associated with counts of lymphocyte subsets.

References

1. Froesch ER, Hussain MA, Schmid C, Zapf J. Insulin-like growth factor I: physiology, metabolic effects and clinical uses. Diabetes Metab Rev. 1996 Oct;12(3):195-215.
2. Baxter JB, Blalock JE, Weigent DA. Characterization of immunoreactive insulin-like growth factor-I from leukocytes and its regulation by growth hormone. Endocrinology. 1991 Oct;129(4):1727-34.
3. Sabharwal P, Varma S. Growth hormone synthesized and secreted by human thymocytes acts via insulin-like growth factor I as an autocrine and paracrine growth factor. J Clin Endocrinol Metab. 1996 Jul;81(7):2663-9.
4. Robbins K, McCabe S, Scheiner T, Strasser J, Clark R, Jardieu P. Immunological effects of insulin-like growth factor-I--enhancement of immunoglobulin synthesis. Clin Exp Immunol. 1994 Feb;95(2):337-42.
5. Foster M, Montecino-Rodriguez E, Clark R, Dorshkind K. Regulation of B and T cell development by anterior pituitary hormones. Cell Mol Life Sci. 1998 Oct;54(10):1076-82.

6. Clark R, Strasser J, McCabe S, Robbins K, Jardieu P. Insulin-like growth factor-1 stimulation of lymphopoiesis. J Clin Invest. 1993 Aug;92(2):540-8.
7. Dorshkind K, Horseman ND. The roles of prolactin, growth hormone, insulin-like growth factor-I, and thyroid hormones in lymphocyte development and function: insights from genetic models of hormone and hormone receptor deficiency. Endocr Rev. 2000 Jun;21(3):292-312.
8. LeRoith D, Yanowski J, Kaldjian EP, Jaffe ES, LeRoith T, Purdue K, et al. The effects of growth hormone and insulin-like growth factor I on the immune system of aged female monkeys. Endocrinology. 1996 Mar;137(3):1071-9.
9. de Mello Coelho V, Villa-Verde DM, Farias-de-Oliveira DA, de Brito JM, Dardenne M, Savino W. Functional insulin-like growth factor-1/insulin-like growth factor-1 receptor-mediated circuit in human and murine thymic epithelial cells. Neuroendocrinology. 2002 Feb;75(2):139-50.
10. Tarantal AF, Hunter MK, Gargosky SE. Direct administration of insulin-like growth factor to fetal rhesus monkeys (Macaca mulatta). Endocrinology. 1997 Aug;138(8):3349-58.
11. Alpdogan O, Muriglan SJ, Kappel BJ, Doubrovina E, Schmaltz C, Schiro R, et al. Insulin-like growth factor-I enhances lymphoid and myeloid reconstitution after allogeneic bone marrow transplantation. Transplantation. 2003 Jun 27;75(12):1977-83.
12. Jardieu P, Clark R, Mortensen D, Dorshkind K. In vivo administration of insulin-like growth factor-I stimulates primary B lymphopoiesis and enhances lymphocyte recovery after bone marrow transplantation. J Immunol. 1994 May 1;152(9):4320-7.
13. Montecino-Rodriguez E, Clark R, Dorshkind K. Effects of insulin-like growth factor administration and bone marrow transplantation on thymopoiesis in aged mice. Endocrinology. 1998 Oct;139(10):4120-6.
14. Hinton PS, Hirvonen MD, Ney DM, Keesey RE. Growth hormone, insulin-like growth factor-1, and the insulin-like growth factor binding proteins in rats maintaining reduced body protein following lesions of the lateral hypothalamus. Physiol Behav. 2000 Mar;68(5):667-72.
15. Hinton PS, Peterson CA, Lo HC, Yang H, McCarthy D, Ney DM. Insulin-like growth factor-I enhances immune response in dexamethasone-treated or surgically stressed rats maintained with total parenteral nutrition. JPEN J Parenter Enteral Nutr. 1995 Nov-Dec;19(6):444-52.
16. Landreth KS, Narayanan R, Dorshkind K. Insulin-like growth factor-I regulates pro-B cell differentiation. Blood. 1992 Sep 1;80(5):1207-12.
17. Tu W, Cheung PT, Lau YL. Insulin-like growth factor 1 promotes cord blood T cell maturation and inhibits its spontaneous and phytohemagglutinin-induced apoptosis through different mechanisms. J Immunol. 2000 Aug 1;165(3):1331-6.
18. Tu W, Cheung PT, Lau YL. IGF-I increases interferon-gamma and IL-6 mRNA expression and protein production in neonatal mononuclear cells. Pediatr Res. 1999 Dec;46(6):748-54.
19. Johnson EW, Jones LA, Kozak RW. Expression and function of insulin-like growth factor receptors on anti-CD3-activated human T lymphocytes. J Immunol. 1992 Jan 1;148(1):63-71.
20. Rosen CJ, Kurland ES, Vereault D, Adler RA, Rackoff PJ, Craig WY, et al. Association between serum insulin growth factor-I (IGF-I) and a simple sequence repeat in IGF-I gene: implications for genetic studies of bone mineral density. J Clin Endocrinol Metab. 1998 Jul;83(7):2286-90.
21. Missmer SA, Haiman CA, Hunter DJ, Willett WC, Colditz GA, Speizer FE, et al. A sequence repeat in the insulin-like growth factor-1 gene and risk of breast cancer. Int J Cancer. 2002 Jul 20;100(3):332-6.
22. Vaessen N, Heutink P, Janssen JA, Witteman JC, Testers L, Hofman A, et al. A polymorphism in the gene for IGF-I: functional properties and risk for type 2 diabetes and myocardial infarction. Diabetes. 2001 Mar;50(3):637-42.
23. Vaessen N, Janssen JA, Heutink P, Hofman A, Lamberts SW, Oostra BA, et al. Association between genetic variation in the gene for insulin-like growth factor-I and low birthweight. Lancet. 2002 Mar 23;359(9311):1036-7.
24. Landmann E, Geller F, Schilling J, Rudloff S, Foeller-Gaudier E, Gortner L. Absence of the wild-type allele (192 base pairs) of a polymorphism in the promoter region of the IGF-I gene but not a poly-

