EPIDEMIOLOGICAL AND GENETIC STUDY OF RESPIRATORY DISTRESS SYNDROME IN PRETERM INFANTS
Specific aspects of twin and multiple births

Abstract in Finnish
RIITTA MARTTILA

EPIDEMIOLOGICAL AND GENETIC STUDY OF RESPIRATORY DISTRESS SYNDROME IN PRETERM INFANTS
Specific aspects of twin and multiple births

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium L12 of the Department of Paediatrics, on December 12th, 2003, at 12 noon.

OUULUN YLIOPISTO, OULU 2003
Marttila, Riitta, Epidemiological and genetic study of respiratory distress syndrome in preterm infants. Specific aspects of twin and multiple births
Department of Paediatrics, University of Oulu, P.O.Box 5000, FIN-90014 University of Oulu, Finland
Oulu, Finland
2003

Abstract
Respiratory distress syndrome, RDS, is a multifactorial lung disease of premature infants. The main cause of RDS is a deficiency of pulmonary surfactant, a lipoprotein mixture required to reduce surface tension at the air-liquid interface and to prevent generalized atelectasis of the alveolar ducts and alveoli. Prematurity is the most important factor predisposing to RDS. During the past decade the number of multiple pregnancies has increased significantly as a result of diversified infertility treatments and advanced maternal age. Due to the considerably higher rate of preterm births of multiples compared to singletons, RDS is one of the major causes of morbidity among them.

The objectives of the present research were to evaluate the incidence and risk factors of RDS in twins compared to singletons, and to assess the role of SP-A and SP-B gene variations and gene-environment interactions in the susceptibility to the disease in a population of preterm twins and higher order multiples.

This research showed that during the past fifteen years the gestational age-specific incidence of RDS has declined. Twin infants do not have increased risk of RDS except when born very immaturely at a very early gestational age. The presenting twin is less susceptible to RDS than the non-presenting twin or singleton infant after 28 weeks of gestation until term. Additionally the SP-B Ile131Thr polymorphism was shown to affect the susceptibility specifically in the presenting twin. The role of SP-A polymorphisms in the risk of RDS in twins turned out to be different from that in singletons. The major allele and genotype of SP-A1 were associated with a decreased risk of RDS in near-term twin infants. The threonine allele in SP-B Ile131Thr appeared interactively with SP-A1 to associate with the risk of RDS both in twins and in singletons: associating with a lower risk of RDS in singletons at very early gestation, but surprisingly associating with a protection in twins and multiples from RDS near term. The risk of RDS, defined by the interaction of SP-A and SP-B alleles, was additionally associated with the fetal mass. Thus, the difference in the susceptibility to RDS in premature singletons and multiples may depend on the size of the conceptus.

In an evaluation of the genetic risk factors for RDS, the classical twin study method comparing the concordance of a disease between MZ and DZ twins underestimates the extent of heredity. Several predominant intrauterine and perinatal environmental factors contribute to disease susceptibility regardless of zygosity and are suspected to override the hereditary components of RDS. Twin gestation was shown to be an effect modifier in the genetic susceptibility to RDS.

Keywords: gene polymorphism, multiple pregnancy, prematurity, preterm birth, pulmonary surfactant-associated proteins, respiratory distress syndrome
Marttila, Riitta, Ennenaikaisena syntyneen lapsen hengitysvaikeusoireyhtymän epidemiologinen ja geneettinen tutkielma: erityisen tarkastelun kohteena kaksos- ja monisikiöiset synnytykset

Lastentautien klinikka, Oulun yliopisto, PL 5000, 90014 Oulun yliopisto

2003

Oulu, Finland

**Tiivistelmä**


Tämän tutkimuksen tarkoituksen oli selvittää RDS-taudin ilmamaantuvuutta ja riskitekijöitä kaksosilla verrattuna yksostiin, tutkia surfaktanttiproteiinien A ja B geenivaihteluita sekä geenien ja ympäristötekijöiden vuorovaikutusta kaksosten ja monisikiöisistä sairauksista syntyneiden lasten RDS-taudissa.


**Asiasanat:** ennenaikainen synnytys, epäkypsyys, geenien monimuotoisuus, hengitysvaikeusoireyhtymä, monisiköiraskaus, surfaktanttiproteiinit
The present work was carried out at the Department of Paediatrics, University of Oulu, during the years 1997–2003.

I express my deepest gratitude to my supervisor, Professor Mikko Hallman, M.D., Head of the Department of Paediatrics for giving me the opportunity to do my thesis work under his guidance. I thank him for his positive attitude, kind patience and trust in this work during these years. I also express my appreciation to Professor Matti Uhari, M.D., for teaching me consistent and critical attitude towards scientific thinking and methodology. I kindly thank Professor Jaakko Kaprio, M.D., Head of the Department of Public Health, University of Helsinki, for guiding me in the field of epidemiology.

I wish to express my cordial thanks to my second supervisor Marja-Leena Pokela, M.D., for her support and encouragement, friendship and guidance in the treatment of the tiniest newborns. Warm thanks are due to my hard-working co-author Ritva Haataja, Ph.D., for her excellent knowledge of genetics and positively critical attitude towards this research. We have shared many moments of hope — and despair — during these years. I am specifically obliged to Mika Rämet, M.D., Johan Löfgren, Med.Cand., and Meri Rova, M.Sc., in the Research Laboratory of Paediatrics for their kind help and co-authorship. Susan Guttentag, M.D., is acknowledged for her contribution. I thank sincerely Ms. Maarit Hännikäinen, Ms. Elsi Jokelainen and Ms. Mirkka Ovaska for their patient and excellent lab work.

I am deeply grateful to Professor Marjo-Riitta Järvelin, M.D., Head of the Department of Epidemiology and Public Health, Imperial College, University of London, and Professor Vineta Fellman, M.D., Head of the Department of Paediatrics and Neonatology, University of Lund, for their prompt review of the manuscript of this thesis and for their expertise. I thank warmly Juha Turtinen, M.Sc., who has helped me numerous times with statistics and computer software. I owe my gratitude to Ms. Eija Rautio and Ms. Marjatta Paloheimo for their kind assistance and supportive attitude towards me and my work. Sirkka-Liisa Lenonen, Lic.Phil., is acknowledged for the skilful revision of English language of the thesis and the original publications.
I wish to express my warm thanks to Docent Maila Koivisto, M.D., my “official” teacher in neonatology. She has taught me a lot with her persistence in struggling for the most preterm infants as well as with her continuous enthusiasm in scientific work. I also thank her for all the help and advice as a co-author. I express my sincere gratitude to Docent Outi Tammela, M.D., University of Tampere, for flexible co-operation and for her contribution to sample collection. Docent Mika Gissler, M.D., and Dr. Martti Virtanen, M.D., in the National Research and Development Center for Welfare and Health Care (STAKES), and Docents Kirsti Heinonen, Sami Ikonen and Pentti Kero are kindly acknowledged for their contribution to this thesis.

As I have been mostly living and working elsewhere, my closest colleagues in the neonatal ward of Oulu University Hospital have helped me a lot with the collection of the study material. I thank especially Eija Anttila, M.D., Tuula Kaukola, M.D., Timo Saarela, M.D., and Marita Valkama, M.D., for valuable help, support and friendship both in clinical practise and in scientific work during these years. During my hasty visits now and then we have had time for inspiring discussions, too. I have enjoyed working in the neonatological ward 55, and I thank the entire nursing staff for smooth and encouraging co-operation. The staff in the Department of Obstetrics and Gynecology also made a great effort to collect the blood samples. I am grateful to my colleagues and friends in the paediatric departments of Seinäjoki Central Hospital and University of Oulu for their flexibility and supportive attitude. I express my sincere thanks to all the tiny newborns and their parents for participating in these studies. Hopefully this work will take us a small step closer to understanding and countering the challenges of preterm birth and the consequent disorders.

I thank warmly my long-standing friends Anu-Maaria Hämäläinen, Merja Joro, Sirpa Koski-Tervo and Tarja Nevala for their concern and care. I owe my warm gratitude to my parents, Terttu and Heimo Juntunen, and my sister Marja-Leena for their loving support and practical help throughout my endless years of studying. Marja-Leena has shared the ups and downs of scientific work with me while finishing her own doctoral thesis.

Finally, I owe my loving gratitude to my family. I thank my husband Timo for his patience and for providing opportunities for me to work where-ever and whenever I needed to. I thank our children Juho, Emmi, Tuukka and Hanna for the joy and optimism they spread around. They are the most precious things in my life and I dedicate this dissertation to them.

This work was supported financially by the Foundation of Paediatric Research in Finland, the Alma and K.A. Snellman Foundation, Oulu, Finland, and the Research Funds of the University of Oulu, the University of Tampere and Seinäjoki Central Hospital, which all are kindly appreciated.

Seinäjoki, October 2003

Riitta Marttila
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>PIF</td>
<td>pulmonary interstitial fibrosis</td>
</tr>
<tr>
<td>PNM</td>
<td>perinatal mortality</td>
</tr>
<tr>
<td>PPROM</td>
<td>preterm premature rupture of fetal membranes</td>
</tr>
<tr>
<td>q</td>
<td>long arm of a chromosome</td>
</tr>
<tr>
<td>RDS</td>
<td>respiratory distress syndrome</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>SGA</td>
<td>small for gestational age</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>surfactant protein</td>
</tr>
<tr>
<td>TM</td>
<td>tubular myelin</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VLBW</td>
<td>very low birth weight (&lt; 1500 g)</td>
</tr>
</tbody>
</table>
List of original papers

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:


* Contributed equally to this work
Contents

Abstract
Acknowledgements
Abbreviations
List of original papers
Contents

1 Introduction .................................................................................................................15
2 Review of the literature................................................................................................16
  2.1 Human lung development .......................................................................................16
  2.2 Composition, function and metabolism of pulmonary surfactant .........................18
  2.3 Pulmonary surfactant protein A ..............................................................................19
    2.3.1 Structure of SP-A .............................................................................................19
    2.3.2 The human SP-A gene .....................................................................................20
    2.3.3 Processing and function of SP-A .................................................................21
  2.4 Pulmonary surfactant protein B ..............................................................................22
    2.4.1 Structure of SP-B .............................................................................................22
    2.4.2 The human SP-B gene .....................................................................................23
    2.4.3 Secretion and function of SP-B .......................................................................24
  2.5 Regulation of SP-A and SP-B expression ...........................................................24
  2.6 Predicting the risk of RDS with surfactant component analysis .........................25
  2.7 Clinical picture of RDS in preterm infants ...........................................................26
  2.8 Pathophysiology of RDS .....................................................................................26
  2.9 Epidemiology of RDS ...........................................................................................27
  2.10 Clinical risk factors for RDS .................................................................................28
    2.10.1 Preterm premature rupture of membranes and intrauterine infection ...........29
    2.10.2 Intrauterine growth .........................................................................................30
    2.10.3 Maternal diseases ..........................................................................................30
    2.10.4 Mode of delivery .........................................................................................31
    2.10.5 Twins and higher order gestations ...............................................................31
      2.10.5.1 Incidence of twins .................................................................................31
      2.10.5.2 Zygosity of twins ....................................................................................32
      2.10.5.3 RDS-related morbidity of twins ............................................................32
2.11 Genetic predisposition to RDS

2.11.1 Epidemiological evidence

2.11.2 Hereditary SP-B and SP-C deficiency

2.11.3 Allelic association studies

2.11.4 SP-A gene polymorphism

2.11.5 SP-B gene polymorphism

2.11.6 Surfactant protein genes associated with other pulmonary diseases than RDS

2.12 Prevention, treatment and prognosis of RDS

3 Objectives of the present research

4 Subjects and methods

4.1 The study populations and the study design

4.2 Epidemiological approach

4.3 Genetic studies

4.4 DNA samples and genotyping

4.5 Ethical considerations

4.6 Statistical analysis

5 Results

5.1 Population-based epidemiology and risk factors for RDS (I, II)

5.1.1 Incidence of RDS (I, II)

5.1.2 Risk factors for RDS in twins (I)

5.1.3 RDS-related mortality and morbidity (II)

5.1.4 Outcome at one year of age (II)

5.2 Concordance of RDS in MZ and DZ twin pairs (III, IV)

5.3 SP-A and SP-B polymorphisms and RDS in twins (III, IV)

5.4 Interaction by the SP-B gene and plurality on the association between the SP-A gene and RDS (V)

6 Discussion

6.1 Epidemiological aspects

6.2 Methods in genetic studies

6.3 Concordance of RDS between MZ and DZ twin pairs

6.4 Role of SP-A and SP-B polymorphisms in RDS of twins

6.5 Interaction by the SP-B gene and plurality on the association between the SP-A gene and RDS

7 Summary and future perspectives

References
1 Introduction

Respiratory distress syndrome, RDS, is a multifactorial lung disease of premature infants. It is characterized by respiratory failure and deficient gas exchange within the first few hours after birth unless effective treatment is instituted. The main cause of RDS is a deficiency of pulmonary surfactant, a lipoprotein mixture that is required to reduce surface tension at the air-liquid interface and to prevent generalized atelectasis of the alveolar ducts and alveoli.

Functional prematurity is the most important factor predisposing to RDS. The incidence of RDS decreases as a function of the length of gestation and RDS only occasionally manifests at term birth. During the 1990s the rate of preterm deliveries, defined as delivery between 22 weeks and 37 completed weeks of gestation, increased from 5.4% in 1990 to 6.1% in 1999 in Finland (Gissler et al 2002). At the same time the number of multiple pregnancies increased significantly as a result of diversified infertility treatments and advanced maternal age. In the late 1990s 1.6% of all births were multiple births. Multiple pregnancies result in much higher rate of preterm births than singleton pregnancies, as almost half of the deliveries occur preterm compared to 4–5% in singleton pregnancies. The high rate of preterm births in twin and higher order gestations leads to disproportionately high overall neonatal morbidity.

As twin and multiple infants represent approximately 20% of all infants born preterm, RDS is one of the major causes of morbidity among them. Maternal diseases, gender and ethnicity are known factors affecting the risk of RDS. There is also evidence of a more specific genetic background, as surfactant protein SP-A and SP-B genes have been suggested to associate with RDS.

The objectives of the present research were to evaluate the incidence and risk factors of RDS in twins compared to singletons, and to assess the role of SP-A and SP-B gene variations and gene-environment interactions in the susceptibility to RDS disease in a population of preterm twins and higher order multiples.
2 Review of the literature

2.1 Human lung development

The human lung is a derivative of the primitive foregut and appears by 22 days of gestation as an enlargement of the caudal end of the laryngotracheal sulcus. In humans, the airways are well formed by 20 weeks gestation. The development of lungs can be divided into five overlapping stages of organogenesis: early embryonic (3–7 weeks of gestation), pseudoglandular (5–17 weeks of gestation), canalicular (16–26 weeks of gestation), saccular (24–38 weeks of gestation) and alveolar (36 weeks of gestation to 2 years of postnatal age) (Burri 1997, Hackett & Gitlin 1997, Kotecha 2000). The stages of organogenesis together with the expression of the surfactant proteins A (Ballard et al 1986) and B (Liley et al 1989) during human gestation are shown in Figure 1.
Fig. 1. The stages of lung organogenesis are shown above (modified from Burri 1997). Lung development continues after preterm delivery. The expression of surfactant protein genes begins early in the fetus and increases towards term gestation.

During the initial embryonic stage, the lung develops as an overgrowth of the foregut endoderm. Epithelial cells from the foregut endoderm invade the surrounding mesoderm to form the proximal structures of the respiratory tract. The trachea, the main bronchi and the major lung lobules are formed, as are also the pulmonary arteries. Epithelial tubules are surrounded by thick mesenchymal tissue during the pseudoglandular phase. By the end of this stage, the conducting airways, terminal bronchioles and primitive acini are completed. During the canalicular phase the gas-exchanging tissue becomes visible in light microscopy, and lamellar bodies containing surfactant proteins and phospholipids in type II pneumocytes can be observed lining the acinar tubules. Vascularization with the concomitant formation of the alveolar-capillary barrier for gas exchange takes place. The secretion of surface-active material enables a prematurely born infant to survive. The saccular phase is characterized by marked enlargement of the peripheral airways and development of primitive saclike alveoli called saccules, which increase the gas-exchanging surface area. Lamellar bodies in type II cells increase, and further maturation into type I alveolar epithelial cells takes place. Capillaries lie close to type I cells, thus reducing the distance at the air-blood interface. The first alveoli begin to appear by 32 weeks of gestation, and by term, an average of 30% of the adult number of alveoli have developed. The formation of thin secondary alveolar septa, remodelling of the capillary bed and marked proliferation of all cell types occur primarily postnatally. During this alveolar phase of lung development, the gas-exchanging surface area increases tenfold, from 20–50 million alveoli at birth to an average of 300 million by adulthood.

A number of hormones and growth factors, induced by fetal breathing movements and other events, interactively regulate normal lung growth and maturation. The exact
mechanisms are still largely unknown (Jobe 1999, Kotecha 2000). Lung development and growth can be altered as a result of various antenatal and perinatal events, such as an inflammation caused by intrauterine infection (Jobe & Ikegami 2001). A deficiency or impaired function of the regulatory elements, such as the vascular endothelial growth factor (VEGF), affects pulmonary vascular growth and alveolarization (Grover et al 2003) as well as the differentiation of type II cells (Compernolle et al 2002).

2.2 Composition, function and metabolism of pulmonary surfactant

The maintenance of alveolar structure during the cyclic changes in lung volume is critical for normal respiration. The tendency of the alveolus to collapse at the end of expiration is mainly due to the high surface tension generated by the aqueous layer lining the alveolar epithelium. Alveolar stability is achieved by the pulmonary surfactant present at the air-liquid interface which reduces surface tension and prevents atelectasis (Hawgood & Clements 1990). A schematic representation of the structure of an alveolus is shown in Figure 2. Surfactant components also participate in the clearance of foreign material and in the host defence mechanisms and innate immunity of the lung (Crouch & Wright 2001).

Fig. 2. Structure of an alveolus (modified from Hawgood & Clements 1990).

Human surfactant consists of approximately 80% phospholipids, 8% neutral lipids (cholesterol and free fatty acids) and 12% proteins and its composition is fairly constant across mammalian species (King 1984). The most abundant phospholipid is
phosphatidylcholine (PC), especially its disaturated form dipalmitoylphosphatidylcholine (DPPC). DPPC is the component of surfactant that reduces surface tension. The other lipid components of the surfactant (phosphatidylglycerol, phosphatidylinositol and cholesterol) and the hydrophobic surfactant proteins facilitate the adsorption of DPPC. The amount of DPPC increases concurrently with the appearance of lamellar bodies after 22 weeks of gestation in the fetal human lung (Clements 1977).

Approximately half of the proteins consists of contaminating protein from plasma or lung tissue (Jobe 1993). In addition there are four surfactant proteins (SPs) expressed by respiratory epithelial cells, designated as SP-A, SP-B, SP-C and SP-D. SP-A and SP-D are large glycosylated water-soluble proteins and members of the calcium-dependent carbohydrate-binding collectin family and they have a role in the host defence of the lung (Crouch & Wright 2001). SP-A is also important in the organization and function of the surfactant complex regulating surfactant recycling and secretion. SP-B and SP-C are highly hydrophobic small peptides that confer surface tension-lowering properties and are important for the adsorption and spreading of the surfactant (Whitsett & Weaver 2002). They are the only surfactant proteins in the animal-based surfactant replacement preparations used in RDS, as the purification process removes the hydrophilic proteins SP-A and SP-D from the completed product.

Surfactant components are synthesized, secreted and recycled by type II epithelial cells in the alveolus (Rooney et al 1994). With the exception of SP-A, surfactant proteins are synthesized in polyribosomes, modified in the endoplasmic reticulum, Golgi apparatus and multivesicular bodies and stored in lamellar bodies before secretion. Surfactant phospholipids are synthesized in the endoplasmic reticulum, transported through the Golgi apparatus into multivesicular bodies and packaged into lamellar bodies. After exocytosis of lamellar bodies, surfactant phospholipids, in the presence of SP-A, SP-B and Ca$^{2+}$, are organized into a lattice structure called tubular myelin (TM), which forms a lipid-rich layer at the air-liquid interface of the alveolus. Most of the extracellular surfactant is taken up by type II cells, catabolized and transported into lamellar bodies for recycling. Alveolar macrophages also take part in the catabolization process of the surfactant components (Gurel et al 2001).

Because only SP-A (the most abundant SP) and SP-B (essential for postnatal lung function) gene polymorphisms were studied for their associations with RDS in the present project, the review of the literature on surfactant proteins concentrates mainly on SP-A and B.

### 2.3 Pulmonary surfactant protein A

#### 2.3.1 Structure of SP-A

Sequence comparison of the cDNAs of SP-A isolated from human and several animal species shows extensive homology (White et al 1985, Boggaram et al 1988, King et al 1989, Korthagen et al 1992). The primary structure of SP-A in humans consists of four
structural domains: a short N-terminal segment, a collagen-like region, a hydrophobic neck domain and a carbohydrate recognition domain (CRD) homologous to all calcium-dependent (C-type) mammalian lectins. The combination of a collagen-like region and a lectin domain is one of the defining structural features of the collectin family of proteins shown to function as antibody-independent opsonins (Hoppe & Reid 1994). Human SP-A is synthesized as 26 kDa monomer, and after processing it forms a large multimeric 650 kDa-protein with a molecular weight of 32–36 kDa consisting of 248 amino acids. It exists as an octadecameric (6 trimers) bouquet-like molecule within the alveolus (McCormack et al 1998). Each trimer is suggested to consist of two SP-A1 and one SP-A2 gene products linked through their collagen-like domains (Voss et al 1991). Two cysteine residues in the N-terminal domain are required for multimer formation.

### 2.3.2 The human SP-A gene

The human SP-A locus on the long arm of chromosome 10 consists of two functional genes, SP-A1 and SP-A2, with a pseudogene in the middle (Hoover & Floros 1998). The functional genes encode isoforms containing minor amino acid differences in the mature protein (Katyal et al 1992). The human SP-A genes differ in their exon-intron organization and are separated by only 40 kb from each other (Hoover & Floros 1998). There is strong linkage disequilibrium between the functional SP-A genes (Floros et al 1996). The schematic construction of the SP-A protein and gene is shown in Figure 3.

![Diagram of the gene and protein structure of SP-A. The Roman numerals designate the coding exons. Both SP-A genes have four coding exons. The thin line represents the introns. The structural domains of the mature SP-A are also shown.](image-url)

Fig. 3. Diagram of the gene and protein structure of SP-A. The Roman numerals designate the coding exons. Both SP-A genes have four coding exons. The thin line represents the introns. The structural domains of the mature SP-A are also shown.
Both SP-A1 and SP-A2 are highly polymorphic. Single-nucleotide polymorphisms (SNPs) occur throughout the coding sequence, resulting in amino acid substitutions or silent changes within the genes (McGormick et al. 1994). The alleles of the SP-A1 gene are denoted as 6A\(^n\) and the SP-A2 alleles as 1A\(^n\). The alleles within each gene differ from each other in one or several SNPs in the coding exons, as shown in Table 1 and Table 2. There are differences between SP-A1 and SP-A2 alleles in their ability to stimulate TNF-α production in cell lines in vitro (Wang et al. 2000), but the impact of allelic differences on the surfactant function and immune responses of the mature SP-A are unknown. There is also splicing variability in the 5′-untranslated region (UTR) and sequence variability in the 3′-UTR of SP-A1. According to an in vitro study the human 3′-UTR variants play a differential role in SP-A mRNA levels and in the response to dexamethasone (Wang et al. 2003a).

**Table 1. SNP combinations of the most frequent alleles of SP-A1 in a Finnish population of newborn infants (Rämet et al. 2000).**

<table>
<thead>
<tr>
<th>SP-A1 alleles</th>
<th>frequency</th>
<th>Val19Ala</th>
<th>Val50Leu</th>
<th>Pro 62</th>
<th>Thr133</th>
<th>Arg219Trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A</td>
<td>0.04</td>
<td>C (Ala)</td>
<td>C (Leu)</td>
<td>G</td>
<td>G</td>
<td>C (Arg)</td>
</tr>
<tr>
<td>6A(^2)</td>
<td>0.60</td>
<td>T (Val)</td>
<td>G (Val)</td>
<td>A</td>
<td>A</td>
<td>C (Arg)</td>
</tr>
<tr>
<td>6A(^3)</td>
<td>0.28</td>
<td>T (Val)</td>
<td>C (Leu)</td>
<td>A</td>
<td>A</td>
<td>C (Arg)</td>
</tr>
<tr>
<td>6A(^4)</td>
<td>0.09</td>
<td>T (Val)</td>
<td>C (Leu)</td>
<td>G</td>
<td>A</td>
<td>T (Trp)</td>
</tr>
</tbody>
</table>

**Table 2. SNP combinations of the most frequent alleles of SP-A2 in a Finnish population of newborn infants (Rämet et al. 2000).**

<table>
<thead>
<tr>
<th>SP-A2 alleles</th>
<th>frequency</th>
<th>Asn9Thr</th>
<th>Ala91Pro</th>
<th>Ser140</th>
<th>Gln223Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>0.04</td>
<td>C (Thr)</td>
<td>C (Pro)</td>
<td>C</td>
<td>C (Gln)</td>
</tr>
<tr>
<td>1A(^0)</td>
<td>0.57</td>
<td>A (Asn)</td>
<td>G (Ala)</td>
<td>C</td>
<td>C (Gln)</td>
</tr>
<tr>
<td>1A(^1)</td>
<td>0.16</td>
<td>C (Thr)</td>
<td>G (Ala)</td>
<td>T</td>
<td>A (Lys)</td>
</tr>
<tr>
<td>1A(^2)</td>
<td>0.13</td>
<td>C (Thr)</td>
<td>G (Ala)</td>
<td>C</td>
<td>C (Gln)</td>
</tr>
<tr>
<td>1A(^3)</td>
<td>0.03</td>
<td>A (Asn)</td>
<td>G (Ala)</td>
<td>T</td>
<td>A (Lys)</td>
</tr>
</tbody>
</table>

### 2.3.3 Processing and function of SP-A

SP-A is the most abundant surfactant protein, accounting for approximately 50% of total protein. Apart from alveolar type II cells, SP-A mRNA and protein are expressed in epithelial Clara cells and serous cells of tracheo-bronchial glands (Khoor et al. 1993, Wong et al. 1996). SP-A expression has also been localized within the Eustachian tube epithelium, and the mature protein has been found in the granules of epithelial cells (Paananen et al. 2001a). The extrapulmonary expression of SP-A may be species-specific (Rubio et al. 1995). In humans, SP-A expression is considered to be limited to the respiratory system (Madsen et al. 2003). SP-A is synthesized as a preprotein and is
extensively modified in the endoplasmic reticulum (ER) and the Golgi apparatus. Only approximately 1% of the surfactant in lamellar bodies consists of SP-A, and there is evidence that it is secreted into the alveolar space via several routes (Ikegami et al 1992).

Most previous studies have focused on the role of SP-A in surfactant structure and function, but further recent research has shown that the main function of SP-A is in pulmonary host defence (Hoppe & Reid 1994). In vitro and in vivo studies have shown SP-A to be important for the formation of tubular myelin (Poulain et al 1992). It improves the surface activity of surfactant by binding to surfactant lipids and enhancing their adsorption at the air-liquid interface (Hawgood et al 1987). It also has a regulatory role in the secretion of surfactant, and specifically, of phospholipids by type II cells (Rice et al 1987).

Surprisingly, studies on SP-A gene-targeted mice (SP-A -/-) revealed no apparent abnormalities in lung function suggesting that other mechanisms take over in the absence of SP-A (Korfhagen et al 1996, Ikegami et al 1998). However, the lungs of SP-A (-/-) mice had markedly decreased tubular myelin formation and cleared bacterial antigens less efficiently than SP-A wild-type mice. According to a recent study on gene-targeted mice SP-A is also necessary for the pulmonary uptake and metabolism of DPPC (Jain et al 2003).

Both lung collectins, SP-A and SP-D, bind to lipids and carbohydrates and interact with specific cell surface receptors, micro-organisms and leukocytes as well as modulate the functions of phagocytic cells in vitro and in vivo (Haagsman 1998, Korfhagen et al 1998, Crouch & Wright 2001). Studies on mice have shown that SP-A inhibits cytokine production in inflamed lung (Chabot et al 2003). It has been suggested that the surfactant-related and immunomodulatory functions of SP-A overlap, and disturbances in either of them lead to altered homeostasis of the lung (Hawgood & Poulain 2001).

### 2.4 Pulmonary surfactant protein B

#### 2.4.1 Structure of SP-B

The structural organization of SP-B mRNA is highly conserved among mammalian species (Whitsett et al 1995). Translation of human SP-B mRNA results in the synthesis of a precursor protein in which the mature SP-B protein is flanked by N- and C-terminal peptides. Both of these peptides contain saposin-like domains that are identified by six conserved cysteine residues (Patthy 1991). These domains may facilitate the processing of the SP-B preproprotein into a mature peptide during its transit through the secretory pathway (Lin et al 1996). Complete processing of a 381 amino acid precursor into a mature 79 amino acid peptide is considered to take place exclusively in type II epithelial cells in the alveoli (Weaver & Whitsett 1989). Recently SP-B mRNA and protein were shown to be expressed in the Eustachian tube epithelium (Paananen et al 2001b), but the phospholipid composition of the surfactant is different from that of the lungs (Paananen et al 2002). Mature SP-B is a small homodimer of approximately 18 kDa with a reduced
molecular mass of 8 kDa, which comprises 1 to 2% of surfactant (Weaver 1998). The precise conformation of SP-B is not known, and the structural domains are presumed to consist of five amphipathic helices (Weaver & Conkright 2001). Figure 4 shows the gene and protein structure of SP-B.

2.4.2 The human SP-B gene

The human SP-B gene on chromosome 2p12-p11.2 consists of 11 exons with the coding sequences for the mature SP-B in the exons 6 and 7 (Pilot-Matias et al 1989). Within the lung the gene is expressed in type II cells in the alveoli and in bronchiolar Clara cells. A number of polymorphisms have been identified in the SP-B gene (Glasser et al 1987, Floros et al 1995, Lin et al 1998, Lin et al 2000). A coding polymorphism at the end of exon 4, T/C +1580, leading to substitution of threonine for isoleucine at codon 131, is presumably the most remarkable, because it forms a consensus signal for N-terminal linked glycosylation at this site of the SP-B propeptide. This, in turn, may affect the processing, folding and secretion of the surfactant protein (Haataja et al 2000, Wang et al 2003b). A length polymorphism with deletion or insertion of specific sequence motifs in intron 4 of SP-B has been characterized as well as SNPs in the 5’ and 3’ untranslated regions (Pilot-Matias et al 1989), but the impacts of these variations on SP-B mRNA and on mature SP-B are unknown.
2.4.3 Secretion and function of SP-B

Glycosylated SP-B precursor protein is synthesized and transported from ER into the Golgi apparatus and multivesicular bodies and packaged into lamellar bodies with surfactant phospholipids (Guttentag et al 1998). The contents of lamellar bodies are then secreted into the alveoli, where they interact with SP-A to form tubular myelin.

Hydrophobic SP-B is the only surfactant-associated protein indispensable for postnatal lung function and survival (Whitsett & Weaver 2002). SP-B is critical to the organization of surfactant phospholipids in lamellar bodies and the generation of the surfactant monolayer bilayers that reduce surface tension. It is essential for maintaining the integrity of alveolar epithelial cells, for tubular myelin formation (Whitsett et al 1995, Pryhuber 1998) and for the processing of SP-C (Vorbroker et al 1995). It promotes surface adsorption of DPPC and enhances the stability of phospholipid molecules (Hawgood et al 1998).

SP-B may also possess anti-inflammatory properties (Miles et al 1999), protect against oxygen-induced lung injury (Tokieda et al 1999) and regulate the immune responses in the airways (van Iwaarden et al 2001). A recent study on transgenic mice showed that SP-B-B protects against endotoxin-induced lung inflammation by enhancing surfactant function, decreasing the influx of inflammatory cells and lowering cytokine levels (Epaud et al 2003).