morphism in the insulin gene variable number of tandem repeat locus is associated with accelerated weight gain in infancy. Pediatrics. 2006 Dec;118(6):2374-9.
25. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol. 2004 Jan;18(1):61-72.
26. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006;21(6):475-84.
27. Weber JL, May PE. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet. 1989 Mar;44(3):388-96.
28. Allen NE, Davey GK, Key TJ, Zhang S, Narod SA. Serum insulin-like growth factor I (IGF-I) concentration in men is not associated with the cytosine-adenosine repeat polymorphism of the IGF-I gene. Cancer Epidemiol Biomarkers Prev. 2002 Mar;11(3):319-20.
29. Frayling TM, Hattersley AT, McCarthy A, Holly J, Mitchell SM, Gloyn AL, et al. A putative functional polymorphism in the IGF-I gene: association studies with type 2 diabetes, adult height, glucose tolerance, and fetal growth in U.K. populations. Diabetes. 2002 Jul;51(7):2313-6.
30. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Boerma GJ, Lotgering FK, et al. Correction for erythroid cell contamination in microassay for immunophenotyping of neonatal lymphocytes. Arch Dis Child Fetal Neonatal Ed. 1999 May;80(3):F226-9.
31. Cole TJ. Sympercents: symmetric percentage differences on the 100 log(e) scale simplify the presentation of log transformed data. Stat Med. 2000 Nov 30;19(22):3109-25.
32. Kooijman R, Willems M, De Haas CJ, Rijkers GT, Schuurmans AL, Van Buul-Offers SC, et al. Expression of type I insulin-like growth factor receptors on human peripheral blood mononuclear cells. Endocrinology. 1992 Nov;131(5):2244-50.
33. Xu X, Mardell C, Xian CJ, Zola H, Read LC. Expression of functional insulin-like growth factor-1 receptor on lymphoid cell subsets of rats. Immunology. 1995 Jul;85(3):394-9.
34. Walenkamp MJ, Wit JM. Genetic disorders in the growth hormone - insulin-like growth factor-I axis. Horm Res. 2006;66(5):221-30.
35. Rietveld I, Janssen JA, Hofman A, Pols HA, van Duijn CM, Lamberts SW. A polymorphism in the IGF-I gene influences the age-related decline in circulating total IGF-I levels. Eur J Endocrinol. 2003 Feb;148(2):171-5.
36. Geelhoed JJ, Mook-Kanamori DO, Witteman JC, Hofman A, van Duijn CM, Moll HA, et al. Variation in the IGF1 gene and growth in foetal life and infancy. The Generation R Study. Clin Endocrinol (Oxf). 2007 Sep 20.
37. Kajantie E, Fall CH, Seppala M, Koistinen R, Dunkel L, Yliharsila H, et al. Serum insulin-like growth factor (IGF)-I and IGF-binding protein-1 in elderly people: relationships with cardiovascular risk factors, body composition, size at birth, and childhood growth. J Clin Endocrinol Metab. 2003 Mar;88(3):1059-65.
38. Rietveld I, Janssen JA, van Rossum EF, Houwing-Duistermaat JJ, Rivadeneira F, Hofman A, et al. A polymorphic CA repeat in the IGF-I gene is associated with gender-specific differences in body height, but has no effect on the secular trend in body height. Clin Endocrinol (Oxf). 2004 Aug;61(2):195-203.
39. Montecino-Rodriguez E, Clark R, Johnson A, Collins L, Dorshkind K. Defective B cell development in Snell dwarf (dw/dw) mice can be corrected by thyroxine treatment. J Immunol. 1996 Oct 15;157(8):3334-40.
40. Montecino-Rodriguez E, Clark RG, Powell-Braxton L, Dorshkind K. Primary B cell development is impaired in mice with defects of the pituitary/thyroid axis. J Immunol. 1997 Sep 15;159(6):2712-9.
41. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Hop WC, Boerma GJ, et al. Neonatal blood lymphocyte subpopulations: a different perspective when using absolute counts. Biol Neonate. 2000 May;77(4):230-5.

42. Arends N, Johnston L, Hokken-Koelega A, van Duijn C, de Ridder M, Savage M, et al. Polymorphism in the IGF-I gene: clinical relevance for short children born small for gestational age (SGA). J Clin Endocrinol Metab. 2002 Jun;87(6):2720.
43. Rechler MM, Nissley SP. The nature and regulation of the receptors for insulin-like growth factors. Annu Rev Physiol. 1985;47:425-42.
44. Isaksson OG, Ohlsson C, Nilsson A, Isgaard J, Lindahl A. Regulation of cartilage growth by growth hormone and insulin-like growth factor I. Pediatr Nephrol. 1991 Jul;5(4):451-3.
45. Chirico G, Gasparoni A, Ciardelli L, Martinotti L, Rondini G. Leukocyte counts in relation to the method of delivery during the first five days of life. Biol Neonate. 1999 May;75(5):294-9.
46. De Amici D, Gasparoni A, Chirico G, Ceriana P, Bartoli A, Ramajoli I, et al. Natural killer cell activity and delivery: possible influence of cortisol and anesthetic agents. A study on newborn cord blood. Biol Neonate. 1999 Dec;76(6):348-54.

Summary and general discussion

5

In this chapter, the main findings of the studies are presented. Subsequently, methodological considerations are discussed and future perspectives on infectious diseases and the adaptive immune system in infants are described.

Main findings

All studies described in this thesis were embedded in the Generation R Study, a population-based prospective cohort study from early fetal life until young adulthood among 9,778 mothers and their children (1, 2). More detailed assessments of fetal and postnatal growth and development were conducted in a subgroup of 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study (1, 2). Studies on infectious diseases in pregnancy and infectious diseases in infancy were performed in the whole cohort study. Studies on nasopharyngeal carriage of *Streptococcus pneumoniae* and the adaptive immune system in infancy were conducted in the Generation R Focus Study.

Infectious diseases in pregnancy

The incidence of low birth weight and premature birth varies from 4.2% to 24.6% and 5 to 15%, respectively, depending on geographical and demographical features of the population studied (3-5). Delivery of low birth weight and premature infants is recognized as a major public health problem, accounting for the majority of neonatal death and morbidity in developed countries. In the last decades, there seems to be an upward trend in the incidence of premature births in Western societies (6-8).

Chlamydia trachomatis infection during pregnancy may influence pregnancy outcomes leading to adverse conditions, including preterm birth and low birth weight. The literature regarding these detrimental effects of *C. trachomatis* infection yields conflicting conclusions that seem primarily due to differences in study design, population and microbiological tests employed (9-19). We found evidence that *C. trachomatis* infection was associated with a 6-fold increased risk of preterm delivery before 32 weeks, but not with low birth weight (**chapter 2.1**). Of all preterm deliveries before 32 weeks gestation in our region 15.8% was attributable to *C. trachomatis* infection. The population attributable risk of *C. trachomatis* infection for preterm delivery before 35 weeks of gestation was 7.5%. Young age, Antillean and Surinamese ethnicity and single marital status were independent risk factors for *C. trachomatis* infection in pregnant women as were Cape Verdian, Surinamese and Moroccan or Turkish ethnicity and promiscuity of their partners.

Presence of urogenital tract infections during pregnancy is associated with low birth weight, preterm delivery, premature rupture of membranes (PROM) and other adverse pregnancy outcomes (10, 15, 20). Not much is known of the effects of urogenital

symptoms in different periods of pregnancy with pregnancy outcomes. This may be relevant for identifying critical time-periods that could be used for preventive strategies. Our study showed that mothers with vaginal bleeding in late pregnancy have an increased risk of preterm birth (**chapter 2.2**). Mothers with urinary tract symptoms present in early or multiple periods of pregnancy had an increased risk of low birth weight.

Infectious diseases in infancy

World wide, respiratory tract infections and gastrointestinal infections are the leading cause of morbidity in children (21, 22). In industrialized countries, prospective cohort studies in the last decade revealed prevalence's of 3.4% to 32.1% for respiratory tract disease and 1.2% to 26.3% for gastrointestinal disease in infancy (23-28). Various risk factors for upper respiratory tract infections, lower respiratory tract infections and gastrointestinal infections have been identified, including gestational age, birth weight, socioeconomic status, ethnicity, number of siblings, daycare attendance, maternal smoking and breastfeeding (23-44). Of these, maternal smoking and breastfeeding are the most modifiable influencing factors.