2.5 Regulation of SP-A and SP-B expression

Expression of the genes encoding SPs is developmentally and hormonally regulated, and complex interactive mechanisms are involved. Several transcription factors that are critical determinants of lung morphogenesis per se are also critical regulators of surfactant gene expression (Boggaram et al 1988, Bohinski et al 1994, DeFelice et al 2003). SP-A gene transcription is initiated in fetal lung in the second trimester and reaches its maximum just prior to birth (Ballard et al 1986). SP-A gene expression in fetal lung tissue is regulated by factors that increase the formation of cyclic AMP by enhancing the rate of type II cell differentiation (Mendelson et al 1998, Gonzales et al 2002).

Corticosteroids appear to have a complex biphasic dose-dependent effect on SP-A gene expression. According to in vitro studies, when present at low levels they cause induction of SP-A gene transcription, but in elevated concentrations, they temporarily decrease SP-A mRNA stability (Boggaram et al 1989). In addition the SP-A1 and SP-A2 genes are regulated differently by corticosteroids (Kumar & Snyder 1998). It has been proposed that with increased lung maturation there is a decrease in the responsiveness of the fetal lung to the inhibitory effects of corticosteroids on SP-A gene expression (Mendelsson et al 1998). The physiological role of endogenous corticosteroids in the regulation of SP-A gene expression in fetal lung remains unclear, however.

During the past few years inflammatory mediators have been studied widely for their role in lung development and in the regulation of surfactant synthesis. In fetal lung
explants, interferon-γ increases total SP-A mRNA, but there are marked differences in the response between explants (Karinch et al. 1998). In the human pulmonary adenocarcinoma cell line recombinant interleukin-6 (IL-6) initiates the synthesis of SP-A mRNA and protein (Shimoya et al. 2000). Recently, inhaled nitric oxide (NO) was shown to increase both SP-A and SP-B mRNA content in newborn lambs (Stuart et al. 2003). The effect of cytokines on surfactant synthesis seems to be gestational age-dependent: in very immature lung, IL-1 strongly upregulates the expression of SP-A, whereas towards term gestation TNF-α, lipopolysaccharide (LPS), and the combination of cytokines and LPS suppress the expression of SP-A (Odom & Ballard 1997, Bry et al. 1997, Väyrynen et al. 2002).

Expression of the SP-B and SP-C genes begins before the differentiation of type II epithelial cells. Both SP-B and SP-C mRNA have been detected as early as the end of the first trimester. By 24 weeks of gestation, the SP-B mRNA level has reached 50% of that of adults (Liley et al. 1989), while the level of mature SP-B is less than 5% of the adult level at that time (Beers et al. 1995). Similarly to SP-A expression, different hormonal, inflammatory and growth factors regulate SP-B gene expression according to in vitro studies. Corticosteroids increase SP-B mRNA and enhance the accumulation of SP-B mRNA in human fetal explants (Liley et al. 1989). IL-1 induces the synthesis of SP-B in immature lung (Väyrynen et al. 2002), whereas TNF-α decreases SP-B mRNA and SP-B protein (Pryhuber et al. 1996).

Expression of surfactant protein genes fades when type II cells undergo terminal differentiation into type I cells, which constitute most of the gas-exchanging surface in the alveolus.

### 2.6 Predicting the risk of RDS with surfactant component analysis

Surfactant components secreted by the fetal lung accumulate in amniotic fluid, and the proportions of phospholipids change during fetal development. The lecithin-to-sphingomyelin (L/S) ratio and the presence of phosphatidylylycerol (PG) and phosphatidylcholine (PC) in amniotic fluid have been used to predict lung maturation (Gluck & Kulovich 1973, Hallman et al. 1976, Torday et al. 1979). In pregnancies complicated by pre-eclampsia, for example, their value in predicting RDS is unreliable. Additionally amniocentesis required for the analysis may lead to acute complications threatening the fetus (Hallman 2002). Gastric aspirate for surfactant analysis immediately after birth have been used for the prediction of RDS (Fiori et al. 2001), but this has not been adopted into clinical use.

Experimentally, the levels of SP-A in amniotic fluid have been used to predict the risk of RDS. SP-A secretion increases sharply with advancing gestational age (Hallman et al. 1988). Cho et al. measured SP-A levels in cord blood sera from 48 infants born at gestational ages < 32 weeks. They found that a SP-A level over 10 ng/ml in cord blood was significantly related to a non-RDS outcome (Cho et al. 2000). In another study the SP-A content in sera from newborn infants was measured with an enzyme-linked immunosorbent assay system. The level of serum SP-A was shown to increase with
advancing gestation, but no definite cut-off level could be pointed out because of inadequate sample size (Kaneko et al 2001).

Mature SP-B is first detectable in amniotic fluid at 31 weeks of gestation and its specificity for the prediction of RDS is considered low (Pryhuber et al 1991).

2.7 Clinical picture of RDS in preterm infants

Respiratory distress syndrome in a newborn develops within the first few hours after preterm birth. It manifests as deficient gas exchange and progressive respiratory failure, unless effective treatment is instituted. Infants with RDS present with clinical signs of respiratory distress that include tachypnea, grunting, retractions and cyanosis accompanied by increasing oxygen requirements. Physical findings include rales, poor gas exchange, use of diaphragmatic and other accessory muscles of breathing, nasal flaring, and abnormal patterns of respiration that may be complicated by apnoea. Chest radiograph is characterized by diffuse reticulogranular infiltrates, atelectasis and air bronchograms, often progressing to severe bilateral opacity. However, radiographic patterns in RDS are variable and may not reflect the degree of respiratory compromise.

The infant attempts to avoid alveolar collapse by prolonging and increasing expiratory pressures by breathing against partially closed glottis, which causes the grunting noise characteristic of RDS, but also seen in other respiratory disorders. The increasing oxygen requirements and the need for continuous positive airway pressure (CPAP) or ventilatory support with continuous distending pressure persist for two to three days or even longer. The clinical course depends on the severity of RDS and the maturity of the infant at birth. In uncomplicated RDS, typically seen in more mature infants, recovery is rapid during the first two weeks of life. In most cases, the surfactant content increases and reaches the normal level by one week of postnatal age (Hallman et al 1994). The administration of early exogenous surfactant has alleviated the course of “classical” RDS. The most premature infants are still at greatest risk for severe RDS and frequently develop complications, including air leak, infection, patent ductus arteriosus and central nervous system hemorrhage, all of which contribute to prolonged requirements for oxygen and ventilatory support.

2.8 Pathophysiology of RDS

Pathologic findings include atelectasis, high-permeability pulmonary edema, pulmonary vascular congestion, pulmonary hemorrhage and direct injury to the respiratory epithelium by inflammatory cells. The previous term “hyaline membrane disease” refers to the histologic findings of pathognomic hyaline membranes in the gas-exchanging alveoli seen post-mortem. Hyaline membranes are fibrin-rich clots representing plasma components and pulmonary epithelial debris. In uncomplicated, mild RDS these membranes disappear quickly. In ventilated newborns, the healing process is altered and
delayed, and hyaline membranes may remain prominent. In most severe cases of RDS, progressive scarring, fibrosis and emphysema of the alveoli and airways lead to chronic lung disease (CLD).

Discovery of high surface tension in the terminal airways of infants dying of hyaline membrane disease was the first demonstration of surfactant deficiency (Avery & Mead 1959). Subsequently, qualitative and quantitative abnormalities in the surfactant complex were demonstrated in infants who developed RDS after preterm birth (Hallman 1982). The importance of the surfactant complex and some of its critical components was further confirmed when surfactant given at birth or in established RDS ameliorated respiratory difficulty considerably (Jobe 1983). The ultimate cause of RDS is a failure to generate an adequate surfactant layer at the alveolar lining after preterm birth. The high capillary permeability allows plasma-derived components and edema fluid to deteriorate surfactant function. Alveolar ventilation is further impaired and RDS complicated by the relatively weak respiratory muscles and the compliant chest wall of the premature infant. Pulmonary vascular resistance is increased due to diminished oxygenation and respiratory and metabolic acidosis. Right-to-left shunting through the ductus arteriosus and foramen ovale and intrapulmonary ventilation-perfusion mismatch further aggravate hypoxemia. The causes of the striking variations in the severity of RDS at a given state of gestation remain largely unknown, however.

2.9 Epidemiology of RDS

Despite the progress made in perinatal care, RDS continues to be the major reason for increased mortality and morbidity among preterm infants. The surviving premature infants are, furthermore, at risk of long-term morbidity, i.e. chronic lung disease of prematurity and neurosensory disorders (Dammann & Leviton 1997, Hallman 1999).

RDS-related mortality represents approximately 10–15% of all neonatal deaths. In the United States, infant mortality (mortality from birth up to one year of age) due to RDS declined from 0.8 in 1987 to 0.4 per thousand live births in 1995 (Malloy et al 2000). In Finland, the overall infant mortality rate declined from 5.6 to 3.4 per thousand live births, perinatal mortality (PNM) (stillbirths and deaths before one week of postnatal age) from 7.8 to 5.6 per thousand births and early neonatal mortality (deaths during the first week of life) from 3.1 to 2.1 per thousand live births between 1990 and 1999 (Gissler et al 2002). There are no published studies on RDS-related mortality in Finland.

The published incidence figures of RDS vary widely. In a Swedish study, the incidence of RDS in newborn infants was only 3.3 per thousand (Hjalmarson 1981), while in an American study it was 18.3 per thousand live-born infants (Robertson et al 1992). In Finland the average incidence of RDS was 6 per thousand live-born infants and 120 per thousand infants born preterm (Koskinen et al 1999). Studies on the influence of advanced perinatal care and the increasing number of multiple gestations to the overall incidence of RDS are scanty, and the analyses are limited to very or extremely low birth weight infants (ELBW, < 1000 g) or singletons (Horbar et al 2002). In Finland, the overall incidence of RDS among those ELBW surviving for more than 12 hours was 760
per thousand (Tommiska et al 2001). Due to the delayed maturation of their lungs male infants have a higher incidence of RDS and higher RDS-related mortality compared to females (Farrell & Wood 1976, Khoury et al 1985).

2.10 Clinical risk factors for RDS

RDS is associated with preterm birth at a time when the biochemical and structural functions of the lung are still underdeveloped. Figure 5 shows an overview of complex components involved in the susceptibility to RDS.

![Diagram of complex relationships between the environmental, inherent and genetic factors that affect the risk of RDS in preterm infants.](image)

Fig. 5. Schematic representation of complex relationships between the environmental, inherent and genetic factors that affect the risk of RDS in preterm infants.

The degree of prematurity is the main factor determining the risk of RDS. The frequency of RDS decreases as a function of the length of gestation, and term infants are very rarely affected by RDS (Usher et al 1971, Wax et al 2002). There is evidence that preterm birth per se results in altered alveolarization that affects lung function (Pelkonen et al 1997, Hjalmarson & Sandberg 2002).

The preterm infant is more prone to asphyxia, hypoxia, hypothermia and hypotension, all of which are likely to impair surfactant synthesis and to increase alveolar capillary permeability. Severe asphyxia is considered to be present when umbilical arterial pH is ≤ 7.10 and/or Apgar score at 5 minutes is ≤ 4. During fetal asphyxia, lung perfusion falls, resulting in ischemic damage to pulmonary capillaries. After recovery pulmonary
hyperperfusion occurs and protein-rich fluid leaks out of the damaged capillaries. This inhibits surfactant activity, leading to RDS.

Agenesis of the urinary tract and subsequent deficient urination by the fetus, lack of fetal breathing and oligohydramnion are conditions leading to diminished lung fluid production and subsequently, to pulmonary hypoplasia and associated RDS.

Spontaneous preterm birth, induced preterm birth due to maternal or fetal condition, and preterm premature rupture of membranes (PPROM) are the main conditions leading to preterm birth. Subclinical infection with intact membranes is presumably a major reason for spontaneous, or idiopathic, preterm birth (Moutquin 2003).

2.10.1 Preterm premature rupture of membranes and intrauterine infection

Preterm premature rupture of membranes is defined as premature rupture of fetal membranes before 37 completed weeks of gestation. Furman and coworkers compared 376 infants with PPROM to 1950 infants without PPROM and found no differences in morbidity from RDS between the groups. They concluded that RDS was affected by prematurity itself rather than by the occurrence of PPROM (Furman et al 2001).

Subclinical infection has been implicated in the mechanism of membrane rupture. Up to 50%, or even more, of fetuses delivered before 30 weeks of gestation have chronic chorioamnionitis, as proved by histologic and microbiologic examinations of amniotic fluid and fetal membranes (Goldenberg et al 2000). Intrauterine infection activates cytokines and other inflammatory mediators that trigger the cascade leading to preterm labour (Gomez et al 1995, Greci et al 1998). The role of infection in the susceptibility to RDS is controversial. Severe and chronic infection is generally considered deleterious. Very early onset of respiratory distress is usually a manifestation of perinatally acquired severe infection leading to ARDS (Becroft et al 1976). However, PPROM and associated infection may expose the fetus to stress and increase the concentrations of different hormones, cytokines and growth factors, which, in turn, induce surfactant synthesis, accelerate lung maturation and protect from RDS (Lassus et al 1999, Shimoya et al 2000, Kallapur et al 2001, Sims et al 2002). Infants born to mothers with histologic chorioamnionitis had a decreased incidence of RDS, but an increased incidence of chronic lung disease, compared to infants of mothers without chorioamnionitis (Watterberg et al 1996). Adjustment for gestational age was not conducted due to the small sample size of 52 preterm infants. Signs of infection and increased cytokine production associated with RDS have also been shown in preterm births with intact membranes (Hitti et al 2001).
2.10.2 Intrauterine growth

Studies on the effect of intrauterine growth on RDS are controversial. Restricted growth of the fetus due to placental insufficiency induces hypoxemia and increases the production of corticosteroids, which, in turn, may facilitate pulmonary maturation (Laatikainen et al 1988, Braems et al 1998). According to Ley and coworkers the effect of intrauterine growth restriction (IUGR) on the risk of RDS depends on the gestational age of the infant. In very premature infants (under 28 weeks of gestation) it increased the risk, whereas between 29 and 32 weeks it decreased the risk (Ley et al 1997). Piper and coworkers reported no differences in the rate of RDS between small for gestational age (SGA) and appropriate for gestational age (AGA) infants after stratification for gestational age (Piper et al 1996). In a large study based on infants delivered at a regional hospital in Dallas, USA between 1988 and 1996, preterm SGA singleton infants had increased risk of RDS compared to AGA infants (McIntire et al 1999).

2.10.3 Maternal diseases

Some studies report a higher incidence of RDS in preterm infants of hypertensive than non-hypertensive mothers, which could, however, be explained by the absence of labour before caesarean section (Tubman et al 1991, Wolf et al 1993). In another study preterm infants of mothers with pre-eclampsia had less RDS compared to infants of non-hypertensive mothers (Shah et al 1995). In a matched cohort study of 225 infants of pre-eclamptic women maternal pre-eclampsia per se did not accelerate lung maturation or have any other salutary effect on the postnatal course of infants born at 24 to 35 weeks of gestation (Friedman et al 1995). Expectant management of pre-eclampsia remote from term in selected patients is recommended to reduce the risk of RDS and other neonatal complications due to preterm birth associated with pre-eclampsia (Friedman et al 1999). In animal models, insulin delayed the maturation of alveolar type II cells and decreased the proportions of saturated phosphatidylglycerol (PG) in surfactant (Gross et al 1980, Ojamo et al 1990). Surfactant protein A mRNA levels were decreased in diabetic pregnancy (Moglia & Phelbs 1996). Abnormal surfactant synthesis, particularly a delay in the appearance of PG, has also been reported in human infants of diabetic mothers (Ojomo et al 1990). The most recent studies report normal surfactant patterns and fetal lung maturation in normoglycemic, metabolically controlled diabetic mothers (Piazzc et al 1999). The improved control of the disease during pregnancy makes it possible to postpone the delivery until term and thus RDS is no longer of such great concern.
2.10.4 Mode of delivery

Caesarean section carried out before the onset of labour is considered to increase the risk of RDS (Reed 1978, Goldberg et al 1981, Ziadeh & Badriat 2000). Infants born by caesarean section have a larger residual volume of lung fluid and a smaller residual capacity and, consequently, secrete less surfactant into the alveolar space. During vaginal delivery, the chest of the infant is squeezed and part of the fetal lung fluid is removed. The adrenergic stimulation associated with vaginal labour also releases surfactant into the airways (Walters & Olver 1978). Cephalic presentation in vaginal deliveries is regarded optimal. Especially in twin deliveries breech presentation of the presenting infant is considered a risk factor for asphyxia and adverse outcome, and caesarean delivery is usually preferred. Trial of vaginal delivery is safe, however, in twin deliveries with the presenting twin in cephalic presentation, and the non-presenting twin in cephalic or breech presentation (Winn et al 2001, Caukwell & Murphy 2002).

2.10.5 Twins and higher order gestations

2.10.5.1 Incidence of twins

The advanced use of ovulation induction and other infertility treatments during the past decade has increased the incidence of twins and higher order multiples. In the United States, between 1980 and 1999, the overall twin and multiple birth ratio increased by 59%. At the same time twin and multiple infant mortality declined more than that of singletons in all birth weight categories. Consequently, the mortality rates of very low birth weight (VLBW) and low birth weight (LBW) infants were lower among multiples than among singletons (Russell et al 2003). Similar decline in the PNM of preterm twins under 32 weeks of gestation was seen in a cohort population study including births between 1985 and 1996 in Canada (Joseph et al 2001). In Finland, the incidence of multiple births was 11 per thousand in the 1980s, but increased up to 17 per thousand in 1998 (Gissler et al 2002).

Twin and higher order gestations have a much higher rate of preterm births than singleton pregnancies. Approximately 40–50% of twins are born prematurely, accounting for 14–15% of all preterm births according to a previous study (Powers & Kiely 1994). Due to the high rate of preterm births, infants from multifetal gestations have much higher postnatal morbidity compared to singletons.

The greater risk of preterm delivery for twins is considered to be due to uterine over-distension (Neilson et al 1988, Rouse et al 1993). Decreased uterine blood flow, hormonal factors and subclinical chorioamnionitis, secondary to ascending infection associated with premature cervical ripening and PPROM, have also been suggested as factors leading to preterm delivery among twins (Romero et al 1990).
2.10.5.2 Zygosity of twins

Identical twins originate at some stage during the first two weeks following fertilization, as the early embryo splits into two parts that subsequently develop separately and give rise to two individuals. These two siblings originate from the same zygote and are thus called monozygotic (MZ) twins. They are genetically identical and, theoretically, any difference existing between MZ co-twins should originate from environmental influences. When at least two embryos originate from two different zygotes, they are called dizygotic (DZ) in the case of twins, and polyzygotic in the case of higher order multiples. Genetically, these individuals are no more similar than any other pair of full siblings. The similarity between two DZ twins can vary widely, but 50% genetic similarity is usually considered an average (Bomsel-Helmreich & Mufti 1995).

2.10.5.3 RDS-related morbidity of twins

The growth and development of a twin fetus is affected not only by the intrauterine factors present in a singleton pregnancy but also by the interaction with the co-twin fetus and by the placental, circulatory or other factors associated with the multiple pregnancy per se. The average weight of a newborn twin is less than that of a singleton of the same gestational age. It has been shown that the divergence in growth pattern between singleton and twin infants becomes visible at 30 weeks of gestation (Alexander et al 1998). After that, the fetal growth of twins slows down markedly, their body weight lagging disproportionately behind head circumference. The fetal growth patterns also differ by gender; female twins are lighter than male twins at each gestational age in both same-sex and opposite-sex pairs.

The considerable birth weight discordance between twins is associated with increased mortality and morbidity (Demissie et al 2002). Severely divergent fetal growth increases risk of fetal demise and, consequently, leads to induction of labour and preterm birth (Hollier et al 1999). Discordance in birth weight is most often attributable to a fetal growth restriction, frequently in the second-born twin (Cooperstock et al 2000). Studies comparing the outcome of the smaller versus the larger twin of the pair are contradictory, but the large twin is usually considered to possess greater risks.

MZ twins are assumed to have higher perinatal mortality and morbidity compared to DZ twins or singletons (Benirschke & Kim 1973). According to recent studies the risk for an adverse outcome depends on chorionicity rather than zygosity (Sherer et al 2001), with monochorionic monozygotic twins being at the greatest risk. Monochorionic twins represent approximately two-thirds of all MZ twins. Specifically alterations in placental circulation and the associated discordance of growth in monochorionic twins have serious implications for development and survival (Victoria et al 2001).

Morbidity from RDS has been shown to be similar (Friedman et al 1997, Gardner et al 1995, Wolf et al 1992) or higher (Donovan et al 1998) in preterm twins compared to preterm singletons and corrected for gestational age. When the L/S ratio was used as an index of fetal lung maturation, amniotic fluid studies revealed that pulmonary maturity
was biochemically comparable between preterm twin and singleton pregnancies (Winn et al 1992). At term gestation comparison of the mean L/S ratios of twins to those of uncomplicated singleton pregnancies showed that fetal lung maturation was concordant in twins and occurred several weeks earlier in them (Leveno 1984).

Most studies have shown that the second-born, or non-presenting twin, has a higher risk of RDS compared to the presenting twin (Lankenau 1976, Ziadeh & Badria 2000), but only in vaginal deliveries (Arnold et al 1987, Hacking et al 2001). Arnold et al concluded that a predisposition to distress at birth does not explain the excess risk, but the non-presenting twin does not benefit from the salutary effects of labour to the same extent as the presenting twin.

2.11 Genetic predisposition to RDS

A genetic risk for respiratory distress in infancy has been suggested in reports of family clusters of affected infants, studies of different ethnic groups and gender, characterization of infants with inherited deficiency of surfactant protein B and association studies using the candidate gene approach on surfactant protein polymorphisms.

2.11.1 Epidemiological evidence

According to a study published in 1965, mothers who gave birth to LBW infants could be divided into low- and high-risk groups based on the risk of RDS in the offspring (Graven & Mesenheimer 1965). An unknown genetic factor was suggested to be involved in the susceptibility to the disease, as the two groups did not differ in any perinatal characteristics.

Lankenau studied retrospectively 111 families with an infant affected by RDS. The risk for maternal half-sibs was the same as that for full-sibs, while the risk for paternal half-sibs was minimal (Lankenau 1976). Infants of maternal aunts possessed an excess risk of RDS as well. The author concluded that there is a genetically determined maternal factor predisposing infants to RDS. There is also recurrence of low birth weight in siblings unrelated to other predisposing perinatal factors (Khoury et al 1989). According to a study of 400 mothers by Nagourney and coworkers, preterm infants born to women with a previous preterm infant affected by RDS are at an increased risk of RDS, suggesting an important genetic predisposing factor (Nagourney et al 1996).

A twin study showed a higher concordance rate (i.e. frequency of twin pairs where both were affected) of RDS in MZ (85%) compared to DZ (44%) twin pairs. The study was done in a rather small study population of 31 twin pairs of known zygosity, including twin pairs of opposite sexes (Myriathopoulos et al 1971).

Ethnicity affects the risk of RDS. Even though black infants have a higher rate of preterm births, the incidence of RDS is lower compared to white infants of the same gestational age (Farrell & Wood 1976, Hulsey et al 1993). Black infants are considered to
be more responsive to antenatal corticosteroid administration and having accelerated lung maturation, or else there are qualitative differences in surfactants between races. However, the difference in the susceptibility to RDS between races was independent of the L/S ratio (Richardson & Torday 1994). RDS-related neonatal mortality has improved more for white than for black infants with very low birth weight after the introduction of surfactant therapy for RDS (Hamvas et al 1996).

2.11.2 Hereditary SP-B and SP-C deficiency

It was noticed a decade ago that term infants unable to produce SP-B due to a genetic defect develop fatal neonatal respiratory disease unresponsive to exogenous surfactant (Nogee et al 1993). Subsequent work demonstrated that these infants were homozygous for a frame-shift mutation that involved a 1-bp -deletion and 3-bp -insertion at codon 121 in exon 4 of the SP-B gene (121ins2). This caused a hereditary SP-B deficiency, one form of congenital alveolar proteinosis (CAP) (Nogee et al 1993). The frequency of the 121ins2 mutation was counted to be one allele per 1000–3300 individuals in two cohorts of American population (Cole et al 2000). A variety of other, even more rare mutations in the SP-B gene have been found which may associate with reduced or no mature SP-B (Nogee et al 2000).

Studies on homozygous gene-targeted mice SP-B (−/−) dying of surfactant deficiency and respiratory distress immediately after birth have shown that complete deficiency of SP-B disrupts tubular myelin and lamellar body formation and the processing of SP-C (Clark et al 1995, Vorbroker et al 1995). Heterozygous SP-B +/− mice have decreased lung compliance and air trapping, but the processing of SP-B and lamellar body formation remain sufficient (Clark et al 1997). Genetic and biochemical studies of heterozygous human infants and adults as well as gene-targeted animal studies suggest that approximately 50% of normal SP-B synthesis may be sufficient for normal pulmonary function at birth (Clark et al 1997, Klein et al 1998).

SP-B influences critically the intracellular processing of SP-C, since, in the absence of SP-B, no mature SP-C is recovered in the airways (Melton et al 2003). Abnormal processing of SP-C may indeed prove to be involved in the pathogenesis of severe respiratory failure, since a mutation in the SP-C gene was recently associated with familial interstitial lung disease (Nogee et al 2001). Gene-targeted SP-C (−/−) mice do not develop neonatal respiratory distress, but the stability of the surfactant isolated from them is significantly decreased (Glasser et al 2001).

Surfactant containing only SP-C has been shown to function well in premature lambs and rabbits suffering from RDS (Davis et al 1998), but SP-C is not able to compensate for a lack of SP-B in humans.
2.11.3 Allelic association studies

The first allelic association study of RDS suggested the presence of HLA-linked susceptibility genes, with over-representation of the HLA antigen alleles A3 and B14 in RDS compared to controls (Hafez et al. 1989). This finding has not been confirmed in later studies, however. The genes coding for SP-A and SP-B have been regarded as the most plausible candidate genes associating with RDS, because these proteins have been considered essential for surfactant function and metabolism.

Table 3 shows case-control allelic association studies and transmission disequilibrium test (TDT) analyses that have been conducted so far in the human SP-A and SP-B genes, and which have been suspected to associate with RDS and other pulmonary diseases.

**Table 3. Studied polymorphisms in the human SP-A and SP-B genes that have been suspected to associate with RDS and other pulmonary diseases**

<table>
<thead>
<tr>
<th>Location of the polymorphism</th>
<th>Study design</th>
<th>Study populations</th>
<th>Result</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-B intron 4</td>
<td>Case-control</td>
<td>82 RDS, 137 non-RDS infants</td>
<td>Insertion/deletion variants ↑ in RDS</td>
<td>Study groups not adjusted for gestational age</td>
<td>Floros et al 1995</td>
</tr>
<tr>
<td>SP-B intron 4</td>
<td>Case-control</td>
<td>40 RDS, 274 non-RDS infants</td>
<td>12 groups of alleles carrying 3 to 18 motifs</td>
<td>Allele frequency differences evident only in racially mixed groups</td>
<td>Veletza et al 1996</td>
</tr>
<tr>
<td>SP-A, SP-B intron 4</td>
<td>Case-control</td>
<td>144 RDS, 98 non-RDS infants</td>
<td>SP-A2 1A0 freq ↑ in RDS in white infants, synergistic association with SP-B intron 4?</td>
<td>Ethnically heterogenous study group, association evident only in subgroups</td>
<td>Kala et al 1998</td>
</tr>
<tr>
<td>SP-A, SP-B intron 4</td>
<td>Matched case-control</td>
<td>88 RDS, 88 non-RDS infants, 225 term non-RDS infants</td>
<td>SP-A 6A2-1A0 freq ↑, 6A1-1A1 freq ↓ in RDS</td>
<td>Associations evident specifically in infants &lt; 32 weeks</td>
<td>Rämet et al 2000</td>
</tr>
<tr>
<td>SP-A, SP-B intron 4, SP-B exon 4</td>
<td>Case-control</td>
<td>188 RDS, 500 preterm 475 term non-RDS infants</td>
<td>SP-A 6A2-1A0 freq ↑, 6A1-1A1 freq ↓ in RDS, association restricted according to gestational age and SP-B Ile131Thr genotype</td>
<td>p-values corrected for multiple testing</td>
<td>Haataja et al 2000</td>
</tr>
<tr>
<td>SP-A, SP-B</td>
<td>Case-control</td>
<td>52 ARDS, 25 at risk, but no ARDS, 46 healthy controls</td>
<td>SP-B exon 4 Thr allele freq ↑ in idiopathic ARDS</td>
<td>Associations evident only in disease subgroups, healthy control group not in H-W equilibrium</td>
<td>Lin et al 2000</td>
</tr>
<tr>
<td>Location of the polymorphism</td>
<td>Study design</td>
<td>Study populations</td>
<td>Result</td>
<td>Comments</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------</td>
<td>-------------------</td>
<td>--------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>SP-A</td>
<td>Case-control</td>
<td>107 tuberculotic, 71 contacts, 101 healthy control adults</td>
<td>SP-A 6A³ and 1A³ freq ↑ in tuberculosis</td>
<td></td>
<td>Floros et al 2000</td>
</tr>
<tr>
<td>SP-B</td>
<td>Case-control</td>
<td>243 RDS, 369 non-RDS infants</td>
<td>SP-B intron 4 deletion variant ↑ in white males, insertion variant↑ in black females</td>
<td>Associations evident only in racial subgroups</td>
<td>Floros et al 2001</td>
</tr>
<tr>
<td>SP-A</td>
<td>Case-control</td>
<td>249 RDS, 335 non-RDS infants TDT: 32 trios</td>
<td>SP-A 6A²-1A³ freq ↑, 6A¹-1A¹ freq ↓ in RDS both in black and white infants</td>
<td>Ethnically heterogenous study group in TDT analysis</td>
<td>Floros et al 2001</td>
</tr>
<tr>
<td>SP-A</td>
<td>TDT-analysis</td>
<td>76 RDS trios</td>
<td>SP-A 6A²-1A³ freq ↑, 6A¹-1A¹ freq ↓ in RDS</td>
<td>p-values corrected for multiple testing</td>
<td>Haataja et al 2001</td>
</tr>
<tr>
<td>SP-A, SP-B</td>
<td>Case-control</td>
<td>97 COPD, 82 smoker control, 99 non-smoker control adults</td>
<td>SP-B exon 4 Thr allele freq ↑ in COPD</td>
<td>Smoking was a significant confounder</td>
<td>Guo et al 2001</td>
</tr>
<tr>
<td>SP-A</td>
<td>Matched case-control</td>
<td>86 RSV, 95 control infants</td>
<td>SP-A2 1A³ freq ↑ and 1A freq ↓ in RSV infants</td>
<td></td>
<td>Löfgren et al 2002</td>
</tr>
<tr>
<td>SP-B intron 4</td>
<td>Observational</td>
<td>29 preterm infants with deletion or insertion, 111 with wild type</td>
<td>RDS ↑ and BPD ↑ in infants with genetic variations</td>
<td></td>
<td>Makri et al 2002</td>
</tr>
<tr>
<td>SP-B intron 4</td>
<td>Matched case-control</td>
<td>118 chronic bronchitis or COPD, 118 matched controls, 118 healthy adults</td>
<td>Size variants ↑ in COPD, specifically in acute respiratory failure</td>
<td>Associations evident only in disease subgroup</td>
<td>Seifert et al 2002</td>
</tr>
<tr>
<td>SP-A, SP-B</td>
<td>Case-control</td>
<td>84 IPF, 194 healthy control adults</td>
<td>SP-B exon 4 Thr allele freq ↑ in IPF</td>
<td></td>
<td>Selman et al 2003</td>
</tr>
</tbody>
</table>
2.11.4 SP-A gene polymorphism

There is evidence that allelic heterogeneity of SP-A is associated with susceptibility to RDS in premature infants. The SP-A1 allele 6A², the SP-A2 allele 1A⁸ and corresponding haplotype 6A²-1A⁸ are most frequently associated with an increased risk of RDS (Kala et al 1998, Rämet et al 2000, Haataja et al 2000, Floros et al 2001, Haataja et al 2001). The problems arising from ethnic heterogeneity and racial differences in allele frequencies can be minimized by studying homogenous populations.