There is extended evidence that breastfeeding protects infants against infections in developing countries. In industrialized countries, the effect remains controversial mainly due to methodological flaws in various studies performed in the nineties. In our review, we described studies performed in industrialized countries from the late nineties until present (**chapter 3.1**). Altogether, it seems that breastfeeding protects infants against overall, gastrointestinal and respiratory tract infections in industrialized countries. A dose-response effect is suggested whereby a longer and more exclusive period of breastfeeding leads to a better protection against infection diseases. The benefits of 4 versus 6 months of exclusively breastfeeding are not completely clarified, although the World Health Organization recommended in 2001 to exclusively breastfeed infants for 6 months in all countries (45). We found that infants exclusively breastfed for 4 months had lower risks for upper and lower respiratory and gastrointestinal tract infections during the first 6 months and for lower respiratory tract infections between 7 and 12 months (**chapter 3.2**). These effects were explained by additional partially breastfeeding for 4 to 6 months. Exclusively breastfeeding for 6 months was only associated with upper respiratory tract infections from birth to 6 months.

The independent effects of maternal smoking in different periods of pregnancy and in the postnatal period on the risks of respiratory tract infections in young infants may be relevant for identifying critical periods for the effect of exposure of maternal smoking on airway and lung development and respiratory tract infections in infants. We observed weak evidence for an association of maternal smoking in the early postnatal period with lower respiratory tract infections in infants in the first 6 months of life (**chapter 3.3**). No effect of maternal smoking of different duration in pregnancy on respiratory tract

infections in infants was found. We did not observe dose-response effects for maternal smoking in different periods on respiratory tract infections.

Nasopharyngeal carriage of *S. pneumoniae* is mostly asymptomatic, but might lead to serious pneumococcal disease when the host-pathogen balance is disturbed (46-49). Population-based prospective cohort studies in infants regarding environmental factors, dynamics and interference of *S. pneumoniae* with other pathogens are scarce. Risk factors for pneumococcal carriage at 6 weeks of age were low birth weight and siblings (**chapter 3.4**). Having siblings was also associated with pneumococcal carriage at 6 months of age as was daycare attendance. Attending a daycare center was the only independent risk factor for pneumococcal carriage at 14 months of age. Furthermore, we found that pneumococcal carriage at 6 months of age predisposes for subsequent pneumococcal carriage at older age. No associations of gestational age, gender, maternal socio-economic status, maternal smoking or breastfeeding with pneumococcal carriage in infancy were found.

Immune system in infancy

One of the major reasons for the high rate of recurrent infections in infants might be their relative immature immune system. Several studies observed changes in absolute numbers of various lymphocyte subsets from birth until adulthood, especially in the first two years of life (50-52). The different maturation phases of the lymphocyte subsets in infancy are assessed in more detail and described in **chapter 4.1**.

Large population-based studies assessing the associations of gestational age, birth weight and fetal growth or other influencing pre-and perinatal factors with the development of the lymphocytes are scarce. We found that with increasing gestational age at birth, absolute numbers of T, B, NK lymphocytes and helper, cytotoxic and naïve T lymphocytes increase (**chapter 4.2**). Increased birth weight and fetal growth in late pregnancy were associated with higher absolute numbers of B lymphocytes, but not with T, NK and T lymphocyte subset counts. Furthermore, we observed that with increasing stress-related mode of delivery, absolute NK and T lymphocyte counts as well as several subset counts within the T lymphocyte population increased, but B lymphocyte counts did not (**chapter 4.3**). Lower 1-minute Apgar scores showed higher B and NK lymphocyte counts. Larger effects on these lymphocyte counts were observed for lower 5-minute Apgar scores and additionally on total T and cytotoxic T lymphocyte counts. Lower umbilical cord blood pH was associated with higher T, B, NK and memory T lymphocyte counts. Effects of Apgar scores and umbilical cord blood pH on other T lymphocyte subsets were not observed.

We hypothesized that variants of the promoter region of the *IGF-I* gene, which are associated with circulating IGF-I levels, may be related to an increase of absolute numbers of lymphocyte subsets. Our study showed that non-carriers of the 192-bp allele had

Table 1. Main findings of the studies presented in this thesis.

1. *Chlamydia trachomatis* infection and vaginal bleeding in late pregnancy are associated with increased risks of preterm delivery, but not with low birth weight.
2. In industrialized countries, breastfeeding protects infants against infectious diseases in infancy. Infants who are exclusively breastfed for 4 months and partially thereafter have lower risks for respiratory tract and gastrointestinal infections. Maternal smoking of different duration in pregnancy not affects respiratory tract infections in infants. Main risk factors for pneumococcal carriage at 6 weeks of age are low birth weight and siblings.
3. Fetal growth, birth weight, gestational age, stress- and hypoxia associated mode of delivery and a known IGF-I promotor region polymorphism are associated with changes in the absolute numbers lymphocytes (T, B and NK) and T lymphocyte subsets (helper, cytotoxic, naïve and memory) in neonates.

lower absolute numbers of T and B lymphocytes compared to 192-bp allele homozygous neonates (**chapter 4.4**). Within the T lymphocyte population, non-carriers had lower absolute numbers of helper T and naïve T lymphocytes. Of the remaining lymphocyte subsets, absolute numbers of NK lymphocytes and cytotoxic T lymphocytes tended to be lower in non-carriers, but memory T lymphocytes did not. No differences in absolute numbers of lymphocyte subsets and T lymphocyte subsets were observed between the 192-bp allele homozygous and 192-bp allele heterozygous groups.

Methodological considerations

The specific methodological considerations of the studies presented in this thesis have been discussed in the separate chapters. In this paragraph, selection bias, information bias and confounding are discussed in general for our studies perfomed within the Generation R Study.

Selection bias

Of all eligible children at birth, 61% participated in the Generation R Study. National and regional registries do not have subject characteristics in all eligible children and their parents that enables detailed non-response analyses (2). However, the percentages of mothers from different ethnicity, lower socio-economic status and the percentages of mothers or children with medical complications were lower among the participants than expected from the population figures in Rotterdam (2, 53). This selection towards a more affluent and healthy study population may be related to some determinants and outcomes separately, affecting the frequency rates and, as a consequence, the statistical power and generalizibility of the results. The prevalence and incidence rates found in the study should therefore carefully be interpreted considering the role of potential selec-

tion mechanisms. This selection leads only to bias in etiological studies if the selection mechanisms are both related to the determinant and outcome.

C. trachomatis could not be tested for 21% of initially enrolled women. However, all risk factors were similarly distributed among tested and untested women and no differences were found in the median gestational age and mean birth weight (40.1 weeks versus 40.1 weeks; p=0.33, 3419 grams versus 3418 grams; p=0.99). Also, no differences were found in the proportion of *C. trachomatis* infection between women included and not included in the analyses of gestational age and birth weight (3.6% versus 4.5%); p=0.57). Therefore, selection bias seems unlikely. Our numbers were too small to properly assess the association of *C. trachomatis* infection with abortion, stillbirth or perinatal death. Of all women enrolled in the study and with information about *C. trachomatis* infection, 62 (1.5%) had an abortion, stillbirth or perinatal death. Data on gestational age and birth weight were not available for women with the latter adverse pregnancy outcomes, but it is likely that these women delivered prematurely relatively more frequently. None of these women had a *C. trachomatis* infection. Theoretically, our effect estimates for the associations of chlamydial infection with gestational age and birth weight could be biased and exaggerated when these associations would differ between all fetuses and fetal `survivors`. This would be the case when *C. trachomatis* infection has a `protective` effect on early fetal death. However, this is most unlikely (12, 18).