In the Finnish population, the SP-A 6A² and 1A⁸ alleles were associated with an increased risk, whereas the 6A³ allele was associated with a decreased risk of RDS, in very premature infants under 32 weeks of gestation (Rämet et al 2000). The associations were further determined by the SP-B Ile131Thr genotype. The homozygous SP-B genotype Thr/Thr interactively with certain SP-A alleles and genotypes was associated with an increased risk of RDS (Haataja et al 2000). A family study based on a transmission disequilibrium test (TDT) confirmed the association between the SP-A gene polymorphism and RDS (Haataja et al 2001). In adults, the SP-A 6A²-1A⁸ genotype may associate with low levels of SP-A mRNA (Karinch et al 1997). Furthermore, a low SP-A level has been shown to associate with the severity of RDS, and infants who died from RDS had very low levels of SP-A (deMello et al 1993, Moya et al 1994).

2.11.5 SP-B gene polymorphism

A highly polymorphic region containing variable nucleotide tandem repeat sequences within intron 4 of the SP-B gene was studied by Floros and coworkers. They observed differences in the distribution of alleles in different ethnic groups (Veletza et al 1996). Either insertion or deletion of extra motifs was found in preterm infants suffering from RDS compared to controls (Floros et al 1995). A subsequent study showed that this intron 4 polymorphism was associated with RDS interactively with an SP-A gene polymorphism (Kala et al 1998).

Neither of the two SP-B polymorphisms studied, i.e the intron 4 variant or the exon 4 Ile131Thr polymorphism, was associated directly with RDS or prematurity in a Finnish population consisting mainly of singleton infants (Rämet et al 2000, Haataja et al 2001).

2.11.6 Surfactant protein genes associated with other pulmonary diseases than RDS

In adults surfactant protein B polymorphism Ile131Thr have been associated with ARDS (Lin et al 2000), chronic obstructive pulmonary disease (COPD)(Guo et al 2001) and idiopathic pulmonary fibrosis (IPF)(Selman et al 2003). SP-A and SP-D gene
polymorphisms have been associated with RSV bronchiolitis (Lofgren et al 2002, Lahti et al 2002) and tuberculosis (Floros et al 2000).

2.12 Prevention, treatment and prognosis of RDS

Prematurity is the most important factor predisposing to RDS and the most efficient way to reduce the incidence of RDS would thus be to prolong gestation. Unfortunately, however, this cannot be always achieved. Furthermore the progress made in antenatal and neonatal intensive care during the past two decades has enabled the survival of even more premature infants.

Corticosteroids were first used antenatally to accelerate the synthesis of surfactant and to prevent RDS in premature infants in 1972 (Liggins & Howie 1972), but for fear of adverse effects they were only adopted into wider use 20 years later. Prophylactic administration of corticosteroids has been shown to reduce the incidence of RDS by 50% in certain gestational age groups (NIH consensus, Crowley 2003). It is debated, however, whether prophylactic corticosteroids have any beneficial effect on preterm twins and higher order multiples (Murphy et al 2002, Crowley 2003). Additionally, corticosteroids have been shown, time- and dose-dependently, to interfere with alveolarization (Jobe et al 1998). Studies on animal models or preterm infants have revealed no beneficial effect of repetitive doses of corticosteroids (Polk et al 1997, McEvoy et al 2002). There are ongoing multicenter studies to find out the optimal dosage of antenatal steroids.

Better control of gestational diabetes, pre-eclampsia and other maternal diseases affecting the pregnancy outcome would also decrease the rate of preterm deliveries (Hauth et al 1995) and the related morbidities of infants born preterm. Expectant management of elective deliveries without labour (Wax et al 2002) and without documented lung maturity reduces the risk of RDS. In a meta-analysis the use of maternal systemic antibiotics in PPROM was associated with a delay in delivery and a reduction in neonatal infections, use of surfactant and oxygen therapy (Kenyon et al 2003). However, another meta-analysis failed to demonstrate any benefit or harm for neonatal outcome in spontaneous preterm labour with intact membranes and without evidence of clinical infection (King & Flenady 2002). The analysis raised concerns about the increased neonatal mortality among those who received antibiotics. In a retrospective study, antenatal corticosteroid significantly decreased the incidence of RDS in preterm infants in the presence of histologic chorionamnionitis (Elimian et al 2000). Associated use of antibiotics and antenatal corticosteroid in PPROM seems to reduce the risk of RDS following preterm delivery (Lovett et al 1997, Vermillion et al 2000), but the optimal timing and dosage of combined therapies remain to be evaluated.

The introduction of surfactant has significantly decreased overall and RDS-related mortality and morbidity in VLBW infants (Hamvas et al 1996). Administration of exogenous surfactant directly into the airways has been a common practice to treat RDS of preterm infants during the last decade and such prophylactic use is increasing among the most premature infants. It has been shown that treatment with exogenous surfactant also stimulates endogenous surfactant synthesis in premature infants suffering from RDS
(Bunt et al. 2000). Advanced ventilatory support, including administration of continuous distending pressure via the nasal route (De Paoli et al. 2003), patient triggered ventilation as well as high frequency ventilation and nitric oxide treatment in elective severely ill infants are all practices used to treat RDS and its complications.

During the 1990s, corticosteroid treatment, mainly dexamethasone in relatively high doses, became into wide use postnatally in order to improve lung function, to facilitate endotracheal extubation and to prevent CLD. Towards the end of the decade, however, follow-up studies provided evidence of abnormal neurodevelopment, especially in infants treated within the first two or three days (Halliday et al. 2003), and the use of postnatal corticosteroids became more deliberate. There are only a few studies that have aimed to evaluate the effects of postnatal inhaled steroids on lung function and their role hence remains to be elucidated (Shah et al. 2003). Despite the progress in care, CLD, or bronchopulmonary dysplasia (BPD), continues to be a common sequel of lung damage in infants born preterm. Late neurosensory disorders also associate with prematurity and RDS-morbidity (Hallman 1999).
3 Objectives of the present research

The complex relationships between genetic, inherent and environmental factors contribute to the development of RDS in premature infants. Multiple pregnancies involve a considerably higher rate of preterm births than singleton pregnancies, and consequently, RDS is the major cause of morbidity among premature twins and multiples.

The objectives of the present research were:

1. To evaluate the incidence and risk factors of RDS in twins compared to singletons
2. To estimate the changes in the incidence of RDS and RDS-related morbidity due to progress made in antenatal and perinatal care during the past decade
3. To estimate the genetic component of RDS in a twin concordance study comparing monzygous and dizygous twin pairs
4. To evaluate the association of SP-A and SP-B polymorphisms and RDS in twin infants
5. To assess the role of SP-A and SP-B gene variations, and the gene-gene and gene-environment interactions on the risk of RDS in a population of premature twins and higher order multiples compared to singletons
4 Subjects and methods

4.1 The study populations and the study design

The present study is based on an epidemiological and a genetic approach to evaluating the incidence and risk factors of RDS in preterm infants. The data on the study populations are shown summarized in Table 4. Additionally the geographical distribution of the study populations and their proportion to each other are demonstrated in Figure 6. Detailed descriptions of the subjects and the methods are also presented in the original articles (I–V).
### Table 4. Summary of infants in the individual studies

<table>
<thead>
<tr>
<th>Study population</th>
<th>Total number of live-born infants</th>
<th>Number of infants with RDS</th>
<th>Gestational age of RDS infants (sd)</th>
<th>Male/Female ratio of RDS infants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Population-based registry study I</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>850 406</td>
<td>3 945</td>
<td>31.4 (4.0)</td>
<td>0.60</td>
</tr>
<tr>
<td>Singleton infants</td>
<td>23 278</td>
<td>1 025</td>
<td>30.0 (3.3)</td>
<td>0.56</td>
</tr>
<tr>
<td>Preterm (≥ 24 weeks and &lt; 37 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singleton infants</td>
<td>36 043</td>
<td>3 535</td>
<td>30.8 (3.4)</td>
<td>0.59</td>
</tr>
<tr>
<td>Twin pairs</td>
<td>9 498</td>
<td>1 003</td>
<td>29.8 (3.1)</td>
<td>0.56</td>
</tr>
<tr>
<td><strong>Regional epidemiologic study II</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singleton infants</td>
<td>57 357</td>
<td>373</td>
<td>31.2 (3.7)</td>
<td>0.54</td>
</tr>
<tr>
<td>Twin pairs</td>
<td>1 633</td>
<td>96</td>
<td>30.8 (2.7)</td>
<td>0.65</td>
</tr>
<tr>
<td>Higher-order multiples</td>
<td>19</td>
<td></td>
<td>31.0 (2.5)</td>
<td>0.47</td>
</tr>
<tr>
<td><strong>Genetic studies III and IV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Altogether 209 preterm twin pairs</td>
<td>418</td>
<td>209</td>
<td>30.5 (2.8)</td>
<td>0.56</td>
</tr>
<tr>
<td>Concordance study of twins III, SP-Blle131Thr polymorphism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 preterm twin pairs</td>
<td>200</td>
<td>149</td>
<td>30.6 (2.8)</td>
<td>0.52</td>
</tr>
<tr>
<td>SP-A polymorphism IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>198 preterm twin pairs</td>
<td>396</td>
<td>189</td>
<td>30.4 (2.7)</td>
<td>0.58</td>
</tr>
<tr>
<td>Interaction between SP-A and SP-B V</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singleton infants</td>
<td>441</td>
<td>119</td>
<td>30.5 (3.3)</td>
<td>0.66</td>
</tr>
<tr>
<td>Twin pairs</td>
<td>366</td>
<td>172</td>
<td>30.4 (2.8)</td>
<td>0.59</td>
</tr>
<tr>
<td>Higher-order multiples</td>
<td>114</td>
<td>73</td>
<td>30.8 (1.9)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

*Twin and higher-order multiples altogether*
Fig. 6. Geographical distribution of the study populations. Retrospective twin pairs for the genetic studies were recruited through each of the five university hospitals (Helsinki, Kuopio, Oulu, Tampere and Turku). The diagram demonstrates the study populations in the individual genetic studies III–V in proportion to each other.

4.2 Epidemiological approach

The population-based registry study (I) was based on data from the Finnish Medical Birth Registry. All twin and singleton pregnancies and births registered since the founding of the register in 1987 until the end of the year 2000 were evaluated. The register covers over 98% of all births in Finland, and it includes data on pregnancy, delivery and the condition of all live-born neonates and stillborn fetuses weighing 500 gram or more or having gestational age of 22 weeks or more. The whole study population consisted of 853 831 singleton and 23 691 twin infant births. Specifically 36 043 singleton and 9 498 twin infants born preterm were evaluated. The diagnosis of RDS was coded according to the International Classification of Diseases ninth revision (ICD9) (769) in 1987–1990, dichotomously as present/not present in 1991–1995 and as ICD10 code P 22.0 in 1996–2000. Gestational age, birth year, mode of delivery, gender, birth order, birth weight and intra-pair difference in the birth weight of twins were recorded. The concordance of RDS, i.e. both twins being affected, was compared between same-sex and opposite-sex pairs.

The regional epidemiologic study (II) was a population-based survey of the incidence, clinical course and outcome of respiratory distress syndrome in the 1990s. The study population consisted of 58 990 infants, 57 357 singletons and 1 633 multiples, born in the catchment area of Oulu University Hospital, in Northern Finland, between January 1st,
1990 and December 31st, 1999. The clinical characteristics of the neonates admitted to intensive care were prospectively recorded on admission, and the diagnosis was coded in each case on discharge. RDS was defined according to the published clinical (grunting respiration, retractions, nasal flaring, need for supplemental oxygen for > 48 h, or need for exogenous surfactant therapy), radiographic (diffuse reticulogranular pattern and air bronchograms) and pathologic criteria (diffuse atelectasis and hyaline membranes). The hospital records of all infants born alive before 33 completed weeks of gestation and all others suspected or documented to have RDS were individually reviewed and evaluated by the authors of the original manuscript. The RDS-associated morbidity of infants and outcome up to one year of age were recorded.

4.3 Genetic studies

The twin study (III) on the concordance of RDS and the role of SP-B gene variation in MZ and DZ twins consisted of twin pairs born in Finland between 1987 and 1996. The power calculations for the size of a study population needed were hampered by the gestational age dependency of RDS. Our calculations were based on a previous twin study by Myrianthropoulos et al. 1971. They found significantly higher concordance of RDS in MZ (0.85) compared to DZ twin pairs (0.44), suggesting a genetic contribution to RDS. They had, however, included both same-sex and opposite-sex DZ twin pairs. Based on our calculations, in order to avoid the confounding effect of gender, we would have needed 90–100 same-sex twin pairs in order to achieve a concordance difference of the same degree as in the previous study with $\alpha = 0.05$ and $\beta = 0.20$. This number of preterm twin pairs was estimated to be attainable only by recruiting twin pairs both prospectively and retrospectively.

Among twin pairs of the same sex born in Finland before 37 weeks of gestation in 1987–1996, those with at least one of them suffering from RDS were identified from the Finnish Medical Birth Register. Altogether 351 twin pairs were found, of whom 188 twin pairs born in one of the five university hospitals in Finland were studied. The clinical data of the twin pairs was reviewed and the diagnosis of RDS was confirmed. None of the newborns were treated with surfactant prophylactically. Of the 188 pairs, 26 were excluded because of stillbirth or the early death of at least one of the infants before the diagnosis of RDS was established ($n = 12$), the parents were not of Finnish origin ($n = 4$) or the diagnosis of RDS was not confirmed ($n = 10$). A total of 162 twin pairs were eligible for analysis. Clinical data for several neonatal variables (gestational age, gender, birth order, birth weight, postnatal surfactant use and duration of oxygen therapy) were obtained. Maternal records were also reviewed for age, parity, underlying diseases, medication, cause of preterm birth, antenatal glucocorticoid treatment, and mode of delivery. Histological findings of the placenta and fetal membranes were recorded when available. The difference in the concordance rates of RDS between monozygotic (MZ) and dizygotic (DZ) twin pairs, as presumed evidence for genetic influence, was evaluated. The parents of the twin pairs were asked to obtain double buccal smear samples for DNA analysis from their children according to written instructions and to
send them to the research laboratory for genetic analysis. Altogether 100 preterm twin pairs of same-sex were successfully genotyped for the SP-B polymorphisms. The 62 pairs with no DNA available for analysis did not differ in population characteristics from the 100 twin pairs analyzed.

The genetic association study on surfactant protein A gene (IV) included premature twin infants born in Finland during 1987–2000. The study was designed to evaluate the associations between SP-A allelic variants and RDS in a population consisting of 198 premature twin pairs. From 102 retrospectively recruited preterm twin pairs born during 1987–1996, buccal smear samples were obtained and analyzed. In addition, 96 premature twin pairs born in the University Central Hospitals of Oulu and Tampere and Seinäjoki Central Hospital during 1997–2000 were prospectively recruited, and umbilical cord blood samples were obtained and analyzed for SP-A1 and SP-A2 SNPs. The medical records of all preterm twins were reviewed to confirm or exclude the diagnosis of RDS based on clinical, radiographic and pathologic criteria.

The interaction by the SP-B gene and plurality on the association between the SP-A gene and RDS (V). Based on our earlier observations in different sets of Finnish neonates, the allelic associations between the SP-A or SP-B genes and RDS were not similar throughout the population (Ramet et al 2000, Haataja et al 2000). The conflicting observations made in singleton and twin subpopulations of the similar ethnicity are likely to be affected by multiple birth. Furthermore, the published studies on differences in the incidence of RDS between singletons and twins matched for gestational age are contradictory (Wolf et al 1992, Donovan et al 1998). Thus, we wanted to assess in further detail the interaction between the SP-A and SP-B genetic variants as a genetic determinant of RDS separately in subpopulations of prematurely born singletons versus multiples. DNA samples from 119 singleton infants with RDS, 322 without RDS and 245 multiple infants with RDS and 235 without RDS were genotyped for the SP-A1 and SP-A2 SNPs, SP-B exon 4 Ile131Thr and SP-B intron 4 size variants and studied for association with RDS, prematurity and fetal size. Blood samples collected prospectively (n = 594) during 1997–2000 and 327 retrospectively recruited premature singletons and multiples born during 1987–1996, from whom buccal smear samples or a blood spot dried on a filter paper were available, were included in the study.

Additionally experimental data on the different glycosylation of preSP-B in human fetal lung tissue is presented to explain the potential differences in the processing of SP-B conferred to the different SP-B exon 4 Ile131Thr genotypes.

Infants were excluded from the genetic studies III–V if one or both of the parents were of non-Finnish origin or if the infants had received intrauterine blood transfusion. All DNA samples were genotyped for SP-A1, SP-A2 and SP-B exon 4 polymorphisms and intron 4 size variants, but occasional failure in genotyping caused slight variation in the number of analyzed cases.
4.4 DNA samples and genotyping

There were altogether 594 umbilical cord blood specimens obtained prospectively from preterm infants during 1997–2000. Samples of 0.5–3.0 ml of whole blood were used to isolate genomic DNA with the Purogene DNA Isolation Kit (Gentra Systems). From 276 twin and multiple infants born during 1987–1996 duplicate buccal smear samples from the cheek mucous membrane were collected retrospectively into sterile swab collection tubes. DNA was extracted from the swabs using Chelex 100 medium (Bio-Rad). Appropriate contamination controls for all reagents were included in each series of DNA preparation. In addition, 51 retrospectively collected samples of blood spots dried on a filter paper were included in the series. The sample disk punched from the paper was purified with DNA Purification Solution (Gentra Systems) and used directly as a template. A blank paper disk treated similarly was included in each series to control for potential DNA cross-contamination.

Polymerase chain reaction (PCR) amplification was used to carry out the genetic analyses. The PCR reactions were controlled for purity by inclusion of a water sample in each series of amplification. The SP-A genotypes were determined using a restriction fragment length polymorphism (RFLP) method based on converted gene-specific PCR primers (Di Angelo et al 1999). The samples were digested with appropriate restriction enzymes. SPs for five codons for the SP-A1 gene, and four codons for the SP-A2 gene were analyzed for the SP-A genotypes. After PCR amplification, the SP-B Ile131Thr genotypes were digested with the restriction enzyme TaqI before analyses. SP-B intron 4 size variants were analyzed directly after PCR amplification (Rämet et al 2000).

Twin zygosity was determined by using four highly informative tetranucleotide repeat markers in addition to genotyping of the SP-A and SP-B polymorphisms. Dizygosity was inferred if twins differed in any of the markers.

Additionally, human fetal lung explants were cultured and genotyped for the SP-B Ile131Thr polymorphism. Homozygous Thr/Thr and Ile/Ile explants were labelled and processed for immunoprecipitation as described (Guttentag et al 1998). The proteins were immunoprecipitated with a rabbit polyclonal antibody to human SP-B and the products separated using a 12% NuPAGE Bis-Tris gel with a MES buffer system. SP-B fragments were visualized by Storm Phosphorimagerr.

4.5 Ethical considerations

The Finnish Ministry for Social Affairs and Health gave a permission to conduct the population study I and genetic studies on a nationwide basis. In order to obtain an adequate population of twins the National Birth Registry was evaluated. The prospective and retrospective samples for genetic studies were collected during 1996–2000 from the districts served by the University Hospitals of Oulu (III–V), Tampere (III–V), Turku (III– V), Kuopio (III–V), and Helsinki (III–V), as well as Seinäjoki central hospital (V). Families with preterm twins or multiples were contacted via the respective university hospitals and asked for buccal smear samples for genetic studies. The ethical committees
of the participating centres approved the study protocols. The clinical data and the neonatal and maternal histories were obtained from the medical records of each hospital. Written informed consent was obtained from the parents of the infants for the blood and buccal smear samples to be used in genetic studies.

4.6 Statistical analysis

In the epidemiological studies I and II the overall differences in the binary and category variables between singletons and twins were tested by $2 \times k$ with trend $\chi^2$ tests. Continuous variables were tested by Student’s t-test for unpaired samples if the variables were normally distributed and by the non-parametric Mann-Whitney U-test if they were not. Odds ratios (ORs) and 95% confidence intervals (CIs) for the differences between singletons and twins, male and female newborns and first-born and second-born twins were calculated with the Mantel-Haenszel method after stratification by gestational age, mode of delivery or year of birth. The independent samples t-test and the binomial test were used to calculate the proportional differences with 95% CIs of the concordance rates of RDS between the different zygosity groups. Multiple regression analyses, with twin pairs clustered, i.e. taking into account the dependence within twin pairs, were used to estimate the available birth characteristics as risk factors for RDS. Discordant twin pairs (only one of the pair being affected) were additionally analyzed with conditional logistic regression analysis (considered as matched case-control pairs).

The t-test for independent samples and the binomial test were used to calculate the proportion differences with 95% CIs in the concordance rates of RDS between MZ and DZ twin pairs.

In the genetic association studies III–V the differences in the allele and genotype frequencies between the RDS and no-RDS infants were evaluated using $2 \times 2$ contingency tables and $\chi^2$ analysis. Fisher’s exact test was used when the expected cell frequency was $< 5$. Bonferroni’s corrections were made for multiple comparisons by multiplying the statistically significant $p$ values by the number of pairwise comparisons being made (V). ORs and 95% CIs were calculated with the Woolf logit method to assess the risk of RDS conferred by a particular allele and genotype. The observed genotype frequencies were compared with the expected Hardy-Weinberg distributions by $\chi^2$ tests. The ORs between genetic factors and RDS, and stratified by zygosity (MZ/DZ), degree of prematurity (< or $\geq$ 32 weeks of gestation), antenatal glucocorticoid treatment (yes/no), birth order (first/second born), and gender (male/female) in order to explore effect modification, were compared with the homogeneity of the odds ratio (HOR) test (IV, V). Multiple logistic regression analyses were used to determine whether the different clinical variables or the homozygosity or heterozygosity of particular alleles/genotypes of SP-A or B influenced the risk of RDS. The twin pairs were clustered in the analyses. The analyses were adjusted for zygosity, degree of prematurity, antenatal glucocorticoid treatment, birth order and gender.

Statistical analyses were performed by the SPSS versions 9.0 to 11.0 for Windows (basic statistical calculations, logistic regression analysis, Mantel-Haenszel
stratifications), Arcus Quick Stat ($\chi^2$-tests for allele frequencies), StatXact-3 for Windows (HOR test), Intercooled STATA version 7.0 (logistic regression analysis with clustered data of twin pairs) and Egret for Windows (conditional logistic regression analysis).
5 Results

5.1 Population-based epidemiology and risk factors for RDS (I, II)

5.1.1 Incidence of RDS (I, II)

Although the overall number of births per year in Finland decreased from 60,223 in 1987 to 57,371 in 2000, the rate of preterm births increased from 5.6% to 6.3%, respectively, ranging from 4.5% to 4.9% in singletons and from 39.0% to 49.0% in twins. 4,970 RDS cases were found among the 873,784 singleton and twin infants born alive during the years 1987–2000, accounting for an overall RDS incidence of 5.7 per 1000 live-born infants: 5.0 for singletons and 39.5 for the first-born and 50.9 for the second-born twin infants, respectively. The incidence of RDS decreased from 114 in 1987–1990 to 102 per 1000 live-born preterm infants in 1996–2000 in the whole population of preterm infants and, specifically, from 114 to 83 among the first-born twin infants.

When the catchment area of Oulu University Hospital was studied, altogether 488 infants with RDS were identified among 58,990 live-born infants, accounting for an overall incidence of RDS 8.3 per 1000 live births. Figure 7 shows birth weight-specific incidence rates of RDS in the two consequent year periods 1990–1995 and 1996–1999. The incidence of RDS was 6.7 per 1000 singletons and 70.4 per 1000 multiple live-born infants, respectively. The overall incidence of RDS was 8.7/1,000 live births in 1990–95 and 7.6 in 1996–99 ($p = 0.15$). The incidence of RDS changed towards lower gestational age due to the decreased incidence rate among preterm infants born after 30 weeks. The gestational age-specific incidence of RDS decreased significantly from 1990–1995 to 1996–1999 (OR 0.7, 95% CI 0.5–0.9, $p = 0.005$). The most marked difference in the RDS incidence rate between the two periods was seen in the infants born at 31 and 32 weeks (58% vs. 36% and 46% vs. 28%) and in the birth weight classes of 1,750–1,999 g (28% vs. 11%) and 2,000–2,249 g (8% vs. 4%).
Fig. 7. Birth weight-specific incidence of RDS in the two periods, 1990–95 and 1996–99. When adjusted for birth weight, the incidence of RDS was significantly lower in the latter compared to the previous period (OR 0.7, 95% CI 0.5–0.9, \( p = 0.001 \)). This was especially obvious in the birth weight groups of 1 750–1 999 g and 2 000–2 249 g. The numbers of infants with RDS and all infants born in each birth weight category are shown above the histograms.

### 5.1.2 Risk factors for RDS in twins (I)

Due to the significantly higher rate of preterm births, twin and multiple infants were over-represented in the overall incidence rates. When the data were stratified by plurality and gestational age, the incidence rate of RDS in twin infants differed significantly compared to that of singletons. The incidence rates were counted per 1000 live-born preterm singleton infants, first-born and second-born twins, respectively. In very preterm infants born before 28 weeks, the incidence of RDS was higher in twins compared to singletons of similar gestational age. On the contrary, in near-term infants born between the weeks 32 and 36, the risk of RDS was lower in twins than in singletons of similar gestational age. Compared across the whole set of data, first-born twins had a lower gestational age-adjusted incidence of RDS compared to second-born twins (OR 0.6, 95% CI 0.5–0.8). As a whole, there were no significant differences between second-born twins and singleton
infants (OR 1.1, 95% CI 0.9–1.2), as shown in Figure 8. This result was even more pronounced when only the latter five-year period of 1996–2000 was considered.

![Graph of Incidence of RDS in premature singleton and first- and second-born twin infants. The gestational age-adjusted incidence of RDS between first-born and second-born twins and between first-born twins and singletons differed significantly (OR 0.6, 95% CI 0.5–0.7 and OR 0.7, 95% CI 0.6–0.8, respectively), but no difference was seen between second-born twins and singletons (OR 1.1, 95% CI 1.0–1.2).]

Same-sex pairs, both males and females, were born more prematurely than opposite-sex pairs (mean difference for gestational age -0.4 weeks, 95% CI -0.5 to -0.2). There was no difference in the gestational age-adjusted risk of RDS (OR 1.1, 95% CI 0.9–1.3) or in the gestational age-adjusted concordance of RDS (OR 1.3, 95% CI 0.9–1.7) between same-sex and opposite-sex twin pairs. When stratified by gender, female infants from opposite-sex pairs had a slightly lower risk of RDS compared to infants from same-sex pairs (OR 0.8, 95% CI 0.6–1.0) regardless of birth order. Multiple regression analysis showed that vaginal delivery (OR 0.7, 95% CI 0.6–0.9), female sex (OR 0.7, 95% CI 0.5–0.9), being born first (OR 0.2, 95% CI 0.1–0.3) and being the lighter of the twins (OR 0.3, 95% CI 0.2–0.5) were independent factors protecting from RDS.
5.1.3 RDS-related mortality and morbidity (II)

According to the regional study II during the 1990s, RDS-related neonatal mortality decreased parallel to overall neonatal mortality, accounting for 15% of all neonatal deaths, (i.e deaths during the first 28 days). All infants who died from RDS or its complications were born before 28 completed weeks, with the exception of four cases in the beginning of 1990s. The mean gestational age at birth of the infants who died during the neonatal period were 27.2 weeks (range 23–33) in 1990–1995 and 25.6 weeks (range 23–27) in 1996–1999.

The duration of oxygen therapy shortened (8.0 vs. 5.5 days) and the incidence of pneumothorax decreased (9.7% vs. 4.1%), whereas the rate of chronic lung disease at 36 weeks of postconceptional age (16.4% vs. 16.7%) and at one year of corrected age (9.2% vs. 8.2%) remained unchanged.

5.1.4 Outcome at one year of age (II)

Only 8 (1.9%) out of 488 RDS infants did not participate the follow-up by one year of corrected age. Late pulmonary and neurosensory sequelae of the infants affected with RDS remained at the same level between the two periods. Somatic growth in terms of height and head circumference expressed as standard deviations from the mean remained below the mean. Weight, which was expressed relative to height as a percentage of normal, showed that the infants settled about two percent below the mean.

5.2 Concordance of RDS in MZ and DZ twin pairs (III, IV)

The recruitment of retrospective twin pairs covered all university hospital districts in Finland, and preterm twins with no DNA available for analysis did not differ in population characteristics from the twin pairs analyzed. Of the 200 twin infants analysed, RDS was diagnosed in 149 (75%). The concordance rates of RDS were 54% and 44% in the MZ and DZ same-sex pairs, respectively. The concordance difference of 10% was not statistically significant (95% CI −0.1 to 0.3, \(p = 0.32\)). Stratification by gender or gestational age did not affect the concordance rates or differences between MZ and DZ twins. When 96 prospectively recruited twin pairs were added to the concordance analysis, the concordance difference between MZ and DZ same-sex pairs was 5% (95% CI -0.1 to 0.2, \(p = 0.62\)).
5.3 SP-A and SP-B polymorphisms and RDS in twins (III, IV)

In the twin concordance study, the SP-A and SP-B genes were evaluated for potential associations with the susceptibility to RDS in 100 same-sex pairs. The SP-B Ile131Thr polymorphism was shown to associate with RDS. The threonine allele was associated with an increased risk of RDS (OR 2.2, 95% CI 1.4–3.5, \( p = 0.0014 \)). This was particularly evident in first-born male twins (OR 6.2, 95% CI 2.4–16.3, \( p < 0.001 \)). Figure 9 shows the allele frequencies in RDS and no-RDS infants by birth order. Neither the SP-A alleles or the SP-B intron 4 polymorphism differed between RDS and no-RDS infants or between MZ and DZ twins in the whole population or in any of the subgroups determined by gender or gestational age. The degree of prematurity (OR 2.0, 95% CI 1.1–3.7, \( p = 0.021 \)) and birth order (second-born OR 3.1, 95% CI 1.3–7.4, \( p = 0.009 \)) were the clinical variables that influenced the risk of RDS, when the data were analyzed with clustered multiple logistic regression.

![Fig. 9. SP-B exon 4 Thr allele frequencies in no-RDS and RDS infants among first- and second-born twins. The horizontal line represents the allele frequency among Finnish premature singletons. The \( p \) values, odds ratios (OR) and 95% confidence intervals (CI), which illustrate the association of the Thr allele with RDS, are shown below the histograms.](image)
The prospectively and retrospectively recruited twin pairs did not differ significantly in any clinical characteristics. When they were pooled together, there were altogether 198 premature twin pairs. DNA samples from all of the 396 twin infants were genotyped for SP-A1 and SP-A2 polymorphisms. Among the twin pairs, the SP-A1 allele frequency distribution as a whole differed significantly between the no-RDS and RDS infants \((p = 0.030)\). The frequencies for 6A\(^2\) were 0.65 in the no-RDS group and 0.57 in the RDS group \((p = 0.027)\), and those for 6A\(^3\) 0.20 and 0.28 \((p = 0.0074)\), respectively. The SP-A1 genotype 6A\(^2\)/6A\(^3\) frequencies were 0.45 in the no-RDS and 0.31 in the RDS twin infants \((p = 0.0042)\), and the SP-A haplotype 6A\(^2\)-1A\(^0\) frequencies were 0.62 and 0.53 \((p = 0.016)\), respectively.

The association between RDS and the SP-A gene locus was dependent on the degree of prematurity. Very preterm twin infants (< 32 weeks of gestation) showed no detectable differences in the distribution of SP-A1 or SP-A2 alleles, SP-A genotypes or haplotypes according to RDS status. However, among the near-term twin infants (from 32 to 36 weeks of gestation), the SP-A1 allele, genotype and SP-A haplotype frequency differences were more pronounced. The frequencies in no-RDS and RDS twin infants were 0.69 and 0.63 \((p = 0.029)\) for allele 6A\(^2\), 0.17 and 0.25 \((p = 0.080)\) for the allele 6A\(^3\), 0.49 and 0.28 \((p = 0.0070)\) for the homozygous genotype 6A\(^2\)/6A\(^2\), and 0.65 and 0.57 \((p = 0.16)\) for the haplotype 6A\(^2\)-1A\(^0\), respectively.