Of all eligible participants of the Generation R Study, questionnaires with data on the main determinants were missing for breastfeeding (35%) and maternal smoking (2.9%). At the age of 6 months, infectious diseases were more present in those without information than those with information about breastfeeding (50.0% and 43.6%). No differences in prevalences of infectious diseases between those with and without information about maternal smoking were found. Selection bias due to non-response seems therefore only applicable to the effects of breastfeeding on infectious diseases at the age of 6 months, which might be underestimated. We do not expect that non-response to breastfeeding questions did affect the associations between breastfeeding and infectious diseases from the age of 7 through 12 months. Of all participants with breastfeeding data, information on infectious diseases was missing at the age of 6 in 9.8% and 12 months in 14.2%. Selection bias due to loss to follow-up seems therefore unlikely for the association of breastfeeding and infectious diseases. However, selection bias due to loss to follow-up could be present in our study on maternal smoking and infectious diseases since infants lost to follow-up more often had smoking mothers than non-smoking mothers (48.4% and 41.9%, respectively).

In our studies on influencing factors of absolute numbers of lymphocyte subsets in neonates, data were mostly missing for mode of delivery (5.3%) and umbilical cord blood pH (50.8%) of the eligible infants. Those with missing data on mode of delivery had lower geometric means of absolute numbers of T, B, NK and helper T lymphocytes.

The observed effects of mode of delivery on numbers of these lymphocytes may therefore be underestimated. Measurements of umbilical cord blood pH in the Netherlands are not routinely performed and neonates without umbilical cord blood pH were more frequently from uncomplicated pregnancies without fetal distress. Thus we expect that missing values in umbilical cord blood pH did not materially change the effect estimated but decreased the power of the study. Participating infants without cord blood samples more often had a lower gestational age, lower birth weight, more often were born by caesarean section and more often had a 192-bp allele homozygous genotype for *IGF-I* than infants with cord blood samples. These differences due to selective loss to follow-up might have led to an underestimation of the observed effects of the influencing factors on absolute numbers of the various lymphocyte subsets.

Information bias

Information about most of the determinants, including maternal urogenital symptoms, smoking, ethnicity, education, infants' feeding habits, siblings and daycare attendance were prospectively obtained by postal questionnaires. This information was recorded without direct reference to adverse birth outcomes, infectious diseases or pneumococcal carriage in infants. The parents were not aware of the specific research questions addressed in this thesis. However, misclassification may occur, especially for self-reported information about maternal smoking and breastfeeding. Assessment of adverse life style habits (i.e. smoking) by questionnaires may lead to underreporting. Random misclassification of the smoking and breastfeeding categories would lead to an underestimation of the effect estimates.

To overcome the limitation of underreporting of smoking, other studies have used biomarkers of tobacco exposure including cotinine in maternal urine, saliva or blood samples or nicotine in indoor air (9, 26, 54, 55). However, so far these studies have demonstrated that these biomarkers are not superior to self-report when studying the effect of maternal smoking on respiratory tract infections of their offspring (19, 26, 35, 37, 56, 57).

Misclassification of breastfeeding habits seems difficult to prevent. A recent review of studies performed between 1966 and 2003 showed that maternal report of breastfeeding is reliable through the age of 3 (58).

The main outcomes in our study assessed by questionnaires were self-reported doctor-attended upper and lower respiratory tract infections and gastointestinal infections. These infections were not compared with data from medical records and could be either under- or overreported by the parents. If independent of the determinant, the differences in effects are usually biased towards the null. It would be ideal to diagnose respiratory and gastrointestinal infections by detection of the pathogens in saliva or stools. In large cohort studies this is rarely done probably due to logistic and financial constraints.

Although assessing infectious diseases by questionnaires is not optimal, questionnaires are widely used in large epidemiological studies to obtain diagnoses and reliably reflect the true incidence of respiratory and gastrointestinal infections (28, 59-62)

Validity of the laboratory tests to detect *C. trachomatis*, *S. Pneumoniae* and absolute numbers of lymphocyte subsets are described in the relating chapters separately.

Confounding

Our interest was in the effects of maternal infectious diseases in pregnancy on birth outcomes and in the effect of breastfeeding and maternal smoking on infectious diseases in infancy. Adjustment for gestational age, birth weight, siblings, daycare attendance, gender, maternal ethnicity or education weakened the strengths of the associations. Misclassification of these variables seems not likely and probably do not cause a bias of the estimated effect in either direction. The adjustment for birth weight in the association of maternal smoking and infectious diseases in infants could be argued. We hypothesized that maternal smoking in pregnancy has its independent effect on lung development thereby increasing the risk of infectious diseases. However, it has been shown that birth weight can also act as an intermediate in the association of maternal smoking in pregnancy and symptoms of the respiratory tract (41). Therefore, the intermediating effect of birth weight on infections of the respiratory tract needs to be further explored.

Our interest was in the effect of pre-and perinatal influencing factors on absolute numbers of lymphocyte subsets in neonates. Regarding the literature and our own data analyses, proper adjustment for known and possible confounders was assessed. However, we were unable to take acute maternal infections during delivery into account which might dilute or exaggerate the observed effects.

Future research

Our study showed that *C. trachomatis* infection in pregnant women increases the risk of preterm delivery, especially early prematurity, such that a significant proportion of preterm deliveries can be attributed to this infection. *C. trachomatis* infection in pregnancy should be regarded as a serious public health problem, especially for young women belonging to certain socio-economic and ethnic groups at increased risk for *C. trachomatis* infection. Population-based screening on *C. trachomatis* infection in pregnancy in the Netherlands seems indicated. Since the prevalence, risk factors and adverse effects of a chlamydial infection are clarified, cost-benefits strategies for implying a chlamydial screening program in high risk pregnant women have to be explored.

Our studies suggest that dose-duration of maternal smoking and breastfeeding influences the risk of respiratory and gastrointestinal infections in infancy. Follow-up studies are needed to examine whether these effects persist at older ages. Furthermore,

the dose-duration effects on other outcomes including growth, development, asthma symptoms and allergy are of interest.

We examined numbers of lymphocyte subsets to express the developmental status of the adaptive immune system. Our studies suggest that fetal growth and development as well as perinatal stress and the *IGF-1* promoter region polymorphism influence absolute numbers of lymphocyte subsets in neonates. These findings underline the importance of focusing on environmental and genetic factors in the development of the adaptive immune system. Whether numbers of lymphocyte subsets are related to function of these cells remains to be assessed.

Further studies are needed to examine whether other influencing factors, including pre-eclampsia, pregnancy induced hypertension or acute urogenital infections of pregnant women during delivery, lead to changes in absolute lymphocyte subset counts in neonates. However, prevalences of these diagnoses are low, leading to less power to detect differences in absolute numbers of lymphocyte subsets in neonates. A nested case-control design in a larger cohort might overcome this issue. A lower gestational age at birth was associated with lower lymphocyte subset counts in neonates and suggests a relative more immature adaptive immune system. To what age these lower counts persist is unknown. If 'catch-up' growth in absolute numbers of lymphocyte subsets occurs, it is interesting to examine whether this might depend on the frequency rate of exposure to pathogens or other influencing factors including vaccination. Follow-up studies are also needed to examine whether the observed prenatal effects of stress-associated perinatal factors and the *IGF-I* promoter region polymorphism on lymphocyte subsets counts in neonates persist at older ages and have consequences for morbidity in childhood. Furthermore, the associations of postnatal factors including infections, vaccinations and nutrition with the development of lymphocyte subsets at older ages would be interesting to assess in this large cohort study. Blood samples of infants until adulthood will be preferable, although the difficulties of obtaining consent for

References

1. Hofman A, Jaddoe VW, Mackenbach JP, Moll HA, Snijders RF, Steegers EA, et al. Growth, development and health from early fetal life until young adulthood: the Generation R Study. Paediatr Perinat Epidemiol. 2004 Jan;18(1):61-72.
2. Jaddoe VW, Mackenbach JP, Moll HA, Steegers EA, Tiemeier H, Verhulst FC, et al. The Generation R Study: Design and cohort profile. Eur J Epidemiol. 2006 Jul 7.
3. Steer P. The epidemiology of preterm labor--a global perspective. J Perinat Med. 2005;33(4):273-6.
4. Slattery MM, Morrison JJ. Preterm delivery. Lancet. 2002 Nov 9;360(9344):1489-97.
5. Kramer MS. The epidemiology of adverse pregnancy outcomes: an overview. J Nutr. 2003 May;133(5 Suppl 2):1592S-6S.