It is known that SP-A gene expression is regulated by glucocorticoids (Boggaram et al. 1989). The results of the HOR tests showed effect modification by antenatal glucocorticoid treatment on the SP-A allele frequencies \((p = 0.015, 0.022, 0.076 and 0.22\) for frequencies of the SP-A1 alleles 6A\(^2\), 6A\(^3\), genotype 6A\(^2\)/6A\(^3\) and SP-A haplotype 6A\(^2\)-1A\(^0\), respectively). When the data were stratified according to the glucocorticoid treatment, the most striking differences were seen in the infants with no steroid therapy. The SP-A1 genotype 6A\(^2\)/6A\(^3\) frequency was 0.51 in the no-RDS infants compared to 0.27 in the infants with RDS (OR 0.35, CI 0.2–0.6, \(p < 0.001\), corrected for multiple comparisons). The SPA1 genotype 6A\(^2\)/6A\(^3\) frequencies in infants with and without RDS in two gestational age groups and stratified by glucocorticoid treatment are illustrated in Figure 10. Stratification according to zygosity, birth order, growth restriction or gender did not have any detectable effect on the association between RDS and SP-A polymorphisms.
Fig. 10. Homozygous genotype 6A2/6A2 frequencies in no-RDS and RDS twin infants according to glucocorticoid treatment in all twins and in the two gestational age groups. The p values, odds ratios (OR) and 95% confidence intervals (CI), which illustrate the association of the SP-A1 6A2/6A2 genotype with RDS, are shown below the histograms. The corrected p value in multiple comparison is shown in parentheses.

In order to control confounding factors, the data were analyzed with logistic regression, with the twin pairs clustered. After adjustment for the clinical variables, the homozygous 6A2/6A2 genotype of SP-A1 compared to any other SP-A1 genotype was associated with a decreased risk of RDS (OR 0.57, CI 0.3–0.9, p = 0.027). This type of association was even stronger in the twin pairs born near term (OR 0.34, CI 0.2–0.7, p = 0.004).

5.4 Interaction by the SP-B gene and plurality on the association between the SP-A gene and RDS (V)

No significant differences in the allele distributions of the SP-A or SP-B genes were shown between the RDS and no-RDS infants when singletons and multiple infants were considered together. However, the HOR test showed the presence of a profound effect modification by multiple birth in the SP-A gene polymorphisms (p = 0.0002, 0.001, 0.0006, 0.0001 and 0.17 for the frequencies of the alleles 6A^2, 6A^3, 1A^0, the genotype
6A^2^/6A^2^ and haplotype 6A^2^-1A^0^, respectively). The SP-B Ile131Thr polymorphism differed significantly between RDS and no-RDS only in the first-born infants from twin and multiple gestations, the threonine allele being directly associated with increased risk of RDS (OR 1.72, 95% CI 1.18–2.52, \( p = 0.005 \)) and the isoleucine allele with decreased risk of RDS (OR 0.58, 95%CI 0.40–0.85, \( p = 0.005 \)), as shown in the twin concordance study. No differences between the RDS and no-RDS infants were found in the distributions of the intron 4 polymorphism in infants born from singleton or multiple gestations even after separate evaluation.

As expected, the distributions of both the SP-A1 and the SP-A2 genes between RDS and no-RDS infants differed significantly when infants from singleton and multiple gestations were analyzed separately. This relationship was influenced by the SP-B Ile131Thr genotype. The SP-A1 allele 6A^2^ was over-represented in RDS of singletons when the SP-B genotype was Thr/Thr (frequencies 0.71 in RDS and 0.50 in no-RDS, \( p = 0.009 \)). In contrast the SP-A1 allele 6A^2^ tended to be under-represented in RDS of multiples when the SP-B genotype was Ile/Thr (frequencies 0.53 in RDS and 0.67 in no-RDS, \( p = 0.012 \)) or Thr/Thr (frequencies 0.54 in RDS and 0.64 in no-RDS, \( p = 0.12 \)). The SP-B Ile131Thr genotype-dependent association between SP-A1 homozygous 6A^2^/6A^2^ genotype and RDS was even more evident, as shown in Figure 11.

Fig. 11. Interaction of the SP-A and SP-B genes in all premature infants born from singleton and multiple gestations: Frequencies of the genotype 6A^2^/6A^2^ among 322 singleton and 235 multiple infants without RDS and 119 singleton and 245 multiple infants with RDS. The infants were divided into subgroups based on their SP-B Ile131Thr genotype. The \( \rho \) values corrected for multiple comparisons, odds ratios (ORs) and 95% confidence intervals (CIs) which illustrate the association of the 6A^2^/6A^2^ genotype with RDS in the different SP-B genotype groups; in singletons with the SP-B Thr/Thr genotype, \( \rho = 0.006 \), OR 4.5, CI 1.8–11.3; in multiples with the Ile/Thr genotype \( \rho = 0.05 \), OR 0.5, CI 0.3–0.9; and in multiples with the Thr/Thr genotype \( \rho = 0.04 \), OR 0.4, CI 0.2–0.8.
In a previous study consisting mainly of singletons (Haataja et al. 2001), the interaction between the SP-A and SP-B exon 4 polymorphisms was strongly dependent on the degree of prematurity. Accordingly, the data were analyzed separately for premature infants born before (very premature) and after 32 weeks of gestation (near term). Among the very premature singleton infants, the SP-B Ile131Thr genotype defined the cases with a significant association between the SP-A allele 6A² and RDS. Regardless of the SP-B Ile131Thr genotype, no significant association was evident between the SP-A1 alleles and RDS among very premature multiples. On the contrary, among near-term multiples, the presence of the SP-B genotype Ile/Thr or Thr/Thr defined the significant association between the SP-A1 allele 6A² and the genotype 6A²/6A² and RDS. In near-term singletons, the SP-A and/or SP-B alleles/genotypes/haplotypes had no detectable association with RDS.

The results of multiple logistic regression analyses supported the findings on allelic and genotype associations. Gestational age (< and ≥ 32 weeks of gestation), birth order and gender were included as confounding factors in the analyses. In singletons, a homozygous 6A²/6A² genotype was associated with an increased risk of RDS when the SP-B genotype was Thr/Thr (OR 0.27, 95% CI 0.06–0.80, p = 0.018). In multiples, on the other hand, a homozygous 6A²/6A² genotype decreased the risk of RDS when the SP-B genotype was Ile/Thr or Thr/Thr (OR 0.42, 95% CI 0.22–0.80, p = 0.008). This was especially obvious in near-term multiples (OR 0.29, 95% CI 0.09–0.90, p = 0.032).

Table 5 demonstrates the main findings of genetic studies III–V. SP-A1 6A²/6A² and SP-B exon 4 Thr/Thr genotype frequencies in twin infants with and without RDS, and stratified by gestational age, are shown.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Non-RDS</th>
<th>RDS</th>
<th>p a</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-B Thr/Thr</td>
<td>0.14</td>
<td>0.28</td>
<td>0.04</td>
<td>2.5 (1.0–6.0)</td>
</tr>
<tr>
<td>&lt; 32 weeks of gestation</td>
<td>0.12</td>
<td>0.27</td>
<td>0.07 (0.1)</td>
<td>2.9 (0.8–10.4)</td>
</tr>
<tr>
<td>≥ 32 weeks of gestation</td>
<td>0.15</td>
<td>0.27</td>
<td>0.2 (0.4)</td>
<td>2.0 (0.6–7.1)</td>
</tr>
<tr>
<td>SP-A1 6A²/6A²</td>
<td>0.45</td>
<td>0.31</td>
<td>0.004</td>
<td>0.5 (0.3–0.7)</td>
</tr>
<tr>
<td>&lt; 32 weeks of gestation</td>
<td>0.32</td>
<td>0.32</td>
<td>0.6 (1.0)</td>
<td>1.0 (0.5–2.1)</td>
</tr>
<tr>
<td>≥ 32 weeks of gestation</td>
<td>0.49</td>
<td>0.28</td>
<td>0.003 (0.007)</td>
<td>0.4 (0.2–0.7)</td>
</tr>
</tbody>
</table>

Table 5. Frequencies of SP-A1 6A²/6A² and SP-B Thr/Thr genotypes in genetic studies III–V

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Non-RDS</th>
<th>RDS</th>
<th>p a</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-B Thr/Thr and SP-A1 6A²/6A²</td>
<td>0.21</td>
<td>0.55</td>
<td>0.002 (0.006)</td>
<td>4.5 (1.8–11.3)</td>
</tr>
<tr>
<td>Singleton infants</td>
<td>0.27</td>
<td>0.65</td>
<td>0.06 (0.4)</td>
<td>4.9 (0.9–25.7)</td>
</tr>
<tr>
<td>&lt; 32 weeks of gestation</td>
<td>0.20</td>
<td>0.43</td>
<td>0.08 (0.5)</td>
<td>3.0 (0.9–10.4)</td>
</tr>
<tr>
<td>≥ 32 weeks of gestation</td>
<td>0.49</td>
<td>0.25</td>
<td>0.01 (0.04)</td>
<td>0.4 (0.2–0.8)</td>
</tr>
<tr>
<td>Twin infants</td>
<td>0.25</td>
<td>0.26</td>
<td>0.6 (1.0)</td>
<td>1.0 (0.2–4.2)</td>
</tr>
<tr>
<td>&lt; 32 weeks of gestation</td>
<td>0.56</td>
<td>0.24</td>
<td>0.01 (0.06)</td>
<td>0.3 (0.1–0.8)</td>
</tr>
</tbody>
</table>

The corrected p values for multiple comparisons are shown in parentheses.

In a comparison of singleton and multiple pregnancies, the major SP-A1 allele 6A² and the SP-B Ile131Thr genotype appeared to have contrasting effects on the risk of RDS defined on the basis of the length of pregnancy. Therefore, the pregnancies were analyzed on the basis of intrauterine size. The birth weight of singletons and the combined birth
weight of multiples were considered, since accurate information on placental weight and intra-amniotic volume was not available. As shown in Figure 12, there was a continuous association between fetal weight and the risk of RDS, which was confined to the carriers of the major SP-A1 allele 6A\textsuperscript{2} and the SP-B genotype Ile/Thr or Thr/Thr.

Fig. 12. Influence of the birth weight of singletons or the combined weight of multiples on the interaction between the SP-A and SP-B genes in predicting susceptibility to RDS. Altogether 313 singleton and 344 twins or multiples with either SP-B Ile/Thr or Thr/Thr genotype were analysed. The birth weights and the combined birth weights were categorised into two groups based on the medians. Both premature singletons and multiples were categorised into two subgroups: below or above the median birth weight / combined birth weight. In the singletons, the median birth weight and the interquartiles were 2220 g, 1565 g, and 2707 g, respectively. In the multiples, the median birth weight and the interquartiles were 3625 g, 2920 g, and 4605 g, respectively. Upward and downward arrows illustrate the increased and decreased risk of RDS associated with the SP-A allele 6A\textsuperscript{2}. The odds ratios (ORs) and 95% confidence intervals (CIs), which illustrate the association of the 6A\textsuperscript{2} allele with RDS in the combined SP-B genotype Ile/Thr and Thr/Thr groups are shown. They were in singletons under the median birth weight they were OR = 1.8, CI 1.1–2.9, p = 0.02; in singletons over the median birth weight OR 1.1, CI 0.6–2.1, p = 0.72; in multiples under the median birth weight OR = 0.7, CI 0.5–1.2, p = 0.18; in multiples over the median birth weight OR 0.6, CI 0.4–1.0, p = 0.04. An asterisk (*) above a plot identifies a significant result. There were no significant associations with SP-A and RDS in the SP-B Ile/Ile group (data not shown).

According to sequence data, the peptide encoded by the Thr allele but not by the Ile allele contains a consensus sequence (Asn-X-Thr) for N-linked glycosylation at Asn\textsuperscript{129}, suggesting that the Ile131Thr variation results in genetic variation of the molecular phenotype of proSP-B. In an experimental design utilizing human lung tissue, the molecular size of human proSP-B was shown to be genotype-specific, with the presumably glycosylated Thr/Thr product migrating more slowly than the unglycosylated
Ile/Ile product. Furthermore, the NH₂-terminal fragment was shown to be glycosylated in proSP-B from Thr/Thr lung, but not from Ile/Ile lung, whereas both forms were detected in heterozygous Ile/Thr lung.
6 Discussion

6.1 Epidemiological aspects

The rate of preterm births increased during the 1990s, due to the increasing incidence of pregnancies with assisted conception and the high rate of multiples in these pregnancies (Blickstein & Keith 2002). It has also been shown that in vitro fertilization twins have more likely a higher incidence of preterm birth and prematurity-related respiratory complications (Nassar et al 2003). According to a Canadian study the increase of preterm deliveries is partly related to the population level phenomenon of delayed childbearing (Tough et al 2002).

During the 1990s, more premature infants survived and the incidence of RDS shifted towards more immature infants. The gestational age-adjusted incidence of RDS declined significantly. The overall incidence of RDS, however, did not decline as much as could have been expected as a consequence of the introduction of novel treatments. During the period studied, however, prophylactic surfactant was not in use. Combined use of antenatal corticosteroid, postnatal early surfactant and distending pressure via the nasal route has been shown to be effective in preterm infants (Verder et al 1999, DePaoli et al 2003). Even though new therapies do not remarkably decrease the incidence of RDS, at least in very premature infants, they alleviate the disease, reduce the risk of pulmonary leakage and decrease the incidence of intraventricular hemorrhage (Horbar et al 2002).

The regional incidence figures of RDS in Oulu University Hospital were somewhat higher than national figures. The incidence rate of RDS recorded in the study based on the National Birth Registry may, to some extent, underestimate the true incidence rate of RDS in the whole population. There may be some missing diagnoses in the registry especially among the most premature infants who died early in the postnatal period. Although RDS is a well known disorder, its definition is not straightforward, and some variation in the diagnostic criteria between neonatal units may occur. The validity of registry-based RDS diagnoses proved to be good in twin pairs recruited retrospectively to the genetic studies, however. The practise of antenatal corticosteroid administration to the mother was quite uniform in Finland during the last decade covered here. However, even though prophylactic surfactant was not given in the delivery room during the study
period, subsequent use of surfactant was one of the diagnostic criteria. Pulmonary infection and the administration of surfactant immediately in response to respiratory distress may result in minor misclassifications.

The risk of developing RDS is strongly dependent on gestational age at birth. The rate of preterm births in Finland is low compared to other industrialized countries (Robertson et al 1992), which explains the rather low incidence figures of RDS. On the basis of regional epidemiological study carried out in the Oulu University Hospital, RDS-related neonatal mortality decreased parallel to the decrease in overall neonatal mortality. However, RDS mortality was still considerable, representing 15% of all neonatal deaths. The proportion of infants with supplemental oxygen at 36 postconceptional weeks remained constant throughout the study period from 1990 to 1999, which is consistent with the reports published elsewhere (Jobe 1999). Additional long-term morbidity remained unchanged and considerably high.

Most previous studies on neonatal outcomes have compared singletons and twins across the whole range of gestational ages. However, because the degree of prematurity is the main risk factor for RDS, it is essential to stratify the data by gestational age (Kilpatrick et al 1996). We found out that, after taking into account the gestational age, twins are not at a substantially higher risk of RDS compared to singletons except at a very early gestational age. On the contrary, after 28 weeks of gestation, first-born twins had a lower incidence of RDS compared to both second-born twins and singletons. Twins have been shown to have higher fetal lung maturity values compared to singletons after 31 weeks of gestation (McElrath et al 2000). In addition, pulmonary maturity is usually synchronous in twins (Leveno et al 1984). It is possible that the maturation of lungs in twins precedes that of singletons due to hormonal, growth or inflammatory factors influencing the intrauterine environment. The second-born, or non-presenting infant may not benefit from the salutary effects of vaginal delivery to the same extent as the presenting twin and may be inherently at a higher risk for respiratory distress (Arnold et al 1987).