6. Langhoff-Roos J, Kesmodel U, Jacobsson B, Rasmussen S, Vogel I. Spontaneous preterm delivery in primiparous women at low risk in Denmark: population based study. Bmj. 2006 Apr 22;332(7547):937-9.
7. Stoelhorst GM, Rijken M, Martens SE, Brand R, den Ouden AL, Wit JM, et al. Changes in neonatology: comparison of two cohorts of very preterm infants (gestational age <32 weeks): the Project On Preterm and Small for Gestational Age Infants 1983 and the Leiden Follow-Up Project on Prematurity 1996-1997. Pediatrics. 2005 Feb;115(2):396-405.
8. Davidoff MJ, Dias T, Damus K, Russell R, Bettegowda VR, Dolan S, et al. Changes in the gestational age distribution among U.S. singleton births: impact on rates of late preterm birth, 1992 to 2002. Semin Perinatol. 2006 Feb;30(1):8-15.
9. Alger LS, Lovchik JC, Hebel JR, Blackmon LR, Crenshaw MC. The association of Chlamydia trachomatis, Neisseria gonorrhoeae, and group B streptococci with preterm rupture of the membranes and pregnancy outcome. Am J Obstet Gynecol. 1988 Aug;159(2):397-404.
10. Gravett MG, Nelson HP, DeRouen T, Critchlow C, Eschenbach DA, Holmes KK. Independent associations of bacterial vaginosis and Chlamydia trachomatis infection with adverse pregnancy outcome. Jama. 1986 Oct 10;256(14):1899-903.
11. Harrison HR, Alexander ER, Weinstein L, Lewis M, Nash M, Sim DA. Cervical Chlamydia trachomatis and mycoplasmal infections in pregnancy. Epidemiology and outcomes. Jama. 1983 Oct 7;250(13):1721-7.
12. Martin DH, Koutsky L, Eschenbach DA, Daling JR, Alexander ER, Benedetti JK, et al. Prematurity and perinatal mortality in pregnancies complicated by maternal Chlamydia trachomatis infections. Jama. 1982 Mar 19;247(11):1585-8.
13. Martius J, Krohn MA, Hillier SL, Stamm WE, Holmes KK, Eschenbach DA. Relationships of vaginal Lactobacillus species, cervical Chlamydia trachomatis, and bacterial vaginosis to preterm birth. Obstet Gynecol. 1988 Jan;71(1):89-95.
14. Sweet RL, Landers DV, Walker C, Schachter J. Chlamydia trachomatis infection and pregnancy outcome. Am J Obstet Gynecol. 1987 Apr;156(4):824-33.
15. Andrews WW, Goldenberg RL, Mercer B, Iams J, Meis P, Moawad A, et al. The Preterm Prediction Study: association of second-trimester genitourinary chlamydia infection with subsequent spontaneous preterm birth. Am J Obstet Gynecol. 2000 Sep;183(3):662-8.
16. Association of Chlamydia trachomatis and Mycoplasma hominis with intrauterine growth retardation and preterm delivery. The John Hopkins Study of Cervicitis and Adverse Pregnancy Outcome. Am J Epidemiol. 1989 Jun;129(6):1247-57.
17. Claman P, Toye B, Peeling RW, Jessamine P, Belcher J. Serologic evidence of Chlamydia trachomatis infection and risk of preterm birth. Cmaj. 1995 Aug 1;153(3):259-62.
18. Gencay M, Koskiniemi M, Ammala P, Fellman V, Narvanen A, Wahlstrom T, et al. Chlamydia trachomatis seropositivity is associated both with stillbirth and preterm delivery. Apmis. 2000 Sep;108(9):584-8.
19. Andrews WW, Klebanoff MA, Thom EA, Hauth JC, Carey JC, Meis PJ, et al. Midpregnancy genitourinary tract infection with Chlamydia trachomatis: association with subsequent preterm delivery in women with bacterial vaginosis and Trichomonas vaginalis. Am J Obstet Gynecol. 2006 Feb;194(2):493-500.
20. Pararas MV, Skevaki CL, Kafetzis DA. Preterm birth due to maternal infection: Causative pathogens and modes of prevention. Eur J Clin Microbiol Infect Dis. 2006 Sep;25(9):562-9.
21. World Health Organization. Major causes of death among children under 5 years of age and neonates in the world, 2000-2003. http://www.who.int/child-adolescent-health/OVERVIEW/CHILD_HEALTH/map_00-03_world.jpg: The World Health Organization; 2003.
22. Chretien J, Holland W, Macklem P, Murray J, Woolcock A. Acute respiratory infections in children. A global public-health problem. N Engl J Med. 1984 Apr 12;310(15):982-4.
23. Wright AL, Taussig LM, Ray CG, Harrison HR, Holberg CJ. The Tucson Children's Respiratory Study. II. Lower respiratory tract illness in the first year of life. Am J Epidemiol. 1989 Jun;129(6):1232-46.

24. Neuspiel DR, Rush D, Butler NR, Golding J, Bijur PE, Kurzon M. Parental smoking and post-infancy wheezing in children: a prospective cohort study. Am J Public Health. 1989 Feb;79(2):168-71.
25. Baker D, Taylor H, Henderson J. Inequality in infant morbidity: causes and consequences in England in the 1990s. ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. J Epidemiol Community Health. 1998 Jul;52(7):451-8.
26. Brunekreef B, Leaderer BP, van Strien R, Oldenwening M, Smit HA, Koopman L, et al. Using nicotine measurements and parental reports to assess indoor air: the PIAMA birth cohort study. Prevention and Incidence of Asthma and Mite Allergy. Epidemiology. 2000 May;11(3):350-2.
27. Panico L, Bartley M, Marmot M, Nazroo JY, Sacker A, Kelly YJ. Ethnic variation in childhood asthma and wheezing illnesses: findings from the Millennium Cohort Study. Int J Epidemiol. 2007 May 24.
28. Quigley MA, Kelly YJ, Sacker A. Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study. Pediatrics. 2007 Apr;119(4):e837-42.
29. Celedon JC, Litonjua AA, Weiss ST, Gold DR. Day care attendance in the first year of life and illnesses of the upper and lower respiratory tract in children with a familial history of atopy. Pediatrics. 1999 Sep;104(3 Pt 1):495-500.
30. Marbury MC, Maldonado G, Waller L. Lower respiratory illness, recurrent wheezing, and day care attendance. Am J Respir Crit Care Med. 1997 Jan;155(1):156-61.
31. Leeder SR, Corkhill R, Irwig LM, Holland WW, Colley JR. Influence of family factors on the incidence of lower respiratory illness during the first year of life. Br J Prev Soc Med. 1976 Dec;30(4):203-12.
32. Fergusson DM, Horwood LJ, Shannon FT, Taylor B. Parental smoking and lower respiratory illness in the first three years of life. J Epidemiol Community Health. 1981 Sep;35(3):180-4.
33. Martinez FD, Wright AL, Holberg CJ, Morgan WJ, Taussig LM. Maternal age as a risk factor for wheezing lower respiratory illnesses in the first year of life. Am J Epidemiol. 1992 Nov 15;136(10):1258-68.
34. Paradise JL, Rockette HE, Colborn DK, Bernard BS, Smith CG, Kurs-Lasky M, et al. Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. Pediatrics. 1997 Mar;99(3):318-33.
35. Margolis PA, Keyes LL, Greenberg RA, Bauman KE, LaVange LM. Urinary cotinine and parent history (questionnaire) as indicators of passive smoking and predictors of lower respiratory illness in infants. Pediatr Pulmonol. 1997 Jun;23(6):417-23.
36. Greenough A, Giffin FJ, Yuksel B. Respiratory morbidity in preschool children born prematurely. Relationship to adverse neonatal events. Acta Paediatr. 1996 Jul;85(7):772-7.
37. Rylander E, Pershagen G, Eriksson M, Bermann G. Parental smoking, urinary cotinine, and wheezing bronchitis in children. Epidemiology. 1995 May;6(3):289-93.
38. Ponsonby AL, Couper D, Dwyer T, Carmichael A, Kemp A. Relationship between early life respiratory illness, family size over time, and the development of asthma and hay fever: a seven year follow up study. Thorax. 1999 Aug;54(8):664-9.
39. Nafstad P, Hagen JA, Oie L, Magnus P, Jaakkola JJ. Day care centers and respiratory health. Pediatrics. 1999 Apr;103(4 Pt 1):753-8.
40. Duncan B, Ey J, Holberg CJ, Wright AL, Martinez FD, Taussig LM. Exclusive breast-feeding for at least 4 months protects against otitis media. Pediatrics. 1993 May;91(5):867-72.
41. Caudri D, Wijga A, Gehring U, Smit HA, Brunekreef B, Kerkhof M, et al. Respiratory symptoms in the first 7 years of life and birth weight at term: the PIAMA Birth Cohort. Am J Respir Crit Care Med. 2007 May 15;175(10):1078-85.
42. Lux AL, Henderson AJ, Pocock SJ. Wheeze associated with prenatal tobacco smoke exposure: a prospective, longitudinal study. ALSPAC Study Team. Arch Dis Child. 2000 Oct;83(4):307-12.
43. Sherriff A, Peters TJ, Henderson J, Strachan D. Risk factor associations with wheezing patterns in children followed longitudinally from birth to 3(1/2) years. Int J Epidemiol. 2001 Dec;30(6):1473-84.
44. Kamper-Jorgensen M, Wohlfahrt J, Simonsen J, Thrane N, Benn CS. Temporal trend in paediatric infections in Denmark. Arch Dis Child. 2006 May;91(5):401-4.

45. World Health Organization, Department of Nutrition for Health and Development. The optimal duration of exclusive breastfeeding: report of an expert consultation. Geneva, Switzerland: World Health Organization; 2001.
46. Hament JM, Kimpen JL, Fleer A, Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. FEMS Immunol Med Microbiol. 1999 Dec;26(3-4):189-95.
47. Mulholland K. Strategies for the control of pneumococcal diseases. Vaccine. 1999 Jul 30;17 Suppl 1:S79-84.
48. Plotkowski MC, Puchelle E, Beck G, Jacquot J, Hannoun C. Adherence of type I *Streptococcus pneumoniae* to tracheal epithelium of mice infected with influenza A/PR8 virus. Am Rev Respir Dis. 1986 Nov;134(5):1040-4.
49. Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis. 2004 Mar;4(3):144-54.
50. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr. 1997 Mar;130(3):388-93.
51. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003 Nov;112(5):973-80.
52. Shahabuddin S, Al-Ayed I, Gad El-Rab MO, Qureshi MI. Age-related changes in blood lymphocyte subsets of Saudi Arabian healthy children. Clin Diagn Lab Immunol. 1998 Sep;5(5):632-5.
53. Center for Research and Statistics (COS). http://www.cos.rotterdam.nl. Rotterdam; 2005.
54. Patrick DL, Cheadle A, Thompson DC, Diehr P, Koepsell T, Kinne S. The validity of self-reported smoking: a review and meta-analysis. Am J Public Health. 1994 Jul;84(7):1086-93.
55. Wang X, Tager IB, Van Vunakis H, Speizer FE, Hanrahan JP. Maternal smoking during pregnancy, urine cotinine concentrations, and birth outcomes. A prospective cohort study. Int J Epidemiol. 1997 Oct;26(5):978-88.
56. O'Connor TZ, Holford TR, Leaderer BP, Hammond SK, Bracken MB. Measurement of exposure to environmental tobacco smoke in pregnant women. Am J Epidemiol. 1995 Dec 15;142(12):1315-21.
57. Gehring U, Leaderer BP, Heinrich J, Oldenwening M, Giovannangelo ME, Nordling E, et al. Comparison of parental reports of smoking and residential air nicotine concentrations in children. Occup Environ Med. 2006 Nov;63(11):766-72.
58. Li R, Scanlon KS, Serdula MK. The validity and reliability of maternal recall of breastfeeding practice. Nutr Rev. 2005 Apr;63(4):103-10.
59. Bachrach VR, Schwarz E, Bachrach LR. Breastfeeding and the risk of hospitalization for respiratory disease in infancy: a meta-analysis. Arch Pediatr Adolesc Med. 2003 Mar;157(3):237-43.
60. Pardo-Crespo R, Perez-Iglesias R, Llorca J, Alvarez-Granda L, Garcia-Fuentes M, Martinez-Gonzalez MA, et al. Breast-feeding and risk of hospitalization for all causes and fever of unknown origin. Eur J Public Health. 2004 Sep;14(3):230-4.
61. Chantry CJ, Howard CR, Auinger P. Full breastfeeding duration and associated decrease in respiratory tract infection in US children. Pediatrics. 2006 Feb;117(2):425-32.
62. Paricio Talayero JM, Lizan-Garcia M, Otero Puime A, Benlloch Muncharaz MJ, Beseler Soto B, Sanchez-Palomares M, et al. Full breastfeeding and hospitalization as a result of infections in the first year of life. Pediatrics. 2006 Jul;118(1):e92-9.

Samenvatting

Het onderzoek beschreven in dit proefschrift is uitgevoerd in het Generation R onderzoek. Het Generation R onderzoek is een populatie-gebaseerd prospectief cohort onderzoek bij 9778 moeders en kinderen vanaf het vroege foetale leven tot de volwassenheid. Het onderzoek gericht op infectieziekten in de zwangerschap en in het eerste jaar is uitgevoerd in dit gehele cohort. Aanvullende, gedetailleerde metingen zijn uitgevoerd in een subgroep van 1232 moeders en kinderen. Dit subgroep onderzoek wordt het Generation R Focus onderzoek genoemd. Het onderzoek gericht op dragerschap van *Streptococcus pneumoniae* en het immuunsysteem is uitgevoerd in deze subgroep.

Infectieziekten in de zwangerschap

Het onderzoek beschreven in **hoofdstuk 2** is gericht op de effecten van een *Chlamydia trachomatis* infectie, één van de belangrijkste seksueel overdraagbare aandoeningen, en subklinische urogenitale symptomen bij moeder in de zwangerschap op het risico voor het krijgen van een kind met een laag geboortegewicht of prematuriteit. Laag geboortegewicht en prematuriteit zijn de belangrijkste risicofactoren voor neonatale sterfte en morbiditeit in Westerse landen. De afgelopen decennia lijkt er een trend zichtbaar naar een toenemende incidentie van prematuriteit in Nederland.