The finding on the lack of concordance difference between same-sex twins compared to opposite-sex twins refutes the proposal that genetic factors uniformly influence the risk of RDS in twin pregnancies. The same-sex twin pairs that were born more preterm than opposite-sex pairs did not have a higher gestational age-specific incidence or concordance rate of RDS. Chorionicity rather than zygosity may affect the risk of RDS. It is likely that among the most premature twins, monochorionic monozygous twin pairs, who are known to possess the greatest risk of pregnancy complications, are overrepresented. It can be concluded that environmental factors predominate over genetic ones in the predisposition of twins to RDS.

### 6.2 Methods in genetic studies

The sample size determination was performed before the twin concordance study. Twin pairs were recruited also retrospectively in order to obtain adequate number of twin pairs. The retrospectively recruited pairs did not differ from prospectively recruited in any of
the clinical characteristics. Even after combining retrospectively and prospectively recruited twin pairs, the concordance difference between MZ and DZ pairs was not significant. Lack of adequate hypothesis concerning the magnitude of genetic influence or the distribution of individual alleles in the study population posed restrictions on power calculations of the genetic studies. Furthermore the limitation to achieve extensive sample size in a population of preterm twins hampered the stratification into subpopulations.

Comparison concerning the concordance of diseases between MZ and DZ twin pairs have been used to define the role of heredity in disease etiology (Kaprio et al. 2000). If MZ twins, with their complete genetic identity, have a significantly higher concordance than DZ twins, with their mean 50% genetic similarity, genetic factors are considered to influence the susceptibility to a disease. The interpretation of classic twin studies is not always so straightforward, however. It is usually assumed that MZ and DZ twins have an equal environment, and there does not exist major genetic-environmental interactions. Under these assumptions, the estimation of heredity, i.e. the proportion of etiology attributable to genetic factors, can be measured as the fraction of population variance (i.e. the underlying liability to disease) due to genetic variance (MacGregor 2000). Thus we could roughly calculate the heredity component as the concordance difference between MZ and DZ twin pairs multiplied by two. Statistical modelling with genetic, shared and individual environmental effects also assumes the absence of interaction effects and is thus not directly applicable to RDS, either.

Even though the concordance study failed to show a major hereditary impact on RDS, it does not mean that genetic analysis is worthless in assessing the etiologic factors of RDS. Twins do not share a completely identical intrauterine environment (Phillips 1993). Intrapair differences due to antenatal events may exist irrespective of zygosity (Martin et al. 1997). The presenting twin is more prone to PPROM and intrauterine infection, and may therefore be less susceptible to RDS (Arnold et al. 1987). When estimating the risk of RDS in twin and higher-order gestations, twinning itself is an effect modifier, which hampers the assessment of the genetic component in the disease risk.

Association studies with candidate genes have been widely used to evaluate the genetic component of complex diseases. Several conflicting aspects must be regarded when interpreting the results. The most common type of genetic variation originates from a single base mutation, when one nucleotide is substituted for another, referred to as a single-nucleotide polymorphism (SNP). An association study aims to find the disease-predisposing or protective alleles by statistically comparing allele frequencies between a case and a control population. Statistical evidence for an association may result from different situations (Cardon & Palmer 2003). Firstly SNP may be functional and directly affect the expression of a phenotype. Both SP-A and SP-B are essential for proper lung function. In populations of mainly singletons the common polymorphisms of the SP-A and SP-B genes have been found out to associate with RDS (Rämet et al. 2000, Haataja et al. 2000, Floros et al. 2001). There is also evidence that the SP-B Ile131Thr polymorphism may affect the glycosylation process of proSP-B and this in turn may affect the processing of mature SP-B (Haataja et al. 2000, Wang et al. 2003b). Thus we considered meaningful to study these polymorphisms in preterm twins and multiples. Secondly the allele studied may correlate with, or be in linkage disequilibrium with, a causative allele located nearby. Thirdly the association may be due to confounding or selection bias. The
recruitment of retrospective twin pairs covered all university hospital districts in Finland, and twins with no DNA available for analysis did not differ in population characteristics from the twin pairs analyzed. Additionally almost all parents of preterm twin pairs, who were recruited prospectively, gave their consent to conduct the genetic studies. Thus we considered the present studies be fairly free from selection bias. The candidate gene approach has also been criticized because of the non-reproducibility of the results and its limited ability to include all possible causative factors in complex diseases. Identical phenotypes resulting from different constellation of genes, a large number of different genes, interactions between genes, and the effect of one gene being only modest are factors that further complicate the interpretation of the results (Tabor et al 2002). Additionally a single gene may contain polymorphisms that influence the risk of several diseases (genetic parsimony). In situations where an association between a candidate gene and a disease is physiologically meaningful, the approach is better than linkage studies detecting genes of minor effect (Tabor et al 2002). Furthermore linkage analysis requires families with affected individuals in multiple generations. A matched case-control setting is usually preferred, and in case of twin pairs, discordant twins would be optimal, if birth order, gender and twinning itself were associated with RDS. Adequately large samples of RDS-discordant preterm twins for genetic studies are hard to obtain. An unmatched case-control design allows the use of a larger study population, and confounding factors can be controlled by stratification and logistic regression analysis (Sham 1998), as proceeded in the present studies. Irrespective of disease status, allele frequencies are known to vary widely between populations. The Finnish population is historically isolated and genetically comparatively homogenous (de la Chapelle et al 1993). In the present studies only infants of Finnish origin were included and population stratification was thus avoided.

6.3 Concordance of RDS between MZ and DZ twin pairs

According to the results of the twin studies III–IV, there was no significant concordance difference between the MZ and DZ twin pairs suggesting a lack of significant overall genetic impact on RDS. The result was similar after correction for gestational age or gender. In contrast, both the SP-B Ile131Thr and the SP-A polymorphisms were significantly associated with RDS in the same twin populations. This apparent discrepancy between the results of the concordance study and the candidate gene analyses can be explained on the basis of the lack of uniformity in the shared intrauterine environment of twins and the overriding role of environmental factors in twins compared to singletons. In addition, the observed low concordance rate in MZ twins may be due to the generally lower risk of RDS in the first-born than in the second-born twin infants. The inflammatory mediators and growth factors released in active preterm labour accelerate fetal lung maturity (Bry et al 1997, Jobe et al 2000) and, regardless of zygosity or genetic contribution, may protect the presenting twin against RDS, even without manifest infection. Therefore, birth order in twin pregnancy is a confounding factor when evaluating the genetic component of RDS in twin infants.
The previous study showing a higher concordance of RDS in 13 MZ compared to 18 DZ twin pairs included DZ twin pairs of opposite sexes (Myrianthropoulos et al 1971). The high concordance rate in MZ compared to DZ twin pairs was likely due to bias, because male sex is associated with an increased risk of RDS (Khoury et al 1985). In the present concordance study, only same-sex twin pairs were included.

6.4 Role of SP-A and SP-B polymorphisms in RDS of twins

Being significantly over-represented among the first-born no-RDS twins the Ile allele and Ile/Ile genotype was associated with a significantly lower rate of RDS than the Thr allele and Thr/Thr genotype in both MZ and DZ twin pairs. The present study is the first to indicate a direct allelic association between the SP-B Ile131Thr polymorphism and RDS. The SP-B Ile131Thr polymorphism was associated with RDS in the presenting twin. The SP-B Ile131Thr polymorphism may be an important determinant of lung maturity, especially in the first-born infant. This may partly explain the first-born infant’s lower risk of RDS. Recently, SP-B has been shown to take part in lung defence against infection (van Iwaarden et al 2001, Epaud et al 2003), but the mechanism by which the SP-B gene polymorphism might influence the function of mature SP-B is speculative.

The substitution of threonine for isoleucine in the SP-B peptide at amino acid 131 affects the N-linked glycosylation site (Asn-X-Ser/Thr) of proSP-B. Glycosylation may influence the proSP-B folding and sorting and thereby the secretion of surfactant (Haataja et al 2000, Wang et al 2003b). This could be tested using pulse-chase experiments on cultures from human fetal lung. SP-B polymorphism and birth order serve as an example of genetic and environmental interaction as a determinant of the phenotype. It can be speculated whether SP-B in the association of the Ile131 Thr polymorphism and RDS in the presenting twin functions as a protein involved in inflammation. The SP-B Thr allele has been associated with pulmonary diseases in adults (Lin et al 2000, Guo et al 2000, Selman et al 2003).

In the study of SP-A polymorphisms on twins we found that, in healthy preterm twin infants, particularly ones born at 32 weeks of gestation or later, the major allele 6A², the homozygous genotype 6A²/6A² of SP-A1 and the 6A²-1A⁰ haplotype of SP-A were over-represented even after adjustment for zygosity, birth order, gender and corticosteroid therapy. Antenatal corticosteroid therapy served as an effect modifier on the association between SP-A polymorphisms and RDS among preterm twin pairs. Since corticosteroid therapy is mainly indicated during imminent very premature birth, gestation and corticosteroid treatment were interdependent factors. Therefore, some of the associations displayed may be due to a selection bias. However, the genotype of the twin individual affected the risk of RDS even after separate adjustments for gestational age and antenatal corticosteroid treatment with the clinical variables. According to in vitro studies, the responsiveness of SP-A expression to corticosteroid is dependent on the length of gestation (Boggaram et al 1989). It has also been shown that the responsiveness of different alleles of SP-A to synthetic corticosteroid is allele-specific (Kumar & Snyder 1998), the SP-A1 alleles being more responsive to corticosteroid than SP-A2 (Hoover et
Corticosteroid suppresses the inflammatory functions frequently associated with premature birth, including the expression of TNF-α (Shima et al. 1999). The SP-A protein may restrict or promote the inflammatory processes potentially influencing the risk of RDS (Song & Phelps 2000). The observed associations between RDS, premature birth, corticosteroid and SP-A genotype may be due to inflammatory events. However, the mechanism by which the major SP-A genotype and haplotype alter the interaction of the inflammatory events and antenatal lung maturation remains open.

In twin pregnancies, the association between the SP-A polymorphism and the risk of RDS is apparently different from that seen in singleton pregnancies. The reason for this remains speculative. In twin pregnancies, birth order and other confounding environmental factors not present in singleton pregnancies potentially contribute to the susceptibility to RDS. The length of gestation is closely associated with fetal size. When the cumulative fetal weight or total intrauterine content is considered, twin and singleton pregnancies are quite different. If expressed on the basis of cumulative fetal weight the genotype-associated susceptibility to RDS appears to make up a continuum. With the low weight in the case of very immature singletons the main SP-A allele is associated with RDS, while with the moderate weight of many near-term singleton infants and very premature twins the genotype has little influence. In the presence of excessive intrauterine distention in near-term twin pregnancies, the SP-A 6A² allele is associated with a lower risk of RDS. Hormones and growth factors associating with uterine distension influence both the onset of labour and the differentiation of the surfactant system (Hallman et al. 2001). Because SP-A is important both for the proper function of surfactant and for the innate immunity of the lungs, it can be speculated that the most common SP-A allele and haplotype may function differently depending on the length of gestation.

### 6.5 Interaction by the SP-B gene and plurality on the association between the SP-A gene and RDS

The interactive associations of SP-A and SP-B polymorphisms with RDS were latent and intertwined with acquired factors, particularly the degree of prematurity, birth order and twinning. Despite the lack of overall direct genetic influence on the risk of RDS, the segments of the study population had strong gene-gene and gene-environment interactions. The degree of prematurity was always an overriding risk factor. SP-A and SP-B have an interactive role in maintaining the surface activity in vitro, and they are both essential components of the highly surface-active tubular myelin fraction (Possmayer et al. 2001). According to an in vitro study low levels of SP-A mRNA, confined to the major SP-A2 allele 1A⁰, have been observed to associate with respiratory distress (Floros & Hoover 1998). According to the present study, the risk of RDS, defined by the SP-A genotype among the carriers of the SP-B Thr allele, is restricted to the subgroup carrying either the homozygous Thr/Thr or also the heterozygous Thr/Ile genotype. This study on interaction between the SP-A and SP-B common genetic variants confirmed that the genetic predisposition to RDS in multiples is different from that seen
in singleton infants. Almost 50% of multiple pregnancies end in preterm birth, whereas preterm birth in singleton pregnancies is uncommon and often associated with placental disease. The risk of RDS, defined by the interaction of SP-A and SP-B polymorphisms, was associated with the fetal mass. Thus, the difference in the susceptibility to RDS in premature singletons and multiples may depend on the size of the conceptus. A single hormone or cytokine may influence the expression of SP-A in very immature lung, whereas the same agonist may have an opposite effect on the same gene towards term (Odom et al. 1997, Liley et al. 1988, Glumoff et al. 2000). Furthermore, specific combinations of hormones or cytokines may have concentration-dependent additive effects (Väyrynen et al. 2002), making the underlying molecular mechanisms even more complex and difficult to elucidate at the phenotypic level.

Figure 13 shows a schematic framework of current knowledge of SP-A and B gene polymorphisms, which have been found to associate with the risk of RDS and other pulmonary diseases.

Fig. 13. Schematic framework illustrating the current knowledge of SP-A and SP-B gene polymorphisms associating with the risk of RDS and other pulmonary diseases. SP-B intron 4 size variants are represented as ∆i4.
7 Summary and future perspectives

Despite the progress made in antenatal and neonatal care, RDS continues to be a major factor causing increased morbidity and mortality among preterm infants. Advances in molecular genetics have enabled identification of candidate genes and interactions between genes affecting the susceptibility to RDS and have clarified some of the genetic background of RDS. Allelic variation and interaction of the SP-A and SP-B genes have been shown to associate with the disease susceptibility.

The present study improves our understanding of factors involved in the susceptibility to RDS in preterm infants. The study confirms that the genetic susceptibility to RDS is heterogenous and involves complex genetic and environmental relationships. The degree of prematurity is the most important determinant of RDS, but there are individual differences between infants of the same gestational age. This research showed that the gestational age-specific incidence of RDS has decreased after the introduction of new treatments. Twin infants do not have an increased risk of RDS, except at very early gestational age. The presenting twin is less susceptible to RDS than the non-presenting infant or singleton infant towards term gestation. A direct allelic association between the SP-B Ile131Thr polymorphism and RDS was evident only in the presenting twin. Both presentation and the polymorphism of the SP-B gene are controlled by a complex network of inflammatory and growth factors modulating lung development and growth. The role of SP-A polymorphisms in the risk of RDS in twins turned out to be different from that in singletons. The major allele and genotype of SP-A1 were associated with a decreased risk of RDS in near term twin infants. The threonine allele in SP-B Ile131Thr appeared to associate, interactively with SP-A1, with the risk of RDS both in twins and in singletons, with an increased risk of RDS in singletons at very early gestation, but with a lower risk of RDS in twins and multiples near term. The risk of RDS, defined by the interaction of SP-A and SP-B alleles, was associated with the fetal mass. Thus, the difference in the genetic susceptibility to RDS in premature singletons and multiples may depend on the size of the conceptus. Twin gestation modified the association between SP-A and SP-B gene polymorphisms and RDS. Environmental factors turned out to predominate over genetic ones in the disease susceptibility among twins.

When evaluating the genetic risk factors for RDS in twins, the classical twin study method of comparing MZ and DZ twins for the concordance of a disease is not directly
applicable. Several predominant intrauterine and perinatal environmental factors contribute to disease susceptibility regardless of zygosity and are suspected to partly override the heredity components of RDS.

The present results explain part of the susceptibility to RDS in different subgroups of preterm infants. There are several open issues concerning the processing of surfactant proteins and the role of surfactant protein gene polymorphisms modulating the process. The precise molecular mechanisms and interactions by which lung surfactant protein genes and their common variants contribute to lung function in health and disease remain to be elucidated. The future perspective into epidemiology, too, would require methods using genetic associations to test hypotheses about the causal pathways in disease etiology. Genome-wide scans, followed by positional cloning, may enable the identification of novel genes influencing the development of RDS and other pulmonary diseases. Genes for corticosteroid receptors, for cytokines and their receptors, for growth factors and for transcription factors that also regulate the expression of surfactant proteins might be potential candidates.
References