Hoofdstuk 2.1 laat zien dat een *C. trachomatis* infectie geassocieerd is met een 6-keer hoger risico op prematuriteit met een zwangerschapsduur korter dan 32 weken. Er werd geen relatie met laag geboortegewicht gevonden. Van alle prematuren met een zwangerschapsduur korter dan 32 weken, lijkt bijna 16% verklaarbaar door een *C. trachomatis* infectie. Van alle prematuren met een zwangerschapsduur van korter dan 35 weken, is dit 7.5%. In ons onderzoek waren bij de moeders een jonge leeftijd, een Antilliaanse of Surinaamse etniciteit en ongetrouwd zijn risicofactoren voor een infectie met *C. trachomatis* infectie. Bij de partners waren Kaap Verdiaanse, Surinaamse en Marokkaanse of Turkse etniciteit en verschillende sexuele contacten risicofactoren.

De aanwezigheid van urogenitale infecties gedurende de zwangerschap lijkt gerelateerd te zijn aan het risico op prematuur gebroken vliezen, prematuriteit en andere ongunstige geboorteuitkomsten. In **hoofdstuk 2.2** wordt aangetoond dat vaginaal bloedverlies in het laatste deel van de zwangerschap leidt tot een hoger risico op prematuriteit. Daarnaast blijkt dat moeders met urogenitale symptomen in de vroege zwangerschap of in verschillende periodes van de zwangerschap een verhoogd risico hebben op het krijgen van een kind met een laag geboortegewicht.

Infectieziekten in het eerste levensjaar

Infectieziekten zijn de belangrijkste oorzaak van morbiditeit in de eerste levensjaren. Eerdere studies hebben gesuggereerd dat meer dan 25% van de kinderen luchtweg en

gastrointestinale infecties in het eerste levensjaar doormaakt. Bekende risico factoren voor luchtweg en gastrointestinale infecties zijn de zwangerschapsduur, het geboortegewicht, de sociaal economische status, de etniciteit, het aantal broers en zussen, bezoek aan het kinderdagverblijf, het roken door moeder en het krijgen van borstvoeding. De laatste 2 risicofactoren lijken het meest beïnvloedbaar.

Wij concludeerden op basis van eerdere studies dat borstvoeding een beschermend effect heeft op het krijgen van luchtweg en gastrointestinale infecties in geïndustrialiseerde landen (**hoofdstuk 3.1**). Een langere periode van exclusieve borstvoeding lijkt het grootste beschermende effect te hebben. Uit het Generation R onderzoek blijkt dat zuigelingen die minimaal 4 maanden exclusief borstvoeding krijgen en daarna borstvoeding combineren met flesvoeding een lager risico hebben op bovenste en onderste luchtweginfecties en gastrointestinale infecties in het eerste levensjaar. Het grootste effect was zichtbaar op infectieziekten in de eerste 6 maanden (**hoofdstuk 3.2**).

In **hoofdstuk 3.3** worden de relaties bestudeerd tussen enerzijds roken door moeder in verschillen perioden in de zwangerschap en in de eerste maanden postnataal en anderzijds het risico op luchtweginfecties in het eerste levensjaar. Hiermee kunnen specifieke perioden in het vroege leven die kritiek zijn voor de ontwikkeling van de luchtwegen en voor het risico op luchtweg infecties geïdentificeerd worden. Wij vonden een zwakke relatie tussen roken door moeder in de vroege postnatale periode en het risico op onderste luchtweginfecties. Wij vonden geen relatie tussen het roken in verschillende perioden in de zwangerschap en het risico op luchtweginfecties.

Eén van de belangrijkste bacteriële verwekkers van luchtweginfecties is de *Streptococcus pneumoniae*. Dragerschap van de *S. pneumoniae* is meestal asymptomatisch maar kan leiden tot milde en ernstige luchtweginfecties. Factoren van invloed op dragerschap van *S. pneumoniae* en de interactie met andere bacteriën is niet uitgebreid bestudeerd in populatie-gebasseerd prospectief cohort onderzoek. Laag geboortegewicht en het hebben van broers of zussen lijken risicofactoren voor dragerschap in de eerste maanden (**hoofdstuk 3.4**). Bezoek aan een kinderdagverblijf is gerelateerd aan *S. pneumoniae* dragerschap op 6 en 14 maanden.

Het immuunsysteem in het eerste levensjaar

Eén van de belangrijkste redenen voor het hoge aantal infectieziekten in het eerste levensjaar is het relatieve onrijpe afweersysteem. In **hoofdstuk 4** worden de ontwikkeling van het afweer systeem en de factoren van invloed daarop bestudeerd.

In **hoofdstuk 4.1** wordt de ontwikkeling en uitrijping van het immuunsysteem bestudeerd. Hiervoor zijn T, B, NK lymfocyten en hun verschillende subsets bepaald in bloedmonsters die verzameld zijn bij de geboorte en op de leeftijd van 6, 14 en 24 maanden. De gepresenteerde gegevens kunnen als normaal waarden voor gezonde kinderen gebruikt worden.

Hoofdstuk 4.2 laat zien dat een langere zwangerschapsduur positief geassocieerd is met de absolute aantallen T, B, NK lymfocyten en helper, cytotoxische and naïeve T lymfocyten. Een hoger geboortegewicht en snellere foetale groei in de laatste fase van de zwangerschap zijn alleen geassocieerd met een hoger aantal B lymfocyten. In **hoofdstuk 4.3** laten we zien dat stress gerelateerde bevallingen geassocieerd zijn met het aantal NK and T lymfocyten en met verschillende aantallen lymfocyten binnen de subset van T lymfocyten. Er was geen relatie met B lymfocyten. Een lage Apgar score na 1 minuut leidt tot een hoger aantal hogere B en NK lymfocyten. Grotere effecten op deze en ook de T lymfocyten zijn zichtbaar voor de Apgar score na 5 minuten. Een lage pH in het navelstrengbloed is geassocieerd met een hoger aantal T, B, NK and memory T lymfocyten. Er waren geen effecten van de Apgar score en van de pH in het navelstreng bloed op andere T lymfocyten.

In **hoofdstuk 4.4** hebben we onderzocht of een bekende variant in de promoter regio van het insuline-like growth factor-I (*IGF-I*) gen geassocieerd is met verschillen in aantallen lymfocyten. Eerder onderzoek heeft aangetoond dat deze genetische variant gerelateerd is aan IGF-1 niveaus in het bloed. Kinderen die geen drager waren van het 192-bp allel (non-carriers) hadden lagere aantallen T and B lymfocyten in vergelijking met 192-bp allel homozygoten. Non-carriers hadden tevens lagere aantallen helper en naïeve T lymfocyten.

In **hoofdstuk 5**, de algemene discussie, worden de resultaten van dit proefschrift beschreven en in een bredere context geplaatst. Bovendien worden relevante methodologische kwesties bediscussieerd en suggesties gegeven voor toekomstig onderzoek.

Dankwoord

'Wat Jantje is, zal Jan worden', prof.dr. H.K.A. Visser, 19 mei 1995.

Als eerste promovendus vanuit de afdeling kindergeneeskunde mocht ik mij vlak na de start van het Generation R onderzoek bij dit grote avontuur aansluiten. Mijn eerste en grootste dank gaat uit naar de vele enthousiaste, kundige, gedreven wetenschappers en medewerkers die betrokken zijn bij de groei, ontwikkeling en gezondheid van het Generation R onderzoek en de duizenden kinderen en hun ouders waarvan wij belangeloos unieke gegevens mogen verzamelen!
Henriëtte Moll, van co-promotor tot promotor, nu voor de eerste maal in de grote commissie als prof, super! Dank voor de bewogen jaren, de vrijheid die je me gaf en je altijd aanwezige optimisme. Ronald de Groot, mijn promotor 'op afstand', bedankt voor het doorploegen van een heel manuscript in korte tijd.
Bert Hofman, dank dat ik met jouw gedrevenheid, enthousiasme, kennis en kunde de wondere wereld van de epidemiologie en het opzetten van een cohort onderzoek mocht ontdekken. Herbert Hooijkaas, het immuunsysteem is een beetje zoals de Generation R populatie, veel subpopulaties die door verschillende factoren op hun eigen manier matureren, kortom ongelofelijk complex. Je hebt mij, als clinicus, daar op een enorm vriendelijke en bereidwillige manier een beetje wegwijs in kunnen maken, bedankt. Johan de Jongste, jouw inzet tot het inhoudelijk en taalkundig beter worden van het manuscript staat niet in verhouding tot het aantal co-auteurschappen op mijn papers…bedankt.
Bert van der Heijden en Matthijs de Hoog, dank voor de mogelijkheid om wetenschappelijk onderzoek met de opleiding tot kinderarts te combineren. Anja Vaessen-Verberne, stimulerend, motiverend en waakzaam om het maximale uit mijn opleiding tot kinderarts te halen, je bent een opleider pur sang! De levendigheid en Brabantse gezelligheid van jou en de andere Bredaase kinderartsen siert jullie. Muppets i.o., dank voor jullie altijd aanwezige begrip en mogelijkheid tot -tig keer ruilen van diensten deze afgelopen maanden.
Henri Verbrugh, Ingrid Rours, Roel Verkooijen en het lab Medische Microbiologie, dank voor jullie bewogen samenwerking en de Chlamydia-substudie. Peter Hermans, Debby Bogaert, Marcel Sluijter en laboranten, peumococcen goeroes, bedankt voor de samenwerking.
Liesbeth B., naam- en schrijfgenoot, ook jij hebt mij veel van de immunologie bijgebracht. Dank voor je inzet, elke keer weer. De mooiste immuno-papers zullen nog volgen!
Romana, Jac, Marja, Ivonne, Anja en Mirjam, immunologie-laboranten, jullie hebben een enorme hoeveelheid werk verricht om de grote hoeveelheid bloedmonsters te analyseren. De taart komt echt…
Het Generation R team, de huidige en oud-medewerkers en promovendi zijn er inmiddels te veel om persoonlijk te bedanken. Mede dankzij jullie is dit proefschrift tot stand

gekomen. Maar bovenal leveren jullie een fantastische ondersteuning aan het Generation R onderzoek! Het Generation R Focus team, de logistiek en uitvoering zijn helemaal vanaf het begin samen met jullie opgezet. Het was spannend, uitdagend en heel gezellig, enorm bedankt! Christi en de bloedmonsters, ach, je weet het wel…super bedankt! Het Generation R Management team en het 'Klein MT', dank voor de samenwerking en de vele leermomenten. Claudia, bedankt voor je positiviteit en je altijd snelle reply's met het zoveelste geupdate databestand. Ernst-Jan, Vincent, Mijke, Sabine en Bero, om als eerste GR-promovendi vanuit diverse afdelingen een onderzoek mee helpen op te zetten vergt gedrevenheid. 'Never ever give up' lijkt jullie slogan te zijn. Bedankt voor de mooie herinnering en de vele borrels…met bittergarnituur!

Karin, zusje van me, paranimf, ben er trots op dat je naast me staat. Als iemand met passie en vuur strijdt voor een doel wat ie voor ogen heeft, dan ben jij het wel! Dank voor je steun de afgelopen maanden!

Joost, paranimf, van frequentie-tabelletjes tot confounder-modelling, van ambitieuze onderzoeker tot fotomodelletje van GR, van Kluun tot 3000 pagina's over 1 pneumoc (lekker ontspannen lezen, duh!), van puinzooi tot Baukje, van 'het allermooiste-Thaise-meisje-wat-ik-ooit-gezien-heb' (elke hoek weer 1) tot 'alive and kicking' Anouk, van poepiedoepiesnoepie Vincent tot Vinnie mét dolcegabbanalightblueman, van strak, marineblauw-wit gestreept t-shirtje (zó 2007!) tot door stof bedekte smoking, van Joost Wilhem Maria Labout tot JLa. We've shared it all…X Roomie.

Troetje, toch ook een klein woordje aan jou, zo na dertien jaar vriendschap en periodiek collega's zijn. Het doet me goed je in die jaren te hebben zien groeien van serieuze, nette, ambitieuze topstudent tot geinfantiliseerde wetenschapper met kwik-kwek-kwak-gelkuifje met bijpassende, kleurrijke, zeg maar, sokken. Bovendien wist je me altijd weer op te vrolijken met je stralende glimlach, gezellige afdelingsroddels, rustige aanwezigheid, en het dagelijks netjes op orde houden van mijn op auteur, jaartal en tijdschrift gerangschikte artikelen, mappen en boeken. Bedankt!

Van origine Leidse medische studie-vrienden, inmiddels aangevuld met private bankers, juristen, managers, verpleegkundigen en veel, heel veel garnalen, dank voor de enorme lol die we elke keer weer hebben! Dikke knuffel van T. Lies. Ook groetjes aan de Jaddoetjes. Emy en Theo, dank voor jullie steun tijdens al mijn opleidingsjaren. Indonesië was een soort van thuiskomen…

Caroline, Karin en Wouter, zussies en Da Lill' Bro, zo verschillend en toch zo hetzelfde. We zien elkaar weinig, maar dat maakt jullie betekenis voor mij niet minder, integendeel!

Pap en mam, om iets op eigen kracht te kunnen doen moet je wel de nodige basis hebben meegekregen. Jullie hebben ieder op je eigen manier daaraan bijgedragen. Knuf!

Vincent, lieffie, partner in science en in nog zo veel meer…bedankt!

'Wat Liesje was, is Liesbeth geworden', tijd voor een feestje!

About the author

Liesbeth Duijts was born in Haarlem, The Netherlands, on September 4th, 1974. She passed her secondary school exam (Gymnasium) in 1992 at the Stedelijk Gymnasium in Haarlem. In the same year, she started to study medicine at the Leiden University Medical Center (LUMC). During her study, she had clinical trainings at the Diaconessen Hospital, Paramaribo, Surinam. She participated in a research project on the effect of pre-emptive treatment of CMV infection during bone marrow transplantation on morbidity and mortality in children at the Department of Pediatrics, LUMC, Leiden (former head prof.dr. J.M. Vossen). After obtaining her medical degree, she worked as a resident in pediatrics (ANIOS) at the Albert Schweitzer Hospital (head dr. R. Schornagel) and the Medisch Centrum Rijnmond-Zuid (former head Dr. E.J.A. Gerritsen). In March 2002, she started with the research project presented in this thesis. She was a member of the Generation R Management Team and coordinator of the Generation R Focus Study in 2003 and 2004. She obtained a Master of Science degree in Clinical Epidemiology from the Netherlands Institute for Health Sciences (Nihes) in Rotterdam in 2004. From August 2004, she combined her research training with a residency in pediatrics at the Erasmus Medical Center, Sophia Children's Hospital (head prof. dr. A.J. van der Heijden, Dr. M. de Hoog). She currently works at the Amphia Hospital, Breda, as part of her training in pediatrics (head dr. A.A.P.H. Vaessen-Verberne).